

Synthesis and *in vitro* metabolism studies of selected steroids for anti-doping analysis

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at the Australian National University*



**Australian
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DECLARATION OF ORIGINALITY

This thesis is composed of my original work and contains no material previously published or written by another person. Previously published data used in comparisons and established methods have been acknowledged by the citation of the original publications from which they derive. The content of my thesis is the result of work I have carried out since the commencement of my research degree candidature (Feb. 2016-Dec. 2019) and has not been previously submitted for another degree or diploma in any university or tertiary institution.

Sumudu Anuradha Weththasinghe

January 2020

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ABSTRACT

Steroids are naturally occurring organic compounds that can be found in all eukaryotes. Anabolic Androgenic Steroids (AAS) are compounds derived from the parent steroid testosterone that were first introduced to treat medical conditions. Later, these synthetic steroids became famous as performance enhancing drugs and were used in human and animal sports to gain an advantage. Though rules are established against the use of AAS, World Anti-Doping Agency (WADA) reports have shown that athletes still use them to gain an advantage. Therefore, advanced analytical techniques and long term markers must be developed to establish a future for sport free of AAS.

The second chapter presents a method to conduct *in vitro* sulfation studies using inexpensive starting materials. Sulfation studies are rarely demonstrated in comparison to phase I metabolism studies and glucuronylation studies as the universal sulfate donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) is expensive. However, sulfate metabolites have been identified as long term markers especially for 17 β -hydroxy-17 α -methyl steroids. In the method developed in this thesis, sodium sulfate and adenosine 5'-triphosphate (ATP) were employed to generate PAPS *in situ*. Therefore, this method can be used to mimic *in vivo* sulfation and potentially to identify new long term markers.

Chapter three discusses an *in vitro* metabolism study of a designer steroid; Δ 6-methyltestosterone that was found in a dietary supplement; junglewaffare. Depending upon the possible phase I metabolic pathways, the synthesis of reference materials was performed alongside an *in vitro* metabolism study. The *in vitro* metabolites generated were compared with synthesised reference materials. This allowed for a number of phase I and phase II metabolites to be identified and out of those, parent glucuronide metabolite and a triply reduced glucuronide metabolite were identified and confirmed using the reference materials.

Chapter four describes work carried out to identify an unknown urinary metabolite observed in an *in vivo* equine study of hemapolin (2 α ,3 α -epithio-17 α -methyl-5 α -androstane-17 β -ol). A mechanistic proposal was developed for the formation of the

suspected urinary metabolite and based on this proposal, four reference materials were synthesised. The retention time of the urinary metabolite matched with one of the synthesised steroid sulfonates according to AORC retention time criteria. Further studies are required to complete the confirmation of the identified urinary metabolite based on AORC MS criteria.

List of Publications

(1) **S. A. Weththasinghe**, C. C. Waller, H. L. Fam, B. J. Stevenson, A. T. Cawley, M. D. McLeod. Replacing PAPS: *In vitro* phase II sulfation of steroids with the liver S9 fraction employing ATP and sodium sulfate. *Drug Test. Anal.*, **2018**, 10, 330–339.

(2) C. C. Waller, **S. A. Weththasinghe**, L. McClure, A. T. Cawley, C. Suann, E. Suann, E. Sutherland, E. Cooper, A. Heather, M. D. McLeod. *In vivo* metabolism of the designer anabolic steroid hemapolin in the thoroughbred horse. *Drug Test. Anal.*, **2020**, 12, 752–762.

LIST OF ABBREVIATIONS

δ	chemical shift
Δ	delta, change
AAS	anabolic androgenic steroids
ABP	Athlete Biological Passport
ADP	adenosine diphosphate
AKR	aldo-keto reductase
AORC	Association of Official Racing Chemists
AP-ESI	atmospheric pressure electrospray ionization
AR	androgen receptor
AS	androsterone 3-sulfate
ATP	adenosine 5'-triphosphate
ARFL	Australian Racing Forensic Laboratory
CPR	Cytochrome P450 reductase
CYP	Cytochrome P450
DEAD	diethyl azodicarboxylate
DHEA	dehydroepiandrosterone
DHT	5 α -dihydrotestosterone
DMDO	Dimethyldioxirane
DMF	<i>N,N</i> -dimethylformamide
EAAS	endogenous anabolic androgenic steroids
EC	etiocholanolone
epistane	2 β ,3 β -epithio-17 α -methyl-5 α -androstan-17 β -ol
ER	estrogen receptor
EtOAc	ethyl acetate
eV	electron volt
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GC-MS	gas chromatography coupled to mass spectrometry
hemapolin	2 α ,3 α -epithio-17 α -methyl-5 α -androstan-17 β -ol
HRMS	high-resolution mass spectrometry

IAAF	International Association for Athletics Federation
IOC	International Olympic Committee
IUPAC	International Union of Pure and Applied Chemistry
LC-MS	liquid chromatography coupled to mass spectrometry
LRMS	low-resolution mass spectrometry
L-Selectride®	lithium tri-sec-butylborohydride
M	madol
madol	desoxymethyltestosterone, 17 α -methyl-5 α -androst-2-en-17 β -ol
MeOH	methanol
<i>m</i> -CPBA	<i>meta</i> -chloroperbenzoic acid
mRNA	messenger RNA
MT	methyltestosterone
N	nandrolone
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NBS	<i>N</i> -bromosuccinimide
NMO	4-methylmorpholine <i>N</i> -oxide
NMR	nuclear magnetic resonance
NOE	nuclear overhauser effect
o/n	over-night
PAP	3'-phosphoadenosine-5'-phosphate
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PaS	<i>Pseudomonas aeruginosa</i> arylsulfatase
PCC	pyridinium chlorochromate
QRIC	Queensland Racing Integrity Commission
SULT	sulfotransferase
SO ₃ .py	sulfur trioxide-pyridine complex
SPE	solid-phase extraction
T	testosterone
t-BuOH	tertiary-butanol
TLC	thin layer chromatography
THF	tetrahydrofuran

UDPGA	uridine-5'-diphosphate glucuronic acid
UGT	uridine 5'-diphospho glucuronosyltransferase
UHPLC	ultra high-performance liquid chromatography
WADA	World Anti-Doping Agency
WAX	weak anion-exchange

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Chapter 1

Introduction

1.1 Steroids

Steroids are organic compounds that consist of four fused rings; three of them are six membered (Rings A, B and C) and one is five membered (Ring D). They can be found naturally in animals, plants and fungi.

Nearly all the natural steroids carry an oxygen atom at C3 in the form of a carbonyl group, a 3α or 3β configured hydroxyl group, or a phenolic hydroxyl group. In addition to that, methyl groups at C10 and C13 positions are another characteristic feature of steroids, except if the A ring becomes fully unsaturated, as in the estrogenic hormones, where the C10 methyl group is absent. Another arguably important feature is C17 substitution. The substituent can be a carbonyl, a hydroxyl group or can be an alkyl substituent.

Therefore, steroids differ from each other, depending on the different functional groups and carbon chains attached to the structure, and depending on the stereochemistry and degree of unsaturation. Those differences determine the chemical and physical properties of the steroids and can lead to considerable differences in biological activity.

Figure 1.1 shown below is a general model for the steroids. This numbering and labelling of the steroidal structure was established by International Union of Pure and Applied Chemistry (IUPAC).

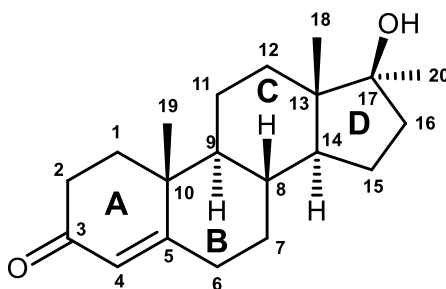
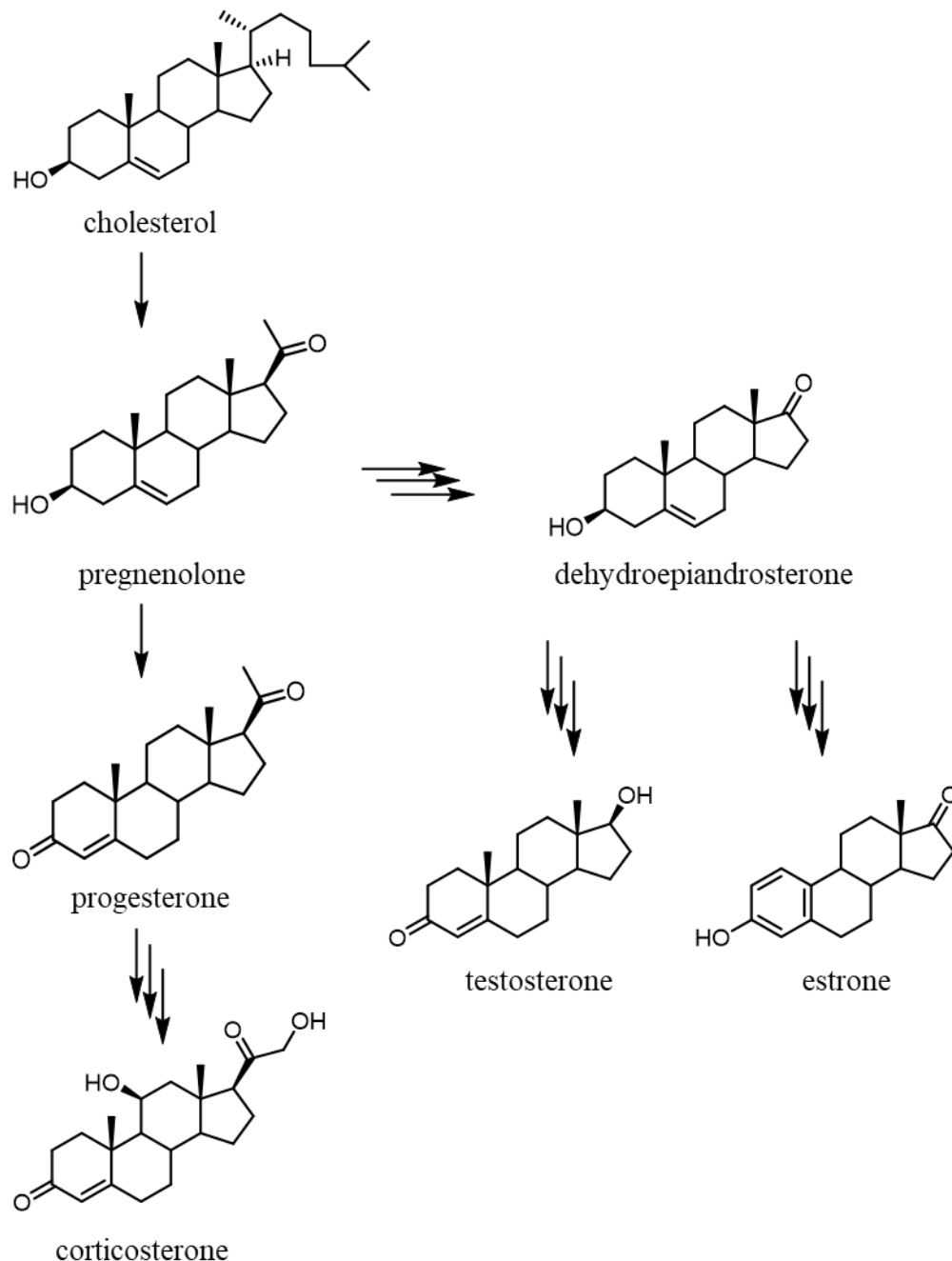


Figure 1.1: 17 α -Methyltestosterone; a typical example showing ring labelling and carbon numbering

Steroids in the human system are categorized as hormones. Those steroid hormones have derived from a common precursor, cholesterol. There are four main types of steroid hormones, progestins, androgens, estrogens and corticoids. A few of those steroids commonly found in humans are testosterone (T), pregnenolone, progesterone, estrone, dehydroepiandrosterone (DHEA) and corticosterone (figure 1.2). Steroid hormones exhibit diverse physiological functions and effects.

Figure 1.2: Steroidogenesis; highlighting the major types of steroids



Testosterone is the major androgen in males and it is important for the development of male reproductive system and secondary sexual characteristics.^[1] After the

discovery and synthesis of T, it became famous for its anabolic activity; increasing muscle size and strength, and for its androgenic properties, including aggression and virilisation.^[2] During the same time, pharmaceutical companies introduced analogues of T, synthetic anabolic androgenic steroids (AAS) in order to treat medical conditions.

1.1.1 Anabolic androgenic steroids (AAS)

Anabolic Androgenic Steroids (AAS) that mimic the endogenic male hormone “testosterone” are used as therapeutics for a number of diseases and are also prescribed for certain deficiencies.^[3] There are several testosterone derivatives that were developed for pharmaceutical purposes^[4] and number of new derivatives are available in the market which are used as performance enhancing substances. As the name suggests, anabolic effects accelerate the muscle mass growth, erythropoiesis and nutritional intake, while androgenic effects increase the male secondary sexual characteristics. Even though AAS were developed as therapeutic drugs, they are also used as performance enhancing drugs due to physiological effects that can benefit athletes. Depending on the origin of the steroids, they can be categorised as either “exogenous” or “endogenous”. The endogenous AAS (EAAS) are the “steroids that are present in the body” such as T and DHEA, and these can be abused by illicit administration. Exogenous AAS are steroids that are not EAAS. These can be abused also. In contrast to EAAS, exogenous AAS are usually easier to detect.

The main attention regarding AAS is towards the illicit use of these agents to gain a competitive advantage in sports. On the other hand, the consequences of using AAS to health is a less addressed topic. There is an increased risk of death and certain disorders such as, metabolic, cardiovascular, endocrine, psychiatric and neurological problems that are associated with the use of AAS.^[5] In 1972, International Olympic Committee banned the use of AASs in sports. However, according to Aguilar *et al.* there has remained a significant number of steroid abuse findings over the years, with the introduction of efficient anti-doping strategies.^[6]

1.1.2 Mechanism of androgenic action

The action of AAS is mediated by the androgen receptor (AR), a type of nuclear receptor, which regulates physiological processes. Each steroid may have different profile of action due to the differences in tissue distribution and the structure and affinity towards the androgen receptor.^[7] Either AAS can bind directly to an AR protein or can convert through metabolism to a more active compound that can then be bound to the AR protein (figure 1.3). For an example, testosterone is transported into the target tissue cell cytoplasm and binds with the androgen receptor either directly or after conversion to 5 α -dihydrotestosterone (DHT) by the enzyme 5-alpha reductase. Steroids, which are categorised as female sex hormones have another type of receptor known as the estrogen receptors (ERs) which act in a similar way to ARs. The steroid-receptor complex then acts on chromosomal DNA in the cell nucleus and activates DNA transcription for specific genes^[1] thereby mediating protein synthesis^[7].

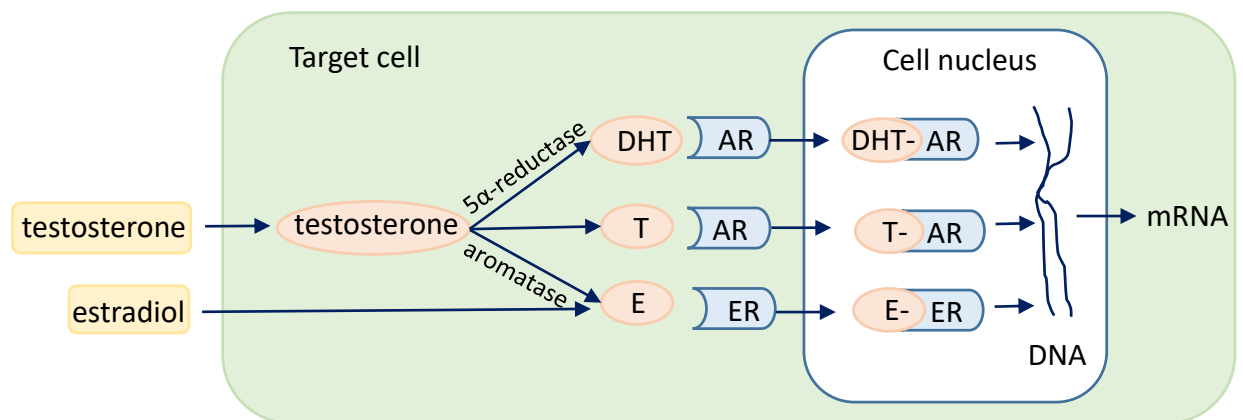


Figure 1.3: Mechanism of action of testosterone. DHT = dihydrotestosterone; E = estradiol; T = testosterone; AR = androgen receptor; ER = estrogen receptor; DHT-AR = dihydrotestosterone-receptor complex; E-ER = estradiol-receptor complex; T-AR = testosterone-receptor complex^[7].

1.2 Steroids as doping agents in sports

1.2.1 History of doping

The history of doping in sports runs for thousands of years ^[8]. It is human nature to strive for fame and success. The same attitudes are applied even with sports. In ancient times athletes used plant extracts (poppy, hippuris), hallucinogenic mushrooms, animal testicles and alcoholic beverages in an effort to gain advantage in sports.^{[8],[9]} Though there were incidents related to doping, and actions against doping, they had not been documented until modern times due to the different attitudes people had towards the performance enhancing drugs.^{[9],[10]}

Later, with the development of medicine and the pharmaceutical industry, new doping agents were introduced. In 1950's amphetamine, a synthetic stimulant was popular among cyclists. Other than amphetamine, analeptics, alkaloids and narcotics were commonly used as doping agents^[9]. After the first isolation and synthesis of testosterone in the mid-1930's, people started to study the properties that testosterone exhibits as an anabolic steroid.^{[10],[11]} Once the structure of testosterone was elucidated, a number of anabolic steroids were synthesized by pharmaceutical companies. These synthetic compounds were analogous to the parent compound "testosterone" and were extensively used for doping purposes. Nowadays a range of anabolic steroids can be included in dietary supplements and are available with a range of different commercial names. According to statistical reports from the World Anti-Doping Agency (WADA) accredited laboratories, exogenous and endogenous anabolic steroids accounted for the majority of doping offences reported between 2003-2015. Apart from anabolic steroids; stimulants, cannabinoids, glucocorticosteroids, masking agents, peptide hormone and growth factors were also reported with lower frequency.^[6]

1.2.2 Insight into doping control

International Association for Athletics Federation (IAAF) was the first to ban doping in 1928, but it did not lead to human tests. In 1967, the International Olympic Committee (IOC) established a medical commission; initiating a medical testing

service for the 1968 Olympics.^[12] After the introduction of GC-MS as a sophisticated tool for steroid detection, systematic doping control analysis was established for the 1972 Olympics.^[12]

Together with these emerging rules and regulations, new markers were introduced to detect the misuse of steroids. The testosterone (T) / epi-testosterone (epi-T) ratio is a well-known marker for the detection of testosterone administration. Initially, a ratio greater than six was considered to indicate a doped condition. Nevertheless, in some cases there are rare exceptions, where naturally occurring levels can lead to false positives. Anti-doping labs now use this ratio in initial screening processes, as an evidence for AAS.^[13] Technically more reliable tests have also been introduced with the establishment of the World Anti-Doping Agency. According to WADA criteria, a ratio greater than four is considered as an adverse analytical finding after which further tests are required for proper confirmation.^[14]

In 1999 WADA was established to fight against doping in sports, by making anti-doping policies more consistent throughout the world. WADA introduced the World Anti-Doping Code in 2004 for the first time; a code, which covers six international standards including prohibited list, testing and investigations, laboratories and therapeutic use of exemptions. The “prohibited list” includes substances and methods banned in sports, and this list, expands annually. Anabolic agents, peptide hormones, growth factors, stimulants, beta-2-agonists, hormones, metabolic modulators are considered as prohibited substances and methods involved in manipulation of blood and blood components are considered as prohibited methods.^[15]

Furthermore, to enhance the efficiency of anti-doping in sports, WADA has introduced a system to monitor selected biological variables over time, named Athlete Biological Passport (ABP). Exogenous steroids can be identified by the parent compound or their metabolites. However, administration of endogenous steroids is harder to detect; as endogenous steroids, are already found in the athletes themselves. The ABP provides the history of a particular athlete, with the help of the two modules: haematological and steroidal. Steroidal markers include six steroids; T, epitestosterone (Epi-T), androsterone (A), etiocholanolone (EC), 5 α -androstane-3 α ,17 β -diol (5 α Adiol), 5 β -androstane-3 α ,17 β -diol (5 β Adiol) and 5 ratios between pairs of those steroids mentioned. Thus, the administration of endogenous steroids can alter one or more of the above markers or ratios. Haematological markers are for

the identification of Erythropoiesis- Stimulating Agents (ESAs), such as haematocrit (HCT), haemoglobin (HGB), red blood cell count (RBC) and reticulocyte count (RET%).

1.3 Steroid metabolism in animal systems

In terms of doping analysis of AAS, it is vital to study the metabolism of steroids. The proper understanding about the steroid metabolism, leads to the accurate detection of the misused steroid. Metabolism aids deactivation and elimination of steroids by converting them to more soluble forms. As steroids undergo extensive metabolism inside the body, a broad range of metabolites are excreted in the urine including free steroids, and conjugated fractions. The majority of steroids are conjugated to form glucuronide or sulfate metabolites which have increased aqueous solubility to facilitate their excretion.

The metabolism of the steroids in different animals can differ. However, in any system steroid metabolism generally proceeds with two independent pathways, known as phase I and phase II. Certain steroids directly undergo phase II metabolism but in many cases phase I metabolism precedes phase II. Steroids undergo phase I metabolism via enzymatic reactions (hydroxylation, reduction, oxidation) to change the steroidal functional groups.

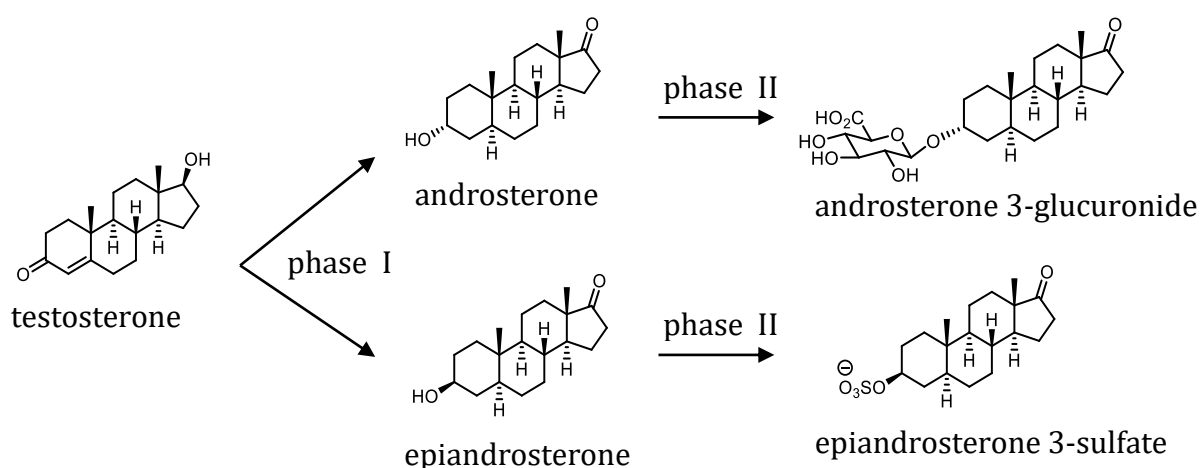


Figure 1.4: Two possible phase I and phase II metabolic pathways of testosterone

Cytochrome P450 reductases (CPRs) use reduced nicotinamide adenine dinucleotide phosphate (NADPH) and mainly perform hydroxylation. Oxidation of an alcohol or

reduction of a carbonyl group is catalysed by the aldo-keto reductase enzymes thereby aiding the interconversion of the alcohol and carbonyl functions. These enzymes belong to the superfamily aldo-keto reductase and depending on the action, the enzyme name differs. Hydroxy steroid dehydrogenases use nicotinamide adenine dinucleotide (NAD) and oxidise alcohols to ketones. The same enzyme, but in the reverse way known as keto steroid reductase, uses reduced NAD (NADH) to reduce ketones to alcohols.

Sulfation and glucuronylation are two major conjugation pathways described in phase II steroid metabolism. The active sulfate donor in sulfation process is 3'-phosphoadenosine 5'-phosphosulfate (PAPS), formed with adenosine triphosphate (ATP) and inorganic sulfate in the presence of ATP-sulfurylase and APS-kinase.^[16] Out of two main classes of sulfotransferases, cytosolic sulfotransferases (SULTs) are involved in sulfation of small endogenous and exogenous compounds including steroids. In the process of glucuronylation, the membrane bound UDP-glucuronosyltransferases (UGTs) catalyse the conjugation of glucuronic acid moiety to the steroid backbone. The co-factor involved in glucuronylation is uridine-5'-disphosphate glucuronic acid (UDPGA)

Figure 1.4 shows the production of two phase I metabolites from T. Both phase I metabolites were formed with reduction of the double bond, 3 ketone and with the oxidation at C17 hydroxy group. Testosterone being a 3-keto-4-ene steroid, the Δ^{4-5} reduction can lead to two isomers, either the 5β or 5α configuration. In the example shown here 5α -reductase, a membrane associated NADH dependent protein^{[17],[18]} has catalysed the reaction to yield the 5α -configured steroid metabolites. The reduction of the 3-keto group of the 5α -configured steroid has made the difference between A and epi-A. In that case 3α -hydroxysteroid dehydrogenase gives rise to the 3α -configured hydroxyl group and 3β -hydroxysteroid dehydrogenase gives rise to the 3β -configured hydroxyl group. Then newly formed 3-hydroxy group has undergone phase II conjugation to produce a steroid glucuronide or a steroid sulfate.

Table 1.1 illustrates some of the selected steroids, their diagnostic phase I metabolites and the trade name used. In order to study steroid metabolism inside mammals, two main methods are used, *in vivo* and *in vitro* test methods.

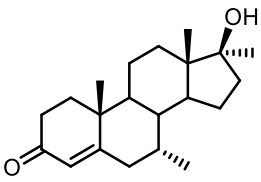
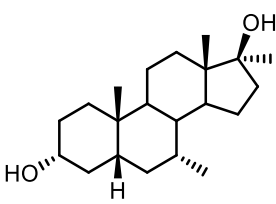
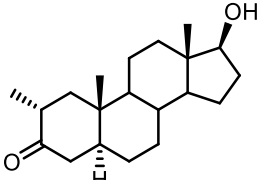
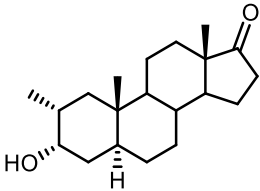
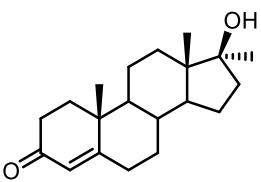
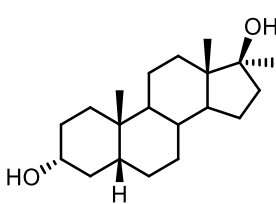
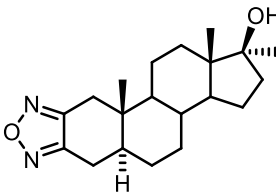
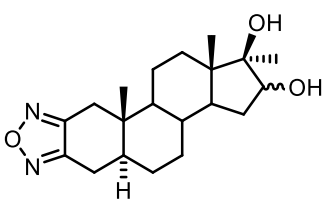
Parent	Metabolite	Trade name
 bolasterone	 7α,17α-dimethyl-5β-androstane-3α,17β-diol	Boldane Equipoise
 drostanolone	 3α-hydroxy-2α-methyl-5α-androstan-17-one	Drolban Methanolone
 methyltestosterone	 17α-methyl-5β-androstane-3α,17β-diol	Android Metandren
 furazabol	 16ξ-hydroxy furazabol	Frazalon Miotolon

Table 1.1: Structures of selected anabolic-androgenic steroids with corresponding diagnostic phase I metabolites and examples of registered trade names^[2]

1.4 *In vivo* and *in vitro* techniques to study steroid metabolism

Most of AAS undergo extensive metabolism to downstream phase II metabolites within the body, hence the recovery of parent compound is very low or absent.^{[18],[19]} Properly studied metabolic profiles can lead to unambiguous detection of the misuse of AAS. These studies can identify long-term markers that can be used in doping control. Thus, metabolism plays an important role in anti-doping analysis.

Metabolism studies are carried out either *in vivo* or *in vitro*. Animal models and human volunteers are used for *in vivo* steroid metabolism. The administration of the steroid can be done using different routes, intravenous, intramuscular^[20] or oral administration^[21] where the oral route is more convenient compared to the others. Once the drug is administered, the metabolism occurs in the body and typically urinary metabolites are investigated. In some cases, blood samples may also be studied.

There are drawbacks associated with *in vivo* metabolism. As most of those illicit drugs are available only in the internet, their toxicological effects and the activity profiles are not known. They may never have been approved for human or animal use. Thus, they can cause adverse effects on those animals or human volunteers. As a result, it is difficult to obtain ethical approval for *in vivo* metabolism studies. Other than this, the cost is comparatively high and it is difficult to identify the metabolites as the metabolites are present in complex matrices such as blood and urine.^[22] However, veterinary preparations or pharmaceuticals can be administered as recommended, orally or by injection.

In vivo studies have shown that the hepatic enzyme activity and the type of metabolites produced differ from species to species.^{[23],[24]} Thus, conducting metabolism studies *in vivo* with different species, such as a dog cannot be used to predict human metabolism.

The application of *in vitro* techniques for the study of steroid metabolism can overcome some of the shortcomings of *in vivo* methods. The beginning of *in vitro* techniques dates back to 1940, before they became an appropriate alternative to *in vivo* studies. It is only in the last two decades that *in vitro* techniques have reached a position where they can replace or reliably complement *in vivo* studies.^[25] *In vitro* studies are quick, simple and cheap compared to *in vivo* studies.

Drug metabolism can occur within any tissue in the body. However, in mammals drug metabolism mainly occurs in the liver. Given this, *in vitro* metabolism studies are mainly carried out with *ex-vivo* liver preparations; hepatocytes, homogenised liver fractions (S9 fractions)^{[26],[27]} or liver microsomes.^[28] The supernatant that contains cytosol and microsomes, after homogenising liver and pelleting solids at 9,000 g is known as the S9 fraction. Further centrifugation of S9 fraction at 100,000 g, separates

the microsomes from the cytosol as a pellet that consists of the endoplasmic reticulum with associated enzymes.

The cytosol mainly contains phase II enzymes such as sulfotransferases and glutathione S-transferase, while the microsomes carry cytochromes P450 and uridine 5'-glucuronosyltransferase (UGT) enzymes. As the S9 fraction consists of the cytosol and microsomes, it contains both phase I and phase II enzymes.

For phase I *in vitro* studies cofactors must be added to the liver extract. For phase I enzymes to be active, energy should be supplied using a NADH or NADPH regenerating system. As mentioned previously phase I metabolism mainly involves oxidation, reduction and hydroxylation. Hydroxysteroid dehydrogenase catalyses the conversion of hydroxy steroids to ketosteroids using NAD⁺ (oxidized NAD) as the cofactor. Reduction of the steroid is catalysed by the same enzyme; in this case it is known as ketosteroid reductase and employs NADH. Cytochrome P450s catalyse the mono-oxygenation of steroids by utilising NADPH. Therefore, NAD-NADP regenerating system (NAD,NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase) is common in most *in vitro* studies performed.^{[29],[30]}

Phase II metabolism studies still have not gained popularity in this field. However, *in vitro* glucuronylation studies are commonly reported among phase II metabolism studies. In contrast to that, *in vitro* sulfation studies are rarely demonstrated. In order to generate phase II glucuronides, uridine 5'-diphosphate glucuronic acid (UDPGA) is used as the cofactor with liver preparations. Few sulfation studies have been reported with PAPS^[31], an expensive cofactor.

The key concern regarding *in vitro* metabolism is what extent it accurately reflects or mimics the *in vivo* metabolism. It has shown that *in vitro* methods can be used to study the phase I metabolites of different steroids. In the metabolism study of furazadrol, enzymatically hydrolysed *in vivo* phase II metabolites have given rise to a number of phase I metabolites observed with *in vitro* metabolism.^[29] Similar to this, another study carried out using stanazolol has revealed that *in vitro* methods using liver S9 could generate major phase I metabolites which were observed *in vivo*.^[33] Moreover in other cases, *in vitro* methods may failed to produce important metabolites observed *in vivo* as will be discussed in chapter 3.

1.5 Screening methods of steroids and their metabolites

1.5.1 Early detection methods

It was a great challenge to develop specific methods to detect individual steroid metabolites that may be distinguished by only small structural differences. The earliest established tests were not for human athletes but were for horseracing. The first established methods used equine saliva to detect most common doping agents used at that time including cocaine, morphine and heroin. In those saliva tests, the drug was extracted from horse's saliva and the presence was observed by the formation of a precipitate or a cloudy appearance after addition of suitable reagents.^[8]

In the late 1960's thin layer chromatography (TLC) became one of the widely used techniques for the detection of steroids and their metabolites.^[34] These methods were known to be better suited to the identification of groups of steroids rather than for individual compound identification. A wide variety of thin layer chromatography support media, different types of spray reagents and thermal conditions were all used to perform these analyses.

Radioimmunoassay is another important method introduced for the detection of steroids and corticosteroids.^{[35],[36]} In 1980's a radioimmuno assay was developed to detect nandrolone metabolites.^[37] There were some drawbacks associated with this method; radioactive materials were involved and expensive equipment was used. Other than that, there are problems with cross reactivity that can lead to the detection of related structures. As a result, new analytical methods were developed with time.

1.5.2 Mass spectrometric methods

To face the challenges in anti-doping studies, chromatographic techniques combined with mass spectrometry became a powerful tool in identification of doping agents and their metabolites.

Gas chromatography-mass spectrometry (GC-MS) screening methods were introduced for the detection of steroid metabolites in the early 1970's. Ever since mass spectrometry has played a dominant role in doping analysis.^[12]

For GC-MS analysis to succeed, the analyte must be thermally stable and volatile. In many cases, GC-MS analysis requires complex sample preparative steps before the analysis.

Steroid metabolism gives rise to free metabolites as well as conjugated metabolites, such as steroid glucuronides and steroid sulfates. The detection of steroids usually focussed on the conjugated metabolites, as unconjugated free steroids are detected at very low concentrations.^[38] Steroid conjugates are less volatile and thermally unstable. Therefore, deconjugation or hydrolysis to obtain the free steroid and then derivatization is performed to enhance the thermal stability, volatility and enhance chromatographic peak shapes (figure 1.5 and scheme 1.1).^[39]

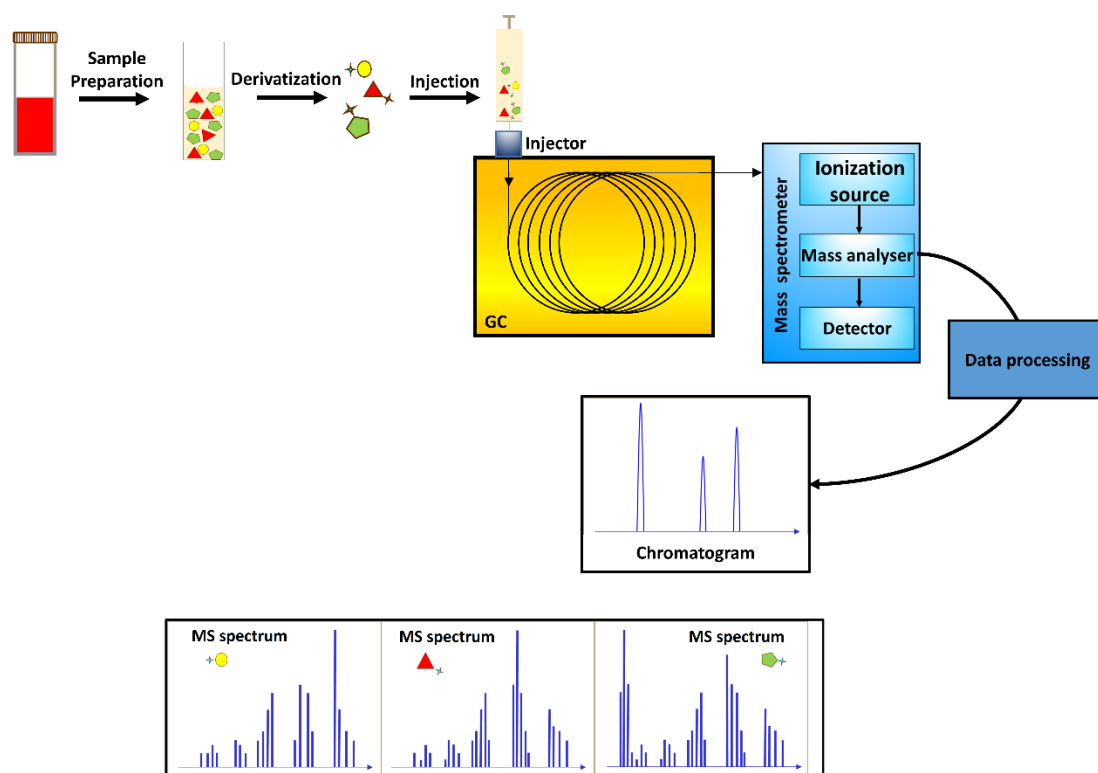
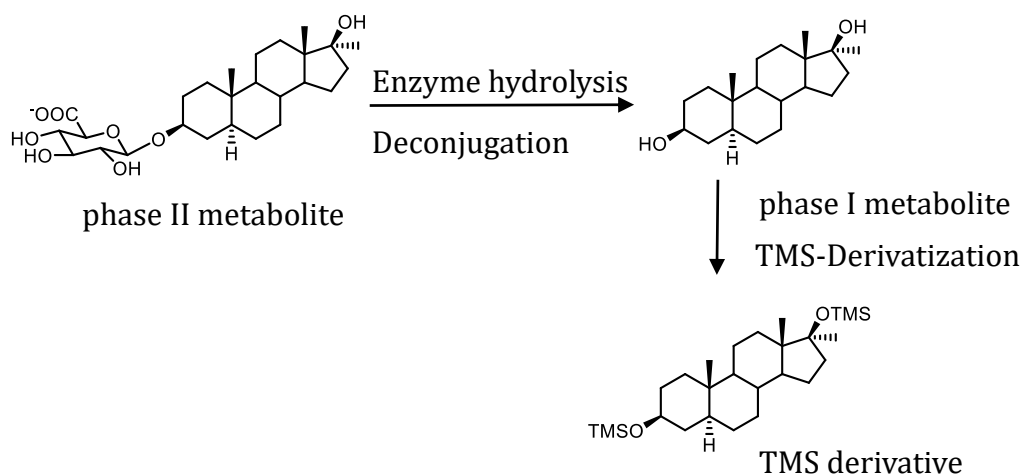


Figure 1.5: Schematic diagram of the GC-MS principle



Scheme 1.1: Two important sample preparative steps associated with GC-MS, deconjugation and derivatization

For GC-MS, deconjugation of sulfates or glucuronides is performed either with chemical hydrolysis or enzymatic hydrolysis in order to obtain the free steroid. Chemical hydrolysis requires strong acids and high temperatures^[41], that can cause degradation of the analytes of interest, increase levels of co-extractants and increase matrix interference from degradation of macromolecules.^[42] Therefore, enzymatic hydrolysis, with β -glucuronidase from *Escherichia coli* is the most commonly used approach. ^{[29],[43], [44]}. Due to this, the detection of steroids is restricted to the glucuronide conjugates and the free forms of the metabolites. So the other phase II conjugates such as sulfates are not usually employed for the detection of doping.^[45]

There are number of factors that affect the efficiency of enzyme hydrolysis: such as, temperature, pH of the medium, incubation time, purity of enzyme, presence or absence of enzyme inhibitors and presence of contaminants.^{[39],[46]} Apart from these the hydrolysis depends on the structure of the glucuronide metabolite.^[46] Therefore, deconjugation with β -glucuronidase is not always 100% effective;^[47] such that it can cause underestimations.^[38] As an example, in a study of testosterone undecanoate, two glucuronide conjugates; $3\alpha,6\beta$ -dihydroxy- 5α -androstane-17-one (6β -hydroxyandrosterone) 3-glucuronide and $3\alpha,6\beta$ -dihydroxy- 5β -androstane-17-one (6β -hydroxyetiocholanolone) 3-glucuronide remained unhydrolysed.^[46] Therefore, it would not be observed in subsequent GC-MS analysis.

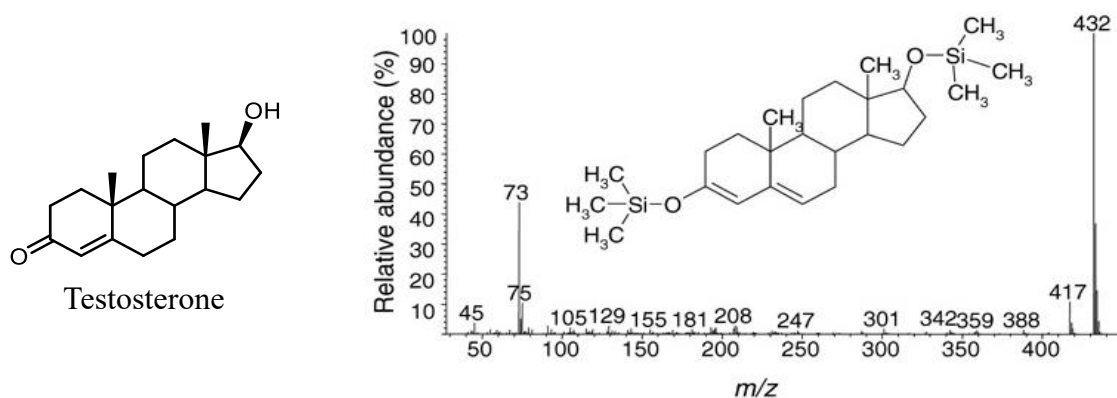
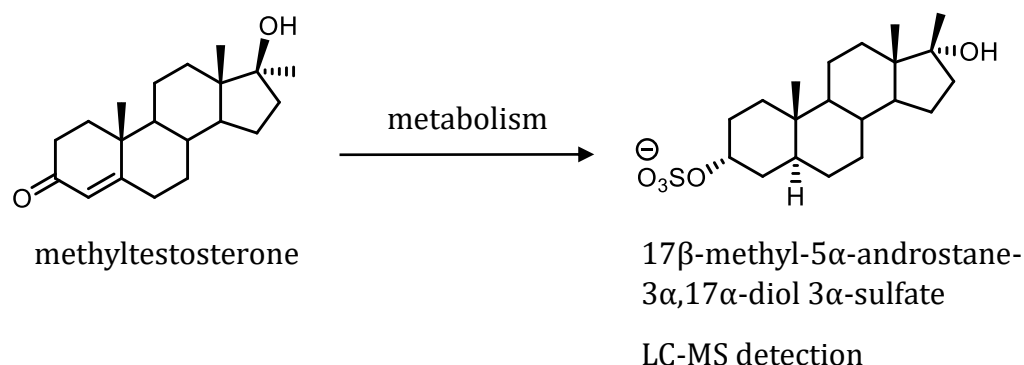


Figure 1.6: Mass spectrum of silylated androsta-3,5-diene-3,17 β -diol, derivatised testosterone.^[48]

In derivatization, polar functional groups are substituted by the derivatizing reagent. The most widespread derivatization technique is silylation.^[49] During this derivatization, active H atoms in OH, NH or SH groups are replaced by silyl group. Silylation occurs via a nucleophilic substitution which is driven by a suitable leaving group present in the derivatizing reagent (figure 1.6).

Even though steroid sulfates represent a significant fraction of steroid metabolites, in the early stages of anti-doping analysis they were neglected. Only glucuronide metabolites were used for analysis as there were few suitable methods to hydrolyse the sulfate metabolites. Pedersen *et al*^[50] demonstrated acid solvolysis as a method for deconjugation of steroid sulfates. As this method generates unwanted by-products and artefacts^{[39],[51]}, enzyme hydrolyses are typically favoured. The crude enzyme preparation derived from *Helix pomatia* is one of the widely used sulfatase enzyme (HpS).^[39] Incomplete hydrolysis and alternative enzyme activities can also lead to the formation of artefacts.^[39] Therefore, it is necessary to develop more efficient substrate specific sulfatases in order to address this issue. In a recent study, Stevenson *et al* revealed that heterologously expressed *Pseudomonas aeruginosa* arylsulfatase (PaS), is advantageous over HpS as it demonstrated relatively high activity towards 3-keto, 17 β -steroid sulfates and was selective for sulfates in the presence of glucuronides.^[52] Another study related to the same PaS enzyme has shown that three mutations on wild-type PaS has enhanced the activity by 150 times towards the hydrolysis of testosterone sulfate.^[53] Although promising, these methods are yet to be widely adopted.

Unlike GC-MS, liquid chromatography with mass spectrometry (LC-MS) assays are capable of measuring conjugated analytes with minimum sample preparation. LC-MS techniques are becoming more popular as they address many problems associated with GC-MS. As a result of this, metabolites resistant to enzyme hydrolysis, or only partially hydrolysed by enzymes and sulfate conjugates can be directly detected with LC-MS techniques.^{[45],[54]} Moreover, in LC-MS electrospray ionization (ESI) leads to high sensitivity, softer ionization.^[55]



Scheme 1.2: Direct detection of a long-term metabolite formed during methyltestosterone metabolism.

LC-MS has led to the direct detection of conjugate metabolites, such as glucuronides and sulfates. Studies have proven that some sulfate metabolites provide extended detection windows by direct detection, compared to the free steroids obtained following glucuronide hydrolysis. For example, the detection of methyl testosterone misuse has improved 2 to 3 times by monitoring a sulfate metabolite (scheme 1.2), 17β-methyl-5α-androstane-3α,17α-diol 3-sulfate.^[56] In an *in vivo* study of methandienone, Gomez *et al.* have observed a sulfate conjugate of 18-nor-17β-hydroxymethyl-17α-methylandrosta-1,4,13-triene-3-one for 26 days after administration. Whereas the glucuronide conjugate of the same metabolite, also reported as the long term metabolite of methandienone^[57], was detected only for 14 days. There are published data where they have shown direct detection of glucuronide metabolites, for stanozolol doping. 17-epistanozolol-N-glucuronide and stanozolol-N-glucuronide were identified as beneficial long-term markers which were detected up to 28 days post administration of stanozolol.^[58]

However, LC-MS cannot completely replace GC-MS. There are certain compounds that cannot be ionized by LC-MS techniques^[22] and when the concentration of the

compounds are very low, derivatization becomes important.^[40] In those incidents, GC-MS becomes the only possible technique to use.

1.6 Project goals

The focus of this project was to understand the metabolism of different types of steroids and build-up strategies to address the problems encountered during anti-doping studies.

Chapter two is an introduction of a new and cheaper *in vitro* sulfation system that can be used to generate phase II sulfate conjugates. As mentioned before, phase II sulfation studies have been rarely demonstrated, due to the unavailability of an inexpensive sulfation system. In contrast, glucuronylation is commonly observed among *in vitro* metabolism studies. Therefore, this gap needed to be filled and as a result, a cheap steroid sulfate generation system was developed by employing ATP and inorganic sulfate as the building blocks to generate PAPS *in situ*. This developed method was applied with six different steroids to see the sulfation potential of the developed system. The steroids were selected in order to have a range of differences in the configuration so that the application of the method would be well demonstrated. Other than that, this was tested among three different species; human, equine and canine to see the applicability of the method among liver extractions from these three species.

Chapter three is a study of an identified potential drug, Δ^6 -methyltestosterone. The human *in vivo* metabolism of the same steroid has been demonstrated previously and has identified four metabolites, including the 17-epimer. That study was carried out with GC-MS by employing β -glucuronidase enzyme from *E.coli*. Our study mainly focused on metabolites that could be identified with direct detection by employing LC-MS techniques. Firstly, upon predicting the possible metabolic pathways, potential metabolites were synthesised as reference materials before moving in to the *in vitro* study of the steroid; thereby giving an opportunity to identify some of the metabolites generated from the study. The study included epimerised metabolites, as 17 β -hydroxy-17 α -methyl steroids are highly prone to undergo 17-epimerisation *via* the formation of the 17 β -sulfate conjugate. This involved developing a synthetic method to obtain some of the predicted epimerised metabolites.

Apart from our study, some of those synthesised substances were given to our collaborators who were willing to run the *in vivo* human, equine and canine studies. This study will be more significant once we can document both *in vivo* and *in vitro* studies.

Chapter four, being one of the most important, is an investigation directed to identify a metabolite that was detected during an *in vivo* study of hemapolin (2 α ,3 α -epithio-17 α -methyl-5 α -androstan-17 β -ol). This metabolite was first predicted as madol sulfate but later identified as a steroid sulfonate. During the study, possible metabolic pathways to form the observed sulfonate metabolite were identified and based on a mechanistic hypothesis, the compounds suspected as the observed metabolites were synthesised. Four steroid sulfonate isomers were synthesised and chromatographic techniques were used to compare the urinary metabolite with the synthesised reference materials.

Ultimately, the project was an attempt to understand the metabolism of some selected steroids and at the same time made an attempt to retrieve better correlation between *in vivo* and *in vitro* test methods by introducing a new sulfate conjugate generating system.

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Chapter 2

Replacing PAPS with ATP and sodium sulfate

2.1 Foreword

The following manuscript published in the journal “Drug Testing and Analysis” describes an economic and facile method for *in vitro* sulfation by utilizing ATP and sulfate. Further, the paper demonstrates the applicability of the method to generate *in vitro* phase II metabolites of AASs for two steroids associated with doping, furazadrol and superdrol. The publisher, John Wiley and Sons have granted the permission to reproduce this manuscript within this thesis (License Number 4670600894973) via RightsLink.

The manuscript was authored by Ms. Sumudu Weththasinghe, Dr. Christopher Waller, Miss Han Ling Fam, Dr. Bradley Stevenson, Dr. Adam Cawley and Associate Professor Malcolm McLeod. The contribution made by myself, Sumudu Weththasinghe, was the development of an alternative and economic method of sulfation by utilizing more stable precursors, ATP and sulfate in order to replace PAPS. The results from my work are depicted in figure 3 and figure 4. Dr. Christopher Waller applied the method in *in vitro* metabolism studies and analysed the metabolites. Miss Han Fam did the pilot studies as an undergraduate project student on using ATP and sulfate. However, those data do not appear in the manuscript.

The metabolism of AAS has already been discussed in chapter one. It is well known that steroids undergo phase I and phase II metabolic pathways inside the body. The latter has two major conjugation pathways, glucuronylation and sulfation. Each of these pathways are involved in inactivation and excretion of steroids in a more water-soluble form.

When it comes to metabolism studies of AAS, this is conventionally performed by *in vivo* studies. In those studies, drugs are administered to a living animal and after that, urine and blood samples are analysed to identify the formed metabolites. However, *in vitro* techniques are gaining popularity due to ease of performing these

studies and reduced ethical concerns. During *in vitro* studies, steroid is incubated in liver preparations and suitable co-factors to generate metabolites.

Liver fractions or liver preparations contain required enzymes for phase I metabolism. Thus, once co-factors are added, phase I metabolites can be easily generated. The phase I regenerating system includes NAD, NADP, d-glucose-6-phosphate (G6P) and G6P-dehydrogenase as co-factors. These phase I co-factors are readily available and they are relatively inexpensive. Thus, *in vitro* phase I metabolism has extensively studied and reported. However, when it comes to *in vitro* phase II metabolism, most phase II studies are restricted to glucuronylation, as the universal sulfate donor, PAPS is extremely expensive and unstable.

Sulfate metabolites have been identified as long term markers for several AAS which provides the motivation for conducting phase II sulfation in *in vitro* metabolism studies. This drove us to develop a cheap alternative method to replace PAPS in order to conduct *in vitro* phase II sulfation.

Most of the markers used in anti-doping are deconjugated glucuronide metabolites. Generally, those metabolites are excreted more rapidly than the sulfate conjugates. As a result, several sulfate conjugates are known that show longer detection windows compared to the glucuronide conjugates for certain AAS, mostly 17-hydroxy-17-methyl steroids.^[1,2] Therefore, this method will open up pathways to reveal novel long-term metabolites, which can be useful in anti-doping studies.

Several published studies have reported the synthesis of PAPS for *in vitro* sulfation. One study discussed PAPS regeneration, carried out using six enzymes.^[3] The method described was successful but also very complex and difficult to implement due to the need for six enzymes.

Both ATP and inorganic sulfate are involved in the biosynthesis of PAPS. The enzymes involved are ATP-sulfurylase and APS-kinase, which are found in liver S9 fractions. Therefore, a study was conducted to generate PAPS *in situ*, by employing ATP and Na₂SO₄ as the starting materials. So, the work presented here was conducted in order to answer the issues mentioned above.

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RESEARCH ARTICLE

Replacing PAPS: *In vitro* phase II sulfation of steroids with the liver S9 fraction employing ATP and sodium sulfate

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In vitro technologies provide the capacity to study drug metabolism where *in vivo* studies are precluded due to ethical or financial constraints. The metabolites generated by *in vitro* studies can assist anti-doping laboratories to develop protocols for the detection of novel substances that would otherwise evade routine screening efforts. In addition, professional bodies such as the Association of Official Racing Chemists (AORC) currently permit the use of *in-vitro*-derived reference materials for confirmation purposes providing additional impetus for the development of cost effective *in vitro* metabolism platforms. In this work, alternative conditions for *in vitro* phase II sulfation using human, equine or canine liver S9 fraction were developed, with adenosine triphosphate (ATP) and sodium sulfate in place of the expensive and unstable co-factor 3'-phosphoadenosine-5'-phosphosulfate (PAPS), and employed for the generation of six representative steroidal sulfates. Using these conditions, the equine *in vitro* phase II metabolism of the synthetic or so-called designer steroid furazadrol ([1',2']isoxazolo[4',5':2,3]-5 α -androstane-17 β -ol) was investigated, with ATP and Na₂SO₄ providing comparable metabolism to reactions using PAPS. The major *in vitro* metabolites of furazadrol matched those observed in a previously reported equine *in vivo* study. Finally, the equine *in vitro* phase II metabolism of the synthetic steroid superdrol (methasterone, 17 β -hydroxy-2 α ,17 α -dimethyl-5 α -androstane-3-one) was performed as a prediction of the *in vivo* metabolic profile.

KEYWORDS

in vitro metabolism, PAPS, steroid, anti-doping, sulfate ester, sulfation

1 | INTRODUCTION

The development of analytical methods to detect the use of illicit substances such as performance-enhancing anabolic androgenic steroids (AAS) requires a detailed knowledge of drug metabolism.¹⁻³ For many agents, the parent compound is extensively metabolized and cannot be detected, so metabolites must be targeted as markers of drug administration. Traditionally, the metabolic profile of a steroid is established by *in vivo* studies that involve drug administration to one or more experimental subjects. Alternatively, *in vitro* technologies based on cultured liver cells or liver extracts provide methods that mitigate many of the ethical concerns regarding human or animal health and safety.^{4,5} These issues are of particular relevance in the case of synthetic or so-called designer steroids since they are often brought to market in a clandestine fashion, and the majority of these compounds do not have available data regarding their purity, safety,

or efficacy.^{6,7} Given this, *in vitro* experiments would offer a safer approach to identifying metabolite markers.

The metabolism of AAS occurs in two complementary phases: phase I metabolism includes chemical modifications of the steroid skeleton such as reduction, oxidation, or hydroxylation, whereas phase II metabolism includes condensation reactions with other small molecules, termed conjugation. Steroid phase II metabolism usually involves conjugation with polar anionic sulfate or glucuronic acid groups to increase aqueous solubility for excretion, and in many cases these phase II conjugates make up the majority of metabolites detected.¹⁻³ As a consequence, phase II metabolites can serve as markers for drug detection, and monitoring directly for these metabolites can offer advantages, such as reduced sample preparation.⁸ Other advantages can also arise from the study of phase II metabolites. Monitoring of phase II sulfate conjugates can increase detection windows for some analytes,⁹⁻¹⁴ and has been used to

distinguish between the endogenous or exogenously administered steroids.^{10,15–17} Further, the formation and subsequent decomposition of phase II sulfate conjugates has also been implicated in epimerization of 17 α -alkyl-17 β -hydroxy steroids, leading to the formation of 17 β -alkyl-17 α -hydroxy compounds and other minor but significant metabolites.^{18–20} Given the importance of phase II metabolism, there is a need for *in vitro* systems that can faithfully replicate *in vivo* metabolism. This is particularly relevant in equine and canine sports, as current Association of Official Racing Chemist (AORC) criteria allow for the use of *in-vitro*-derived reference materials in confirmatory analysis.²¹

In vitro technologies typically make use of enzymatic products derived from liver tissue, as the liver is the primary organ involved in AAS metabolism. Homogenized liver can be centrifuged at 9000 g for 20 min to isolate a supernatant commonly referred to as the S9 fraction.²² This fraction includes disrupted membranes of the endoplasmic reticulum (microsomes) and the soluble components of the cytosol. The S9 fraction can be fractionated further by ultracentrifugation at 100 000 g to isolate the microsomal fraction (pellet) from the cytosolic fraction (supernatant) and all three preparations are commercially available. From the perspective of AAS metabolism: microsomes are a concentrated source of cytochrome P450, flavin monooxygenase, and uridine 5'-diphosphoglucuronosyltransferase (UGT) enzymes; liver cytosol contains aldehyde oxidase and sulfotransferase (SULT) enzymes; and S9 fraction contains all these components. Cofactors must be added to these liver extracts for *in vitro* metabolism: glucuronylation by S9 fraction or microsomes with uridine 5'-diphosphoglucuronic acid (UDPGA), or sulfation (sometimes called sulfonation) by S9 fraction or cytosol with 3'-phosphoadenosine-5'-phosphosulfate (PAPS).⁴ However, the PAPS cofactor required for sulfation is prohibitively expensive (US\$1,645 for 25 mg of PAPS, Sigma-Aldrich²³) and chemically unstable (PAPS $t_{1/2}$ = 20 h at pH 8.0²⁴), and as a result *in vitro* studies are typically limited to phase I metabolism.^{5,25} There have been some reports detailing the study of phase II steroid metabolism using *in vitro* systems,^{26–28} but to date these have not been widely adopted by laboratories.

The limitations associated with PAPS can be overcome by *in situ* synthesis based on the physiological biosynthetic pathway (Figure 1).^{24,29–31} An *in vitro* approach for the preparation of sulfate metabolites has been described by Burkart *et al.*²⁴ and employs a series of six bacterial enzymes derived from *Rhizobium meliloti* and *Escherichia coli* for the *in situ* generation of PAPS. Using this approach, ATP-sulfurylase catalyzes the sulfation of ATP to generate adenosine-5'-phosphosulfate (APS). This compound is subsequently phosphorylated by APS-kinase to generate PAPS and adenosine diphosphate (ADP). In animal cells, these two enzymes are expressed as a bifunctional protein molecule named PAPS synthase (PAPSS).³² Following PAPS synthesis, a sulfotransferase (SULT) can then catalyze the sulfation of a target molecule hydroxyl group. In addition to the sulfated metabolite, 3'-phosphoadenosine-5'-phosphate (PAP) is released and is subsequently dephosphorylated and re-phosphorylated in several enzyme catalysed steps to afford ATP. Overall this protocol generated a number of sulfate compounds in high yield, but may be difficult to implement due to the requirement for a number of bacterial enzymes, many of which are not readily available to laboratories.³³

Sulfation of xenobiotics is known to occur in the liver, suggesting that the enzymes required for PAPS production would be present in liver preparations. The PAPSS isoform PAPSS2b localizes in the cytoplasm and is therefore available for PAPS generation in the liver S9 fraction or cytosol.^{34–37} As a result, this study explored the possibility of employing ATP (US\$53 for 1000 mg, Sigma Aldrich³⁸ Castle Hill, Australia) and sodium sulfate as inexpensive precursors for the generation of PAPS *in situ* for *in vitro* metabolism targeting sulfate metabolites. In particular, the study sought to optimize the experimental conditions and provide a metabolic platform capable of faithfully replicating *in vivo* sulfation. The optimized conditions have been used to generate equine *in vitro* phase II metabolic profiles of the synthetic anabolic steroids furazadrol (Figure 2, [1',2']isoxazolo[4',5':2,3]-5 α -androstan-17 β -ol, F)²⁵ and superdrol (methasterone, 17 β -hydroxy-2 α ,17 α -dimethyl-5 α -androstan-3-one, S),⁵ so demonstrating an economical approach for the study of phase II drug metabolism.

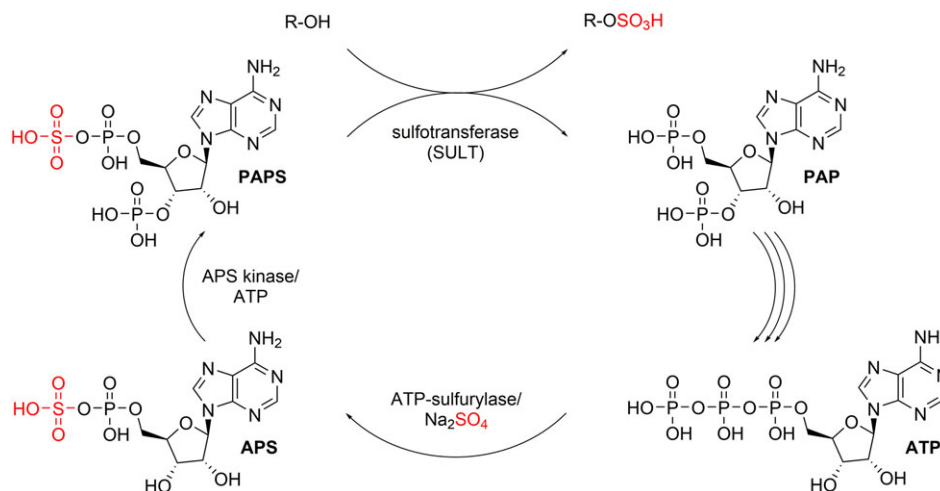


FIGURE 1 Sulfotransferase promoted sulfation of an acceptor alcohol by PAPS together with the two-step biogenesis of PAPS from ATP and sodium sulfate promoted by ATP sulfurylase and APS kinase. In animal cells, these two enzymes are expressed as a bifunctional protein molecule PAPSS [Colour figure can be viewed at wileyonlinelibrary.com]

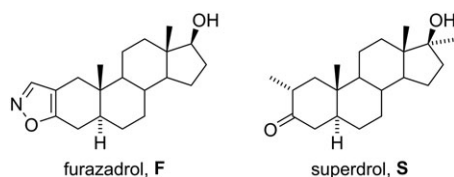


FIGURE 2 Chemical structures of furazadrol, **F** and superdrol, **S**

2 | MATERIALS AND METHODS

2.1 | Materials

Chemicals and solvents including lithium tri-*sec*-butylborohydride (L-Selectride®) solution in anhydrous tetrahydrofuran (THF), anhydrous *N,N*-dimethylformamide (DMF), magnesium chloride, sulfur-trioxide pyridine complex (SO₃.py), *tertiary*-butanol (*t*-BuOH), glucose-6-phosphate (G6P), NAD-dependant glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides*, adenosine triphosphate (ATP), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), nicotinamide adenine dinucleotide phosphate (NADP), uridine 5'-diphosphoglucuronic acid (UDPGA), and estrone (3-hydroxyestra-1,3,5(10)-trien-17-one, **E**) were purchased from Sigma-Aldrich, and were used as supplied unless otherwise stated. Neutral ATP stock solutions were prepared at 0.1 M with aqueous sodium hydroxide solution. Nicotinamide adenine dinucleotide (NAD) was purchased from Amresco (Solon, OH, USA). Formic acid was purchased from Ajax Chemicals (Auburn, Australia). 1,4-Dioxane (dioxane) was purchased from Merck (Darmstadt, Germany). Epiandrosterone (3 β -hydroxy-5 α -androstane-17-one, **EA**), androsterone (3 α -hydroxy-5 α -androstane-17-one, **A**), etiocholanolone (3 α -hydroxy-5 β -androstane-17-one, **EC**), testosterone (17 β -hydroxyandrost-4-en-3-one, **T**) and nandrolone (17 β -hydroxyestra-4-en-3-one, **N**) were purchased from Steraloids (Newport RI, USA). Superdrol (**S**) was purchased from the National Measurement Institute (North Ryde, Australia). Epiandrosterone (17 α -hydroxyandrost-4-en-3-one, **ET**) was prepared from **T** according to literature methods,³⁹ Epiandrosterone 3-sulfate (**EAS**), androsterone 3-sulfate (**AS**), etiocholanolone 3-sulfate (**ECS**), estrone 3-sulfate (**ES**), testosterone 3-sulfate (**TS**) and nandrolone 17-sulfate (**NS**) were prepared according to literature methods.⁴⁰ Pooled equine, canine, or human liver S9 fractions were purchased from Sekisui XenoTech (Kansas City, KS, USA). Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis WAX 6 cc cartridges (PN 186004647), or Waters Sep-Pak C18 (3 cc, 500 mg) cartridges (PN WAT020805) as specified. *Escherichia coli* glucuronylsynthase, and α -D-glucuronyl fluoride were prepared according to literature methods.⁴¹ *Escherichia coli* NADP-dependent G6PDH was expressed with an N-terminal hexa-histidine tag purified according to previously described methods.⁴² The *E. coli* BL21 (DE3) with pETMCSIII-g6pdh was kindly provided by Professor David Ollis at the Research School of Chemistry, Australian National University.

2.1.1 | Furazadrol reference materials

A range of previously synthesized furazadrol reference materials were employed to aid the identification of phase I and phase II metabolites.^{25,40}

These were **F**, isofurazadrol ([1',2']isoxazolo-[4',3':2,3]-5 α -androstane-17 β -ol, **IF**), epifurazadrol ([1',2']isoxazolo[4',5':2,3]-5 α -androstane-17 α -ol, **EF**), oxidized furazadrol ([1',2']isoxazolo[4',5':2,3]-5 α -androstane-17-one, **OF**), oxidized isofurazadrol ([1',2']isoxazolo[4',3':2,3]-5 α -androstane-17-one, **OIF**), furazadrol 17-sulfate (**FS**), isofurazadrol 17-sulfate (**IFS**), epifurazadrol 17-sulfate (**EFS**), furazadrol 17-glucuronide (**FG**), isofurazadrol 17-glucuronide (**IFG**), and epifurazadrol 17-glucuronide (**EFG**).

2.1.2 | Superdrol reference materials

A range of superdrol reference materials were employed to aid the identification of phase I and phase II metabolites. Superdrol (**S**) was sourced commercially, however the remaining materials were prepared synthetically, including 2 α ,17 α -dimethyl-5 α -androstane-3 α ,17 β -diol (**3 α -RS**), 2 α ,17 α -dimethyl-5 α -androstane-3 β ,17 β -diol (**3 β -RS**), 2 α ,17 α -dimethyl-5 α -androstane-3 α ,17 β -diol 3-sulfate (**3 α -RSS**), 2 α ,17 α -dimethyl-5 α -androstane-3 β ,17 β -diol 3-sulfate (**3 β -RSS**), and 2 α ,17 α -dimethyl-5 α -androstane-3 β ,17 β -diol 3-glucuronide (**3 β -RSG**). Experimental details, characterization data, and copies of the ¹H NMR, selected ¹³C NMR, and +EI LRMS or -ESI LRMS spectra are provided in the Supporting Information.

2.2 | Analytical methods

2.2.1 | LC-MS assay for the optimization of the *in vitro* sulfation reaction

The liquid chromatography-mass spectrometry (LC-MS) analysis for the optimization of the *in vitro* sulfation reaction was performed on an Agilent (Mulgrave, Australia) 1260 ultra high-performance liquid chromatography (UHPLC) system coupled to an Agilent 6120 quadrupole mass spectrometer equipped with an Agilent Poroshell 120 EC-C18 column (2.1 mm $\times$ 30 mm, 2.7 μ m) incubated at 30 $^{\circ}$ C and eluting with a gradient consisting of the following mobile phases, A: 10% methanol in aqueous ammonium acetate solution (10 mM), B: 90% methanol in aqueous ammonium acetate solution (10 mM), gradient: 0–5 min A–B (70:30 v/v) to B (100%), 5–6 min, B (100%), 6–9 min B (100%) to A–B (70:30 v/v), 6 min re-equilibration, flow rate 0.2 mL min⁻¹. Steroid sulfate conjugates were monitored for the anion, [M-H]⁻, using atmospheric pressure electrospray ionization (AP-ESI) and selected ion monitoring.⁴³

2.2.2 | Furazadrol LC-MS analysis

The LC-MS analysis for furazadrol metabolites was performed as previously reported.²⁵ Positive mode liquid chromatography-high resolution accurate mass (LC-HRAM) spectrometry analysis was undertaken using a Thermo Fisher Scientific (Bremen, Germany) Ultimate 3000 HPLC coupled to an Q Exactive™ Hybrid Quadrupole Orbitrap mass spectrometer equipped with a Waters SunFire C18 column (100 mm $\times$ 2.1 mm, 3.5 μ m) eluting with a gradient consisting of the following mobile phases, A: 0.1% formic acid in water, B: 0.1% formic acid in methanol, gradient: 0–1 min A–B (95:5 v/v), 1–15 min A–B (95:5 v/v) to A–B (5:95 v/v), 15–19 min A–B (5:95 v/v), 5 min re-equilibration, flow rate 0.4 mL min⁻¹. Unconjugated steroids and steroid glucuronides were monitored for the proton adduct ([M + H]⁺) using Heated Electrospray Ionisation (HESI) in positive full scan or targeted tandem mass spectrometry (MS/MS) mode at a resolution of 70 000 (FWHM). Negative mode LC-HRAM spectrometry analysis was undertaken using a Q Exactive™

Hybrid Quadrupole-Orbitrap mass spectrometer equipped with a Phenomenex (Torrance, CA, USA) Gemini C18 column (50 mm × 2 mm, 5 µm), eluting with a gradient consisting of the following mobile phases, A: aqueous ammonium acetate (0.01 M, pH 9.0), B: 0.1% acetic acid in acetonitrile, gradient: 0–2 min A–B (99:1 v/v), 2–8.5 min A–B (99:1 v/v) to A–B (20:80 v/v), 2.7 min re-equilibration, flow rate 0.5 mL min⁻¹. Steroid glucuronide and sulfate conjugates were monitored for the anion ([M-H]⁻) using HESI in negative full-scan or targeted MS/MS mode at a resolution of 70 000 (FWHM).

2.2.3 | Superdrol LC–MS analysis

The LC–MS analysis for superdrol metabolites was performed using an Agilent 1290 Infinity II LC system coupled to an Agilent 6545 Q-ToF mass spectrometer equipped with a Phenomenex Gemini C18 column (50 mm × 2 mm, 5 µm) eluting with the gradient outlined for negative mode LC–MS analysis in the section Furazadrol LC–MS analysis. Steroid glucuronide and sulfate conjugates were monitored for the anion ([M-H]⁻) using HESI in negative full-scan or targeted MS/MS mode.

2.3 | *In vitro* phase II metabolism

2.3.1 | Optimizing sulfation reactions with liver S9 fraction

Reactions to test and optimize steroid sulfation were prepared with final concentrations of: 50 mM Tris.HCl for pH 7.4, 0.55 mg mL⁻¹ total protein from equine liver S9 fraction, 30 µM steroid, 5 mM MgCl₂, and various sulfation reagents. Nandrolone sulfate (NS) was used as an internal standard with a final concentration of 4 µM.

Sulfation reagents consisted of: 80 or 200 µM PAPS, or ATP and Na₂SO₄ at a range of final concentrations. The ratio of ATP to sulfate was kept at 2:1, in keeping with the stoichiometry for PAPS synthesis (Figure 1). The final ATP concentration was tested from 2 to 32 mM with EA as a substrate to demonstrate 16 mM as optimal (Supporting Information Figure S1). The optimal MgCl₂ concentration was then determined to be 20 mM with 16 mM ATP and 8 mM sulfate (Figure S2).

Reactions were started by adding PAPS or sulfation reagent, with a final volume of 400 µL, and incubated at 37 °C. Aliquots of 50 µL were taken from reactions or standards at defined time points and quenched by mixing with 150 µL of 66% (v/v) methanol in water. The quenched sample was centrifuged at 21 000 g for 10 min, the supernatant transferred to a new vial and 20 µL injected for LC–MS analysis (section on LC–MS assay for the optimization of the *in vitro* sulfation reaction). The EAS peaks were integrated with ChemStation software (Agilent), and the response ratio of EAS to NS (internal standard) was determined and compared to the response ratio for a 30 µM EAS external standard.

2.3.2 | Comparison among species and steroids

To test the general utility of ATP and Na₂SO₄ as a source of PAPS the amount of sulfation for six steroids at 30 µM concentration: A, EA, EC, E, T, and ET was investigated. These were tested with three different liver S9 fractions: human, canine and equine. Reactions and analysis were performed as described above (section LC–MS assay for the optimization of the *in vitro* sulfation reaction), except that the optimized concentrations were used for ATP (16 mM), sulfate (8 mM) and MgCl₂

(20 mM). Reactions were performed in triplicate and were incubated at 37 °C for 24 h. Steroid sulfate peaks were integrated with ChemStation software, and the response ratio of steroid sulfate to NS (internal standard) was determined and compared to the response ratio for 30 µM steroid sulfate external standard.

2.3.3 | *In vitro* phase II metabolism of furazadrol using PAPS

In vitro phase II metabolism was performed by modification of a reported method.²⁵ A solution containing steroid (120 µM, 250 µL) in sodium phosphate buffer (100 mM, pH 7.4) and methanol (0.4%) was treated in order with the following solutions: aqueous magnesium chloride (1.0 M, 2.3 µL), aqueous G6P (100 mM, 37.5 µL), aqueous NAD (50 mM, 15 µL), aqueous NADP (50 mM, 15 µL), aqueous NAD-dependant G6PDH (40 units mL⁻¹, 12.5 µL), aqueous NADP-dependant G6PDH (40 units mL⁻¹, 12.5 µL), aqueous PAPS (1.6 mM, 22.5 µL), additional aqueous magnesium chloride in association with PAPS (1.0 M, 2.5 µL), aqueous UDPGA (610 µM, 31 µL), water (74 µL), and equine liver S9 fraction (20 mg mL⁻¹, 25 µL). The final solution (500 µL) was then incubated in an open tube with agitation for 16 h at 37 °C. The reaction was quenched with acetonitrile (1 mL), centrifuged (2000 rpm, 5 min) to pellet solids, and the supernatant was decanted. Concentration of the supernatant under a stream of nitrogen at 60 °C afforded a residue which was reconstituted in methanol–water (5:95 v/v, 200 µL) and transferred to a sealed vial for subsequent LC–MS analysis as per the section on Analytical methods. Control experiments excluding PAPS, UDPGA, both UDPGA and PAPS, all phase I co-factors, equine liver S9 fraction, and steroid respectively were performed alongside the above reaction, with addition of water or buffer as required to maintain a constant final reaction volume and buffer concentration.

2.3.4 | *In vitro* phase II metabolism of furazadrol and superdrol using ATP and Na₂SO₄

In vitro metabolism with ATP and Na₂SO₄ was performed as per section *In vitro* phase II metabolism of furazadrol using PAPS with the following substitutions: the PAPS solution was replaced by addition of aqueous ATP (250 mM, 32 µL), aqueous Na₂SO₄ (100 mM, 40 µL), and additional aqueous magnesium chloride (1.0 M, 5.7 µL). The volume of water added was reduced to maintain a constant final reaction volume (500 µL). Control experiments analogous to those reported in the section *In vitro* phase II metabolism of furazadrol using PAPS, were also performed alongside this reaction.

3 | RESULTS AND DISCUSSION

3.1 | Steroid sulfation with liver S9 fraction, ATP and sodium sulfate

Sulfation of EA was examined to test the hypothesis that stable and readily available reagents, ATP and Na₂SO₄, could be added to liver S9 fraction leading to PAPS synthesis and SULT-catalyzed sulfation. Sulfation was detected as EAS production and the concentrations of ATP, Na₂SO₄ and MgCl₂ were subsequently optimized (with ATP: Na₂SO₄ fixed at 2:1) as 16, 8, and 20 mM, respectively (section on

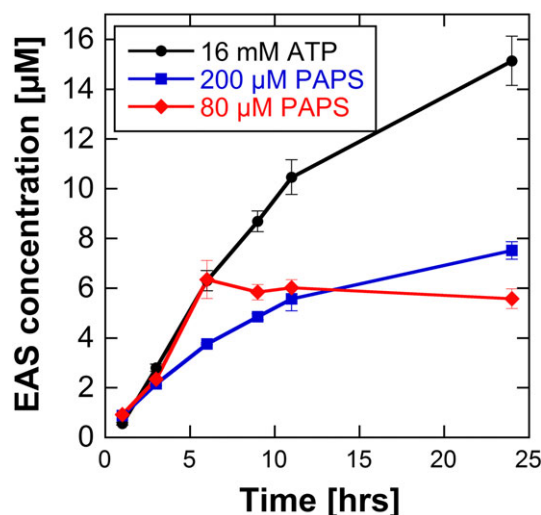


FIGURE 3 A temporal analysis of **EAS** production *in vitro* using equine S9 fraction and different phase II cofactors. Reactions were performed as described in the section *Optimizing sulfation reactions with liver S9 fraction* with 30 μM **EA** and either: 80 μM PAPS (red diamonds), 200 μM PAPS (blue squares), or 16 mM ATP, 8 mM Na₂SO₄ and 20 mM MgCl₂ (black circles) as PAPS precursor. The error bars represent the standard deviation from three independent reactions [Colour figure can be viewed at wileyonlinelibrary.com]

Optimizing sulfation reactions with liver S9 fraction). This optimized reaction was then compared with reactions using PAPS at 80 or 200 μM with 5 mM MgCl₂ (Figure 3). Equine liver S9 fraction with ATP and Na₂SO₄ displayed steady **EAS** production over the first 6 h, with continued **EAS** production but declining rates up to 24 h. Meanwhile, 80 μM PAPS afforded a similar initial rate over the first 6 h but **EAS** production ceased thereafter. Productivity with PAPS was not greatly enhanced by increased concentration; 200 μM PAPS gave the lowest initial rate and only resulted in a slight increase in **EAS** production relative to 80 μM PAPS after 24 h.

The three conditions of ATP and Na₂SO₄, 80 μM and 200 μM PAPS produced approximately 15, 6, and 7.5 μM **EAS**, respectively, after 24 h, with ATP and Na₂SO₄ displaying the greatest **EAS** production beyond 6 h. However, even after 24 h, this reaction only reached about 50% completion. In all cases excess sulfation reagents were insufficient to generate 30 μM **EAS** over 24 h and aside from limited time this could be due to competing substrates for SULTs present in the liver S9 fraction, PAPS instability or inactivation for SULTs over the course of the incubations. Alternatively, the PAPS precursor ATP may be subject to other biochemical transformations, thus limiting the PAPS synthesis and sulfation. Substrate and product inhibition also play a role in the reaction profiles. Substrate inhibition of human SULT2A1 has been observed for PAPS at a saturating concentration of dehydroepiandrosterone (DHEA), an unsaturated congener of **EA**.⁴⁴ Although this phenomenon is highly dependent on DHEA concentration, it could be the basis for the lower initial rates observed for 200 μM PAPS compared with 80 μM PAPS for **EAS** production.

The general applicability of ATP and Na₂SO₄ for *in vitro* PAPS synthesis and steroid sulfation was demonstrated for the six steroids and equine, human and canine S9 fractions (Figure 4). There were distinct differences among the three species for steroid sulfation. Both human and equine systems were capable of sulfating all six steroids with peak

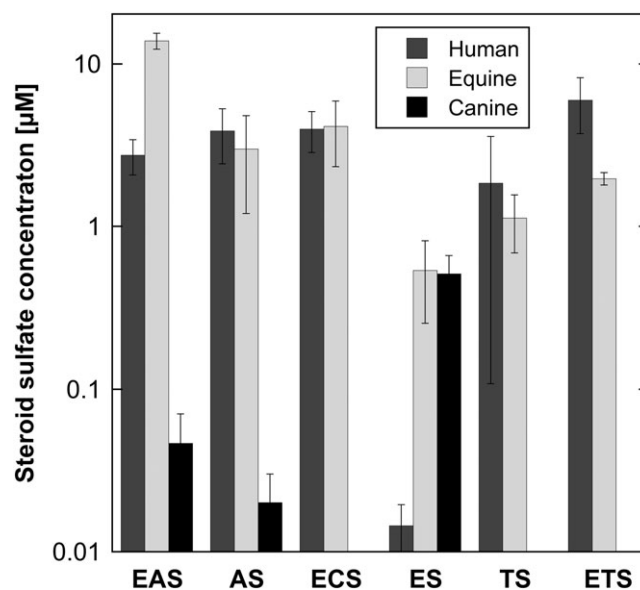


FIGURE 4 Comparing sulfation of six different steroids (30 μM, indicated on the x-axis) with liver S9 fraction from three different species: Human (dark grey), equine (light grey), and canine (black). Reactions were performed as described in section *Comparison among species and steroids*, with ATP and Na₂SO₄ as precursors for PAPS. The production of each steroid sulfate (μM) is presented on a logarithmic scale with the error bars indicating the standard deviation from three independent reactions

activity for **EAS** with equine and **ETS** with human S9 fractions. Production of **ES** was lowest for the S9 fractions from both these species. This result reflects the expression profile for human liver where the SULT responsible for androgen sulfation (SULT2A1) predominates and that for estradiol sulfation (SULT1E1) is only a minor component.^{36,37} In contrast, canine S9 fraction had greatest activity for **ES** and no detectable activity for **ECS**, **TS**, or **ETS** production. This observation is in agreement with previous studies into canine metabolism where glucuronylation is the predominant form of phase II conjugation for steroids with sulfation making only a minor contribution.³ Furthermore, the major SULT expressed in canine liver, SULT1A1, has been expressed recombinantly and shown to sulfate estradiol, but not DHEA. In summary, ATP and Na₂SO₄ gave sulfate ester synthesis for a range of steroids using the liver S9 fractions derived from humans, horses and dogs and therefore serves as a suitable replacement for PAPS for *in vitro* metabolism studies. Attention then turned to the use of ATP and Na₂SO₄ as a replacement of PAPS for the *in vitro* metabolism of the synthetic steroids **F** and **S**.

3.2 | Synthesis of steroid reference materials

The synthesis of the furazadrol reference materials used in this study has been described in a previous publication.²⁵ A number of phase I and II superdrol reference materials were prepared based on predicted patterns of equine metabolism² or the phase I metabolites tentatively assigned in a previous equine *in vitro* metabolism study.⁵ The reduction of **S** with sodium borohydride gave rise to a mixture of 3α/β-hydroxy isomers **3α-RS** and **3β-RS**, favouring the 3β-isomer **3β-RS**.⁴⁵ On the other hand, reduction with L-Selectride®, which is a bulky reducing agent, exclusively gave rise to the 3α-alcohol isomer **3α-RS**.

Performing these reactions on the milligram-scale proceeded smoothly but did not allow these isomers to be readily separated. As a result, the 3 β -isomer **3 β -RS** was prepared with a minor 3 α -impurity **3 α -RS**, which was carried through subsequent reaction steps. The pure 3 α -isomer **3 α -RS** was used to identify the presence of minor 3 α -isomer products generated during reactions of the 3 β -isomer **3 β -RS**.

Mono-sulfation of the 3 α / β ,17 β -diols to give **3 α -RSS**, and mixed **3 α -RSS/3 β -RSS** proceeded in high conversion, using established methodology.⁴⁰ On the other hand, enzymatic glucuronylation afforded only the 3 β -isomer **3 β -RSG**.⁴¹ This was highlighted by the reaction of a mixture of 3 α / β -hydroxy isomers **3 α -RS/3 β -RS**, which afforded 2 α ,17 α -dimethyl-5 α -androstane-3 β ,17 β -diol 3-glucuronide **3 β -RSG** as the sole product after purification by SPE. Unreacted **3 α -RS** was isolated from the reaction mixture. The selectivity for the glucuronylation of 3 β -hydroxy steroids using this enzyme has been documented, and presumably reflects the substrate binding within the enzyme active site.⁴¹

3.3 | *In vitro* phase II metabolism of furazadrol

In vitro studies typically involve only phase I metabolism, and phase II metabolism is not routinely explored, despite the fact that for *in vivo* studies, phase II conjugates are usually the major metabolites observed.¹⁻³ The addition of phase II co-factors has the potential to modify phase I metabolism by selectively intercepting phase I intermediates, and as a result, the inclusion of *in vitro* phase II metabolism has the potential to generate a more detailed metabolic profile that can better match the *in vivo* profile.

To explore these issues, the phase I and phase II equine metabolism of the synthetic steroid **F** was investigated. This compound was chosen as it allowed comparison with a recently reported *in vivo* equine administration study, and a comparative phase I *in vitro* study (**F:IF** 9:1).²⁵ In this work, the metabolism reactions were conducted as previously reported with minor variations.²⁵ Together with the addition of phase II cofactors UDPGA and PAPS or ATP and sodium sulfate, the phase I cofactors NADH and NADPH were regenerated using G6P and appropriate G6PDH enzymes. Metabolism data were examined using mass filters for predicted metabolites formed from up to three metabolic transformations including oxidation, reduction, and

hydroxylation, with or without subsequent sulfation or glucuronylation. Metabolite peaks were identified where exact masses were observed within ± 10 ppm of the predicted mass, and by comparison with control experiments. Metabolites were also matched against synthesized reference materials where available. A comparison of the previously reported study²⁵ with the present study is outlined below (Table 1). A table of the observed metabolites, retention times, precursor ions and MS/MS fragments are reported in the Supporting Information, together with copies of extracted ion chromatograms and MS/MS spectra.

The *in vitro* metabolism of furazadrol (**F:IF** 9:1) was conducted using both UDPGA with PAPS, and UDPGA with ATP and Na₂SO₄ (Table 1). Employing UDPGA and PAPS, major phase II metabolites were observed including **FS**, **IFS**, **FG**, **IFG**, and **EFG**, which were matched to reference materials. Additionally, **EFS** was not observed by comparison to the reference material, identical to observations from the *in vivo* study.²⁵ Minor unidentified phase II metabolites were observed including six hydroxylated furazadrol sulfate metabolites (**S1-S6**), one oxidized and hydroxylated furazadrol sulfate metabolite (**S7**), five hydroxylated furazadrol glucuronide metabolites (**G1-G4**, **G6**), and two oxidized and hydroxylated furazadrol glucuronide metabolites (**G9-G10**). Of these minor metabolites, **S2** was identified as a match with the previously reported *in vivo* study.²⁵ On the other hand, the *in vitro* phase II study employing UDPGA, ATP, and Na₂SO₄, identified the same major phase II metabolites including **FS**, **IFS**, **FG**, **IFG**, and **EFG**. Again, **EFS** was not observed. Minor unidentified phase II metabolites were observed including six hydroxylated furazadrol sulfate metabolites (**S1-S6**), one oxidized and hydroxylated furazadrol sulfate metabolite (**S8**), two hydroxylated furazadrol glucuronide metabolites (**G3**, **G5**), and three oxidized and hydroxylated furazadrol glucuronide metabolites (**G7**, **G8**, **G10**). Of these minor metabolites, **S2**, and **S8** were identified as matches with the previously reported *in vivo* study.²⁵

A comparison of the phase II metabolites observed using both PAPS or ATP and Na₂SO₄ *in vitro* systems (Table 1) indicated that a majority of the metabolites were common. The major phase II metabolites **FS**, **IFS**, **FG**, **IFG**, and **EFG** were common to both systems and in both instances **EFS** was not observed. Thus the *in vitro* studies

TABLE 1 Comparison of *in vivo* and *in vitro* (UDPGA and PAPS, or UDPGA, ATP and Na₂SO₄) metabolism of furazadrol (**F:IF** 9:1); ^AWaller *et al.*²⁵; ^BN/D not detected.

Metabolic transformation	<i>In vivo</i> (previous study) ^A	Phase II <i>in vitro</i> (UDPGA and PAPS)	Phase II <i>in vitro</i> (UDPGA, ATP, and Na ₂ SO ₄)	Phase I <i>in vitro</i> (previous study) ^A	Phase I <i>in vitro</i> (UDPGA and PAPS)	Phase I <i>in vitro</i> (UDPGA, ATP, and Na ₂ SO ₄)
Furazadrol (major)	FS , IFS , FG , IFG , EFG	FS , IFS , FG , IFG , EFG	FS , IFS , FG , IFG , EFG	EF	EF	EF
Oxidized furazadrol (major)	N/D ^B	N/D ^B	N/D ^B	OF , OIF	OF , OIF	OF , OIF
Hydroxylated furazadrol (minor)	Sulfate ($\times 1$)	Sulfate ($\times 6$) S1-S6 Glucuronide ($\times 5$) G1-G4 , G6	Sulfate ($\times 6$) S1-S6 Glucuronide ($\times 2$) G3 , G5	($\times 8$)	($\times 7$) M1-M5 , M8 , M9	($\times 8$) M2-M9
Oxidized and hydroxylated furazadrol (minor)	Sulfate ($\times 2$) Glucuronide ($\times 2$)	Sulfate ($\times 1$) S7 Glucuronide ($\times 2$) G9 , G10	Sulfate ($\times 1$) S8 Glucuronide ($\times 3$) G7 , G8 , G10	($\times 1$)	($\times 2$) M10 , M12	($\times 2$) M11 , M12
Dihydroxylated furazadrol (minor)	N/D ^B	N/D ^B	N/D ^B	($\times 2$)	($\times 4$) M13-M16	($\times 3$) M13 , M15 , M16

clearly indicate that **EF** serves as substrate for UGT promoted glucuronylation but not SULT promoted sulfation. This could arise due to different enzyme-substrate affinities or reaction rates for the competing conjugation processes. It is consistent with observed patterns of equine *in vivo* metabolism where 17 α -hydroxy metabolites are commonly found in the glucuronide, but not the sulfate, fraction.²

Although the major phase II metabolites were common to both *in vitro* systems, the minor phase II metabolites appeared to vary between systems, particularly for the glucuronides (**S1-S6**, **G3**, **G10** were common, **S7**, **G1**, **G2**, **G4**, **G6**, **G9** PAPS only, **S8**, **G5**, **G7**, **G8** ATP and Na₂SO₄ only). The differences in the glucuronide profile may be attributed to the different sulfation reagents used in these systems. In the experiment utilizing 80 μ M PAPS, the co-factors are added at the start of the metabolism reaction, and PAPS is then consumed or decays in solution over time (Figure 3). This results in a burst of phase II sulfation towards the beginning of the reaction, which ceases relatively quickly. This is in contrast to the experiments utilizing ATP and Na₂SO₄ in which a relatively steady rate of sulfation was observed. Of particular interest were the minor metabolites **S2** and **S8**, which were both observed in the previous *in vivo* study.²⁵ The *in vitro* phase II study employing PAPS identified **S2** only, while the study employing ATP and Na₂SO₄ identified both **S2** and **S8** as minor metabolites, suggesting that *in vitro* platforms employing ATP and Na₂SO₄ could offer advantages for generating some phase II metabolites.

A comparison of the phase I metabolites observed using both PAPS, and ATP/Na₂SO₄ *in vitro* systems indicated that a majority of the metabolites were common (**EF**, **OF**, **OIF**, **M2-M5**, **M8**, **M9**, **M12**, **M13**, **M15**, **M16**), with a few exceptions (**M1**, **M10**, **M14** PAPS only; **M6**, **M7**, **M11** ATP and Na₂SO₄ only). The number of phase I metabolites observed did not appear to change significantly in control experiments where phase II co-factors were excluded, suggesting that phase II metabolism was not fully intercepting the phase I metabolites. The extent of phase I metabolism in the present study appeared to be somewhat greater than reported previously.²⁵ The present study used a combination of NADH and NADPH co-factors with G6P and both *E. coli* and *L. mesenteroides* G6PDH enzymes for regeneration of both. In contrast, the previous study used NADH G6P and *L. mesenteroides* G6PDH. Although liver extracts enable NADH to reduce NADP⁺ and vice versa, additional NADPH for cytochrome P450 oxidation could explain the modest increase in the number of hydroxylated metabolites identified in the present study. For both systems, metabolites, **EF**, **OF**, **OIF**, **M2-M4**, and **M8** were identified as matches with the phase I study reported previously.²⁵ Although no unconjugated metabolites were identified in the previous *in vivo* study,²⁵ the hydroxylated furazadrol **M4** was observed to match a phase I metabolite generated after *Pseudomonas aeruginosa* arylsulfatase hydrolysis of the *in vivo* samples.^{25,43}

It appears that the substitution of PAPS with ATP and Na₂SO₄ is capable of generating comparable *in vitro* metabolic profiles, and can serve as an economical alternative for the generation of *in vitro* phase II sulfate conjugates. The major metabolites **FS**, **IFS**, **FG**, **IFG**, and **EFG**, which include the most useful markers for anti-doping screening²⁵ were all detected at higher levels (response ratios 10–10³ times) than the minor phase II metabolites in these studies. On the other hand, the minor *in vivo* metabolites **S2**, and **S8** identified in the *in vitro* study

would likely be difficult to distinguish from the other minor phase II metabolites observed. The long-term metabolites most suitable for use as screening markers are not always the most abundant,² and so may be difficult to identify solely from *in vitro* data. Although it could be argued from these results that the reported conditions are capable of generating an adequate representation of the major *in vivo* metabolites, further work refining these conditions to better match the complete *in vivo* profile solely from *in vitro* results is likely to be beneficial. For equine and canine sports, AORC criteria allow for the use of in-vitro-derived materials as reference materials in confirmatory analysis.²¹ Improved methods to mimic the *in vivo* profile using *in vitro* techniques will generate new reference materials and allow additional instances of AAS misuse to be detected and confirmed.

3.4 | *In vitro* phase II metabolism of superdrol

Given the success of investigations into the metabolism of **F**, attention turned to the equine *in vitro* phase II metabolism of **S**. The equine *in vivo* metabolism of **S** has not been previously reported and in the absence of this information this study serves as a prediction of the likely metabolic profile of this steroidal agent. Previous reports of the equine *in vitro* phase I metabolism of **S** provided some comparison with the present study.⁵ Several human *in vivo* and *in vitro* studies of **S** metabolism have also been reported.^{46–49} Metabolism of **S** with UDPGA, ATP, Na₂SO₄, and equine liver S9 fraction afforded a range of phase II sulfate and glucuronide metabolites which are summarized in Figure 5. A table of the observed metabolites, retention times, precursor ions, and MS/MS fragments are reported in the Supporting Information, together with copies of extracted ion chromatograms and MS/MS spectra.

Major phase II metabolites were observed including **3 β -RSS** which was matched to the available reference material, and a reduced superdrol glucuronide metabolite **G12**.^{48,49} Although no reference material was available, **G12** was tentatively assigned as 2 α ,17 α -dimethyl-5 α -androstane-3 α ,17 β -diol 3-glucuronide (or its 17-glucuronide regioisomer), as this metabolite did not match the reference material for **3 β -RSG** but exhibited similar mass spectrometry behaviour including the proton loss species m/z 495 ([M-H][–]) and the glucuronide derived fragments m/z 113 (C₅H₅O₃[–]), m/z 85 (C₄H₅O₂[–]) and m/z 75 (C₂H₃O₃[–]).⁵⁰ A metabolite corresponding to 2 α ,17 α -dimethyl-5 α -androstane-3 α ,17 β -diol 3-glucuronide (or its 17-glucuronide regioisomer) has also been indirectly observed in human *in vivo* and *in vitro* studies of **S** metabolism.^{48,49} Despite this, some caution is required as the formation of stereoisomeric 17 β -methyl-17 α -hydroxy metabolites through formation and hydrolysis of the tertiary sulfate conjugate provides alternative metabolic pathways.^{18–20} Minor phase II metabolites were observed including two hydroxylated superdrol sulfate metabolites (**S9**, **S10**), a reduced and hydroxylated superdrol sulfate metabolite (**S11**), superdrol glucuronide (**G11**), **3 β -RSG**, and two reduced and hydroxylated superdrol glucuronide metabolites (**G13**, **G14**). Although the majority of minor phase II metabolites remain unidentified in this study, the minor metabolite **3 β -RSG** was confirmed, and **3 α -RSS** was not observed, by comparison to the corresponding reference materials.

The formation of the 3 β -hydroxy metabolites is known to be favoured in horses,² and this is followed by sulfation to give major

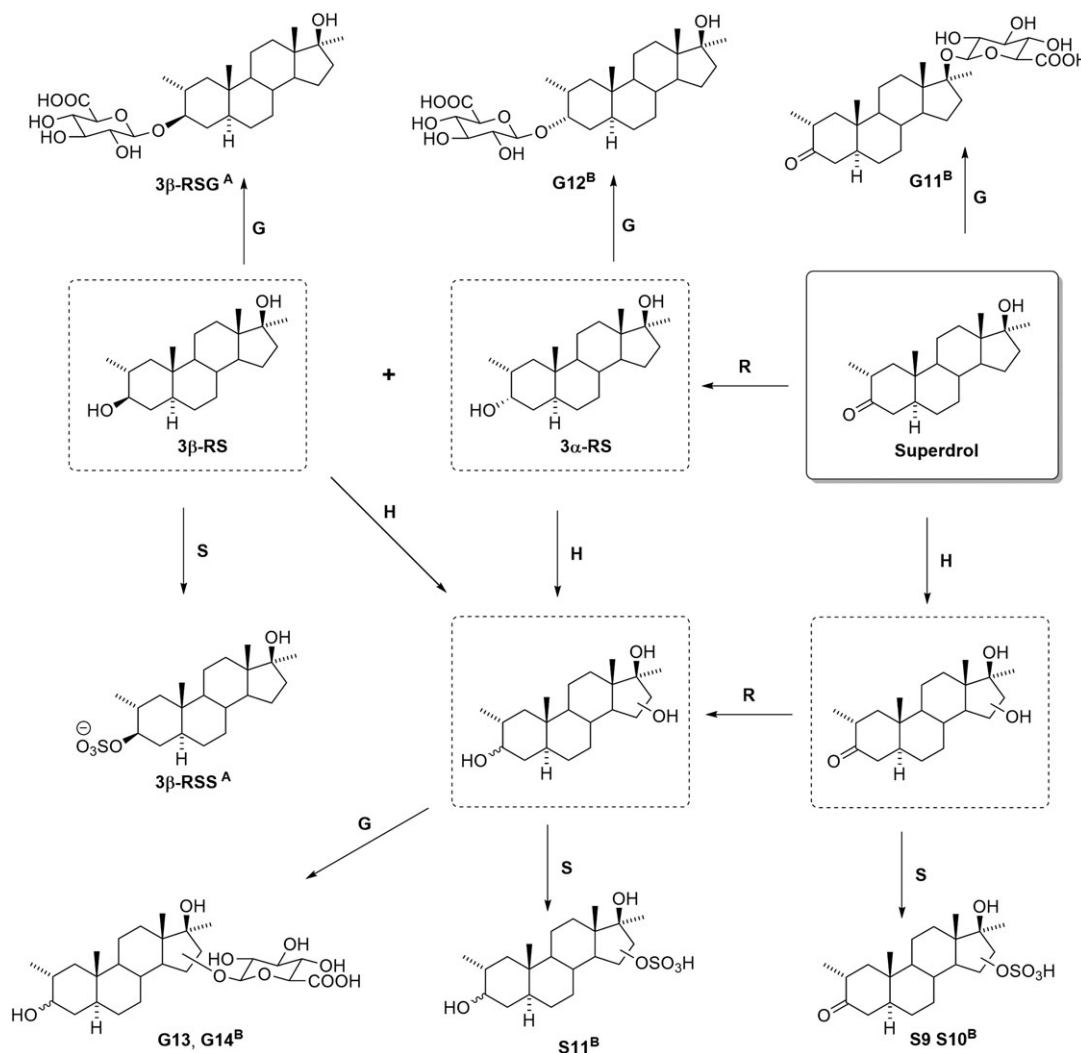


FIGURE 5 Proposed *in vitro* equine metabolism of superdrol (**S**); ^A matched to reference material; ^B unconfirmed regio- and stereo-chemistry. H, hydroxylation; R, reduction; G, glucuronylation; S, sulfation. Dotted boxes indicate presumed intermediates that were not observed in this LC-MS study

3 β -RSS and glucuronylation to give minor **3 β -RSG**. Alternatively, the tentative assignment above suggests that formation of the 3 α -hydroxy metabolite **3 α -RS** is followed by glucuronylation to give major **G12**^{48,49} but not sulfation to **3 α -RSS**, in a pattern similar to that observed in humans.¹ Although no unconjugated phase I metabolites were identified directly in this LC-MS study, the phase I metabolism of **S** is known to afford a number of steroid diol, and triol metabolites which ionize poorly under +ESI conditions.⁵ Instead, these metabolites were identified indirectly through analysis of their intact phase II conjugates. Future studies of the *in vivo* metabolism of **S** are likely to benefit from access to a wider variety of reference materials and the application of both gas chromatography-mass spectrometry (GC-MS) and LC-MS for sample analysis. Overall, the phase I metabolism of **S** appears to be quite simple, and the major phase II conjugates observed correlate well with the major phase I metabolites previously reported for the equine *in vitro* studies.⁵ The reduced superdrol isomers **3 β -RS**, and **3 α -RS** appear to be the most abundant *in vitro* metabolites observed, and would likely be among the major metabolites observed *in vivo*. Additionally, these compounds are also easily prepared from the parent compound, and so should be available as reference materials to

assist in confirmation. As such, it is recommended that anti-doping laboratories monitor for **3 β -RSS**, and 2 α ,17 α -dimethyl-5 α -androstane-3 α ,17 β -diol 3-glucuronide (tentatively identified as **G12**), or their phase I counterparts **3 β -RS**, and **3 α -RS**, until such time as a comparative *in vivo* study can be undertaken.

4 | CONCLUSION

In this work, alternative conditions for *in vitro* phase II sulfation using human, equine or canine liver S9 fraction were developed and employed for the generation of six representative steroidal sulfates, with ATP and Na₂SO₄ in place of the expensive and unstable co-factor PAPS. Using these conditions, the equine *in vitro* phase II metabolism of the synthetic steroid **F** was investigated, with ATP and Na₂SO₄ found to offer comparable metabolism to reactions using PAPS. The major *in vitro* metabolites of **F** also correlated closely with the metabolites observed in a previously reported equine *in vivo* study.²⁵ Although these *in vitro* methods do not fully replicate the *in vivo* metabolic profile, it is expected that future work will refine these methods to more accurately predict the metabolism of new compounds from

in vitro studies. Finally, the equine *in vitro* phase II metabolism of the synthetic steroid **S** was also performed that serves as a prediction of the *in vivo* metabolic profile. Notably the equine *in vitro* phase II metabolites observed in this study correspond well with previously reported equine *in vitro* phase I studies and so are recommended as suitable screening markers until *in vivo* studies can be undertaken.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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CHAPTER 3

The *in vitro* metabolism of “Junglewarfare” ($\Delta 6$ – methyltestosterone)

3.1 Introduction

After the chemical synthesis of testosterone (T), and the study of its anabolic and androgenic properties, pharmaceutical companies introduced a wide variety of synthetic analogues of testosterone. Nandrolone (N) is considered to be the first synthetic analogue of T.^[1] These synthetic steroids share the same steroid backbone but include slight differences in the structure. Furazadrol, boldenone, superdrol, methenolone, chlorodehydromethyltestosterone, oxymesterone and trenbolone are several examples of those modified analogues (figure 3.1). Only a minority of those steroids have been developed as therapeutic agents and others are clearly designed to evade anti-doping efforts.

With the growing popularity of steroids as performance enhancing agents, an arms race has emerged to improve AAS activity or evade detection through alterations to the structure. Some AAS have modified A rings, such as furazadrol, which has a fused isoxazole ring. In addition, there can be double bonds, methyl groups, hydroxyl groups, or heteroatoms like chlorine. Another major modification is the introduction of a methyl group at the C17 position.

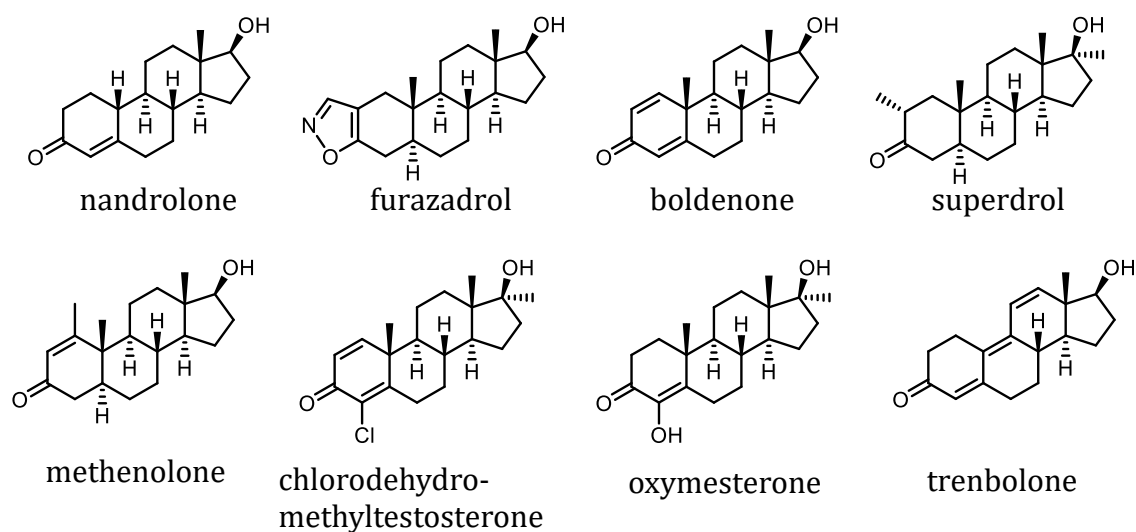


Figure 3.1: Nandrolone, the first synthetic analogue of testosterone^[1] and some other synthetic analogues of T.

3.1.1 17 α -Methyl steroids

The 17 β -hydroxy-17 α -methyl steroids are more common among performance enhancing drugs and AAS. It is known that 17 α -methyl steroids have reduced activity compared with testosterone.^[2] However, oxidation of 17 α -methyl-17 β -hydroxy steroids to the 17-ketone is not possible and conjugation of the 17 β -hydroxy group is slowed down. Therefore, the 17 α -methyl group slows metabolism and as a result, the AAS acts for longer.

An important phenomenon observed with 17 β -hydroxy-17 α -methyl steroids is the formation of the C17 epimer during metabolism.^{[2],[3]} The first occurrence of a C17-epimeric steroid; 17-epimethandienone, was in a cancer patient who was administered methandienone.^{[4],[5]} It took time to understand the mechanism of epimerisation. The mechanism was first suggested by Edlund *et al.* in 1989.^[6] It was revealed that the epimer is a degradation product of the sulfate conjugate derived from 17 β -hydroxy-17 α -methyl steroids.

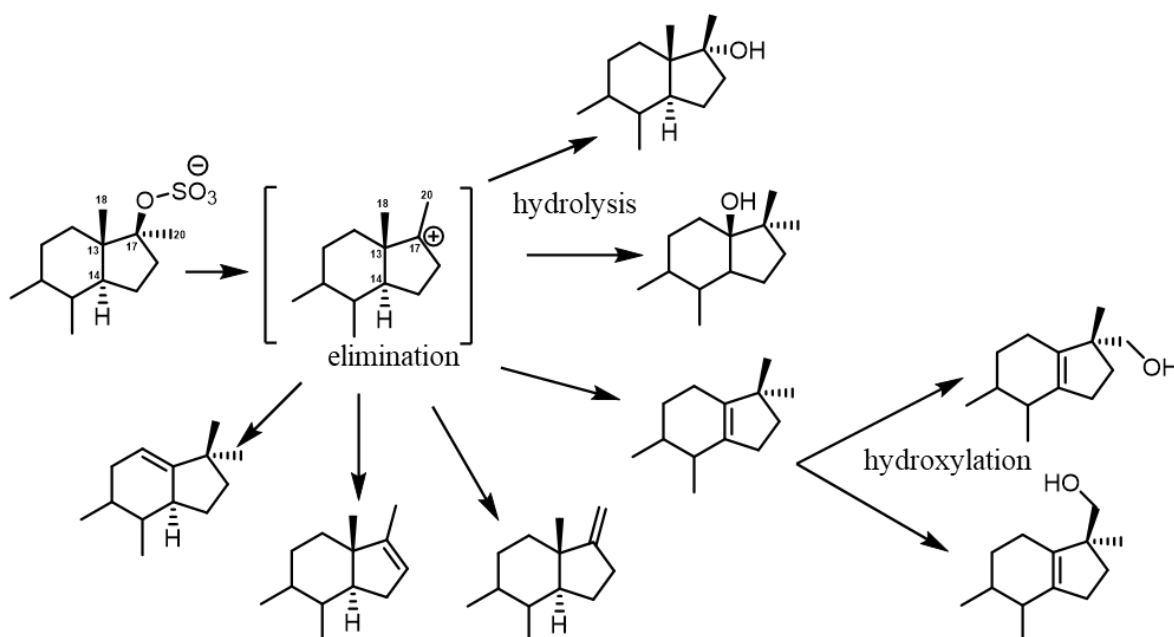
3.1.2 Sulfation of 17 β -hydroxy-17 α -methyl steroids

When steroids undergo metabolism, this typically results in glucuronide and sulfate conjugates. Generally, steroid sulfates are relatively stable metabolites. Even though sulfate metabolites are stable, the 17-sulfate conjugates of 17 β -hydroxy-17 α -methyl steroids are an exception. With these 17-methylated steroids, the 17-sulfate was not detected. Instead, 17-epimers and a range of hydrolysis and elimination products appeared during the metabolism.

Studies have proven that epimerisation occurs *via* the formation of the 17-sulfate.^{[4],[6],[7]} They demonstrate that the sulfate formed eliminates in aqueous media to yield a tertiary carbocation. Further, nucleophilic addition by water gives rise to the 17-epimer by addition to the less hindered α -face. However, the epimer is not the only outcome of this elimination as the tertiary carbocation, can lead to rearranged products. Through the migration of the 18-CH₃ group from C13 to C17, 17,17 di-methylated products are formed (scheme 3.1) and these methyl groups can undergo further hydroxylation to form 17 α - or 17 β - hydroxymethyl

derivatives.^{[3],[8],[9]} Through these pathways, alkene formation through elimination is also possible and other hydrolysis products are observed at minor levels.^[2]

A notable feature of the rearranged products is that they have increased the detection windows for particular steroids. 18-Nor-17-hydroxymethyl derivatives of dehydrochloromethyltestosterone^{[10],[11]}, metandienone^[9], oxandrolone^[12], and a C17 epimer of methyltestosterone^[13] have been identified as long term metabolite markers of those steroids. Therefore, the epimers and rearranged products are of interest due to the potential to increase detection windows.



Scheme 3.1: Epimerisation, elimination and further rearranged metabolites observed on decomposition of 17-sulfated 17 β -hydroxy-17 α -methyl steroids.

3.1.3 Metabolism of Δ 6-methyltestosterone (Δ 6-MT, 17 β -hydroxy-17 α -methylandrosta-4,6-dien-3-one)

Δ 6-Methyltestosterone is an anabolic steroid and was first reported in 1961.^[14] Later in 2011 it was detected in a dietary supplement known as “Junglewarfare”, with human *in vivo* metabolism studies then performed and studied using GC-MS analysis.^[15] Following the oral administration of the steroid, the parent compound was excreted for 60-70 h in urine, with two reduced metabolites, one reduced and epimerised metabolite and the 17-epimer of the parent compound (figure 3.2) also observed. Out of those metabolites, three were identified as major metabolites and

one as the minor metabolite (M2, figure 3.2). Major metabolites were detected up to 181-189 hours and the minor one was detected only up to 20 hours following administration. At the same time, Cooper *et al.*^[16] showed that this steroid has an androgenic potency greater than DHT using an *in vitro* androgen bioassay.

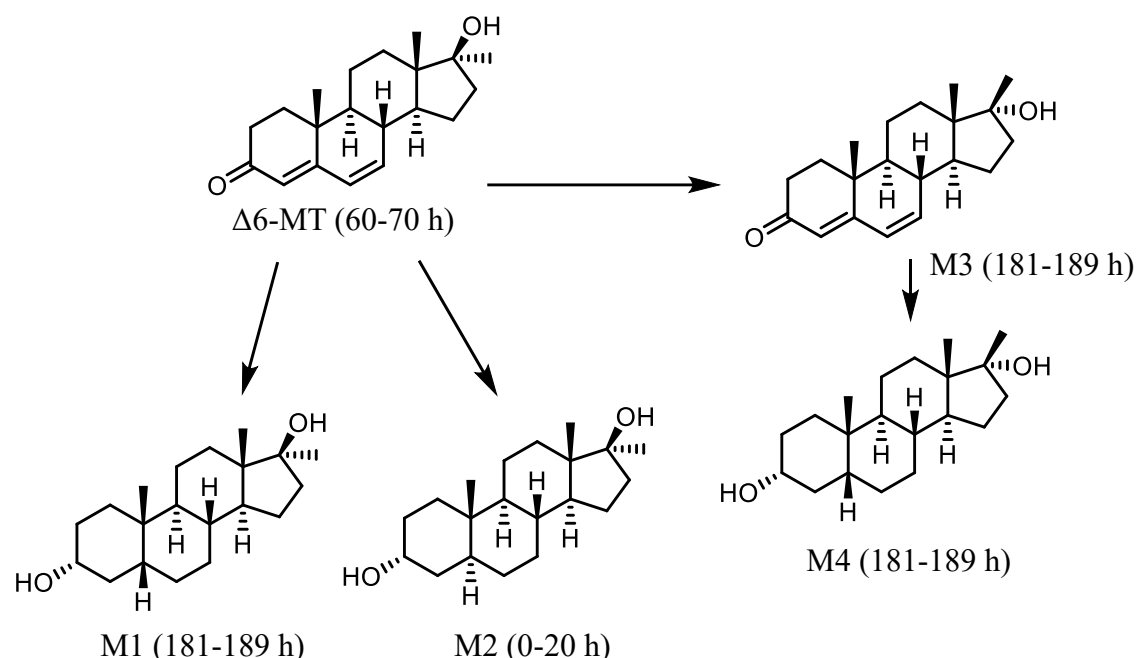


Figure 3.2: Chemical structure of $\Delta 6$ -methyltestosterone ($\Delta 6$ -MT) and human *in vivo* metabolites,^[15] 17 α -methyl-5 β -androstane-3 α ,17 β -diol (M1), 17 α -methyl-5 α -androstane-3 α ,17 β -diol (M2), $\Delta 6$ -epi-methyltestosterone (M3) and 17 β -methyl-5 β -androstane-3 α ,17 α -diol (M4) with time of latest detection in brackets.

3.1.4 Project goals

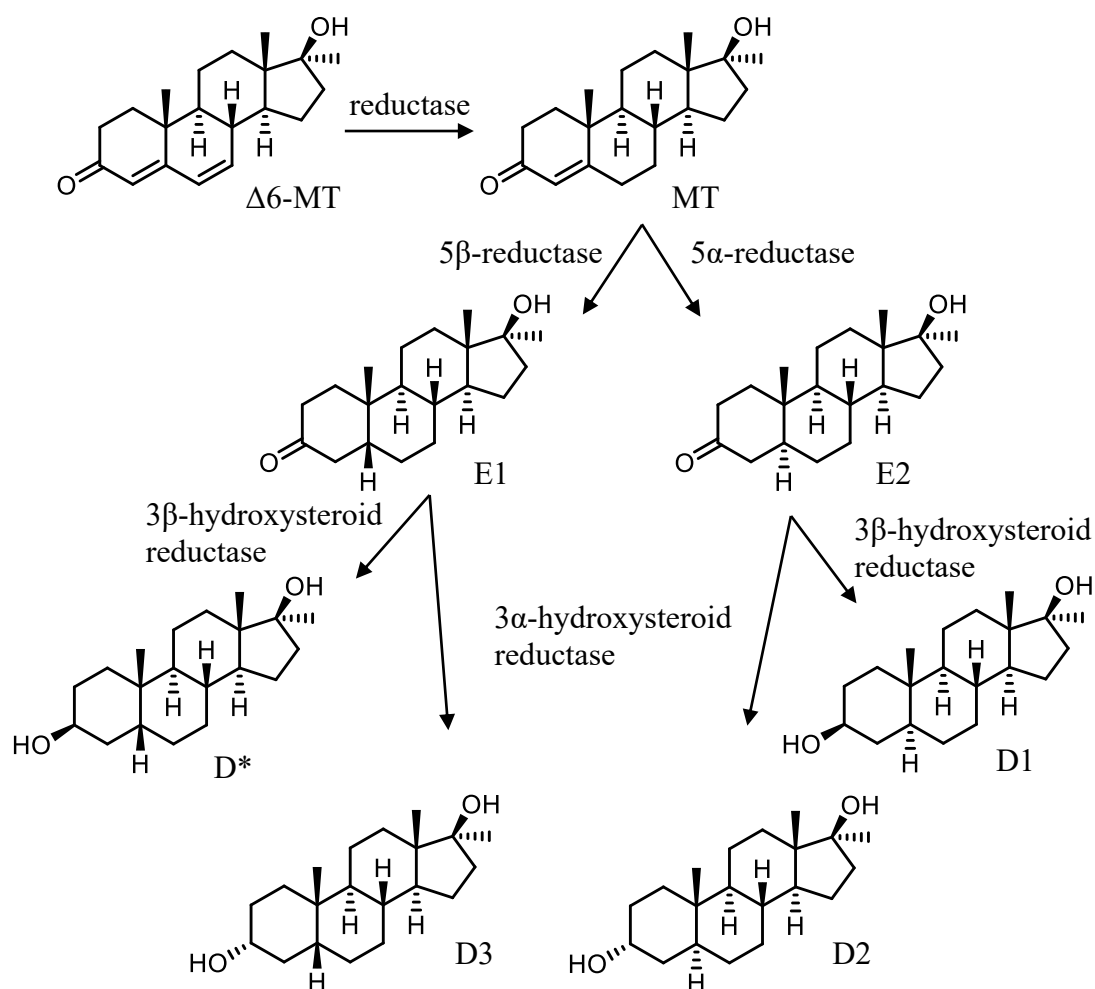
The *in vitro* metabolism of this steroid is not yet reported. In this study, the *in vitro* metabolism of $\Delta 6$ -MT was performed with human, canine or equine liver S9 fractions. The primary goal of this study was to identify the metabolites that can be detected directly with LC-MS and confirm the structures of these by comparison to synthetically derived reference materials. The earlier *in vivo* study described above, targeted the glucuronide conjugates and free steroid metabolites. The research presented here aimed to demonstrate both glucuronylation and sulfation. As $\Delta 6$ -MT is a 17 β -hydroxy-17 α -methyl steroid, ATP-sulfate method of sulfation (chapter 2) was applied to target the epimers that could be generated from 17-sulfation; similar to those observed during human *in vivo* metabolism.

3.2 *In vitro* study of Δ 6-methyltestosterone

3.2.1. Potential metabolic pathways

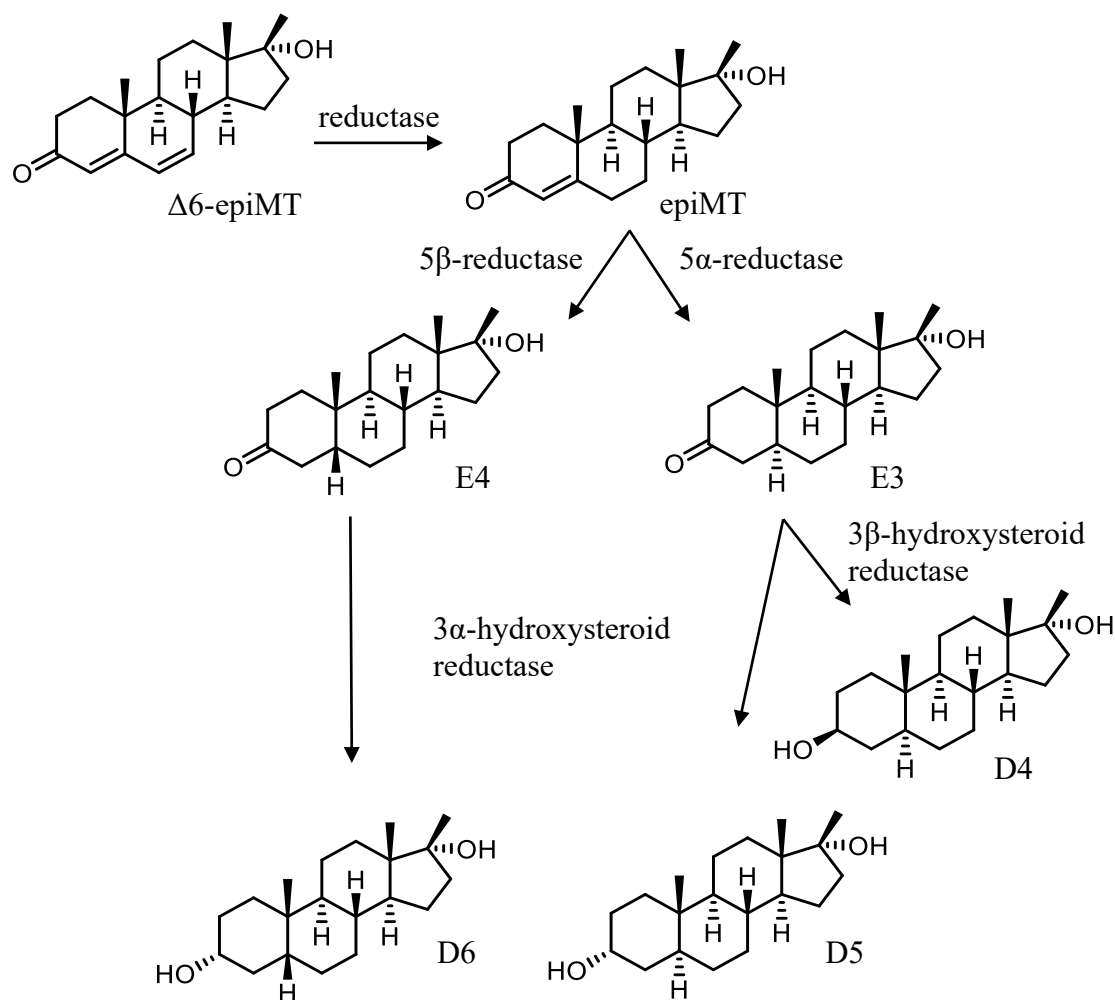
In order to predict metabolites arising from Δ 6-MT, the common metabolic changes of reduction, hydroxylation and oxidation were taken into account. These pathways can occur individually or in concert, such as “reduction+hydroxylation”, to generate metabolites. It was decided to synthesise several potential metabolites as reference materials, as it would create a much more detailed picture of the metabolism of Δ 6-MT. In the course of synthesis, the main concern was the reduction pathway of the steroid. As Δ 6-MT has two double bonds and a ketone at C3, it has three functionalities that can undergo reduction. Therefore, those reductions can form a set of partly reduced and completely reduced metabolites as observed in the human *in vivo* metabolism.

Two of the reduction steps could potentially generate isomers that are different at one stereo centre. As an example, after reducing the 6,7 double bond MT is formed and further reduction of the 3,4 double bond can give rise to two isomers 5 α -androstan-3-one and 5 β -androstan-3-one compounds. Then the reduction of 3-keto group can yield 3 α -hydroxy and 3 β -hydroxy isomers (Scheme 3.2). Out of those; the 3 β ,5 β isomer (**D***) is not typically observed during metabolism.^[17] Thus, during the synthesis of reference materials this reference material was not pursued.



Scheme 3.2: The reduction pathways of Δ^6 -methyltestosterone

This project also targeted 17-epimers that have been observed in human in vivo studies. The parent steroid itself can potentially undergo sulfation and hydrolysis to produce Δ^6 - epi-MT. Similar to the reduction pathway mentioned for the parent steroid, the epimerised parent steroid can also undergo a single reduction to form epi-MT. With the reduction of 3,4-ene of epi-MT, two steroid ketones can be formed, E3 and E4. Similarly three consecutive reductions from the epimerised parent steroid can give rise to three epimerised steroid diols; D4, D5 and D6.



Scheme 3.3: The reduction pathways of Δ^6 -epi-methyltestosterone

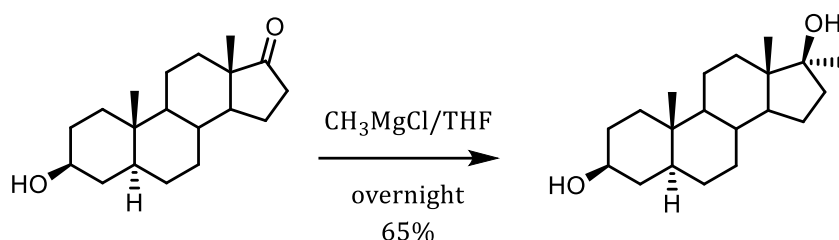
3.2.2 Synthesis of Δ^6 -methyltestosterone reference materials

According to phase I and phase II metabolic pathways discussed above, a range of reference materials were synthesised to aid the identification of *in vitro* metabolites by LC-MS analysis. These reference materials included free forms; steroid ketones, steroid diols and their conjugates; steroid sulfates and steroid glucuronides.

3.2.2.1 Synthesis of steroid diols

Synthesised steroid diols were placed in to two classes; epimerised diols or non-epimerised diols. The non-epimerised diols; 17 α -methyl-5 α -androstane-3 β ,17 β -diol **D1**, 17 α -methyl-5 α -androstane-3 α ,17 β -diol **D2** and 17 α -methyl-5 β -

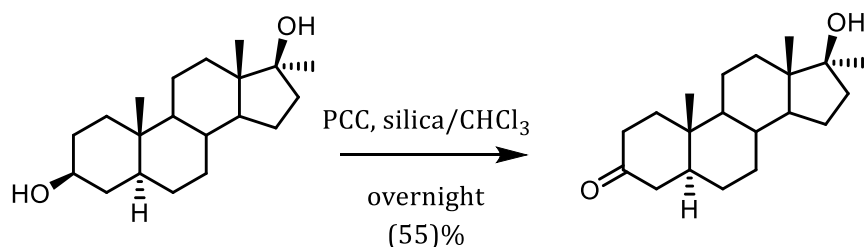
androstane-3 α ,17 β -diol **D3**, were directly synthesised from the commercially available steroids: epi-androsterone (EA), androsterone (A), and etiocholanolone (EC) respectively, which are 3-hydroxy, 17-keto steroids. In order to obtain these 17 methylated steroid diols (**D1**, **D2** and **D3**), the Grignard reaction was employed, which stereoselectively introduces a new C-C bond (methyl group) and an alcohol to the steroid backbone (scheme 3.4). This allowed production of the three times reduced products of Δ^6 -MT steroid.



Scheme 3.4: Synthesis of 17 α -methyl-5 α -androstane-3 β ,17 β -diol (D1**)**

3.2.2.2 Synthesis of 17 β -hydroxy, 17 α -methylated 3-keto steroids

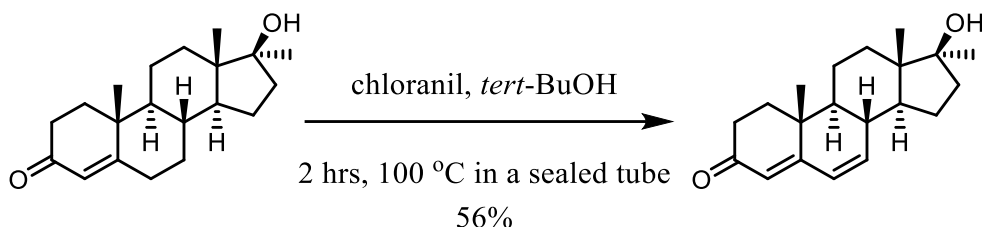
As mentioned in the previous section, if the parent steroid loses the two alkenes; $\Delta^{3,4}$ and $\Delta^{5,6}$, it can give rise to two 17-methylated steroid ketones. The steroid diols prepared above were used to synthesise these doubly reduced products; 17 β -hydroxy-17 α -methyl-5 α -androstan-3-one **E1** from diol **D1**, and 17 β -hydroxy-17 α -methyl-5 β -androstan-3-one **E2** from diol **D3**. The secondary hydroxy group was oxidized using pyridinium chlorochromate (PCC) as the oxidant (scheme 3.5).



Scheme 3.5: Synthesis of 17 β -hydroxy-17 α -methyl-5 α -androstan-3-one

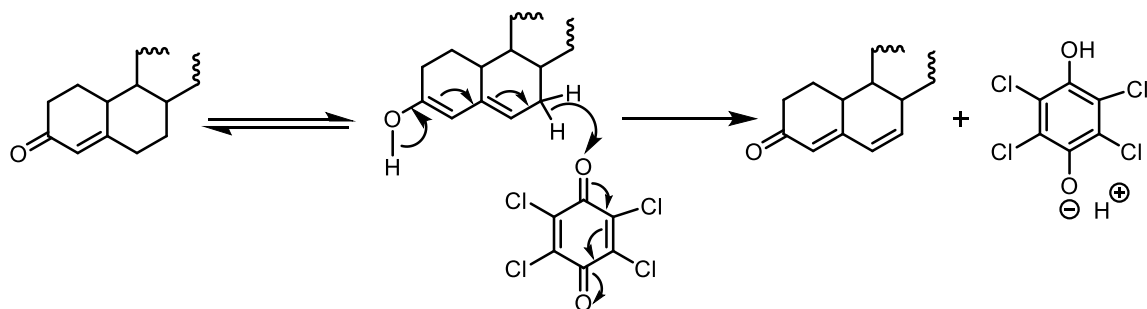
3.2.2.3 Synthesis of parent steroid Δ^6 -methyltestosterone

When the parent steroid undergoes reduction of the Δ^6 double bond, the resulting product is known as methyltestosterone (the reverse reaction of scheme 3.6). Therefore, MT is was purchased and used for this project.



Scheme 3.6: Synthesis of Δ^6 -methyltestosterone from methyltestosterone

The parent steroid, Δ^6 -methyltestosterone was synthesised using commercially available MT (scheme 3.6). During this synthesis, high temperature and pressure were employed, together with chloranil to perform a dehydrogenation giving the 4,6-dien-3-one compound. As MT has a conjugated enone system, tautomerisation followed by hydride abstraction at C7 leads to the 4,6-dien-3-one. Parent steroid was synthesised in large scale as it was used for the *in vitro* study and for an *in vivo* equine administration study (~200 mg).



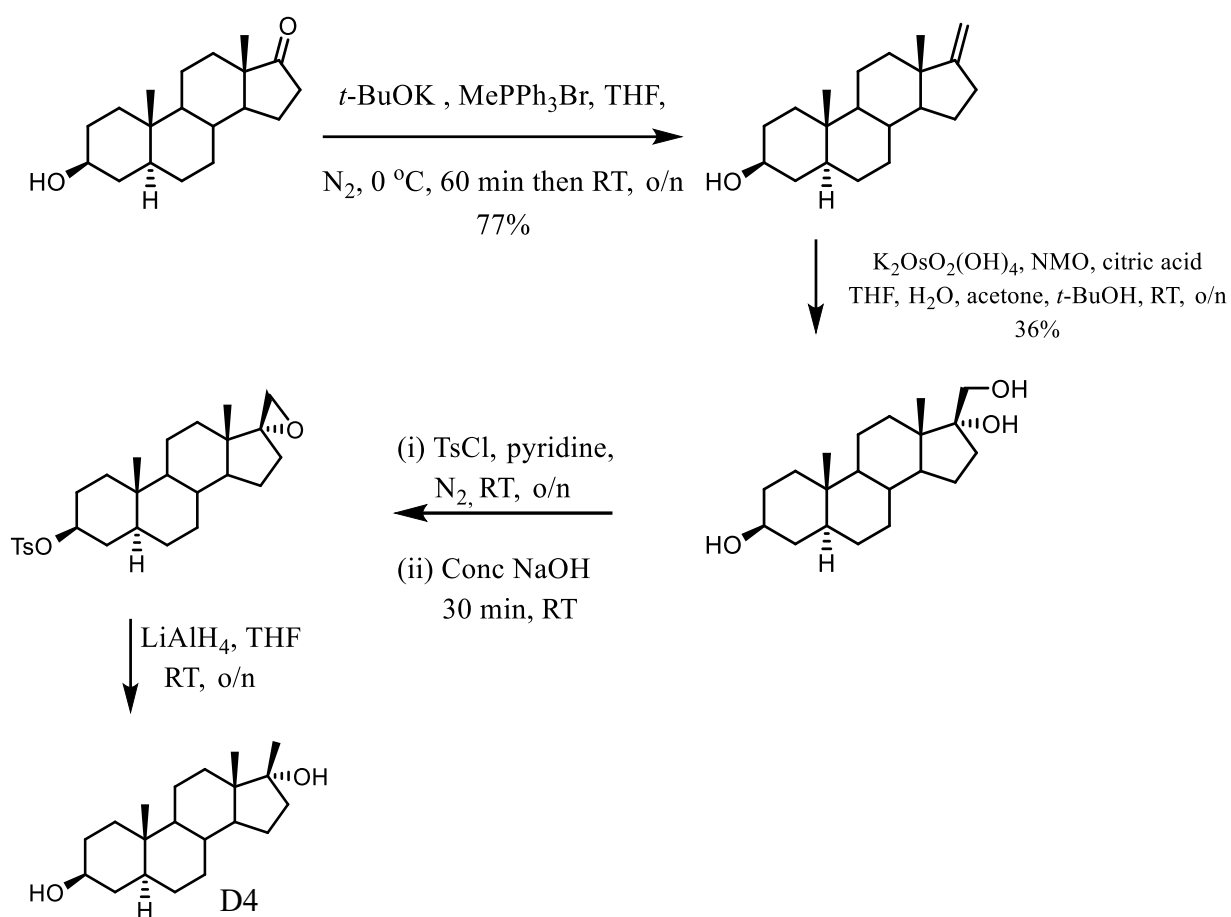
Scheme 3.7: Proposed mechanism for the synthesis of Δ^6 -methyltestosterone in the presence of chloranil.

Quinones are widely used for dehydrogenation. Since quinones bear multiple electron withdrawing groups they can act as hydride abstracting agents. As MT has a conjugated enone system, tautomerisation to the conjugated di-enol and hydride abstraction at C7 leads to the 4,6-dien-3-one (scheme 3.7).

3.2.2.4 Synthesis of epimerised steroid diols

As epimerisation is commonly observed with 17 β -hydroxy-17 α -methylated steroids, it was important to synthesise them during this study. In order to synthesise the epimerised diols, two approaches were employed. Epimerization has been studied with the hydrolysis of 17-sulfates^[2]. However, there was lack of an established synthetic route for the synthesis of some 17-epimers. Therefore, in this chapter, establishment of a multi-step synthesis approach was studied on a relatively large scale with selected steroids and the other approach, 17-sulfation and hydrolysis to synthesise the targets on a small scale.

3.2.2.4.1 Large scale synthesis

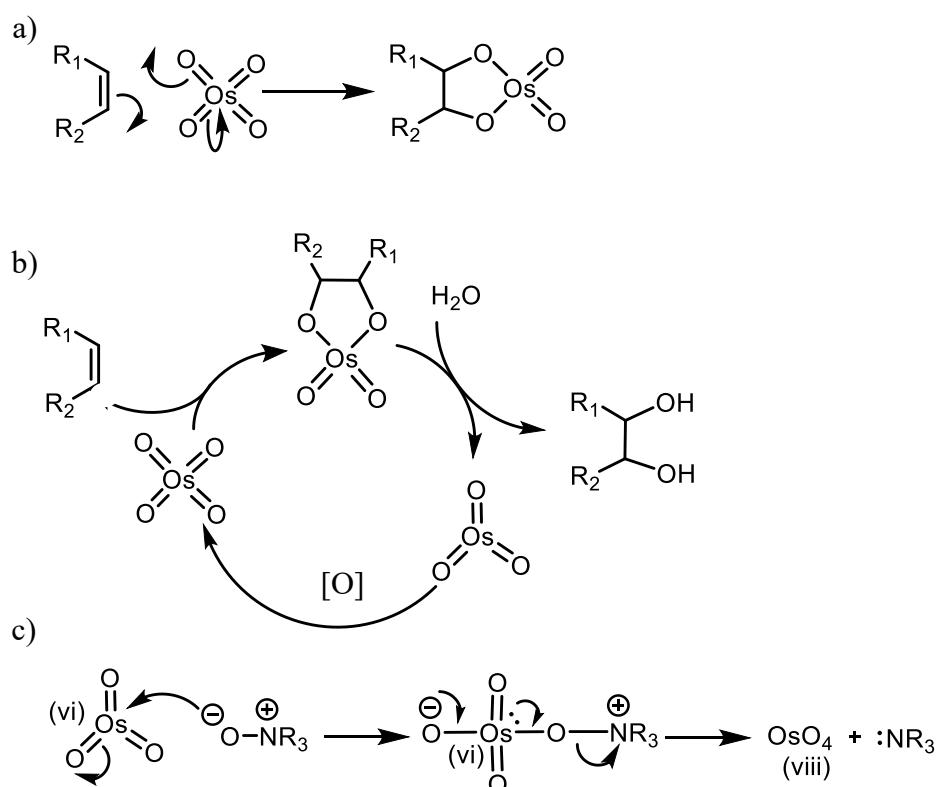


Scheme 3.8: Synthesis of 17 β -methyl-5 α -androstane-3 β ,17 α -diol, D4

17 β -Methyl-5 α -androstane-3 β ,17 α -diol **D4** was synthesised in four steps (scheme 3.8). The carbonyl group at C17 of epiandrosterone was converted to an exocyclic methylenide group using the Wittig reaction. The stereochemistry of the following epoxide formation was a major concern here as an α -configured epoxide was

required in order to achieve the desired 17 α -hydroxy-17 β -methyl stereochemistry. Peroxy acids are very common reagents used for epoxidation. Out of those, *meta*-chloroperbenzoic acid (*m*-CPBA) is commonly used for epoxidations, as it is stable and easy to handle. Previous studies have shown that the addition favours the less hindered face.^[18] Since the β -face is hindered by the C18 methyl group, oxidation was expected to occur from the α -face to have the 17 α ,17 β -(epoxymethano) steroid. However, the epoxidation with *m*-CPBA was not 100% selective as it generated both α and β epoxide isomers; in 4:1 ratio. As it was desirable to obtain the epoxide in stereochemically pure form, an alternative synthesis pathway was investigated.

Dihydroxylation of the 17-methylidene group was performed with potassium osmate dihydrate (K₂OsO₂(OH)₄). Osmium tetroxide (OsO₄) is one of the most common reagents used for *cis*-dihydroxylation. However, since osmium tetroxide is volatile and highly toxic, catalytic amounts of OsO₄ are used with stoichiometric amounts of co-oxidants to minimize the hazard. Al-Fouti and Hanson^[19] have used osmium tetroxide to synthesise the 17 β -hydroxymethyl-5 α -androstane-3 β ,17 α -diol. In the same manner during this synthesis, OsO₄ was prepared *in situ* from K₂OsO₂(OH)₄ with N-methylmorpholine-N-oxide (NMO). The NMO reoxidizes Os(VI) species to Os(VIII) species which then reduces back to Os(VI) by reacting with an alkene to produce a diol, after hydrolysis.



Scheme 3.9: a) Formation of cyclic ester with Os(VI) during the dihydroxylation, b) catalytic cycle of OsO₄ catalysed dihydroxylation c) proposed mechanism of re-oxidation of OsO₃.^[20]

The mechanism of this reaction involves the concerted (3+2) addition, where O=Os=O comes in to contact with the C=C bond that give rise to an intermediate, five membered cyclic ester. During this (3+2) addition, simultaneous movement of three electron pairs gives rise to the five membered cyclic ester intermediate (scheme 3.9). Then hydrolysis yields the *cis* diol compound forming osmium (VI) oxide. The reduced osmium (VI) oxide is then re-oxidized with NMO to get OsO₄, which can start another catalytic cycle.

The facial selectivity plays an important role in the synthesis of 17 β -hydroxymethyl-5 α -androstane-3 β ,17 α -diol. There are number of factors that affect the facial selectivity, such as bulkiness of the reagent, steric effects and even hyperconjugative effects of adjacent C-H bonds. If there are hydroxyl groups nearby, the hydrogen bonding may also effect the facial selectivity.^[21]

When *m*-CPBA is used, it adds to the middle of the π -bond via a so-called “butterfly” transition state^[45]. In contrast OsO₄ is larger and extends outside the ends of the π -bond (figure 3.3). For this reason, the steric effect of the C18-methyl is greater for OsO₄ addition. The intermediate formed has a fairly rigid structure by forming two bonds simultaneously with the alkene carbons. Therefore, this yields 17 β -hydroxymethyl-5 α -androstane-3 β ,17 α -diol as the sole product of this reaction without the 3 β , 17 β epimer. The results obtained here, confirm the results reported for the synthesis of same compound by Al-Fouti and Hanson.^[19] However, osmylation was not completely efficient. Whenever the dihydroxylation was performed, it provided mixtures of the product and the starting material, which were later separated by flash chromatography.

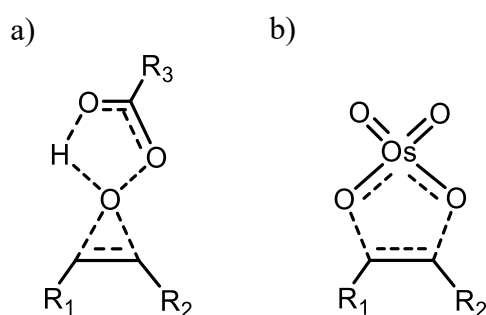
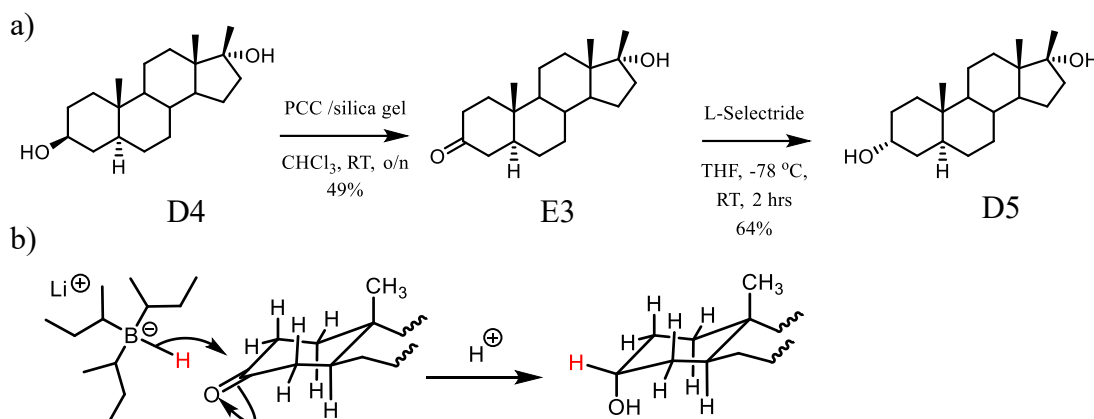


Figure 3.3: a) Addition of perbenzoic acid to the π -bond. b) Addition of OsO_4 to the π -bond.

In order to obtain the 17β -methyl group from 17β -hydroxymethyl group, the hydroxyl group at C20 must be removed. As hydroxyl groups are poor leaving groups, tosylation was used to make the hydroxyl a better leaving group. Upon tosylation, both 3β -hydroxy and 20 -hydroxy groups were converted to their tosyl esters. As tosylates are good leaving groups, it was possible to obtain the $17(20)$ epoxide easily under basic conditions. This method retains stereochemistry at C17 leading to the α -epoxide. Finally LiAlH_4 reduction was used to open the epoxide and also cleave the 3 -tosyl group leaving $3\beta,17\alpha$ -hydroxy diol (**D4**), which is the 17 -epimer of diol **D1**.

The 17β -methyl- 5α -androstane- $3\alpha,17\alpha$ -diol (**D5**), isomer of steroid diol **D4** was synthesised by oxidation and reduction of diol **D4** (scheme 3.10). Oxidation proceeded without issue to provide the 3 -ketone, **E3**. The key step of this synthesis was the stereoselective reduction. Steroids possess a rigid structure as they have a four fused ring system. As a result, the steric influences can usually be clearly defined. Given this it was necessary to select the appropriate reducing agent for the 3 -keto reduction.



Scheme 3.10: a) Synthesis of 17β -methyl- 5α -androstane- $3\alpha,17\alpha$ -diol **D5 b) equatorial addition of lithium tri-sec-butylborohydride (L- Selectride®)**

The reduction was performed to obtain the axial hydroxyl group. Generally, large nucleophiles such as L-Selectride® approach equatorially, whereas small nucleophiles such as LiAlH_4 approach axially. Compared to the equatorial face, the axial face is more hindered to the nucleophile due to developing $1,3$ -diaxial

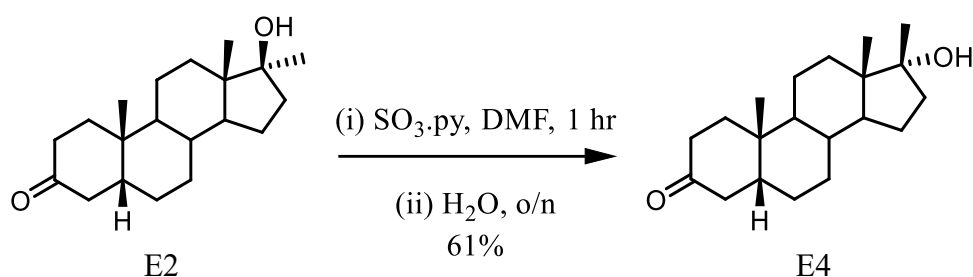
interactions. Thus, lithium tri-sec-butylborohydride (L- Selectride®), a large nucleophile was used to reduce the 3-carbonyl group, to afford diol **D5**.

3.2.2.4.2 Small scale synthesis

The large scale synthesis pathways above, all started from the 5 α -configured steroid, EA. When it comes to the 5 β -configuration, the starting material, EC is expensive and so less readily available for lengthy synthetic sequences. Therefore, it was essential to find a procedure to synthesise the 5 β -configured 17-epimerised diols using fewer steps.

A published protocol by Schanzer *et al.*^[2] was used for the synthesis of epimerised steroids. They obtained the epimeric steroids *via* the sulfation and subsequent hydrolysis of the 17-hydroxy group. A slightly altered method was adapted from the published procedure.

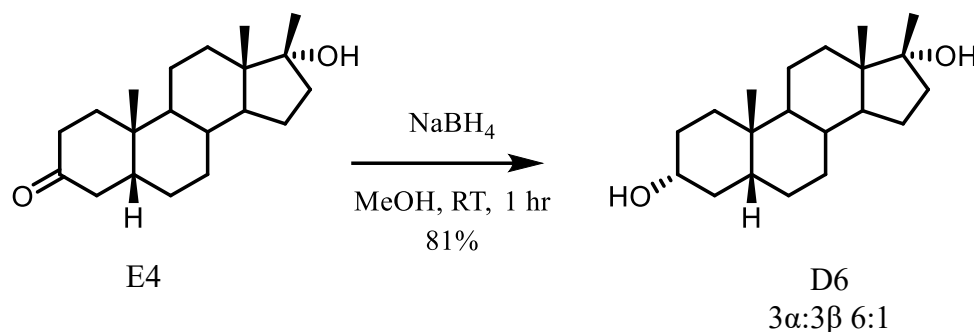
Steroid ketone **E2** derived from **EC** was dissolved in dimethylformamide (DMF), and treated with excess sulfur trioxide pyridine (SO₃.Py) complex. The reaction was stirred for one hour and water was added to the same reaction mixture and stirring continued for another 12 hours. The product was extracted with ethyl acetate, and purified by flash column chromatography to provide the epimerised 17 α -hydroxy-17 β -methyl-5 β -androstan-3-one (**E4**, scheme 3.11).



Scheme 3.11: Synthesis of 17 α -hydroxy-17 β -methyl-5 β -androstan-3-one (**E4**)

The epimeric ketone was then selectively reduced to obtain the 17 β -methyl-5 β -androstan-3 α ,17 α - diol (**D6**). The main concern here was to prepare the 3 α ,5 β -configured compound as the 3 β ,5 β arrangement is not typically detected in metabolism studies.^[17] According to literature, when 3-keto steroids that have 5 β -configuration are treated with small nucleophiles they provide the 3 α -isomer and when they are treated with bulky nucleophiles such as L-Selectride® they provide the 3 β -isomer.^{[22],[23]} Therefore, sodium borohydride was employed to generate **D6**

diol which was purified with C18 to yield a mixture of the two isomers 3 α :3 β in 6:1 ratio (scheme 3.12).



Scheme 3.12: Synthesis of 17 β -methyl-5 β -androstane-3 α ,17 α -diol, D6

3.2.2.5 Synthesis of epi-methyltestosterone (epi-MT) and Δ 6-epimethyltestosterone (Δ 6-epi-MT)

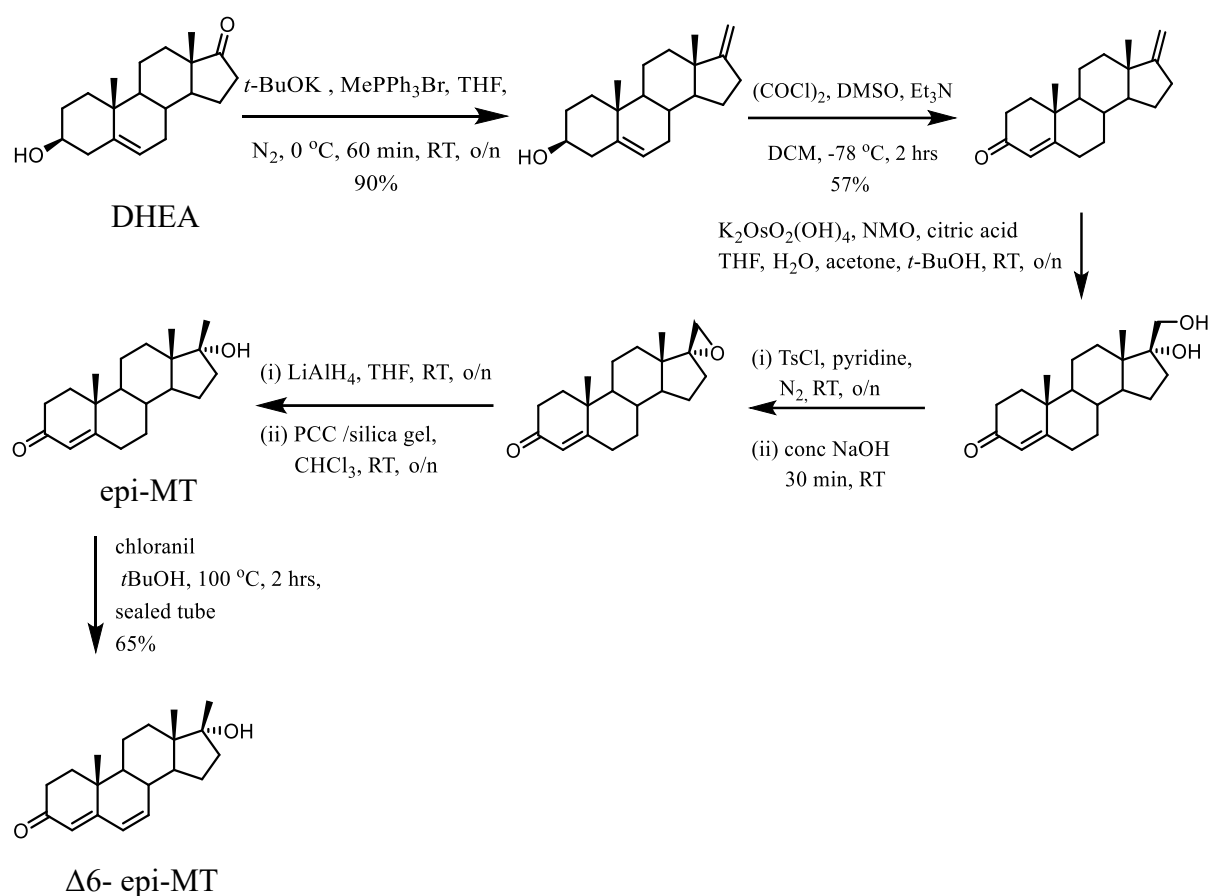
Epi-methyltestosterone and Δ 6-epi-methyltestosterone were successfully prepared with 5 and 6 steps respectively (scheme 3.13), starting with dehydroepiandrosterone (DHEA). The epi-methyltestosterone synthesis was similar to that reported in scheme 3.8, yet had slight differences.

In the first step, formation of the alkene at C17 was easily achieved. The next requirement was to obtain the 4-en-3-one conjugated system from 3-hydroxy-5-ene system. The synthetic strategy was to oxidise followed by base promoted tautomerisation. The first attempt was carried out with PCC. However, this produced the 4-ene-3,6-dione instead^[47]. To avoid this over oxidation, the Swern oxidation was investigated as a suitably mild alternative.

Mechanistically the Swern oxidation starts with activation of DMSO to produce chlorosulfonium ion that releases both carbon dioxide and carbon monoxide as by-products. The steroid alcohol then substitutes the chlorosulfonium cation by releasing hydrogen chloride and forms an alkoxy-sulfonium ion. Deprotonation and elimination occurs to form the steroid ketone, and finally a base catalysed tautomerisation leads to the desired 4-en-3-one conjugated system. This enone, has two alkenes, one in A ring and the other a C17, exocyclic methylenide group. The electron deficient 4-en-3-one was more hindered and less reactive for

dihydroxylation by OsO₄. Therefore, the conjugated double bond was not affected during the dihydroxylation.

Dihydroxylation, and tosylation were performed in similar manner to the previous synthesis. Then the α -epoxide was obtained under base promoted conditions. In order to open the epoxide ring, LiAlH₄ reduction was performed and this gave a mixture of 3 β ,17 α diol and 3 α ,17 α diol. Once this mixture was oxidized with PCC epi-MT was obtained as the sole product. In the previous incident, the dienolate formed gives the diketone and therefore, PCC oxidation was not successful. Dehydrogenation of epi-MT with chloranil then afforded Δ 6-epi-MT in reasonable yield.



Scheme 3.13: Synthesis of epi-methyltestosterone and Δ 6-epimethyltestosterone

When 17 β -hydroxy-17 α -methyl steroids are compared to their 17 epimers, the chemical shifts of the -CH₃ groups differ slightly as the configuration of the steroid has changed with the epimerisation. The chemical shifts of -CH₃ groups are compared in table 3.1. The chemical shifts for E1, E3, MT, epi-MT, E2 and E4 are in

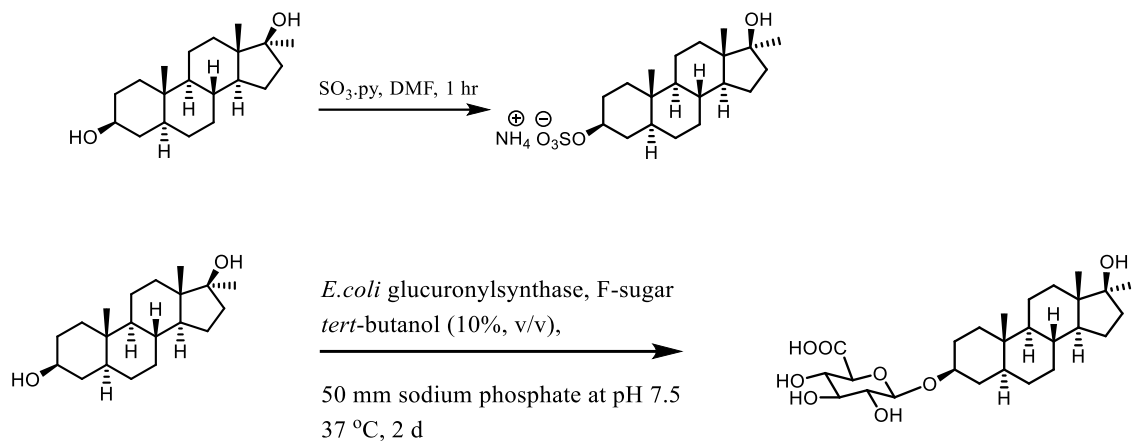
accordance with the published data provided by Schänzer *et al.*^[2] When the steroid is epimerised, 18-CH₃ chemical shifts show the greatest deviation compared to the other two methyl groups. When the 17 β -hydroxyl group is on the same face as the 18-CH₃, the CH₃ protons show a greater chemical shift. In contrast, epimers have the 18-CH₃ and 17-hydroxyl groups facing in opposite directions and the 18-CH₃ chemical shift is reduced because it is relatively shielded. This pattern of shielding and deshielding was also observed for the diol epimers **D1** and **D4**, **D2** and **D5**, and **D3** and **D6**.

Steroid	C18	$\Delta\delta$	C19	C20
17α-methyl-5α-androstane-3β,17β-diol (D1)	0.826	+0.157	0.846	1.206
17β-methyl-5α-androstane-3β,17α-diol (D4; epi D1)	0.669		0.820	1.180
17α-methyl-5α-androstane-3α,17β-diol (D2)	0.797	+0.126	0.845	1.208
17β-methyl-5α-androstane-3α,17α-diol (D5; epi D2)	0.671		0.795	1.182
17α-methyl-5β-androstane-3α,17β-diol (D3)	0.833	+0.169	0.939	1.219
17β-methyl-5β-androstane-3α,17α-diol (D6; epi D3)	0.664		0.937	1.186
17β-hydroxy-17α-methyl-5α-androstan-3-one (E1)	0.875	+0.176	1.031	1.215
17α-hydroxy-17β-methyl-5α-androstan-3-one (E3)	0.699		1.024	1.195
17β-hydroxy-17α-methyl-5β-androstan-3-one (E2)	0.861	+0.164	1.027	1.221
17α-hydroxy-17β-methyl-5β-androstan-3-one (E4)	0.697		1.030	1.202
17β-hydroxy-17α-methylandrost-4-en-3-one (MT)	0.906	+0.178	1.203	1.214
17α-hydroxy-17β-methylandrost-4-en-3-one (epi-MT)	0.728		1.197	1.208
17β-hydroxy-17α-methylandrost-4,6-dien-3-one (Δ6-MT)	0.958	+0.190	1.133	1.231
17α-hydroxy-17β-methylandrost-4,6-dien-3-one (Δ6-epiMT)	0.768		1.120	1.225

Table 3.1: ¹H-NMR chemical shifts of C18, C19 and C20 protons of synthesised steroids and their epimers with $\Delta\delta$ showing 18-CH₃ chemical shift differences of the epimers ([δ 17 α -methyl]-[δ 17 β -methyl])

3.2.2.6 Synthesis of steroid sulfates and steroid glucuronides

The conjugated reference materials of Δ^6 -MT were synthesised using methods established within the group; small scale synthesis of steroid sulfates^[24] and enzymatic synthesis of steroid glucuronides.^[25]



Scheme 3.14: Synthesis of 17 α -methyl-5 α -androstane-3 β ,17 β -diol 3-sulfate (S1), ammonium salt, and 17 α -methyl-5 α -androstane-3 β ,17 β -diol 3-glucuronide (G1)

3.2.2.6.1 Synthesis of steroid sulfates

Sulfation was performed with sulfur trioxide pyridine complex (scheme 3.14). The steroid sulfate product was purified by solid-phase extraction (SPE). The method developed (mentioned in chapter two), is only used with *in vitro* studies, as it is harder than the chemical method and was done in lower scale. The sulfation at the 3-hydroxy group is faster than that of the 17-hydroxy group. As the 3-hydroxy group is a secondary alcohol it is less hindered and more reactive. The 3-substituted mono sulfates were easily synthesised within an hour using the above mentioned protocol. Once the starting material was consumed the reactions were quenched without allowing it to react further. This yielded the mono-sulfates in preference to 3,17-bis-sulfates or epimerized products. The synthesised steroid sulfates are shown in the table 3.2.

This study was qualitative in nature. Thus, the percentage conversions for sulfates were not reported. These methods are well established in our group and as these sulfates are considered as derivatives, the starting steroid defines the product. However, according to the literature, 3 α -hydroxy-5 α -configured, 3 α -hydroxy-5 β -

configured and 3 β -hydroxy-5 α -configured steroid alcohols have shown > 98% conversion in sulfation.^[24]

3.2.2.6.2 Synthesis of steroid glucuronides

Glucuronylation was performed using an enzymatically promoted system (scheme 3.14). The reaction employed the steroid, glucuronylsynthase enzyme and α -D-glucuronyl fluoride dissolved in sodium phosphate buffer and *tert*-butanol co-solvent. The reaction mixture was incubated without agitation at 37 °C for 2 days to afford the product. Steroid glucuronides were then purified by SPE.

In contrast to the sulfates, 3 β -hydroxy-5 α -configured steroid alcohols have shown the highest conversion, 90% during the formation of glucuronides. Conversion of 3 α -hydroxy-5 β -configured steroids is between 5%-25% whereas 3 α -hydroxy-5 α -configured steroids have shown no conversion. The conversion of the glucuronide synthesis depends on the substrate and those effects are well studied in our group and have been discussed by Ma *et al.*^[25] and Pranata *et al.*^[46]. The enzymatic method does not succeed for the synthesis of 3 α -hydroxy-5 α -androstane 3-glucuronides^[25], so was not attempted for these steroids. Apart from those 3-substituted glucuronides, two 17-tertiary glucuronides were also prepared. The conversion of those glucuronides were low and they were only detected with mass spectrometry. The synthesised steroid glucuronides are shown in the table 3.3.

Structure	Name
	17 α -methyl-5 α -androstane-3 β ,17 β -diol 3-sulfate, ammonium salt (S1)
	17 β -methyl-5 α -androstane-3 β ,17 α -diol 3-sulfate, ammonium salt (S2)
	17 α -methyl-5 α -androstane-3 α ,17 β -diol 3-sulfate, ammonium salt (S3)
	17 β -methyl-5 α -androstane-3 α ,17 α -diol 3-sulfate, ammonium salt (S4)
	17 α -methyl-5 β -androstane-3 α ,17 β -diol 3-sulfate, ammonium salt (S5)
	17 β -methyl-5 β -androstane-3 α ,17 α -diol 3-sulfate, ammonium salt (S6)

Table 3.2 Synthesised steroid sulfates (confirmed with proton NMR and high-resolution mass spectrometry)

Structure	Name
	17 α -methyl-5 α -androstane-3 β ,17 β -diol 3-glucuronide (G1)
	17 β -methyl-5 α -androstane-3 β ,17 α -diol 3-glucuronide (G2)
	17 α -methyl-5 β -androstane-3 α ,17 β -diol 3-glucuronide (G3)
	17 β -methyl-5 β -androstane-3 α ,17 α -diol 3-glucuronide (G4)
	17 β -hydroxy-17 α -methylandrosta-4,6-dien-3-one 17-glucuronide (G5)
	17 α -hydroxy-17 β -methylandrosta-4,6-dien-3-one 17-glucuronide (G6)

Table 3.3 Synthesised steroid glucuronides (confirmed with high-resolution mass spectrometry)

3.3 *In vitro* metabolism of Δ 6-methyltestosterone

3.3.1 *In vitro* phase I metabolism of Δ 6-methyltestosterone

The *in vitro* phase I metabolism study of Δ 6-methyltestosterone was carried out with liver S9 fractions of three different species: human, horse (equine) or dog (canine). As *in vitro* metabolism is enzyme driven, pH and temperature were maintained at 7.5 and 37 °C respectively, to mimic physiological conditions. Negative controls were run alongside samples with the absence of steroid, liver S9 fraction or co-factors. After incubating the samples for 24 hours at 37 °C, metabolites were extracted using C18 cartridges, and subjected to positive mode ESI UHPLC-high resolution MS analysis.

The scan data obtained were examined using mass filters, previously created using three combinations of metabolic transformations: reduction, oxidation and hydroxylation. The *in vitro* metabolism samples were compared with the negative controls to identify metabolites by subtracting the signals present in the negative controls. Firstly, metabolites were identified using the mass filters, and where possible, metabolites were confirmed with the reference materials synthesised.

Δ 6-Methyltestosterone contains two alkenes and a carbonyl group. Phase I metabolism can lead to enzymatically driven reduction reactions and as a result of that, Δ 4 and Δ 6 double bonds can be reduced (scheme 3.2 and 3.3). Reduction of Δ 6 double bond leads to methyltestosterone. Reduction of Δ 4 double bond gives rise to two isomers, one with a 5α -hydrogen and the other isomer with a 5β -hydrogen which is governed by 5α -reductase and 5β -reductase enzymes respectively^[26] (scheme 3.3). Further, the reduction of the 3-keto group of 5α -isomer can yield either the 3α or 3β isomer. For reduction of the 5β -3-keto steroid, only the $3\alpha,5\beta$ isomer has been reported, but not $3\beta,5\beta$ -isomer, suggesting that the formation of that isomer occurs at lower levels compared to the others.^{[26],[13]}

After comparing with the negative controls, the *in vitro* phase I study identified a variety of phase I metabolites resulting from hydroxylation, reduction, oxidations or combinations thereof (table 3.4). Of the three species, only one mono-reduced phase I metabolite was observed with equine S9 incubations. This mono-reduced metabolite was expected to match with MT or epi-MT, but did not. This suggests that it has not lost the Δ 6 double bond, and instead could arise from reduction of the Δ 4

double bond. Similar to this observation, the human *in vivo* study reported the absence of MT and epi-MT as metabolites.^[15]

No doubly reduced phase I metabolites were present in the phase I study. Nevertheless, one doubly reduced glucuronide metabolite was observed in the phase II study (see below). When considering the completely reduced steroid diols mentioned above, these are typically not detected by LC-MS due to their poor ionization efficiency. However, if those diols undergo phase II conjugation, this may allow them to be detected.

Hydroxylation is another significant phase I metabolic pathway of steroids. 17 β -Hydroxy-17 α -methyl steroids can undergo hydroxylation at different positions. Schänzer has shown that for humans, 6 β hydroxylation is more pronounced in 17 β -hydroxy-17 α -methyl-steroids where the A-ring is not saturated.^[26] As well, C16 is prone to hydroxylation in 17-methylated steroids. In metabolism studies the 17 β -hydroxy-17 α -methyl steroids: stanozolol^[27], oxandrolone^[28], turinabol ^{[29],[30]}, methyltestosterone^[31] and metandienone^[32], all displayed the formation of hydroxy metabolites with 16 α and 16 β configurations.

In phase I metabolism of Δ 6-MT, it was possible to observe a number of mono- and di-hydroxylated metabolites with three different liver S9 fractions. The number of mono-hydroxylated metabolites was seven with canine, six with human and five with equine. Out of eight mono-hydroxylated metabolites, three were common among all three species, and three metabolites were common between at least two species. The other hydroxylated metabolites varied among species (table 3.4). Hydroxylation was a significant *in vitro* metabolic transformation for the three species.

Importantly the hydroxylated metabolites could arise by a more complicated process involving a combination of hydroxylation with oxidation and reduction at distinct locations.

Some metabolites showed two distinct metabolic pathways, hydroxylation and oxidation, and hydroxylation and reduction in concert. When considering the oxidized metabolites, the possibility arises from hydroxylated metabolites that then undergo oxidation to form a ketone rather than oxidation of the already highly unsaturated parent steroid. The presence of numerous hydroxylated metabolites

suggests that the metabolites observed *in vitro* do not correspond to those observed in human *in vivo* metabolism that primarily arose from reduction, and for this reason they do not match the reference materials that do not have hydroxylation.

The *in vitro* study produced only one mono-reduced metabolite in the equine metabolism which did not match with MT or epi-MT, and all the other metabolites observed across all species involved hydroxylation.

Proposed metabolic pathway	Number of phase I metabolites			Number of common metabolites
	Human	Equine	Canine	
reduced $\Delta 6$ -MT		1		
hydroxylated $\Delta 6$ -MT	6	5	7	3
2*hydroxylated $\Delta 6$ -MT	7	5	6	2
reduced hydroxylated $\Delta 6$ -MT	5	4	5	3
hydroxylated oxidized $\Delta 6$ -MT	6	3	7	2
2*hydroxylated reduced $\Delta 6$ -MT		1		
2*hydroxylated oxidized $\Delta 6$ -MT			1	

Table 3.4: *In vitro* Phase I metabolites observed

3.3.2 *In vitro* phase II metabolism of $\Delta 6$ -methyltestosterone

During the study, phase I metabolism was followed by phase II metabolism. First, the samples were incubated with phase I cofactors and liver S9 for three hours and then the same reaction mixture was treated with phase II co-factors and incubated for 24 hours. Negative control experiments were run without steroid, liver S9 fraction or cofactors. After 24 hours of incubation, the reactions were extracted and purified with C18 SPE. These were analysed both under positive mode and negative mode ESI UHPLC high-resolution MS scan mode and mass filters were first used to

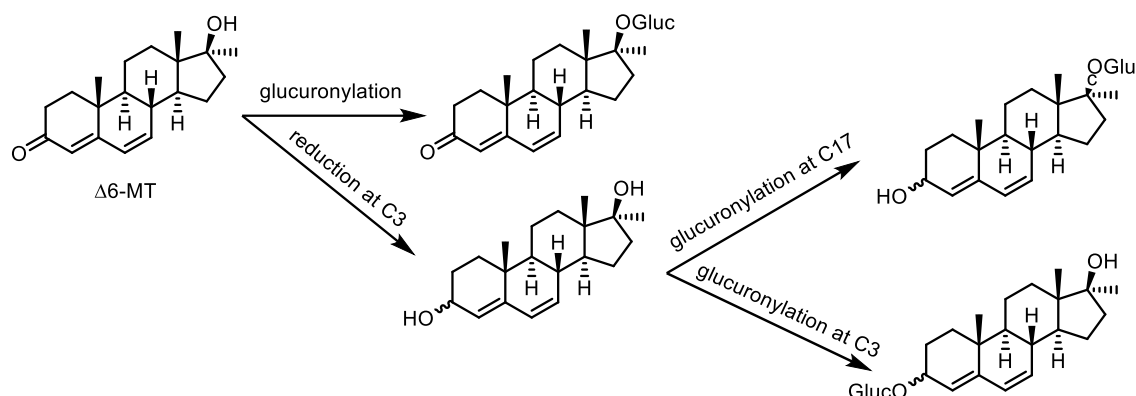
identify the metabolites by comparing against negative controls. Then product ion scans were performed to compare fragmentation behaviour for the verified precursor ions where necessary.

Phase II glucuronylation was promoted by the addition of uridine diphosphate glucuronic acid as the cofactor and sulfation by the addition of ATP, sulfate and MgCl_2 as co-factors. The liver fractions contained the enzymes required for the synthesis of PAPS, the universal sulfate donor. Hence, PAPS can be synthesised *in situ* in the presence of ATP and sulfate, and then be used in sulfation as described in chapter two.

Phase II metabolism generated numerous glucuronides but no sulfates were observed. The parent glucuronide was observed with all three species. Human liver S9 metabolism afforded the highest number of phase II glucuronides compared to the other two liver S9 systems (table 3.5). There were a number of mono- and di-hydroxylated glucuronide metabolites present with all the three species. However, it was surprising that no reduced and hydroxylated glucuronides were present with canine metabolism, as there were 4 reduced and hydroxylated metabolites during phase I. Apart from that, hydroxylated and oxidized phase I metabolites were present in all species, but during phase II, hydroxylated and oxidized glucuronides were present only with canine liver S9.

In contrast to reactions with phase I only, a number of mono-reduced glucuronides were observed when phase II was included. As mono-reduced metabolites were not detected with canine and human phase I metabolism and the only detected mono-reduced metabolite in equine metabolism did not match with MT or epi-MT, this strongly suggests that, these reduced glucuronides are either 3- or 17-glucuronides of 17 β -methyl-5 α -androsta-4,6-diene-3,17-diol (scheme 3.15). As the reduction at C3 can give rise to two isomers, 3 α and 3 β , theoretically it is possible to observe four reduced glucuronide metabolites through conjugation at C3 or C17. Four mono-reduced glucuronide metabolites were observed with human S9 and two with each of other liver S9 fractions. Out of those, the two metabolites observed with canine and equine S9 fractions were present in human S9 incubation as well. Similarly, there were two mono-hydroxylated glucuronide metabolites which were common among all the species. Apart from those, hydroxylated-oxidized, hydroxylated-

reduced, doubly reduced and triply reduced glucuronide conjugates were also present.



Scheme 3.15: Proposed mono-reduced glucuronide metabolites and parent glucuronide

Observed metabolite	Number of phase II metabolites		
	Human	Equine	Canine
parent glucuronide	1	1	1
reduced $\Delta 6$ -MT glucuronide	4	2	2
2*reduced $\Delta 6$ -MT glucuronide	1	-	-
3*reduced $\Delta 6$ -MT glucuronide	1	1	-
hydroxylated $\Delta 6$ -MT glucuronide	4	5	3
2*hydroxylated $\Delta 6$ -MT glucuronide	1	3	2
reduced hydroxylated $\Delta 6$ -MT glucuronide	5	6	-
hydroxylated oxidized $\Delta 6$ -MT glucuronide	-	-	2
2*hydroxylated reduced $\Delta 6$ -MT glucuronide	2		
3*reduced hydroxylated $\Delta 6$ -MT glucuronide	1		

Table 3.5: *In vitro* phase II glucuronide metabolites observed using liver S9 fraction with cofactors

The parent compound includes only one hydroxyl group at C17, a tertiary hydroxyl group that is relatively hindered. As it is sterically more hindered the glucuronide

conjugation of 17 β -hydroxyl group is expected to be less efficient.^[26] However, in this study, the parent glucuronide was detected with all three species and was confirmed against the reference material synthesised (figure 3.4). Even though the epi-glucuronide was prepared as a reference material, it was not observed in this phase II metabolism study.

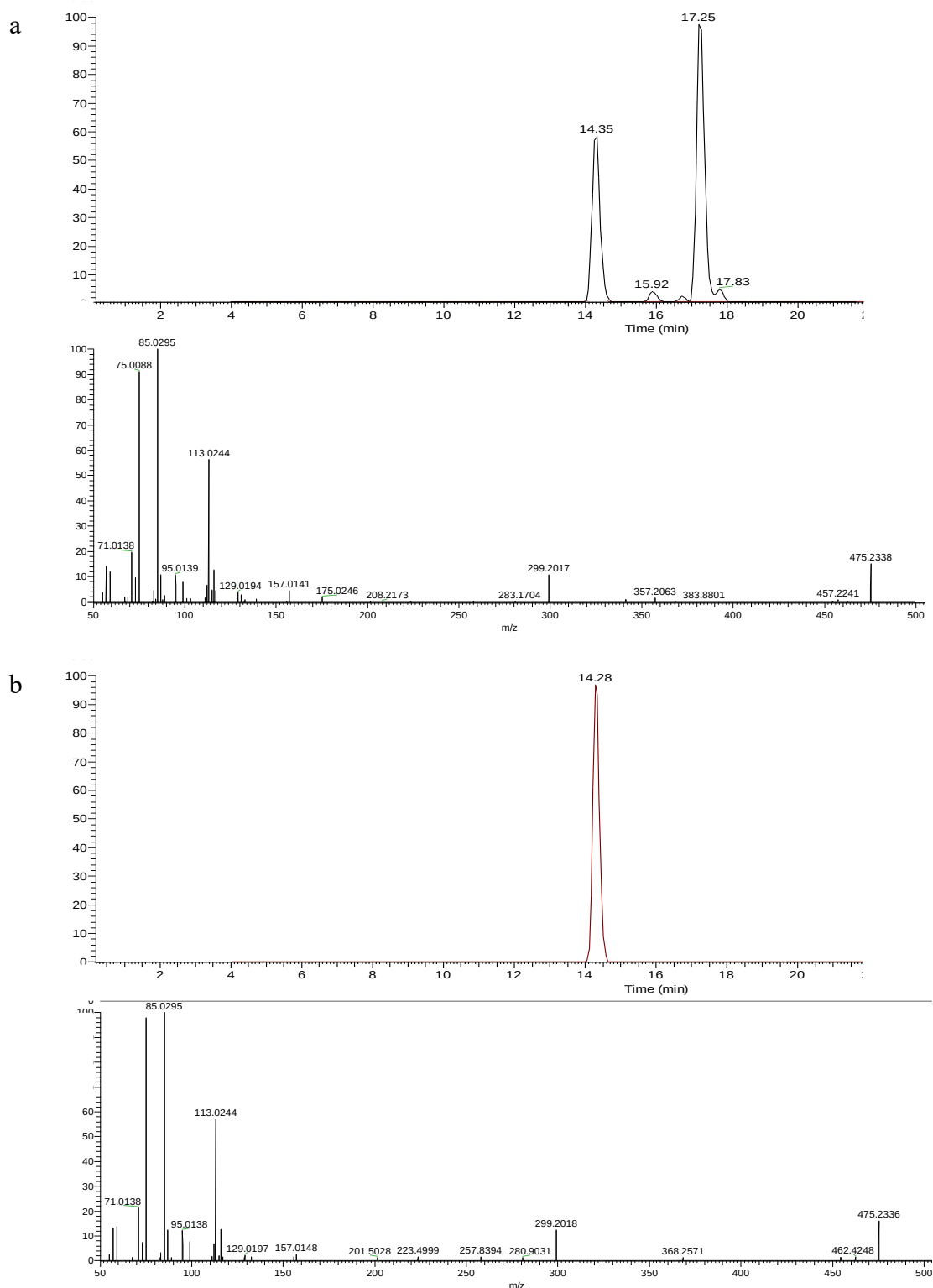


Figure 3.4: extracted chromatogram (m/z 475.2338) and targeted MS/MS spectrum (40 eV) of a) Δ 6-methyltestosterone glucuronide and Δ 6-

epimethyltestosterone glucuronide reference materials; b) human S9 metabolite.

When it comes to the confirmation of the identified metabolites with human liver S9 fraction, WADA has introduced a set of rules or criteria that must be followed before making the final decision. According to the WADA technical document^[33] there are chromatographic and mass spectrometric criteria in order to identify the metabolites using reference materials. Reference materials must be run in the same batch as the samples. The retention time of analytes and their mass spectra are then compared as part of the confirmation.

The retention time of the metabolite in the sample, should not be differ more than 1% or ± 0.1 minutes from that of the reference material. The mass of the metabolite must be with in ± 0.5 Da of the mass of the reference material. When MS/MS fragmentation is used, two transitions are required (figure 3.5) and their relative abundance should lie within the specified tolerances calculated using the relative abundance of the peaks of the reference sample spectrum (table 3.6)

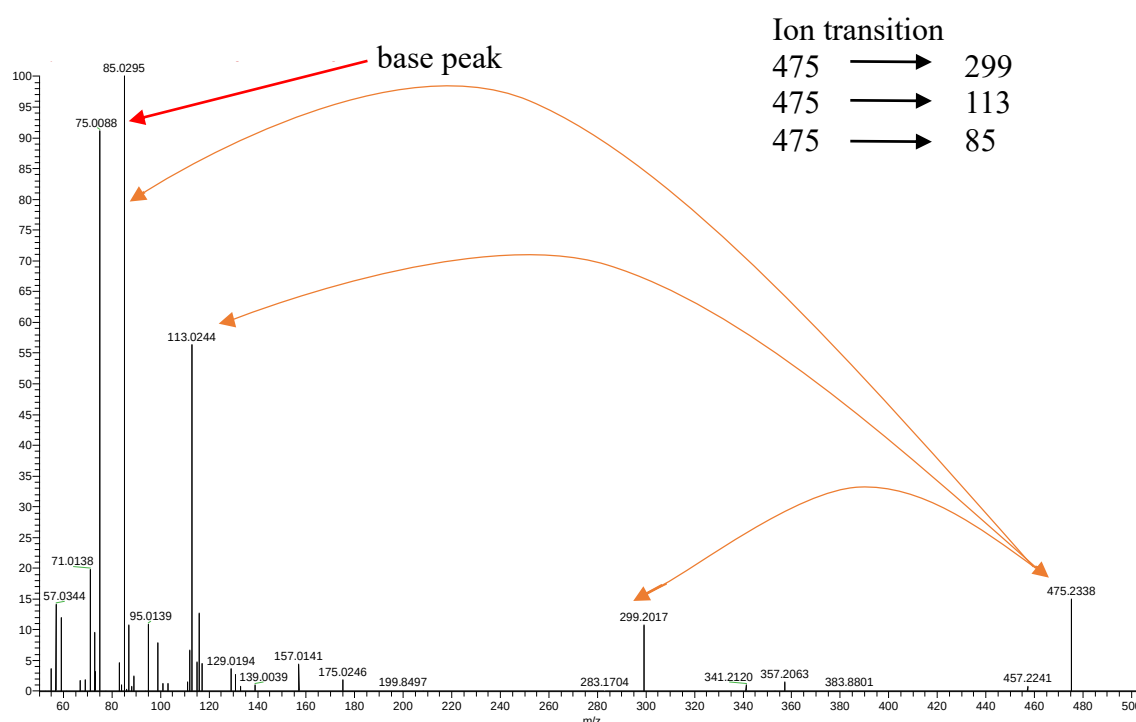


Figure 3.5: The base peak and ion transitions of the MS/MS spectrum of the parent glucuronide ($m/z = 475$) in human S9 metabolism.

Relative Abundance in the reference specimen* (% of base peak)	Maximum Tolerance Windows for the Relative Abundance in the Sample	Examples	
		Relative Abundance (% of base peak)	Tolerance Window (% of base peak)
50 - 100	(±10; absolute)	60 95	50-70 85-100
25 - 50	(± 20%; relative)	40	32-48
1 - 25	(±5; absolute)†	10 3	5-15 (>0†- 8)

Table 3.6: WADA's criteria for maximum tolerance windows for relative abundances leads to the confirmation of metabolites^[33]. *Reference material analysed in the same analytical batch. †The diagnostic ions must always be detected in the sample (S/N>3:1)

Product ion (transitions)	RT reference material	RT sample (tolerance)	Reference material % of base peak	Sample % of base peak (tolerance)
299	14.35	14.28 (14.18-14.38)	10	12 (5-15)
113			56	59 (46-66)
85			100	100 (90-100)
75			91	99 (81-100)

Table 3.7: The comparison of four selected transitions of the parent glucuronide in human S9 metabolism with tolerances, in parentheses, according to WADA criteria.

An example for the comparison of chromatograms and MS/MS spectra of the synthesised parent glucuronide and parent glucuronide metabolite observed with human S9 is shown in figure 3.5 and table 3.7. The retention time of the metabolite is 14.28 minutes, which is within the WADA tolerance window, conformation was taken to the next level. The mass spectra are compared in the negative mode and in this case four transitions were compared (table 3.7), exceeding the minimum requirement of two. One transition m/z 299 is responsible for the loss of glucuronide moiety and the other three fragments: m/z 75 [$\text{HOCH}_2\text{CO}_2^-$], m/z 85 [$\text{gluc-H-H}_2\text{O-CO}_2\text{-CO}^-$], and m/z 113 [$\text{gluc-H-H}_2\text{O-CO}_2^-$], are common fragments observed with glucuronide metabolites in negative mode ESI-MS/MS.^{[3],[34]} The selected transitions of the reference material and the metabolites are within tolerance. Thus, $\Delta 6$ -methyltestosterone 17-glucuronide was confirmed as an *in vitro* phase II metabolite in the human study.

	Product ion (transitions)	RT reference material	RT sample (tolerance)	Reference material % of base peak	Sample % of base peak (tolerance)
Equine parent glucuronide	283	12.87	12.87 (12.82- 12.92)	98	82 (59-100)
	173			30	23 (10-50)
	147			100	100 (60-100)
Canine parent glucuronide	283	13.56	13.58 (13.51- 13.61)	98	89 (59-100)
	173			30	23 (10-50)
	165			28	27 (8-48)
	147			100	100 (60-100)

Table 3.8: The comparison of selected transitions of the parent glucuronide in equine S9 and canine S9 metabolism, AORC criteria.

The parent glucuronide was observed in equine S9 and in canine S9 metabolism as well. In these cases, the presence of the parent glucuronide was confirmed using positive mode ESI-MS/MS fragmentation data, according to the AORC criteria^[44] (table 3.8) used by racing laboratories for the confirmation of equine and canine metabolites. The comparison method has slight differences compared to WADA criteria. The retention time of the metabolite should be within +/- 50% of the half height-peak width or 3 seconds, whichever is greater of the reference material. Similar to WADA, three ion transitions were required to be selected for matching. The tolerances for the selected ions were also different from the WADA criteria.

	Product ion (transitions)	RT reference material	RT sample (tolerance)	Reference material % of base peak	Sample % of base peak (tolerance)
Tri-reduced equine glucuronide	113	14.57	14.52 (14.52- 14.62)	40	17* (20-60)
	85			58	43 (35-81)
	75			87	89 (52-100)
Tri-reduced human glucuronide	113	14.85	14.80 (14.70- 14.90)	41	52* (33-49)
	85			62	99* (52-72)
	75			90	100 (80-100)

Table 3.9: The comparison of selected transitions of the tri-reduced glucuronide (G2) in equine S9 and human S9 metabolism, according to AORC and WADA criteria.

*** Out of tolerance.**

A di-reduced metabolite was observed only with human S9. A tri-reduced glucuronide metabolite was observed with human and equine S9 metabolism. The

retention time of the tri-reduced metabolites matched with the reference material 17 α -methyl-5 β -andostane-3 α ,17 β -diol 3-glucuronide (**G3**); which is a glucuronide conjugate of the reported human *in vivo* metabolite 17 α -methyl-5 β -androstane-3 α ,17 β -diol (**M1**).^[15] However, the confirmation was not possible as the relative abundance of the transitions of the product ions were outside of permitted tolerance (table 3.9). The intensity of the detected metabolites were considerably lower than that of the reference material (ion count of the reference material is 10⁶ whereas the ion count of the metabolite is 10³) and therefore, even at the same collision energy the level of fragmentation may have varied. Despite of this, both metabolites showed characteristic fragments of glucuronic acid moiety m/z 75, 85 and 113 (figure 3.6 and figure 3.7).

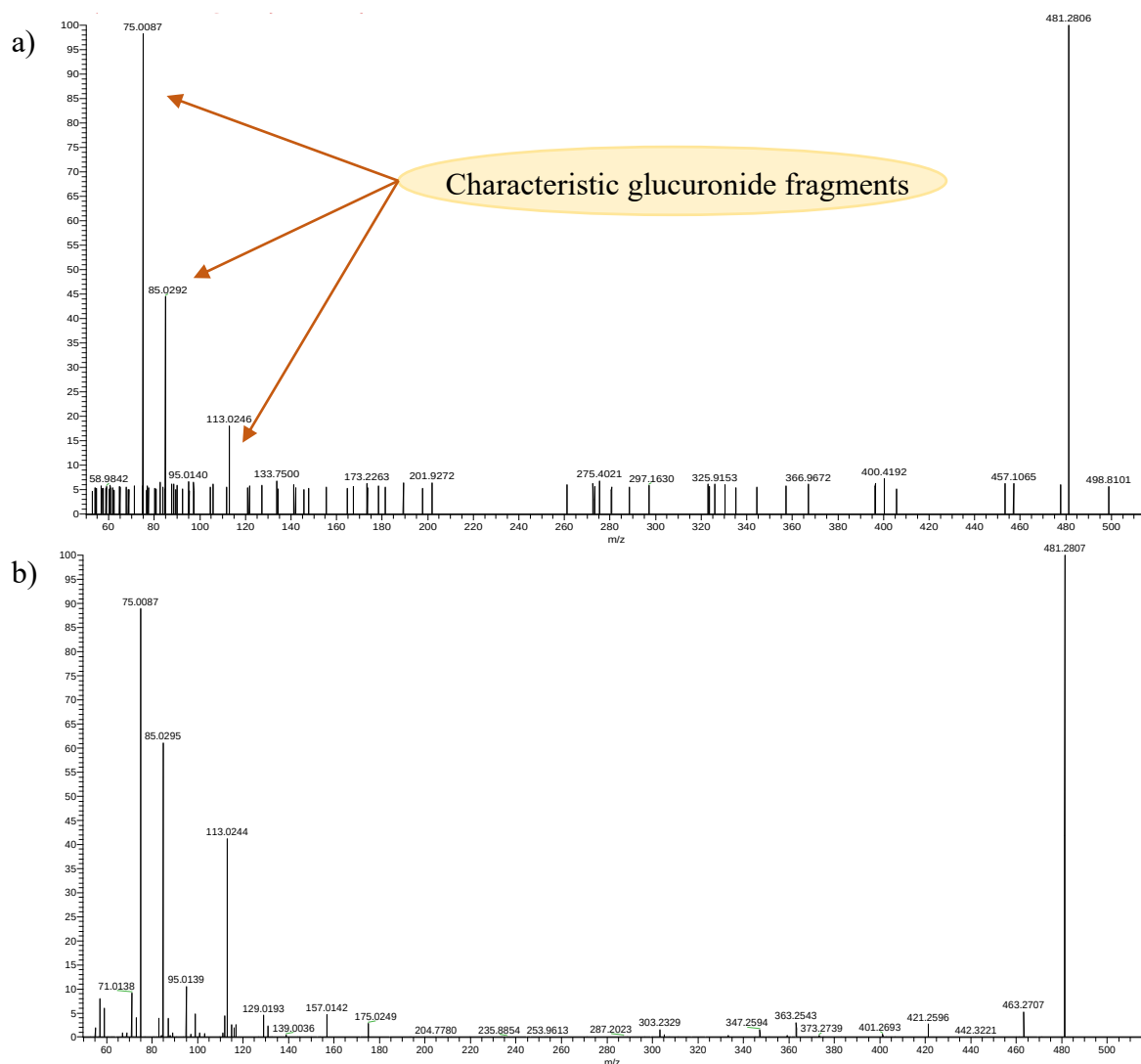


Figure 3.6: Targeted MS/MS spectrum (40 eV) of 17 α -methyl-5 β -andostane-3 α ,17 β -diol 3-glucuronide (G3**) a) metabolite in equine S9 metabolism; b) reference material**

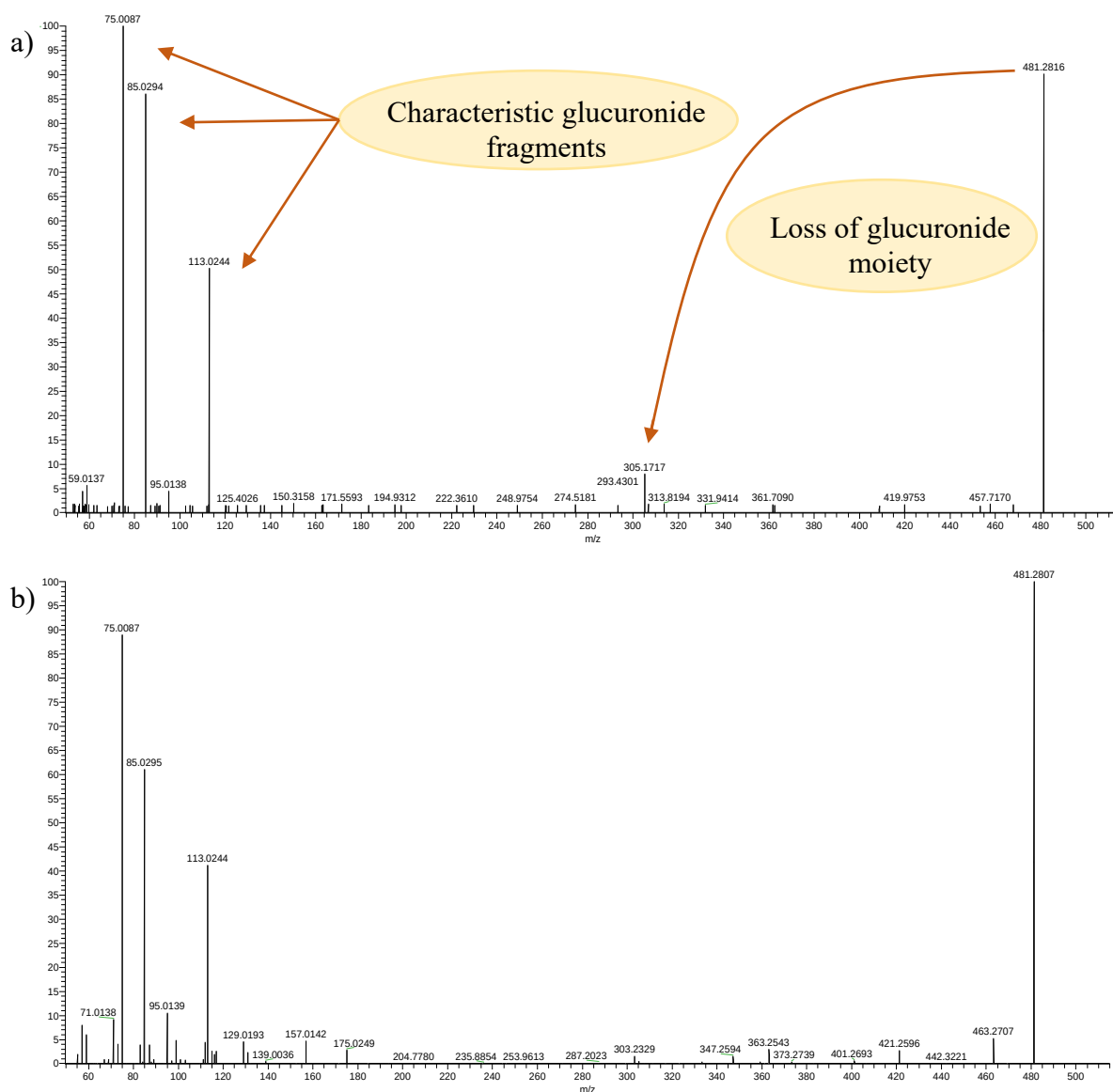


Figure 3.7: Targeted MS/MS spectrum (40 eV) of 17 α -methyl-5 β -androstane-3 α ,17 β -diol 3-glucuronide (G3) a) metabolite in human S9 metabolism; b) reference material

A number of other reduced and hydroxylated glucuronide metabolites were also observed with all the three species, but fewer with the canine S9 fraction (table 3.5).

3.4 Conclusions and future work

In summary, Δ 6-methyltestosterone, originally detected in a dietary supplement, was synthesised and provided to two institutions, Australian Racing Forensic Laboratory (ARFL) and Queensland Racing Integrity Commission (QRIC) to carry out the equine and canine *in vivo* metabolism studies.

While they perform the *in vivo* studies, we synthesised a range of reference materials that could potentially be used to identify the abuse of $\Delta 6$ -methyltestosterone. Based on the results of an earlier human *in vivo* drug administration study, attention focused on reduced reference materials. Therefore, with a sequence of reductions, MT, two 3-keto steroids and three diols were first synthesised. As $\Delta 6$ -MT was a 17β -hydroxy- 17α -methyl steroid, the C17-epimer was expected to be one of its common metabolites. Therefore, the epimer and mono-, di- and tri- reduced forms of epimer were also targeted as reference materials and synthesised. In order to complete the story, six sulfate conjugates and six glucuronide conjugates were also prepared.

After completing the synthesis in the lab, a pilot *in vitro* metabolism study of this steroid was conducted. The phase I and phase II *in vitro* study of $\Delta 6$ -MT with three different liver S9 fractions yielded number of phase I metabolites and phase II glucuronide conjugates. Of the glucuronides, the parent glucuronide was confirmed against the synthetic reference material and three times reduced glucuronide metabolite was observed with human and equine S9 metabolism. In this case the retention time matched with the synthesised reference material but could not be fully confirmed due to differences in the ion ratios.

This three times reduced metabolite was previously identified after glucuronide hydrolysis in a human *in vivo* study as a long term metabolite. Despite the lack of confirmation, the mass spectra of the metabolite provides clear evidence to show that it is a glucuronide metabolite. In the *in vivo* study, limited numbers of metabolites were observed; $\Delta 6$ -MT, epi- $\Delta 6$ MT, and three tri-reduced metabolites one of which is also C17-epimer. In contrast, the *in vitro* study generated a variety of mono and di-hydroxylated metabolites together with metabolites derived from combinations of hydroxylation, reduction and oxidation.

Although this pilot study targeted sulfate conjugates and the epimerised steroid metabolites formed as a result of sulfation, none of those were identified by the study. From chapter two it is known that the S9 fraction promotes sulfation. The failure to observe sulfate metabolites could arise if the sulfation system was not competitive enough to generate sulfates in the presence of glucuronylation.

To address this issue, sulfation can be carried out without glucuronylation as a combination of phase I and phase II metabolism. Thus, it is possible to eliminate the competition caused by glucuronylation.

However, due to the time and costs associated with these *in vitro* metabolism studies additional work was not conducted. Once the *in vivo* studies are completed, we can carry out this study again and analyse *in vivo* and *in vitro* alongside each other. Further work may be needed to confirm the capacity for sulfation utilizing ATP and sulfate in the presence of phase I cofactors and glucuronylation.

3.5 Experimental

3.5.1 *In vitro* metabolism

3.5.1.1 Phase I only studies

For phase I only studies, steroid (30 μmol) was treated with human liver S9 fraction (0.55 mg ml^{-1}), NADP (1.5 mM), G6P (7.5 mM), G6PDH (1 u/mL), MgCl_2 (5 mM) and Tris.HCl buffer (50 mM, pH 7.5). The final volume was made up to 2 mL and was incubated at 37 °C for 3 hours. Then the reaction was diluted with 50 mM tris.HCl buffer (2 mL), centrifuged (4000g, 10 min) to pellet solid. The supernatant was then loaded on to C18 SPE cartridge which was preconditioned with MeOH (3 mL) and water (3 mL). Then the column was washed with water (3 mL), briefly dried and metabolites were eluted with MeOH (3 mL). This methanolic fraction was dried down under nitrogen and reconstituted in a 200 μL mixture of MeOH:H₂O 65:35.

Three negative control experiments were included; without steroid, liver S9 fractions or co-factors.

3.5.1.2 Phase I and phase II combined

For phase I and phase II combined studies phase I metabolism was performed as above. for three hours, and then 500 μL containing rest of the reagents required for phase II metabolism was added to the same reaction vial and incubated for further 21 hours at 37 °C. Phase II mixture contained the cofactors UDPGA, ATP and Na_2SO_4 (8 mM, 16 mM, 8 mM respectively) required for sulfation and glucuronylation. Then the reaction was diluted with 50 mM Tris.HCl buffer (2 mL), centrifuged (4000g, 10 min) to pellet solid. The supernatant was then loaded on to C18 SPE cartridge, which

was preconditioned with MeOH (3 mL) and water (3 mL). Then the column was washed with water (3 mL), briefly dried and metabolites were eluted with MeOH (3 mL). This methanolic fraction was dried down under nitrogen and reconstituted in a 200 μ L mixture of MeOH:H₂O 65:35.

Negative controls were carried along with these positive experiments in the absence of S9 liver fractions, steroid or co-factors. For *in vitro* sulfation, PAPS (3'-phosphoadenosine-5'-phosphosulfate) is commonly used as the cofactor. Yet, in this study instead of PAPS, we used a method developed in our group; ATP and sulfate to generate the sulfate metabolites. [43]

3.5.1.3 Δ 6-Methyltestosterone LC-MS analysis

The liquid chromatography-high resolution accurate mass (LC-HRMS) analysis was performed with Thermo Fisher Scientific Dionex Ultimate 3000 UHPLC coupled to Q Exactive plus Quadrupole-Orbitrap mass spectrometer equipped with an Agilent Poroshell 120 EC-C18 column (2.1 mm x 50 mm, 2.7 μ m) incubated at 30 °C and eluting with a gradient of the following mobile phases, A: 10% methanol in aqueous ammonium acetate solution (5 mM), B: 90% methanol in aqueous ammonium acetate solution (5 mM), gradient: 0-16 min A-B (99:1 v/v) to B (100%), 16-26 min B (100%), 26-27 min B (100%) to A-B (99:1 v/v), 5 min re-equilibration, flow rate 0.2 mL min⁻¹. Unconjugated steroids were monitored in positive mode using [M+H]⁺ with full scan MS and sulfate conjugates were monitored for the anion [M-H]⁻ with full scan MS and targeted MS/MS. Glucuronide conjugates were monitored both in positive and negative mode ESI.

3.5.1.4 Fragmentation data for phase I and phase II metabolites generated with human S9, equine S9 and canine S9 liver fractions.

Metabolite	Precursor ion and MS/MS fragments (% of base peak), [collision energy]	RT (min)	Precursor ion	Theoretical m/z
hydroxylated Δ6-MT M1	317.2094 (54%), 299.1996 (73%), 281.1887 (100%), 263.1769 (19%), 209.1311 (12%), 146.7748 (12%), 81.0693 (11%), [10 eV] ^h	12.10 ^H	[M+H] ⁺	317.2111
hydroxylated Δ6-MT M2	317.2091 (40%), 299.1999 (6%), 281.1888 (10%), 173.0956 (22%), 145.1007 (19%), 121.1008 (37%), 97.0647 (100%), [50 eV] ^h	13.18 ^{H, E, C}	[M+H] ⁺	317.2111
hydroxylated Δ6-MT M3	317.2094 (23%), 299.199 (43%), 281.1886 (45%), 241.1575 (97%), 197.1316 (55%), 173.0954 (70%), 97.0647 (100%), [50 eV] ^h	13.66 ^{H, C}	[M+H] ⁺	317.2111
hydroxylated Δ6-MT M4	317.2094 (17%), 299.1991 (8%), 281.1887 (13%), 173.0954 (33%), 163.111 (100%), 145.1006 (75%), 121.1008 (59%), [50 eV] ^h	14.92 ^{H, E, C}	[M+H] ⁺	317.2111
hydroxylated Δ6-MT M5	317.2095 (85%), 299.1991 (67%), 281.1886 (100%), 241.1576 (34%), 225.1263 (35%), 171.0797 (26%), 135.0799 (26%), 121.0644 (22%), [30 eV] ^h	15.18 ^{H, E, C}	[M+H] ⁺	317.2111
hydroxylated Δ6-MT M6	317.2094 (13%), 299.199 (100%), 281.1885 (85%), 225.1263 (65%), 171.1158 (15%), 147.1161 (61%), 121.1007 (39%), [40 eV] ^h	15.40 ^{H, C}	[M+H] ⁺	317.2111
hydroxylated Δ6-MT M7	317.2108 (17%), 299.2003 (14%), 281.1900 (23%), 173.0961 (37%), 163.1117 (100%), 145.10012 (74%), 97.0651 (63%), [50 eV] ^e	14.92 ^{E, C}	[M+H] ⁺	317.2111
hydroxylated Δ6-MT M8	317.2106 (6%), 299.2003 (51%), 281.1899 (45%), 241.1584 (100%), 223.1480 (26%), 197.1325 (13%), 97.0651 (25%), [40 eV] ^e	13.66 ^{E, C}	[M+H] ⁺	317.2111
2*hydroxylated Δ6-MT M9	333.2063 (85%), 315.1954 (14%), 297.1847 (100%),	11.44 ^{E, C}	[M+H] ⁺	333.2060

	279.1744 (79%), 225.1269 (68%), 171.0806 (34%), 121.0648 (19%), [30 eV] ^e			
2*hydroxylated Δ6-MT M10	333.2067 (39%), 315.1957 (10%), 297.1846 (74%), 279.1747 (100%), 225.1277 (63%), 171.0806 (52%), 163.1115 (65%), [40 eV] ^e	11.91 ^E	[M+H] ⁺	333.2060
2*hydroxylated Δ6-MT M11	333.2057 (40%), 297.1829 (31%), 279.1737 (100%), 256.8318 (14%), 239.1425 (65%), 185.2183 (15%), 171.08 (37%), [30 eV] ^h	12.15 ^{H, C}	[M+H] ⁺	333.2060
2*hydroxylated Δ6-MT M12	333.2028 (100%), 297.1833 (17%), 279.1749 (15%), 251.7819 (12%), 202.5766 (13%), 155.8181 (12%), 117.8879 (13%), [30 eV] ^h	12.63 ^{H, E, C}	[M+H] ⁺	333.2060
2*hydroxylated Δ6-MT M13	333.2017 (10%), 297.1835 (56%), 279.1731 (71%), 227.1418 (62%), 171.0799 (100%), 161.0954 (62%), 95.0854 (52%)' [40 eV] ^h	12.97 ^{H, E, C}	[M+H] ⁺	333.2060
2*hydroxylated Δ6-MT M14	333.2059 (53%), 315.1939 (24%), 297.1844 (77%), 279.1741 (45%), 267.1744 (69%), 209.1321 (100%), 135.0803 (50%) [40eV] ^e	13.50 ^E	[M+H] ⁺	333.2060
2*hydroxylated Δ6-MT M15	333.2050 (100%), 297.1845 (30%), 279.1729 (23%), 194.1429 (15%), 131.343 (15%), 107.3061 (15%), 59.6646 (16%), [20eV] ^h	13.74 ^H	[M+H] ⁺	333.2060
2*hydroxylated Δ6-MT M16	333.2041 (25%), 315.1942 (100%), 297.1844 (20%), 285.1844 (5%), 255.1725 (3%), 225.898 (3%), 133.1014 (3%), [20eV] ^h	14.39 ^H	[M+H] ⁺	333.2060
2*hydroxylated Δ6-MT M17	333.2057 (4%), 315.1946 (11%), 297.1842 (13%), 279.2315 (100%), 261.2209 (45%), 109.1012 (47%), 95.0857 (60%), [30 eV] ^c	14.53 ^C	[M+H] ⁺	333.2060
reduced hydroxylated Δ6-MT M18	319.2249 (14%), 301.2144 (62%), 283.2039 (100%), 265.1932 (62%), 225.1619	10.49 ^{H, E, C}	[M+H] ⁺	319.2268

	(30%), 173.0952 (24%), 121.1008 (18%), [30 eV] ^h			
reduced hydroxylated Δ6-MT M19	319.2251 (5%), 301.2146 (95%), 283.2039 (100%), 265.1934 (55%), 225.1625 (27%), 159.1163 (28%), 121.1003 (16%), [30 eV] ^h	10.99 ^{H, E, C}	[M+H] ⁺	319.2268
reduced hydroxylated Δ6-MT M20	319.2253 (24%), 301.215 (80%), 283.2038 (100%), 265.1932 (28%), 227.0296 (13%), 186.8593 (14%), 58.9278 (14%), [20 eV] ^h	11.94 ^{H, E}	[M+H] ⁺	319.2268
reduced hydroxylated Δ6-MT M21	319.2254 (16%), 301.2155 (18%), 283.2044 (20%), 265.1944 (9%), 225.1629(9%), 125.0593 (15%), 97.0646 (100%), [40 eV] ^h	12.84 ^{H, E, C}	[M+H] ⁺	319.2268
reduced hydroxylated Δ6-MT M22	319.2269 (19%), 301.2179 (19%), 283.2054 (100%), 265.1962 (14%), 225.1641 (13%), 176.053 (44%), 97.0651 (88%), [40 eV] ^c	13.81 ^C	[M+H] ⁺	319.2268
reduced hydroxylated Δ6-MT M23	319.2258 (9%), 301.2152 (34%), 301.1848 (8%), 283.2051 (100%), 265.1963 (33%), 243.1737 (79%), 225.1637 (85%), [40 eV] ^c	14.55 ^C	[M+H] ⁺	319.2268
hydroxylated oxidized Δ6- MT M24	315.1941 (100%), 297.1844 (4%), 279.1739 (23%), 223.1097 (7%), 171.0793 (39%), 147.0799 (18%), 121.0645 (18%), [30 eV] ^h	12.78 ^{H, C}	[M+H] ⁺	315.1954
hydroxylated oxidized Δ6- MT M25	315.1943 (37%), 297.1831 (100%), 279.1722 (5%), 257.3363 (4%), 257.1531 (19%), 239.1427 (16%), 229.1579 (6%), (216.6841 (4%), [30 eV] ^h	13.24 ^{H, C}	[M+H] ⁺	315.1954
hydroxylated oxidized Δ6- MT M26	315.1939 (100%), 257.1531 (16%), 229.1587 (10%), 173.0957 (10%), 161.0943 (8%), 97.0648 (35%), 87.0441 (14%) [40 eV] ^h	13.84 ^{H, E, C}	[M+H] ⁺	315.1954

hydroxylated oxidized Δ6-MT M27	315.1927 (24%), 279.1713 (7%), 223.1098 (4%), 185.0951 (15%), 173.0956 (43%), 147.0798 (100%), 121.1011 (7%), [40 eV] ^h	14.16 ^{H, C}	[M+H] ⁺	315.1954
hydroxylated oxidized Δ6-MT M28	315.1954 (8%), 297.1844 (4%), 279.1744 (10%), 171.0804 (42%), 121.0649 (41%), 147.0804 (100%), 107.0858 (15%), [40 eV] ^h	14.36 ^{E, C}	[M+H] ⁺	315.1954
hydroxylated oxidized Δ6-MT M29	315.1933 (57%), 297.1837 (100%), 279.1729 (26%), 269.1895 (31%), 257.1527 (23%), 171.0795 (95%), 133.1007(27%), [30 eV] ^h	14.76 ^H	[M+H] ⁺	315.1954
hydroxylated oxidized Δ6-MT M30	315.1942 (91%), 297.1842 (35%), 279.1754 (15%), 257.1528 (14%), 171.0798 (44%), 163.0747 (100%), 137.0589 (63%), 121.1012 (15%), [30 eV] ^h	16.30 ^H	[M+H] ⁺	315.1954
reduced Δ6-MT M31	303.2305 (45%), 285.2204 (83%), 267.2105 (100%), 245.1887 (12%), 227.1794 (30%), 159.1165 (42%), [30 eV]	12.96 ^E	[M+H] ⁺	303.2319

Table 3.10: *In vitro* human, equine and canine metabolites of Δ6-methyltestosterone – phase I. ^h Fragmentation in human S9. ^e Fragmentation in equine S9. ^c Fragmentation in canine S9. ^H Metabolites observed with human S9. ^E Metabolites observed with equine S9. ^C Metabolites observed with canine S9.

Metabolite	Precursor ion and MS/MS fragments (% of base peak), [collision energy]	RT (min)	Precursor ion	Theoretical m/z
Δ6-MT glucuronide^a	475.234 (100%), 457.223 (3%), 299.2018 (4%), 175.0248 (4%), 157.0141 (7%), 113.0244 (43%), 85.0295 (40%), 75.0087 (49%), [30 eV] ^h	13.09 ^{H, E, C}	[M-H] ⁻	475.2338
reduced	477.2531 (9%), 113.0245 (58%), 85.0295 (86%), 75.0088 (100%), [40 eV] ^h	12.47 ^H	[M-H] ⁻	477.2495

Δ6-MT glucuronide G1				
reduced Δ6-MT glucuronide G2	477.2487 (16%), 299.1983 (18%), 113.0246 (39%), 160.084 (12%), 85.0293 (83%), 75.0087 (100%), [40 eV] ^h	12.98 ^{H, E, C}	[M-H] ⁻	477.2495
reduced Δ6-MT glucuronide G3	477.2488 (38%), 457.992 (3%), 299.2019 (16%), 113.0245 (35%), 85.0295 (47%), 75.0087 (100%), [40 eV] ^h	14.06 ^{H, E, C}	[M-H] ⁻	477.2495
reduced Δ6-MT glucuronide G4	477.2497 (40%), 459.2375 (2%), 299.2011 (2%), 157.0143 (5%), 113.0243 (56%), 85.0294 (100%), 75.0087 (93%), [40 eV] ^h	15.18 ^{H, E, C}	[M-H] ⁻	477.2495
hydroxylated Δ6-MT glucuronide G5	491.2295 (16%), 113.024 (74%), 85.0292 (100%), 75.0083 (89%), [40 eV] ^h	11.10 ^{H, E, C}	[M-H] ⁻	491.2287
hydroxylated Δ6-MT glucuronide G6	491.2272 (19%), 315.195 (82%), 297.1838 (7%), 112.016 (62%), 85.0291 (44%), 75.0084 (100%), [40 eV] ^h	12.24 ^{H, E, C}	[M-H] ⁻	491.2287
hydroxylated Δ6-MT glucuronide G7	491.2293 (100%), 473.2179 (1%), 315.1967 (33%), 175.0249 (10%), 113.0243 (32%), 87.0087 (19%), 75.0087 (14%), [30 eV] ^e	12.52 ^{E, C}	[M-H] ⁻	491.2287
hydroxylated Δ6-MT glucuronide G8	491.2292 (100%), 315.1962 (38%), 113.0244 (29%), 87.0088 (15%), 75.0088 (14%), [40 eV] ^e	13.13 ^{E, C}	[M-H] ⁻	491.2287
hydroxylated Δ6-MT glucuronide G9	491.2294 (100%), 315.1962 (4%), 175.0244 (5%), 113.0245 (34%), 85.0294 (12%), 75.0087 (27%), [30 eV] ^e	13.38 ^E	[M-H] ⁻	491.2287
hydroxylated Δ6-MT glucuronide G10	491.2267 (29%), 315.1949 (26%), 297.1849 (17%), 267.1741 (28%),	13.62 ^H	[M-H] ⁻	491.2287

	113.0239 (85%), 85.0292 (81%), 75.0084 (100%), [40 eV] ^h			
reduced hydroxylated Δ6-MT glucuronide G11	493.2452 (20%), 113.0238 (40%), 85.0292 (98%), 75.0084 (100%), [40 eV] ^h	10.99 ^{H, E}	[M-H] ⁻	493.2444
reduced hydroxylated Δ6-MT glucuronide G12	493.2449 (63%), 175.0247 (3%), 113.0244 (81%), 85.0295 (100%), 75.0088 (93%), [40eV] ^e	11.50 ^E	[M-H] ⁻	493.2444
reduced hydroxylated Δ6-MT glucuronide G13	493.2413 (24%), 475.2323 (2%), 299.2005 (100%), 175.0236 (2%), 113.0239 (31%), 85.0292 (26%), 75.0084 (51%), [40eV] ^h	11.70 ^H	[M-H] ⁻	493.2444
reduced hydroxylated Δ6-MT glucuronide G14	493.2448 (22%), 356.2926 (5%), 317.2108 (47%), 113.0241 (43%), 112.0162 (62%), 85.0292 (44%), 75.0085 (100%), [40 eV] ^h	12.13 ^{H, E}	[M-H] ⁻	493.2444
reduced hydroxylated Δ6-MT glucuronide G15	493.2431 (33%), 317.2046 (22%), 299.2018 9 (12%), 113.0244 (43%), 85.0295 (58%), 75.0087 (100%), [40 eV] ^e	12.55 ^E	[M-H] ⁻	493.2444
reduced hydroxylated Δ6-MT glucuronide G16	493.2427 (39%), 299.201 (3%), 193.0345 (9%), 113.0239 (97%), 85.0291 (79%), 75.0084 (100%), [40 eV] ^h	13.21 ^H	[M-H] ⁻	493.2444
reduced hydroxylated Δ6-MT glucuronide G17	493.2444 (55%), 299.2013 (4%), 175.0249 95%), 113.0245 (87%), 85.0295 (86%), 75.0088 (100%), [40 eV] ^e	13.32 ^E	[M-H] ⁻	493.2444
reduced hydroxylated Δ6-MT glucuronide G18	493.242 (100%), 299.1992 (5%), 113.0238 (25%), 85.0291 (12%), 75.0084 (20%), [30 eV] ^h	13.79 ^H	[M-H] ⁻	493.2444

reduced hydroxylated Δ6-MT glucuronide G19	493.2451 (68%), 175.0251 (2%), 113.0244 (82%), 85.0295 (100%), 75.0088 (912%), [40 eV] ^e	13.89 ^E	[M-H] ⁻	493.2444
reduced hydroxylated Δ6-MT glucuronide G20	493.2445 (3%), 317.2107 (49%), 193.0339 (18%), 175.0238 (37%), 113.0239 (100%), 85.0292 (18%), [20 eV] ^h	17.44	[M-H] ⁻	493.2444
2*reduced Δ6-MT glucuronide G21	479.265 (100%), 461.2567 (3%), 113.0243 (21%), 75.0087 (25%), [30 eV] ^h	14.56 ^H	[M-H] ⁻	479.2652
3*reduced Δ6-MT glucuronide G22	481.282 (75%), 305.1725 (16%), 113.0245 (54%), 85.0294 (85%), 75.0087 (100%), [40 eV] ^h	14.85 ^{H,E}	[M-H] ⁻	481.2809
2*hydroxylated Δ6-MT glucuronide G23	507.2235 (40%), 331.1927 (12%), 285.0928 (7%), 113.0244 (79%), 85.0295 (100%), 75.0087 (75%), [40 eV] ^e	9.63 ^{E, C}	[M-H] ⁻	507.2236
2*hydroxylated Δ6-MT glucuronide 24	507.2228 (68%), 331.1921 (100%), 283.3628 (5%), 175.0251 (21%), 113.0245 (77%), 85.0295 (28%), 71.0138 (19%), [30 eV] ^e	9.95 ^{E, C}	[M-H] ⁻	507.2236
2*hydroxylated Δ6-MT glucuronide G25	507.2228 (27%), 331.1917 (100%), 175.0244 (3%), 113.0245 (43%), 87.0089 (46%), 85.0296 (26%), 75.0089 (32%), [40 eV] ^e	10.49 ^{E, C}	[M-H] ⁻	507.2236
2*hydroxylated Δ6-MT glucuronide G26	507.2245 (9%), 331.1904 (42%), 283.1689 (7%), 113.0239 (93%), 85.0291 (91%), 75.0084 (100%), 71.0135 (77%), [40 eV] ^h	12.87 ^H	[M-H] ⁻	507.2236
hydroxylated oxidized Δ6-MT glucuronide G27	489.214 (100%), 313.1811 (28%), 175.0247 (6%), 113.0245 (34%), 85.0294 (10%), 75.0088 (31%), [30 eV] ^c	11.43 ^C	[M-H] ⁻	489.2124

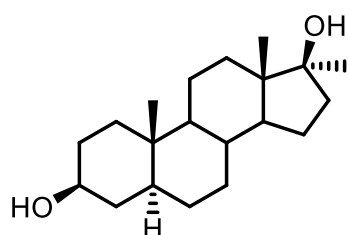
hydroxylated oxidized Δ6-MT glucuronide G28	489.2714 (87%), 313.2389 (51%), 175.0245 (18%), 113.0244 (100%), 85.0294 (44%), [30eV] ^c	13.16 ^c	[M-H] ⁻	489.2124
2*hydroxylated reduced Δ6-MT glucuronide G29	509.2405 (33%), 297.1864 (6%), 267.1759 (6%), 175.2159 (4%), 113.0242 (73%), 85.0294 (73%), 75.0087 (100%), [40 eV] ^h	11.12 ^h	[M-H] ⁻	509.2393
3* reduced hydroxylated Δ6-MT glucuronide G30	497.2715 (70%), 321.2427 (83%), 113.0239 (100%), 85.0291 (48%), 75.0084 (67%), [30 eV] ^h	16.88 ^h	[M-H] ⁻	497.2758

Table 3.11: *In vitro* human, equine and canine metabolites of Δ 6-methyltestosterone – phase II. ^a Structure confirmed against reference materials. ^h Fragmentation in human S9. ^e Fragmentation in equine S9. ^c Fragmentation in canine S9. ^h Metabolites observed with human S9. ^E Metabolites observed with equine S9. ^c Metabolites observed with canine S9.

During phase II metabolism, none of sulfated conjugates were observed. The steroid, Δ 6-methyltestosterone is a 17β -hydroxy- 17α -methyl steroid and epimerisation was expected with sulfation as mentioned in previous section. However, this study did not reveal the presence of epi- Δ 6-MT or any other rearrangement products starting with the migration of methyl group from C13 to C17.

3.5.2 Synthesis data

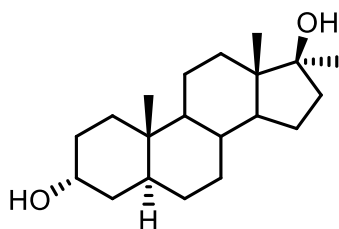
3.6.2.1 17α -Methyl- 5α -androsterane- 3β , 17β -diol (D1)^{[35],[36]}



A solution of epiandrosterone (435 mg, 1.49 mmol) in THF (10 ml) under nitrogen in flame dried glassware was treated with MeMgCl (2.5 mL, 7.5 mmol, 3 M in THF). The resulting cloudy solution was stirred overnight. The reaction was diluted with EtOAc (20 mL) and washed with saturated aqueous ammonium chloride solution (20 mL), water

(20 mL) and brine (20 mL). The organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (60% EtOAc/hexane) to afford the title compound (309 mg, 67%) as a colourless solid. *R_f* 0.61 (40% EtOAc/hexane); mp 180-185 °C. (lit^[35] 209-210 °C); [α]^{25_D} -1 (c 1.0, THF) (lit^[36] -13, THF); ¹H NMR (100 MHz, CDCl₃): 3.59 (m, 1H, C3-H), 1.85-1.63 (m, 5H), 1.63-1.23 (m, 12 H), 1.21 (s, 3H, C20-H₃), 1.19-1.05 (m, 2H), 1.03-0.89 (m, 2H), 0.85 (s, 3H, C18-H₃), 0.83 (s, 3H, C19-H₃), 0.62 (m, 1H), 2 x OH not observed; ¹³C NMR (100 MHz, CDCl₃): 81.87 (C17), 71.47 (C3), 54.57, 50.85, 45.70, 45.13, 39.17, 38.36, 37.21, 36.55, 35.75, 31.96, 31.83, 31.69, 28.79, 25.95, 23.42, 21.04, 14.14 (C18), 12.51 (C19); LRMS (+EI): *m/z* 306 (40%, [C₂₀H₃₄O₂]⁺), 292 (20%), 291 (100%), 290 (20%), 255 (20%), 246 (25%), 233 (35%), 217 (35%), 215 (55%), 165 (35%), 163 (20%), 161 (25%), 147 (25%), 135 (20%), 133 (20%), 121 (30%), 123 (25%), 109 (25%), 108 (30%), 107 (60%), 105 (25%), 95 (25%), 93 (75%), 91 (50%), 81 (40%), 79 (75%), 77 (30%), 71 (25%), 67 (30%), 55 (30%), 43 (30%), 41 (20%); HRMS (+EI): found 306.2549 [C₂₀H₃₄O₂]⁺ requires 306.2559.

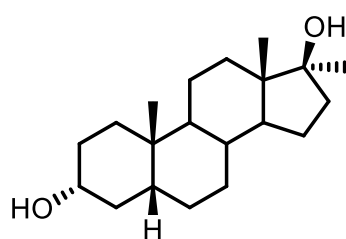
3.6.2.2 17 α -Methyl-5 α -androsterane-3 α ,17 β -diol (D2)^[37]



A solution of androsterone (52.1 mg, 179 μ mol) in THF (1 mL) under nitrogen in flame dried glassware was treated with MeMgCl (300 μ L, 900 μ mol, 3 M in THF). The resulting cloudy solution was stirred overnight. The reaction was diluted with EtOAc (20 mL) and washed with saturated aqueous ammonium chloride solution (20 mL), water (20 mL) and brine (20 mL). The organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (20% EtOAc/hexane) to afford the title compound (35 mg, 64 %) as a colourless solid. *R_f* 0.61 (40% EtOAc/hexane); mp 177-182 °C. (lit^[37] 184-185 °C); [α]^{25_D} -31.2 (c 1.0, CHCl₃) (lit^[37] -12.33, c 0.940); ¹H NMR (400 MHz, CDCl₃): 4.04 (s, 1H, C3-H), 1.84-1.22 (m, 20H), 1.20 (s, 3H, C20-H₃), 0.97-0.68 (m, 2H), 0.85 (s, 3H, C18-H₃), 0.80 (s, 3H, C19-H₃), 2 x OH not observed; ¹³C NMR (100 MHz, CDCl₃): 81.9 (C17), 66.7 (C3), 54.5, 50.9, 45.7, 39.4, 39.1, 36.5, 36.3, 36.0, 32.4, 31.9, 31.8, 29.2, 28.6, 26.0, 23.4, 20.6 (C20), 14.1 (C18), 11.4 (C19); LRMS (+EI): *m/z*

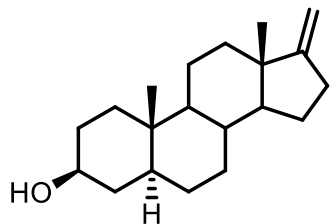
306 (35%, $[\text{C}_{20}\text{H}_{34}\text{O}_2]^+$), 292 (20%), 291 (100%), 288 (30%), 273 (70%), 270 (35%), 255(70%), 248 (20%), 233 (60%), 231 (65%), 230(90%), 217 (60%), 216(25%), 215 (95%), 165 (55%), 161 (40%), 149 (35%), 148(30%), 147 (40%), 145(25%), 135 (50%), 133 (35%), 123 (20%), 122 (20%), 121(30%), 119(35%), 109 (30%), 108 (30%), 107 (75%), 105 (55%), 95 (30%), 93 (40%), 91 (35%), 81 (40%), 79 (40%), 71 (40%), 67 (35%), 57 (35%), 55(45%), 43 (70%), 41 (35%); HRMS (+EI): found 306.2554 $[\text{C}_{20}\text{H}_{34}\text{O}_2]^+$ requires 306.2559.

3.6.2.3 17 α -Methyl-5 β -androsterane-3 α ,17 β -diol (D3)^[35]



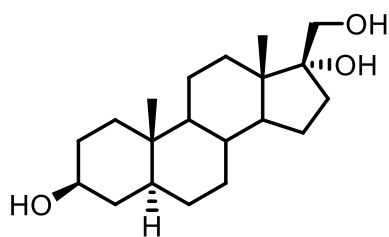
A solution of etiocholanolone (71.7 mg, 247 μmol) in THF (2 mL) under nitrogen in flame dried glassware was treated with MeMgCl (425 μL , 1.28 mmol, 3 M in THF). The resulting cloudy solution was stirred overnight. The reaction was diluted with EtOAc (20 mL) and washed with saturated aqueous ammonium chloride solution (20 mL), water (20 mL) and brine (20 mL). The organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (60% EtOAc/hexane) to afford the title compound (49 mg, 65%) as a colourless solid. R_f 0.38 (40% EtOAc/hexane); mp 155-160 $^{\circ}\text{C}$ (lit 164-165 $^{\circ}\text{C}$); $[\alpha]^{25}_{\text{D}}$ 15.27 (c 0.55, MeOH); ^1H NMR (400 MHz, CDCl_3): 3.63 (m, 1H, C3-H), 1.91-1.62 (m, 6H), 1.61-1.23 (m, 14 H), 1.21 (s, 3H, C20-H₃), 1.14-0.96 (m, 2H), 0.93 (s, 3H, C18-H₃), 0.83 (s, 3H, C19-H₃), 2 x OH not observed; ^{13}C NMR (100 MHz, CDCl_3): 81.9 (C17), 71.9 (C3), 50.9, 45.8, 42.2, 40.7, 39.2, 36.9, 36.6, 35.6, 34.8, 32.0, 30.7, 27.2, 26.3, 26.0, 23.5, 23.4, 20.6 (C18), 14.1 (C19); LRMS (+EI): m/z 306 (3%, $[\text{C}_{20}\text{H}_{34}\text{O}_2]^+$), 288 (30%), 273 (25%), 270 (35%), 255(60%), 231 (70%), 230 (90%), 217 (55%), 215 (100%), 175 (25%), 161 (35%), 149 (30%), 147 (35%), 145 (25%), 135 (40%), 137 (25%), 121(40%), 123 (25%), 109 (20%), 107 (55%), 105 (30%), 95 (25%), 93 (60%), 91 (60%), 81 (30%), 79 (60%), 71 (25%), 67 (50%), 57 (30%), 55 (35%), 43 (55%), 41 (35%); HRMS (+EI): found 306.2553 $[\text{C}_{20}\text{H}_{34}\text{O}_2]^+$ requires 306.2559.

3.6.2. 4 17-Methylidene-5 α -androstan-3 β -ol (A1)^[38]



A solution of potassium tert-butoxide (2.18 g, 19.4 mmol) in dry THF (20 mL) under nitrogen was added to a slurry of epiandrosterone (EA) (1.13 g, 3.88 mmol) and MePPh₃Br (4.24 g, 11.9 mmol) in THF (30 mL) under nitrogen which had been cooled at 0 °C (brine/ice). The reaction was stirred at 0 °C for 60 minutes and then at room temperature overnight. The reaction was diluted with water (100 mL) and extracted with ethyl acetate (3 x 100 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (20% EtOAc/hexane) to afford the title compound (860 mg, 77%) as a colourless solid. *R*_f 0.80 (40% EtOAc/hexanes); mp 140-145 °C [lit^[38] 144-145 °C]; [α]_D²⁵ +2 (c 1.0, MeOH) [lit^[38] +12, MeOH]; ¹H NMR (400 MHz, CDCl₃): 4.62 (m, 1H, C20-H), 4.61 (m, 1H, C20-H), 3.59 (m, 1H, C3-H), 2.48 (m, 1H, C16-H), 2.22 (m, 1H, C16-H), 1.85-1.77 (m, 2H), 1.76-1.66 (m, 3H), 1.66-1.17 (m, 10H), 1.17-1.06 (m, 1H), 1.04-0.86 (m, 3H), 0.83 (s, 3H, C18-H₃), 0.77 (s, 3H, C19-H₃), 0.66 (m, 1H), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 162.2 (C17), 100.8 (C20), 71.5 (C3), 54.7, 54.6, 45.1, 44.3, 38.4, 37.2, 35.9, 35.8, 35.6, 32.1, 31.7, 29.6, 28.8, 24.3, 21.3, 18.7 (C18), 12.5 (C19); LRMS (+EI): *m/z* 288 (35%, [C₂₀H₃₂O]⁺), 273 (100%), 255 (60%), 163 (35%), 162 (50%), 147 (25%), 108 (20%), 107 (35%), 93 (40%), 91 (35%), 81 (20%), 79 (30%), 67 (20%); HRMS (+EI): found 288.2456, [C₂₀H₃₂O]⁺ requires 288.2453

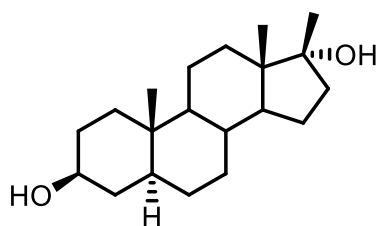
3.6.2. 5 17 β -Hydroxymethyl-5 α -androstane-3 β ,17 α -diol (A2)^[19]



A solution of 17-methylidene-5 α -androstan-3 β -ol **S1** (70.1 mg, 243 μ mol) in THF/*t*-BuOH/Acetone/H₂O (1:1:1:1, 4 mL) was treated with citric acid dihydrate (91.2 mg, 434 μ mol), potassium osmate dihydrate (4.1 mg, 11 μ mol), and NMO (25.7 mg, 219 μ mol) and stirred at room temperature overnight. The reaction was treated with Na₂SO₃ (10% w/v, 10 mL) and extracted with ethyl acetate (3 x 10 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (70%

EtOAc/hexane) to afford the title compound (28 mg, 36%) as a colourless solid. R_f 0.13 (40% EtOAc/hexane); mp 190-195 °C (lit^[19] 179-180 °C); $[\alpha]^{25}_D$ -7.0 (c 1.0, MeOH); ^1H NMR (400 MHz, $CDCl_3$): 3.73 (d, J = 10.9 Hz, 1H, C20-H), 3.57 (d, J = 10.9 Hz, 1H, C20-H), 3.59 (m, 1H, C3-H), 1.85-1.20 (m, 17H), 1.20-1.05 (m, 2H), 1.03-0.88 (m, 2H), 0.82 (s, 3H, C18-H₃), 0.74 (s, 3H, C19-H₃), 0.71-0.63 (m, 1H), 3 x OH not observed; ^{13}C NMR (100 MHz, $CDCl_3$): 84.0 (C17), 71.4 (C3), 67.0 (C20), 54.3, 50.7, 46.5, 45.0, 38.3, 37.2, 35.7, 35.7, 34.7, 32.3, 31.8, 31.7, 28.8, 24.0, 20.9, 15.3 (C18), 12.5 (C19); LRMS (+EI): m/z 322 (10%, $[C_{20}H_{34}O_3]^+$), 304 (15%), 291 (20%), 281 (30%), 274 (20%), 273 (100%), 272 (35%), 255 (40%), 215 (50%), 207 (35%), 161 (40%), 145 (35%), 143 (65%), 133 (20%), 131 (25%), 130 (25%), 129 (30%), 121 (20%), 119 (25%), 105 (20%), 93 (45%), 91 (45%), 79 (25%), 55 (25%), 43 (20%), 41 (25%); HRMS (+EI): found 322.2511 $[C_{20}H_{34}O_3]^+$ requires 322.2508.

3.6.2.6 17 β -Methyl-5 α -androstan-3 β ,17 α -diol (D4)^[35]

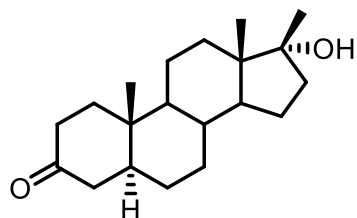


17 β -Hydroxymethyl-5 α -androstane-3 β ,17 α -diol **S2**

(111 mg, 344 μ mol) and p-toluenesulfonyl chloride (332 mg, 1.74 mmol) under nitrogen were dissolved in dry pyridine (4 mL) and stirred at room temperature overnight. The reaction was then treated with aqueous NaOH solution (5M, 10 mL, 0.05 mol) and stirred for 30 minutes. Then it was diluted with EtOAc (20 mL) and washed with water (3 x 20 mL) and saturated aqueous NaCl solution (20 mL). The organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure to afford 17 α ,17 β -(Epoxy-methano)-5 α -androstan-3 β -yl tosylate which was used without further purification. R_f 0.48 (40% EtOAc/hexane); ^1H NMR (400 MHz, $CDCl_3$): 7.78 (d, J = 8.3 Hz, 2H, C22-H), 7.32 (d, J = 8.0 Hz, 2H, C23-H), 4.39 (m, 1H, C3-H), 2.72 (d, J = 4.5 Hz, 1H, C20-H), 2.64 (d, J = 4.5 Hz, 1H, C20-H), 2.44 (s, 3H, C25-H₃), 2.24 (m, 1H, C16-H), 1.86-1.45 (m, 9H), 1.40-1.03 (m, 9H), 0.98-0.83 (m, 2H), 0.78 (s, 6H, C18-H₃, C19-H₃) 0.64 (m, 1H). Then lithium aluminium hydride (19.5 mg, 514 μ mol) under nitrogen was treated with 17 α ,17 β -epoxy-methano-5 α -androstan-3 β -yl tosylate (60.1 mg, 131 μ mol) dissolved in dry THF (6 mL) and stirred overnight. The reaction was

diluted with ethyl acetate while keeping the reaction at 0 °C (ice/brine) and Rochel solution (15% w/v, sodium potassium tartrate tetra hydrate, 20 mL) was added. The reaction mixture was extracted with EtOAc (3 x 20 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (30% EtOAc/hexane) to afford the title compound (18 mg, 17% over two steps) as a colourless solid. Rf 0.51 (40% EtOAc/hexane); mp 185-188 °C (lit^[35]194-195); $[\alpha]^{25}_D$ -6.0 (c 0.1, MeOH) ; ¹H NMR (400 MHz, CDCl₃): 3.59 (m, 1H, C3-H), 1.88-1.76 (m, 2H), 1.75-1.52 (m, 7H), 1.48-1.06(m, 10H), 1.18 (s, 3H, C20-H₃), 1.03-0.89 (m, 2H), 0.82 (s, 3H, C19-H₃), 0.73-0.60 (m, 1H), 0.67 (s, 3H, C-18₃), 2 x OH not observed; ¹³C NMR (100 MHz, CDCl₃): 82.3 (C17), 71.5 (C3), 54.4, 50.1, 46.9, 45.1, 38.5, 38.4, 37.2, 36.1, 35.7, 32.4, 31.7, 30.1, 28.9, 24.1, 22.8, 21.0(C20), 16.1 (C18), 12.5 (C19); LRMS (+EI): *m/z* 306 (20%, [C₂₀H₃₄O₂]⁺), 291 (30%), 273 (100%), 255 (25%), 215 (20%), 161 (25%), 147 (25%), 107 (35%), 105 (30%), 93 (35%), 91 (35%), 79 (25%), 67 (25%), 43 (20%); HRMS (+EI): found 306.2557 [C₂₀H₃₄O₂]⁺ requires 306.2559.

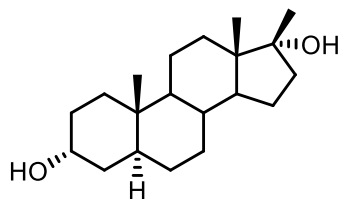
3.6.2.7 17 α -Hydroxy-17 β -methyl-5 α -androstan-3-one (E3)^[35]



A solution of 17 β -methyl-5 α -androstan-3 β ,17 α -diol (**D4**) (64.2 mg, 0.21 mmol) in CHCl₃ (2 mL), was treated with PCC (228 mg, 1.06 mmol), silica (300 mg) and stirred at room temperature overnight. The reaction was diluted with diethyl ether (20 mL) and filtered. The solid residue was washed with diethyl ether (5 x 20 mL) and combined organic extract was washed with aqueous HCl solution (2 M, 2 x 50 mL), water (2 x 50 mL) and saturated aqueous NaCl solution (50 mL). Then the organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexane) to afford the title compound (31 mg, 49%) as a white solid. Rf 0.63 (40% EtOAc/hexane); mp 202-205 °C. (lit^[35] 224-226 °C); $[\alpha]^{25}_D$ +20.7 (c 0.10, CHCl₃); ¹H NMR (400 MHz, CDCl₃): 2.44-2.22 (m, 3H), 2.12-1.99 (m, 2H), 1.86 (m, 1H), 1.75-1.60 (m, 5H), 1.57-1.23 (m, 8H), 1.19 (s, 3H, C20-H₃), 1.18-1.10 (m, 1H), 1.02 (s, 3H, C18-H₃), 1.01-0.92 (m, 1H), 0.78 (m, 1H), 0.70 (s, 3H, C19-H₃), OH not observed; ¹³C NMR

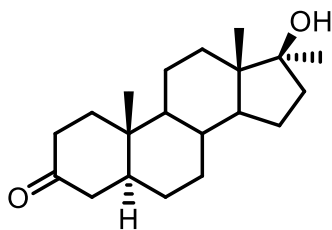
(100 MHz, CDCl₃): 212.2 (C3), 82.3 (C17), 53.8, 49.9, 46.9, 46.8, 44.9, 38.8, 38.5, 38.3, 36.0, 35.9, 32.0, 30.0, 29.1, 24.1, 22.8, 21.1 (C20), 16.1 (C18), 11.7 (C19). LRMS (+EI): *m/z* 304 (10%, [C₂₀H₃₂O₂]⁺), 289 (30%), 286 (25%), 247 (25%), 231 (30%), 163 (20%), 161 (30%), 107 (20%), 105 (25%), 93 (25%), 91 (30%), 79 (30%), 67 (20%), HRMS (+EI): found 304.2401 [C₂₀H₃₂O₂]⁺ requires 304.2402.

3.6.2.8 17β-Methyl-5α-androstane-3α,17α-diol (D5)^[35]



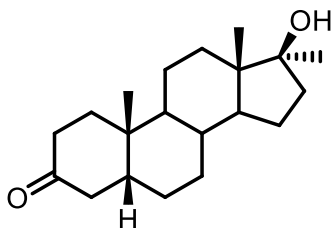
A solution of 17α-hydroxy-17β-methyl-5α-androstan-3-one (**E3**) (8.3 mg, 27 μmol) in THF (1 mL) was treated with a solution of L-Selectride® in anhydrous THF (1.0 M, 140 μL, 140 μmol), and stirred at room temperature for 2 h. The reaction was quenched with aqueous hydrochloric acid (2 M, 1 mL) and purified by SPE. A C18-SPE cartridge was pre-conditioned with methanol (3 mL) and water (3 mL), and the reaction mixture was then loaded. The sample was washed with water (2 mL) until neutral, and eluted with methanol (2 mL). Concentration of the methanol fraction afforded the title compound **D5** and its 3β-isomer in 7:1 ratio. (5.3 mg, 64%) as a white solid. *R_f* 0.7 (40% EtOAc/hexane); mp 195-198 °C. (lit 188-189 °C); [α]_D²⁵ +26.7 (c 0.10, [CHCl₃]); ¹H NMR (400 MHz, CDCl₃): 4.04 (m, 1H), 1.84 (m, 1H), 1.75-1.20 (m, 19H), 1.18 (s, 3H, C20-H₃), 1.16-1.08 (m, 1H), 1.06-0.94 (m, 1H), 0.80 (s, 3H, C19-H₃), 0.67 (s, 3H, C18-H₃), 2 x OH not observed; ¹³C NMR (100 MHz, CDCl₃): 82.3 (C3), 66.7 (C17), 54.3, 50.1, 46.8, 39.4, 38.5, 36.4, 36.1, 36.1, 32.4, 32.3, 30.1, 29.2, 28.7, 24.1, 22.8, 20.5 (C20), 16.1 (C19), 11.4 (C18). LRMS (+EI): *m/z* 306 (15%, [C₂₀H₃₄O₂]⁺), 291 (42%), 273 (100%), 255 (75%), 233 (25%), 230 (32%), 215 (35%), 161 (32%), 147 (35%), 135 (25%), 121 (30%), 105 (42%), 93 (50%), 79 (48%), 67 (35%), 43 (28%) HRMS (+EI): found 306.2558 [C₂₀H₃₄O₂]⁺ requires 306.2559.

3.6.2.9 17 β -Hydroxy-17 α -methyl-5 α -androstan-3-one (E1)^[39]



A solution of 17 α -methyl-5 α -androstan-3 β ,17 β -diol **D1** (72.1 mg, 0.23 mmol) in CHCl₃ (10 mL), was treated with PCC (258 mg, 1.20 mmol), silica (500 mg) and stirred at room temperature overnight. The reaction was diluted with diethyl ether (20 mL) and filtered. The solid residue was washed with diethyl ether (5 x 20 mL) and combined organic extract was washed with aqueous HCl solution (2 M, 2 x 30 mL), water (2 x 30 mL) and saturated aqueous NaCl solution (50 mL). Then the organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexane) to afford the title compound (37 mg, 52%) as a white solid. *R*_f 0.57 (40% EtOAc/hexane); mp 178-183 °C (lit^[39] 188-190 °C); [α]²⁵_D +16.7 (c 0.17, CHCl₃) (lit^[39] [α]²⁵_D 10.5°[CHCl₃]); ¹H NMR (400 MHz, CDCl₃): 2.45-2.20 (m, 3H), 2.12-1.99 (m, 2H), 1.86-1.69 (m, 3H), 1.65-1.15 (m, 12H), 1.21(s, 3H, C20-H₃), 1.03 (s, 3H, C18-H₃), 0.95-0.82 (m, 1H), 0.88 (s, 3H, C19-H₃) 0.72 (m, 1H), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 212.1 (C3), 81.8 (C17), 54.0, 50.7, 47.0, 45.7, 44.9, 39.1, 38.8, 38.3, 36.4, 35.9, 31.8, 31.6, 29.0, 26.0, 23.4, 21.2 (C20), 14.1 (C19), 11.7 (C18); LRMS (+EI): *m/z* 304 (40%, [C₂₀H₃₂O₂]⁺), 290 (20%), 289 (100%), 286 (45%), 271 (70%), 247 (75%), 246 (25%), 244 (30%), 231 (80%), 230 (20%), 229 (35%), 215 (25%), 163 (50%), 161 (30%), 165 (20%), 147 (20%), 133 (20%), 123 (40%), 121 (25%), 109 (25%), 107 (35%), 105 (35%), 95 (30%), 93 (40%), 91 (50%), 81 (50%), 79 (50%), 77 (25%), 71 (35%), 67 (45%), 55 (60%), 43(55%), 41 (35%) ; HRMS (+EI): found 304.2407 [C₂₀H₃₂O₂]⁺ requires 304.2402.

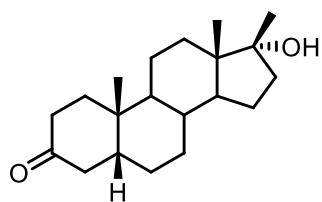
3.6.2.10 17 β -Hydroxy-17 α -methyl-5 β -androstan-3-one (E2)^{[35],[40]}



A solution of 17 α -methyl-5 β -androstan-3 α ,17 β -diol **D3** (66.2 mg, 0.21 mmol) in CHCl₃ (2 mL), was treated with PCC (240 mg, 1.11 mmol), silica (300 mg) and stirred at room temperature overnight. The reaction was diluted with diethyl ether (20 mL) and filtered. The solid residue was washed with diethyl ether (5 x 20 mL) and combined organic extract was washed with aqueous HCl solution (2 M, 2 x 50 mL), water (2 x 50 mL) and saturated

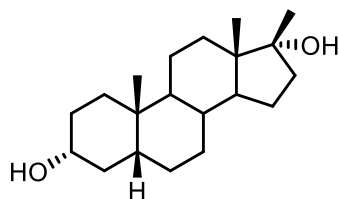
aqueous NaCl solution (50 mL). Then the organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexane) to afford the title compound (26 mg, 40%) as a white solid. Rf 0.73 (40% EtOAc/hexane); mp 145-150°C. (lit^[40] 119-121 °C); $[\alpha]^{25}_{\text{D}} +56.3$ (c 0.10, CHCl₃); ¹H NMR (400 MHz, CDCl₃): 2.67 (m, 1H), 2.32 (m, 1H), 2.16 (m, 1H), 2.07-1.98 (m, 2H), 1.92-1.68 (m, 4H), 1.65-0.8 (m, 13H), 1.22 (s, 3H, C20-H₃), 1.03 (s, 3H, C18-H₃), 0.86 (s, 3H, C19-H₃), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 213.3 (C3), 81.8 (C17), 50.8, 45.8, 44.4, 42.5, 41.0, 39.1, 37.3, 37.2, 36.6, 35.1, 31.9, 26.7, 25.9, 25.7, 23.4, 22.8 (C20), 20.9 (C18), 14.1 (C19). LRMS (+ESI): *m/z* 305 (37%, [C₂₀H₃₃O₂]⁺), 287 (85%), 269 (85%), HRMS (+ESI): found 327.2308 [C₂₀H₃₂O₂+Na]⁺ requires 327.2300.

3.6.2.11 17 α -Hydroxy-17 β -methyl-5 β -androstan-3-one (E4)^[35]



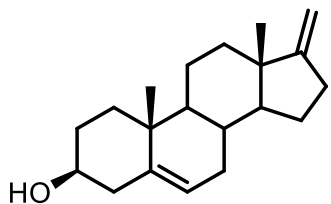
A solution of 17 β -hydroxy-17 α -methyl-5 β -androstan-3-one **E2** (62 mg, 0.204 mmol) in DMF (3 mL), was treated with SO₃.Py (300 mg, 1.92 mmol), and stirred at room temperature for one hour. The reaction was treated with water (5 mL) and stirred overnight. The reaction was diluted with water (5 mL) and extracted with EtOAc (3x10 mL). Then the organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (20% EtOAc/hexane) to afford the title compound (30 mg, 61%) as a white solid. Rf 0.26(40% EtOAc/hexane); mp 150-156°C. (lit 133-134 °C); ¹H NMR (400 MHz, CDCl₃): 2.67 (m, 1H), 2.32 (m, 1H), 2.16 (m, 1H), 2.09-1.99 (m, 2H), 1.92-1.78 (m, 4H), 1.76-1.62 (m, 3H), 1.59-1.49 (m, 5H), 1.47-1.33 (m, 4H), 1.20 (s, 3H, C20-H₃), 1.03 (s, 3H, C19-H₃), 1.00-0.86 (m, 1H), 0.70 (s, 3H, C18-H₃), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 213.4 (C3), 82.3 (C17), 50.1, 46.2, 44.6, 42.5, 40.8, 38.6, 37.4, 37.3, 36.1, 35.1, 30.2, 26.78, 26.1, 24.0, 22.9, 22.8 (C20), 20.9 (C19), 16.1 (C18). LRMS (+ESI): *m/z* 327 (100%, [C₂₀H₃₂O₂Na]⁺); HRMS (+EI): found 304.2401 [C₂₀H₃₂O₂]⁺ requires 304.2402.

3.6.2.12 17 β -methyl-5 β -androstan-3 α ,17 α -diol (**D6**)^[35]



A solution of 17 α -Hydroxy-17 β -methyl-5 β -androstan-3-one (**E4**) (6 mg, 19.7, μ mol) in methanol (1.2 mL) was treated with sodium borohydride (8 mg, 211 μ mol) and stirred at room temperature for 15 minutes. The reaction was quenched with aqueous hydrochloric acid (2 M, 1 mL) and purified by SPE. A C18-SPE cartridge was pre-conditioned with methanol (3 mL) and water (3 mL), and the reaction mixture was then loaded. The sample was washed with water (2 mL) until neutral, and eluted with methanol (2 mL). Concentration of the methanol fraction afforded the title compound **D6** and its 3 β -isomer in 6:1 ratio. (5 mg, 81%) as a white solid. *R*_f 0.21 (40% EtOAc/hexane); mp 180-182 °C. [lit^[35] 164-165 °C]; [α]_D²⁵ +30.2 (c 0.10, CHCl₃), ¹H NMR (400 MHz, CDCl₃): 3.62 (m, 1H, C3-H), 1.90-1.58 (m, 8H), 1.55-0.85 (m, 14H), 1.19 (s, 3H, C20-H₃), 0.94 (s, 3H, C19-H₃), 0.66 (s, 3H, C18-H₃), 2 x OH not observed; ¹³C NMR (100 MHz, CDCl₃): 82.4 (C3), 72.0 (C17), 50.2, 46.9, 42.4, 40.6, 38.6, 36.7, 36.5, 35.6, 34.8, 30.7, 30.3, 27.3, 26.8, 24.1, 23.5, 22.8 (C20), 20.5 (C19), 16.1 (C18). LRMS (+EI): *m/z* 306 (3%, [C₂₀H₃₄O₂]⁺), 288 (30%), 273 (45%), 270 (30%), 255 (60%), 231 (30%), 230 (100%), 217 (35%), 215 (50%), 147 (28%), 135 (30%), 121 (30%), 107 (30%), 105 (33%), 93 (32%), 91 (50%), 81 (33%), 79 (48%), 67 (30%), HRMS (+EI): found 306.2555 [C₂₀H₃₄O₂]⁺ requires 306.2559.

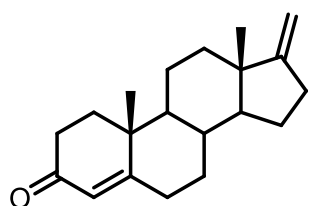
3.6.2.13 17-Methylidene-5-en-3 β -ol (**A3**)^[38]



A solution of potassium tert-butoxide (1.98 g, 17.7 mmol) in dry THF (30 mL) under nitrogen was added to a slurry of dehydroepiandrosterone (DHEA) (1.03 g, 3.56 mmol) and MePPh₃Br (3.78 g, 10.6 mmol) in THF (20 mL) under nitrogen which had been cooled at 0 °C (brine/ice). The reaction was stirred at 0 °C for 60 minutes and then stirred at room temperature overnight. The reaction was diluted with water (100 mL) and extracted with ethyl acetate (3 x 100 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (20% EtOAc/hexane) to afford the title compound (898 mg, 90%) as a colourless solid. *R*_f 0.65 (40% EtOAc/hexane); mp 130-135 °C [lit^[38] 132-133

$^{\circ}\text{C}$); $[\alpha]^{25}_{\text{D}} -81.6$ (c 1.0, MeOH) (lit^[38] -66, MeOH); ^1H NMR (400 MHz, CDCl_3): 5.36 (d, $J = 5.3$ Hz, 1H, C6-H), 4.69-4.60 (m, 2H, C20-H), 3.53 (m, 1H, C3-H), 2.50 (m, 1H, C16-H), 2.34-2.19 (m, 3H), 2.04 (m, 1H), 1.91-1.81 (m, 3H), 1.76-1.44 (m, 8H), 1.34-1.20 (m, 2H), 1.15-0.90 (m, 2H), 1.03 (s, 3H, C18- H_3), 0.80 (s, 3H, C19- H_3); ^{13}C NMR (100 MHz, CDCl_3): 161.9 (C17), 141.01 (C5), 121.6 (C6), 101.0 (C20), 71.9 (C3), 54.9, 50.5, 44.0, 42.5, 37.4, 36.8, 35.7, 32.0, 31.9, 31.8, 29.6, 24.4, 21.1, 19.6 (C19), 18.4 (C18); LRMS (+EI): m/z 286 (60%, $[\text{C}_{20}\text{H}_{30}\text{O}]^+$), 271 (35%), 268 (50%), 254 (30%), 253 (85%), 227 (30%), 201 (40%), 175 (35%), 173 (20%), 171 (20%), 160 (25%), 159 (35%), 158 (30%), 147 (30%), 145 (55%), 143 (30%), 133 (25%), 131 (25%), 128 (20%), 121 (20%), 119 (35%), 117 (45%), 115 (20%), 107 (70%), 105 (60%), 93 (35%), 91 (100%), 79 (60%), 77 (35%), 67 (35%), 57 (45%), 55 (30%), 41 (30%); HRMS (+EI): found 286.2299, $[\text{C}_{20}\text{H}_{30}\text{O}]^+$ requires 286.2297.

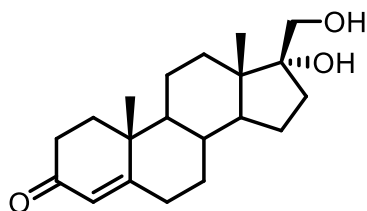
3.6.2.14 17-Methylideneandrost-4en-3-one (A4)^[41]



Oxalyl chloride (450 μl , 5.32 mmol) was added to a solution of DMSO (700 μl , 9.85 mmol) in CH_2Cl_2 (2.5 mL) cooled to -78°C in a dry ice/acetone bath, and was stirred for 15 min. Then 17-methylidene-androst-5-en-3 β -ol **A3** (238 mg, 0.83 mmol) dissolved in CH_2Cl_2 (3.5 mL) was added dropwise within 1 min, continued stirring for 2 hours. Triethylamine (1.5 mL, 10.8 mmol) was added to quench the reaction and stirred for further 15 min at -78°C . Then it was allowed to reach room temperature, diluted with water (20 mL) and extracted with CH_2Cl_2 (3 x 20 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated under required pressure. The crude residue was purified by column chromatography (10% EtOAc/hexane) to afford the title compound (135 mg, 57%) as an off-white solid. R_f 0.8 (40% EtOAc/hexanes); mp $120-125^{\circ}\text{C}$ (lit^[41] $134-135^{\circ}\text{C}$); $[\alpha]^{25}_{\text{D}} +85.3$ (c 1.0, MeOH) (lit^[41] $+131$, EtOH); ^1H NMR (400 MHz, CDCl_3): 5.73 (s, 1H, C4-H), 4.65 (m, 1H, C20-H), 4.63 (m, 1H, C20-H), 2.58-2.18 (m, 6H), 2.04 (m, 1H), 1.95-1.83 (m, 2H), 1.80-1.40 (m, 6H), 1.36-1.22 (m, 2H), 1.20 (s, 3H, C18- H_3), 1.12-0.90 (m, 2H), 0.83 (s, 3H, C19- H_3); ^{13}C NMR (100 MHz, CDCl_3): 199.7 (C3), 171.4 (C5), 161.3 (C17), 124.0 (C4), 101.3 (C20), 54.1, 54.0, 44.0, 38.8, 35.9, 35.7, 35.5, 34.1, 33.0, 32.0, 29.4, 24.3, 21.1, 18.5 (C18), 17.6 (C19); LRMS

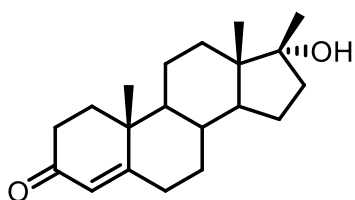
(+EI): m/z 284 (50%, $[C_{20}H_{28}O]^+$), 266 (60%), 171 (20%), 161 (35%), 160 (25%), 147 (25%), 131 (25%), 119 (35%), 107 (25%), 105 (60%), 93 (50%), 91 (100%), 79 (60%), (30%), 41 (20%); HRMS (+EI): found 284.2144, $[C_{20}H_{28}O]^+$ requires 284.2140.

3.6.2.15 17 α -Hydroxy-17 β -hydroxymethylandro-4-en-3-one (A5)^[41]



A solution of 17-methylideneandro-4-en-3-one **A4** (315 mg, 1.10 mmol) in THF/*t*-BuOH/Acetone/H₂O (1:1:1:1, 8 mL) was treated with citric acid dihydrate (513 mg, 2.44 mmol), potassium osmate dihydrate (19.8 mg, 53.7 μ mol), and NMO (131 mg, 1.12 mmol) respectively and stirred at room temperature overnight. The reaction was treated with Na₂SO₃ (10% w/v, 30 mL) and extracted with ethyl acetate (3 x 30 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated under required pressure. The crude residue was purified by column chromatography (70% EtOAc/hexane) to afford the title compound (210 mg) as a pale yellow oil. *R*_f 0.10 (40% EtOAc/hexane); $[\alpha]^{25}_D +72.8$ (c 1.0, MeOH) (lit^[41] +51, EtOH); ¹H NMR (400 MHz, CDCl₃): 5.73 (s, 1H, C4-H), 3.75 (d, *J* = 10.9 Hz, 1H, C20-H), 3.58 (d, *J* = 10.9 Hz, 1H, C20-H), 2.50-2.20 (m, 4H), 2.06-1.96 (m, 3H), 1.90-1.52 (m, 10H), 1.43 (m, 1H), 1.28-1.16 (m, 1H), 1.19 (s, 3H, C19-H₃), 1.09 (m, 1H), 0.98 (m, 1H), 0.79 (s, 3H, C18-H₃); ¹³C NMR (100 MHz, CDCl₃): 199.8 (C3), 171.5 (C5), 124.0 (C4), 83.7 (C17), 66.7 (C20), 53.8, 50.1, 46.3, 38.8, 35.8, 35.8, 34.6, 34.1, 33.0, 32.2, 31.4, 24.0, 20.7, 17.6 (C19), 15.1 (C18); LRMS (+EI): m/z 318 (100%, $[C_{20}H_{30}O_3]^+$), 300 (45%), 287 (100%), 269 (40%), 229 (65%), 147 (30%), 143 (20%), 124 (25%), 119 (25%), 105 (45%), 91 (70%), 79 (35%), 77 (30%), 55 (30%), 41 (25%); HRMS (+EI): found 318.2200 $[C_{20}H_{30}O_3]^+$ requires 318.2195.

3.6.2.16 17 α -Hydroxy-17 β -methylandro-4-en-3-one(epi-MT)^[41]

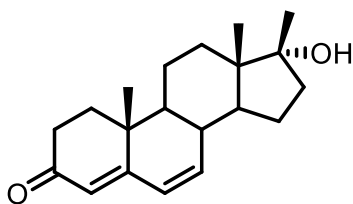


17 α -Hydroxy-17 β -hydroxymethylandro-4-en-3-one **A5** (130 mg, 408 μ mol) and *p*-toluenesulfonyl chloride (390 mg, 2.04 mmol) under nitrogen were dissolved in dry pyridine (4 mL) was stirred at room temperature

overnight. The reaction was then treated with aqueous NaOH solution (5M, 10 mL, 0.05 mol) and stirred for 30 minutes. Then it was diluted with EtOAc (20 mL) and washed with water (3 x 20 mL) and saturated aqueous NaCl solution (20 mL). The organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue, 17 α ,17 β -(epoxymethano)-androst-4-en-3-one was obtained as a yellow colour solid (150 mg) and this was used without further purification. R_f 0.53 (40% EtOAc/hexane); ¹H NMR (400 MHz, CDCl₃): 5.71 (s, 1H, C4-H), 2.73 (d, *J* = 4.4 Hz, 1H, C20-H), 2.65 (d, *J* = 4.4 Hz, 1H, C20-H), 2.45-2.23 (m, 4H), 2.01 (m, 1H), 1.93-1.82 (m, 2H), 1.69 (m, 1H), 1.63-1.49 (m, 3H), 1.45-1.23 (m, 4H), 1.21-1.18 (m, 1H), 1.12-1.03 (m, 1H), 1.01-0.91 (m, 1H), 0.81-0.66 (m, 1H), 1.17 (s, 3H, C19-H₃), 0.84 (s, 3H C18-H₃), OH not observed. Lithium aluminium hydride (85.0 mg, 2.24 μ mol) under nitrogen was treated with crude 17 α ,17 β -(epoxymethano)-androst-4-en-3-one (150 mg, 449 μ mol) dissolved in dry THF (6 mL) and stirred overnight. The reaction was diluted with ethylacetate while keeping the reaction at 0 °C (ice/brine) and Rochel solution (15% w/v, sodium potassium tartrate tetra hydrate, 20 mL) was added. The reaction mixture was extracted with EtOAc (3 x 20 mL). The combined organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was used without further purification. R_f 0.35 (40% EtOAc/hexane); ¹H NMR (400 MHz, CDCl₃): 5.28 (d, 1H, C4-H), 4.18-4.12 (m, 1H, C3-H), 2.20 (m, 1H), 2.10-0.60 (m, 18H), 1.19 (s, 3H, C20-H₃), 1.07(s, 3H, C19-H₃), 0.70 (s, 3H, C18-H₃), 2xOH not observed. Crude mixture of 17 α -hydroxy-17 β -methylandrost-4-en-3-ol (mixture of 3 α , 3 β isomers) (191 mg, 627 μ mol) in CHCl₃ (30 mL), was treated with PCC (676 mg, 3.14 mmol), silica (1 g) and stirred at room temperature overnight. The reaction was diluted with diethyl ether (20 mL) and filtered. The solid residue was washed with diethyl ether (5 x 20 mL) and combined organic extract was washed with aqueous HCl solution (2 M, 2 x 50 mL), water (2 x 50 mL) and saturated aqueous NaCl solution (50 mL). Then the organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexane) to afford the title compound (134 mg) as a colourless solid. R_f 0.65 (40% EtOAc/hexane); mp 174-176 °C. (lit^[41] 181-182 °C); [α]_D²⁵ +65.0 (c 0.3, EtOH) (lit^[41] +68, EtOH); ¹H NMR (400 MHz, CDCl₃): 5.73 (s, 1H, C4-H), 2.48-2.34 (m, 3H), 2.29 (m, 1H), 2.03 (m, 1H), 1.93-1.83 (m, 2H), 1.8-1.34 (m, 10H), 1.21 (s, 3H, C20-H₃), 1.20 (s, 3H, C19-H₃), 1.12-1.02

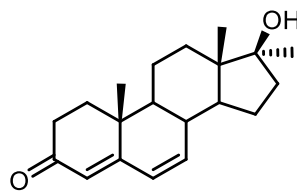
(m, 1H), 0.97 (m, 1H), 0.73 (s, 3H, C18-H₃), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 199.71 (C3), 171.57 (C5), 124.01 (C4), 82.10 (C17), 53.8, 49.5, 46.6, 38.8, 38.5, 36.2, 35.9, 34.1, 33.1, 32.3, 29.8, 24.0, 22.9, 20.7 (C20), 17.6 (C19), 16.0 (C18); LRMS (+EI): *m/z* 302 (100%, [C₂₀H₃₀O₂]⁺), 284 (20%), 269 (50%), 245 (20%), 229 (25%), 124 (25%), 105 (25%), 93 (25%), 91 (25%), 79 (30%), 77 (25%), 43(25%); ; HRMS (+EI): found 302.2245 [C₂₀H₃₀O₂]⁺ requires 302.2246.

3.6.2.17 17 α -Hydroxy-17 β -methylandrosta-4,6-dien-3-one^[15]



A solution of 17 α -hydroxy-17 β -methylandrost-4-en-3-one (80 mg, 0.26 mmol) in *tert*-butanol (6 mL) was treated with chloranil (322 mg, 1.31 mmol). The resulting slurry was heated to 100 °C in a sealed tube and stirred for 2 hours. Excess chloranil was filtered off and filtrate was extracted with DCM (3 x 30 mL). The combined extract was washed with aqueous NaOH solution (1M, 30 mL) and aqueous NaOH fraction was re-extracted with DCM (2 x 30 mL). The combined organics were washed with water (50 mL), saturated aqueous NaCl solution (50 mL), dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexane) to afford the title compound (52 mg, 65%) as a yellow solid. *R_f* 0.48 (40% EtOAc/hexane); mp 155-160 °C; [α]_D²⁵ -18.3 (c 0.3, MeOH) ; ¹H NMR (400 MHz, CDCl₃): δ 6.15 (.dd, *J* = 9.8, 1.8 Hz, 1H, C7-H), 6.09 (dd, *J* = 9.8, 2.5 Hz, 1H, C6-H), 5.66 (s, 1H, C4-H), 2.57 (m, 1H, C2-H), 2.42 (m, 1H, C2-H), 2.23 (t, *J* = 10.5 Hz, 1H, C8-H), 2.00 (m, 1H), 1.95-1.82 (m, 2H), 1.77-1.61 (m, 3H), 1.58-1.30 (m, 5 H), 1.28-1.18 (m, 1H), 1.23 (s, 3H, C20-H₃), 1.12 (s, 3H, C19-H₃), 0.77 (s, 3H, C18-H₃), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 199.8 (C3), 164.1 (C5), 141.6 (C7), 127.9 (C6), 123.7 (C4), 81.8 (C17), 50.7, 47.6, 47.2, 38.5, 38.2, 36.3, 34.1 (2C), 29.8, 23.5, 22.9, 20.3 (C20), 16.5 (C19), 15.8 (C18); LRMS (+EI): *m/z* 300 (1%, [C₂₀H₂₈O₂]⁺), 282 (45%), 267 (100%), 147 (15%), 173 (10%), 131 (15%), 129 (10%), 128 (10%), 117(10%), 115 (15%), 91 (15%), 79 (10%), 43 (10%); HRMS (+EI): found 300.2083 [C₂₀H₂₈O₂]⁺ requires 300.2089.

3.6.2.18 17 β -Hydroxy-17 α -methylandrosta-4,6-dien-3-one^{[15],[42]}



A solution of 17 β -hydroxy-17 α -methylandrost-4-en-3-one (80 mg, 0.26 mmol) in *tert*-butanol (6 mL) was treated with chloranil (321.5 mg, 1.308 mmol). The resulting slurry was heated to 100 °C in a sealed tube and stirred for 2 hours. Excess chloranil was filtered off and filtrate was extracted with DCM (3 x 30 mL). The combined extract was washed with aqueous NaOH solution (1M, 30 mL) and aqueous NaOH fraction was re-extracted with DCM (2 x 30 mL). The combined organics were washed with water (50 mL), saturated aqueous NaCl solution (50 mL), dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexane) to afford the title compound (52 mg, 65%) as a yellow solid. R_f 0.48 (40% EtOAc/hexane); mp 178-182 °C (lit^[42] 193-196 °C); [α]_D²⁵ +20 (c 1.0 CHCl₃) (lit^[42] +35, CHCl₃); ¹H NMR (400 MHz, CDCl₃): 6.15 (d, *J* = 10.9 Hz, 1H, C7-H), 6.09 (dd, *J* = 9.8, 2.4 Hz, 1H, C6-H), 5.66 (s, 1H, C4-H), 2.57 (m, 1H, C2-H), 2.42 (m, 1H, C2-H), 2.23 (t, *J* = 10.5 Hz, 1H, C8-H), 2.05-1.82 (m, 4H), 1.77-1.61 (m, 3H), 1.58-1.28 (m, 6 H), 1.23 (s, 3H, C20-H₃), 1.12 (s, 3H, C19-H₃), 0.77 (s, 3H, C18-H₃); ¹³C NMR (400 MHz, CDCl₃): 199.7 (C3), 163.9 (C5), 140.7 (C7), 128.0 (C6), 123.8 (C4), 81.4 (C17), 50.9, 48.0, 46.5, 38.9, 38.5, 36.3, 34.1 (2C), 31.5, 26.0, 22.9, 20.4 (C20), 16.5 (C19), 13.9 (C18); LRMS (+EI): *m/z* 300 (1%, [C₂₀H₂₈O₂]⁺), 282 (45%), 267 (100%), 147 (15%), 173 (10%), 131 (15%), 129 (10%), 128 (10%), 117 (10%), 115 (15%), 91 (15%), 79 (10%), 43 (10%); HRMS (+EI): found 300.2083 [C₂₀H₂₈O₂]⁺ requires 300.2089.

3.6.2.19 Synthesis of steroid sulfates - general method

A solution of steroid (5.0 mg) in DMF (1ml) was treated with SO₃.Py (25 mg) and stirred for 1 hour. The reaction products were purified by SPE WAX cartridge. First, WAX SPE cartridge (6 cc) was preconditioned with methanol (6 mL) followed by water (12 mL). The reaction mixture was loaded followed by washes with formic acid in water (2% v/v, 12 mL), water (12 mL), methanol (12 mL) and saturated aqueous ammonia solution in methanol (5% v/v, 12 mL) were then loaded and eluted under pressure of N₂ respectively. The methanolic ammonia fraction was

concentrated under reduced pressure to yield the desired steroid sulfate as the corresponding ammonium salt.

High resolution MS data and chemical shifts of C3-H and C20-CH₃ for the six sulfates are given in table 3.12.

3.6.2.20 Synthesis of steroid glucuronides - general method

Steroid 1 mg was dissolved in *tert*-butanol 500 μ L and sodium phosphate buffer (3.22 mL, 50 mM, pH 7.5). Glucuronylsynthase (0.92 mL, 1.09 mg mL⁻¹, final concentration 0.2 mg mL⁻¹) and α -D-glucuronyl fluoride (5 equivalents) dissolved in sodium phosphate buffer (365 μ L, 50 mM) were added and the reaction was incubated without agitation at 37 °C for 2 days. The reaction was then subjected to solid-phase extraction. An Oasis WAX SPE cartridge (3 mL) was pre-conditioned with methanol (3 mL) and milliQ water (3 mL). The crude reaction was loaded onto the cartridge and eluted under pressure of N₂. Then the column was washed with aqueous formic acid (3 mL, 2% v/v), milliQ water (3 mL), and finally the products were eluted with saturated aqueous ammonium hydroxide in methanol (6 mL, 5% v/v). The ammonium hydroxide methanol fraction was concentrated under reduced pressure to yield the desired steroid glucuronide as the corresponding ammonium salt.

High-resolution MS data for the six glucuronides are given in table 3.13.

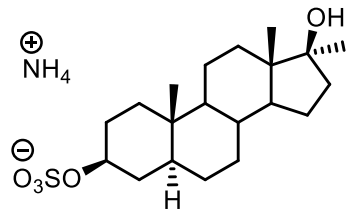
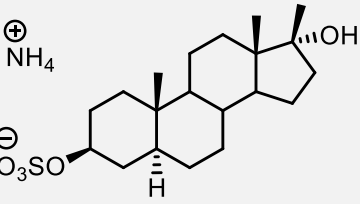
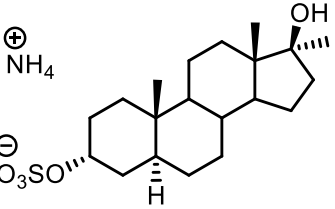
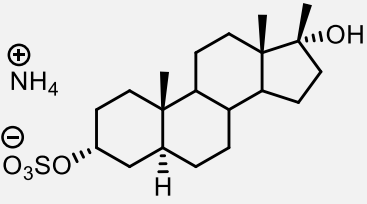
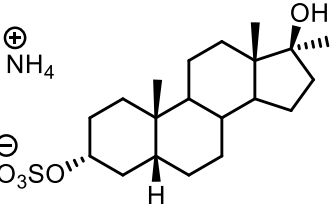
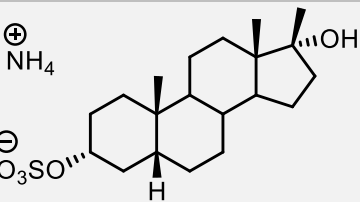
Structure	Shortened name given before	High resolution MS	¹ H-NMR shifts	
			C3-H	C20-CH ₃
	S1	found 385.2056 requires 385.2054	4.25	1.18
	S2	found 385.2060 requires 385.2054	4.25	1.15
	S3	found 385.2056 requires 385.2054	4.49	1.19
	S4	found 385.2057 requires 385.2054	4.49	1.15
	S5	found 385.2056 requires 385.2054	4.28	1.21
	S6	found 385.2056 requires 385.2054	4.29	1.00

Table 3.12: High-resolution MS data and chemical shifts of C3-H and C20-CH₃ of the six sulfates synthesized

Structure	Shortened name given before	High resolution MS
	G1	found 481.2816 requires 481.2809
	G2	found 481.2812 requires 481.2809
	G3	found 481.2815 requires 481.2809
	G4	found 481.2806 requires 481.2809
	G5	found 475.2344 requires 475.2338
	G6	found 475.2341 requires 475.2338

Table 3.13: High-resolution MS data of the six glucuronides synthesised

3.6 References

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CHAPTER 4

Synthesis of a phase I hemapolin metabolite

4.1 Introduction

Episulfides also known as thiiranes, are a class of substances not found in typically nature, but instead are the products of chemical synthesis. Among those episulfide containing compounds, steroid episulfides play an important role in medicinal chemistry and the pharmaceutical industry.^[1]

4.1.1 Steroid episulfides

Steroid episulfides or epithio steroids are steroids having an epithio ring attached to the steroid backbone. There are number of studies that have been carried out on epithio steroids.^{[2],[3],[4]} These steroids exhibit number of fascinating physiological and biological properties.^[5] Out of those, epitiostanol is a steroid that has been investigated in the most detail. Previous studies have shown that epitiostanol and a prodrug of epitiostanol, mepitiostane (shown in figure 4.1), have anti-estrogenic activity ^{[6],[7],[8]} that can be used in the treatment of breast cancer. Most of those studies were carried out from the 1970's to 1990's and were related to the anti-cancer activity of the epithio steroids as experimental drugs.

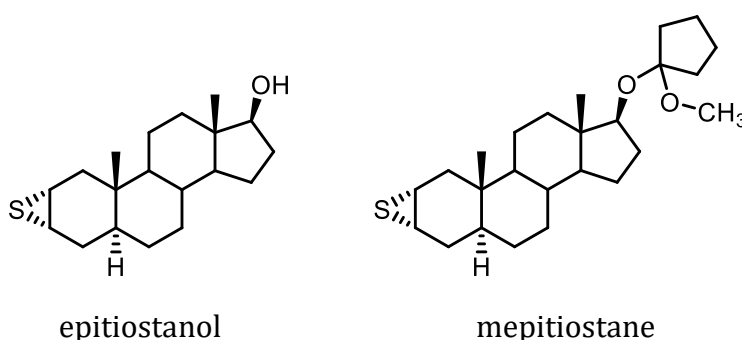


Figure 4.1: Epitiostanol and a pro-drug mepitiostane

Later in 2015, Okano *et al.* conducted a human drug administration study for mepitiostane and identified the major urinary metabolites by LC-MS. In this study, three male volunteers were given 10 mg of the steroid and urine was collected for the first 48 hours. The main metabolites observed by this study were derived from

acetal cleavage to give epitiostanol, together with oxidation to provide epitiostanol *S*-oxide and dethionylation to give 5 α -androst-2-en-17 β -ol.^[9] The sulfoxide was detected up to 48 hours in all three volunteers. This revealed that epitiostanol *S*-oxide was a relatively stable metabolite of mepitiostane.

Even though epitiostanol *S*-oxide and epitiostanol were detected shortly after administration, they did not serve as long-term markers, presumably due to metabolic conversion to 5 α -androst-2-en-17 β -ol. Nevertheless, 5 α -androst-2-en-17 β -ol cannot be used as a definite marker for the detection of mepitiostane or epitiostanol misuse, as it could also arise through administration of 5 α -androst-2-en-17 β -ol itself.

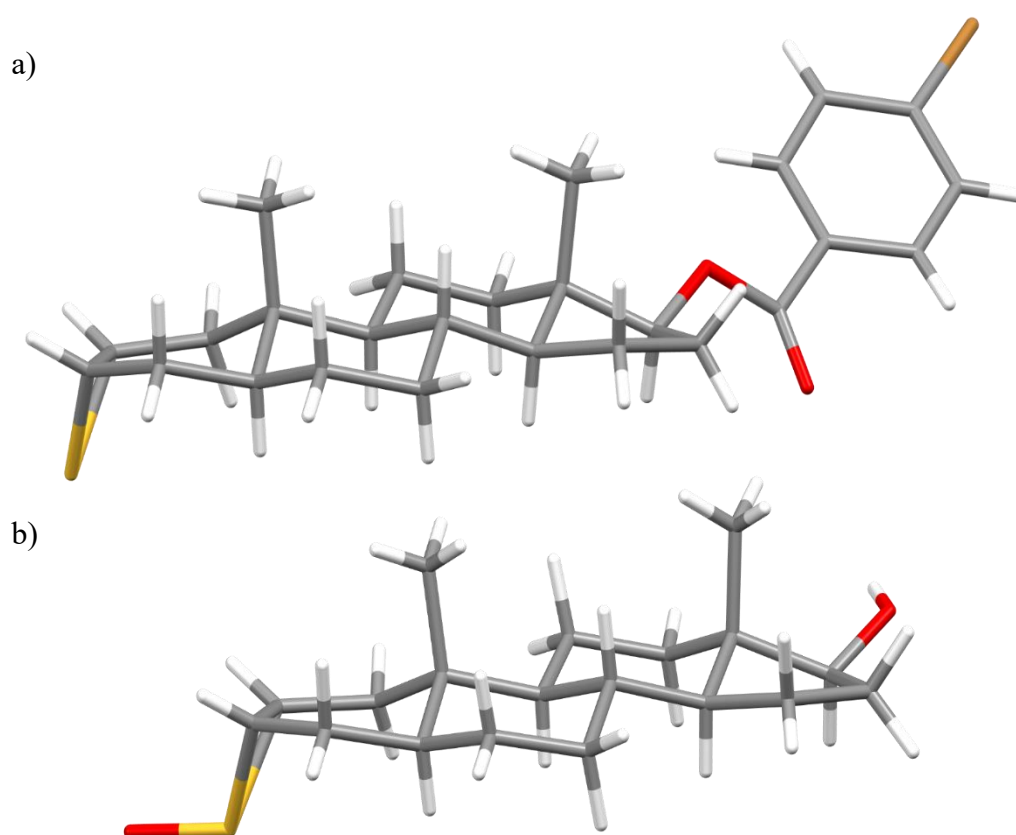


Figure 4.2: a) Single crystal X-ray structure of epitiostanol 17-*p*-bromobenzoate (CSD entry ETANDB) shown in stick representation. The figure was generated from the CIF using Mercury 4.2.0 (build 257471) and required recalculation of hydrogen atom coordinates as these were absent in the CSD record. b) Single crystal X-ray structure of epitiostanol *S*-oxide (CSD entry EPTANO) shown in stick representation. The figure was generated from the CIF using Mercury 4.2.0 (build 257471).

The introduction of the epithio ring creates an impact on the structure of the steroid. Koyama and Utsumi-Oda published the crystal structure of 2 α ,3 α -epithio-5 α -androstan-17 β -yl *p*-bromo-benzoate.^[5] According to this crystal structure, the epithio group is slightly pointing away from the steroid backbone. As shown in the figure 4.2a the episulfide is attached to C2 and C3. In the A-ring, C1 and C4 are adjacent to the episulfide ring, with C1 presenting a pseudo-axial hydrogen which is pointing downwards on the α -face, and C4 presenting a pseudo-axial hydrogen pointing upwards on the β -face. Similar to this, the single crystal X-ray structure of 2 α ,3 α -epithio-5 α -androstan-17 β -ol *S*-oxide (figure 4.2b) was reported by Koyama *et al.* in 1977, which shows similar bond distances and A-ring angles with the *S*-oxide in the *R*-configuration.^[10]

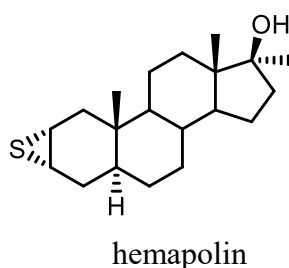


Figure 4.3 Hemapolin, 17 α -methylepithiostanol

In another recent study, Okano *et al.* identified 17 α -methyl-2 α ,3 α -epithio-5 α -androstan-17 β -ol (hemapolin, figure 4.3) and 17 α -methyl-2 β ,3 β -epithio-5 α -androstan-17 β -ol (epistane) in a dietary supplement known as EPISTANETM.^[11] As the steroid A-ring contains the epithio ring, it could result in the steroid not being detected during anti-doping screening. To date, only equine metabolism of hemapolin has been investigated.^[12]

4.2 Hemapolin *in vivo* metabolism

As mentioned above, hemapolin is an epithio steroid. In the steroid structure, the sulfur atom is on the α -face of the steroid skeleton and attached to the C2 and C3 carbons. The GC/MS study^[12] revealed a number of phase I metabolites and several of those were confirmed against the synthesised reference materials. In the horse system, the steroid episulfide was completely metabolized and the parent steroid was not detected. Madol (M) was observed as a metabolite, formed by the elimination of the episulfide group. Other than madol, a reduced and dihydroxylated madol metabolite, two steroid enones, and a number of other mono, di, and tri-

hydroxylated and reduced metabolites were observed in the phase I analysis. Some of those were confirmed with reference materials (figure 4.4).

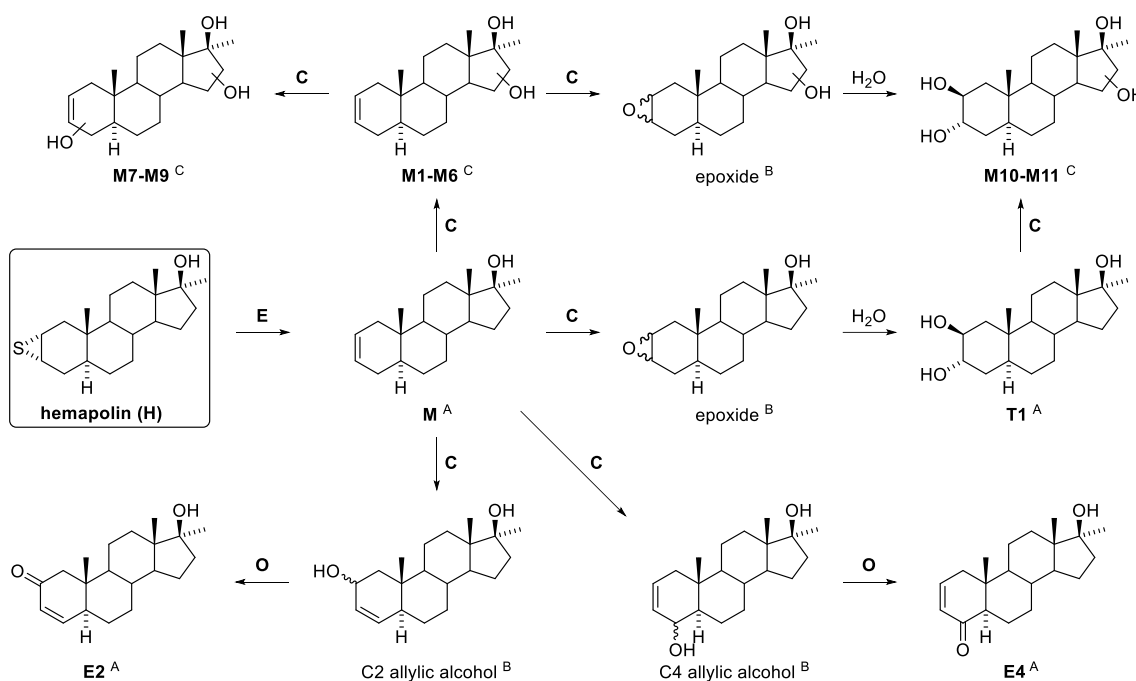


Figure 4.4: Proposed pathways for urinary phase I metabolites of hemapolin in the horse; ^AMatched to reference material, ^BNot observed, ^CStructure undefined. E, elimination; C, Cytochrome P450 oxidation; O, oxidation.^[12]

An unpublished LC-MS study of hemapolin metabolism by one of our group members, Dr. Chris Waller, revealed the absence of hemapolin *S*-oxide, which was expected to be present by analogy with the human metabolism of mepitiostane previously described. Hemapolin shares the same structure as epitio stanol, except for the 17 α -methyl group. Therefore, it could undergo the same metabolic pathway to produce hemapolin *S*-oxide. According to Koyama *et al.* chemical oxidation produces the (*R*)-*S*-oxide. In Okano's human *in vivo* study, the chemically synthesised *S*-oxide matched with the urinary metabolite observed.

Another unusual finding of this LC-MS metabolism study was the observation of a metabolite, with an *m/z* corresponding to madol sulfate. As madol is a 17 β -hydroxy-17 α -methyl steroid, the 17-sulfated products were expected to be unstable and therefore eliminate or hydrolyse to form different structures as described in previous chapter (section 3.1.2).

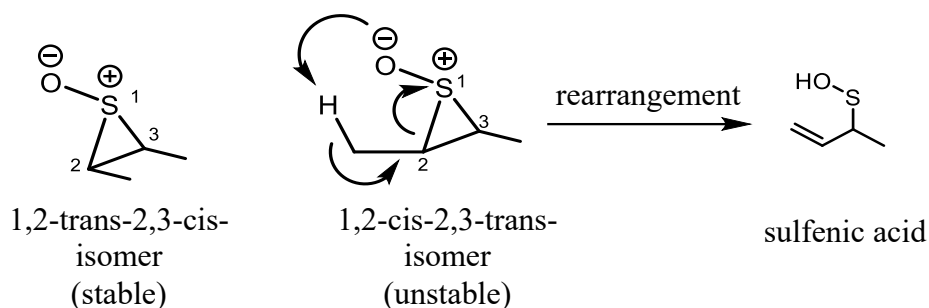
Furthermore, this putative sulfate metabolite afforded an m/z 80 fragment corresponding to sulfur trioxide radical anion [$\bullet\text{SO}_3$] $^-$, as the only product ion following collision induced dissociation. The saturated steroid sulfates generally exhibit m/z 97 [HSO_4] $^-$ as the major fragment with minor m/z 80 [$\bullet\text{SO}_3$] $^-$ only observed at high energies. In contrast, unsaturated sulfates like estrone sulfate gives m/z 80 as the major fragment. Therefore, m/z 80 is not expected as a product ion derived from a structure corresponding to “madol sulfate”.

In order to understand this unusual behaviour, it was necessary to identify alternative pathways that led to the formation of this unusual metabolite. As it didn't exhibit the [HSO_4] $^-$ fragment, the metabolite could not be a saturated sulfate conjugate. Instead of that, one hypothesis was that hemapolin must have undergone episulfide oxidation, ring opening and further oxidation to give rise to a sulfonate group that fragments to form m/z 80.

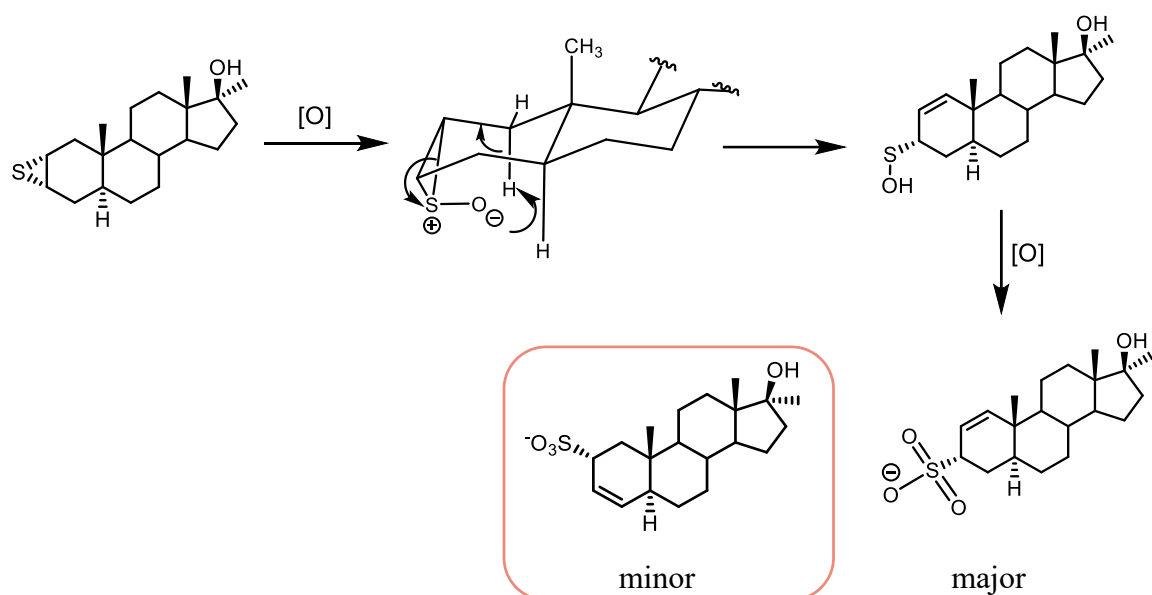
In this chapter, we describe a study that has been carried out to identify this unusual “madol sulfate” metabolite that was found during the equine *in vivo* study.

4.3 Mechanistic proposal for the formation of a putative sulfonate metabolite

Episulfides and episulfoxides are known to be moderately chemically unstable compounds.^{[13],[14]} They decompose to form the resulting olefin by elimination of sulfur or sulfur monoxide.^[13] It has also been proposed that intramolecular proton transfer and ring opening for episulfoxides can lead to rearrangement and formation of a sulfenic acid when the stereochemistry is favourable (scheme 4.1).^{[13],[15]}



Scheme 4.1: Intramolecular proton transfer and ring opening can lead to the relevant sulfenic acid from episulfoxides.



Scheme 4.2 Proposed metabolic pathway for formation of the “madol sulfate” metabolite starting with hemapolin.

Considering these ideas, the formation of the steroid sulfonate was possible with the appropriate stereochemistry. According to the published crystal structure of epitio stanol *S*-oxide, the proposed (*R*)-sulfoxide stereoisomer has the oxygen pointing away from the steroid back-bone with no β hydrogens in proximity to the sulfoxide oxygen. However, oxidation to form the (*S*)-sulfoxide stereoisomer as shown in scheme 4.2, followed by intramolecular proton transfer can produce the 1-ene-3 α -sulfenic acid. Further oxidation then yields the 1-ene-3 α -sulfonate. Opening of the epithio ring to obtain the 3 α product is predicted to be favoured due to the proximity of the pseudo-axial C1-H (see figure 4.5) Nevertheless, the 3-ene-2 α -sulfonate was also considered as a possible minor product.

The epi-thio rings have different bond angles, bond lengths and distances between atoms. The proposed ring opening of hemapolin (*S*)-*S*-oxide depends on the distance between sulfoxide oxygen and C1 or C4 hydrogens. These distances are slightly different in hemapolin (*S*)-*S*-oxide. The distance between the sulfoxide oxygen to the pseudo axial C1-H, is 2.6 Å and for the sulfoxide oxygen to pseudo equatorial C4-H it is 3.0 Å (figure 4.5). However, the differences between dihedral angles were more significant and allowed the prediction of the major and minor ring opened products. For hemapolin (*S*)-*S*-oxide the dihedral angle between the sulfoxide sulfur and the pseudo equatorial H₄ (S-C3-C4-H_{4Eq}) is 57.4°. This is greater than that to the sulfoxide sulfur and the pseudo axial H₁ (S-C2-C1-H_{1Ax}) which is 21.4°. Thus,

alignment suggests that the 1-ene-3 α - sulfonate would become the major product over 3-ene-2 α - sulfonate for hemapolin *S*-oxide.

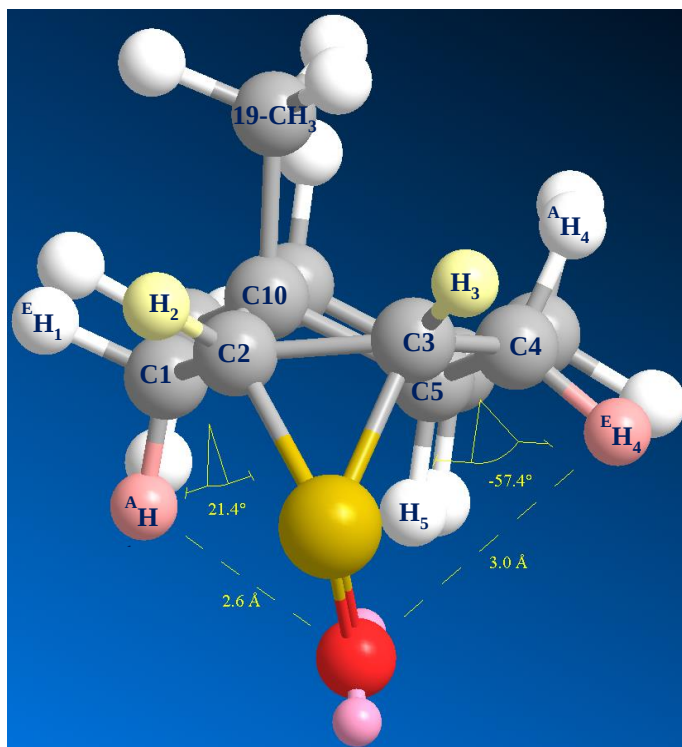


Figure 4.5: Chem3D model showing the dihedral angles and distances that influence the ring opening of the hemapolin (*S*)-*S*-oxide stereoisomer. ^A pseudo-axial. ^E pseudo-equatorial (only A and B rings of the steroid are shown here).

There is an important point that requires raising at this point before moving on to the next section. When the project was underway, the above-mentioned 1-ene-3 α -sulfonate was synthesised and sent to compare with the urinary metabolite. The results obtained did not match. Therefore, it was necessary to revisit the proposed metabolic pathway to solve this puzzle.

On reinvestigating the previous synthesis, it was revealed that the epoxide used for the synthesis of hemapolin was a mixture of 2 β ,3 β and 2 α ,3 α isomers. The administered 2 α ,3 α epithio steroid was then compared with a newly synthesised sample of the 2 β ,3 β epithio steroid, epistane. Once the spectrum was carefully studied, it was revealed that the administered hemapolin sample contained the 2 β ,3 β -epithio steroid, epistane in a ratio of 1:10 with hemapolin (figure 4.6).

This new finding opened a path to synthesise additional sulfonates, the 3-ene-2 α -sulfonate mentioned above (scheme 4.2) and the other two sulfonates, that could be formed from epistane; the 3-ene-2 β -sulfonate and 1-ene-3 β -sulfonate.

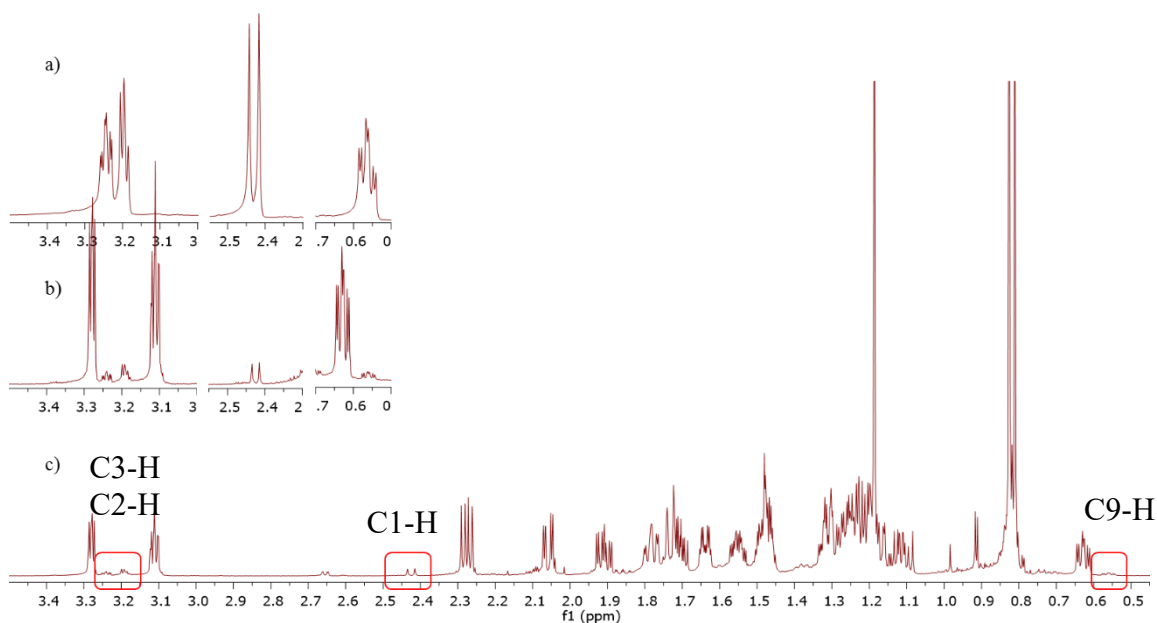
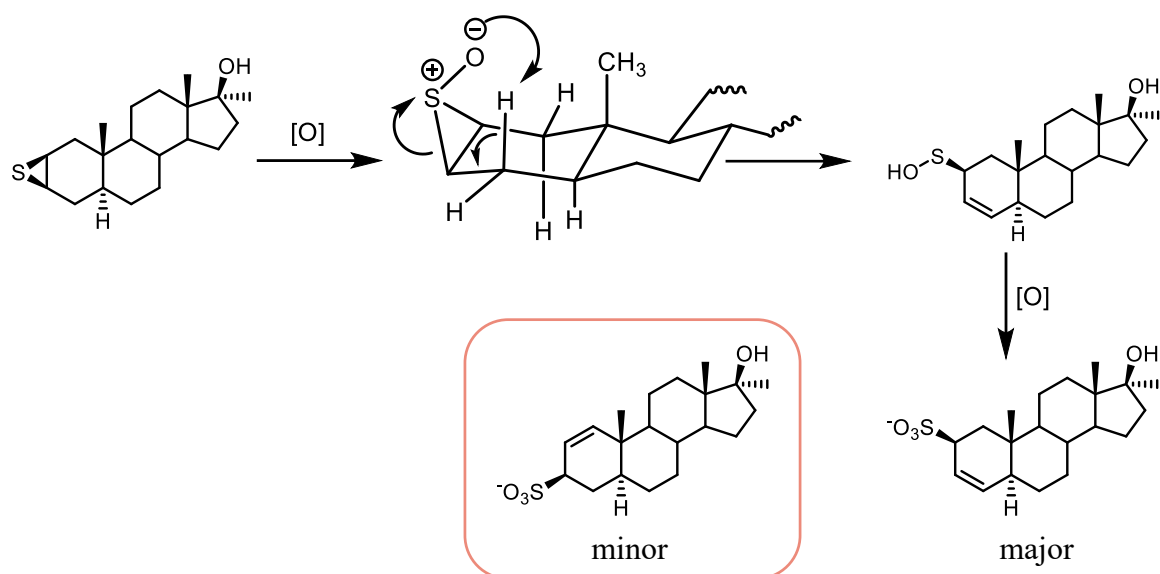


Figure 4.6: a) ¹H-NMR signals for C2-H, C3-H, C1-H and C9-H of a pure epistane sample b) ¹H-NMR signals for C2-H, C3-H C1-H and C9-H of the administered mixture, major isomer-hemapolin c) ¹H-NMR of administered mixture of isomers (0.5-3.5 ppm)

For the epimeric 2 β ,3 β epithio steroid epistane a similar pathway can be proposed. Epistane can either form the relatively stable (*S*)-sulfoxide or can form the (*R*)-sulfoxide which can undergo rearrangement and oxidation to form the 3-ene-2 β -sulfonate. Similar to hemapolin, the ring opening of epistane depends upon the distances between the sulfoxide oxygen and pseudo equatorial C1-H or pseudo axial C4-H where the values are 3.3 Å and 2.6 Å respectively, which have a difference of 0.7 Å. However, the dihedral angles also showed a significant difference between the sulfoxide sulfur and the pseudo axial H₄ (S-C3-C4-H_{4Ax}) which is 32.6°, smaller than that to the sulfoxide sulfur and the pseudo equatorial H₁ (S-C2-C1-H_{1Eq}) which is 68.9° as shown in figure 4.7. This suggests that the 3-ene-2 β -sulfonate becomes the major product (scheme 4.3). Therefore, all four isomers were synthesised as shown in scheme 4.2 and 4.3.



Scheme 4.3: Proposed metabolic pathway for formation of the “madol sulfate” metabolite starting with epistane.

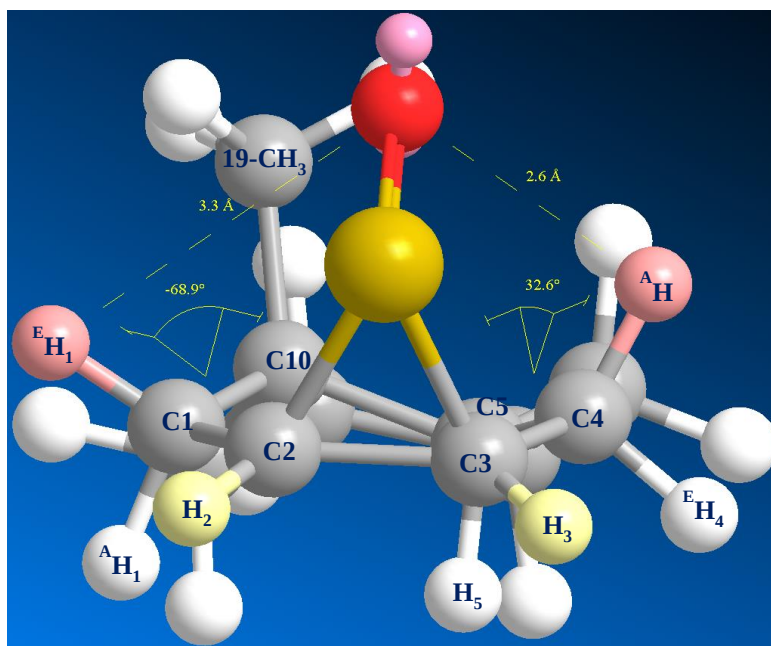


Figure 4.7: Chem3D model showing the dihedral angles and distances that influence the ring opening of epistane (*R*)-*S*-oxide. ^A pseudo axial. ^E pseudo equatorial.

4.4 Synthesis of epithio-steroids and sulfonates

4.4.1 Synthesis of epithio-steroids

As mentioned before, there are two epithio steroids engaged in the work related to this chapter, hemapolin and epistane. In order to synthesise the 2 α ,3 α -epithio steroid, hemapolin the 2 β ,3 β -epoxide derived from madol was used as the starting material.

The synthesis of madol from EA is a three-step reaction as shown in scheme 4.5. Tosylation of epiandrosterone followed by elimination using diazobicyclo[5.4.0]undec-7-ene gave a mixture of Δ^{2-3} and Δ^{3-4} olefins in 15:1 ratio. The mixture was obtained as there are two ways to eliminate the tosyl group. Grignard reaction then afforded madol.

The stereoselectivity of the epoxide determined the epithiosteroid formed. In order to synthesise hemapolin, 2 β ,3 β -epoxide was required. Therefore, the next requirement was bromohydrin formation which leads to the desired 2 β ,3 β -epoxide. The bromine source used here was *N*-bromosuccinimide (NBS), a crystalline solid that is easy to handle and it is less toxic than liquid bromine. The bromonium ion intermediate formed in this reaction undergoes addition by a water molecule. NBS prefers addition from α -face to generate the bromonium ion leading to the formation of 3 α -bromo-17 α -methyl-5 α -androsterane-2 β ,17 β -diol as the major product. However, at this point the ratio between the bromohydrin isomers could not be distinguished clearly using NMR. After that, base promoted elimination gave a mixture of 2 β ,3 β - and 2 α ,3 α -epoxides in a 10:1 ratio.

The epoxide was treated with triphenylphosphine sulfide in the presence of trifluoroacetic acid. The conversion of epoxide to episulfide gives an inversion of configuration^[16] as this involves an S_N2 substitution.

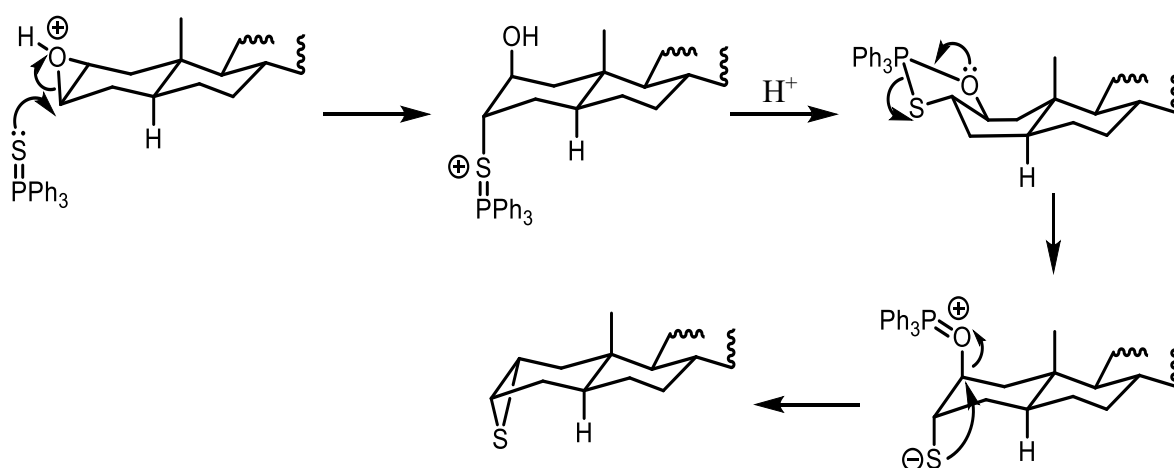
The proposed mechanism involves Ph₃PS substitution at C3 (scheme 4.4). The reaction is driven by the exceptional affinity of phosphorous towards oxygen. The pentavalent phosphorus species is considered as the intermediate of this reaction.^[16] The five membered ring formed is not stable and collapses in a way to transfer the phosphorous atom from sulfur to oxygen. Finally, elimination of

triphenylphosphine oxide gave the steroid episulfide with inverted stereochemistry as a mixture of hemapolin and epistane in 10:1 ratio.

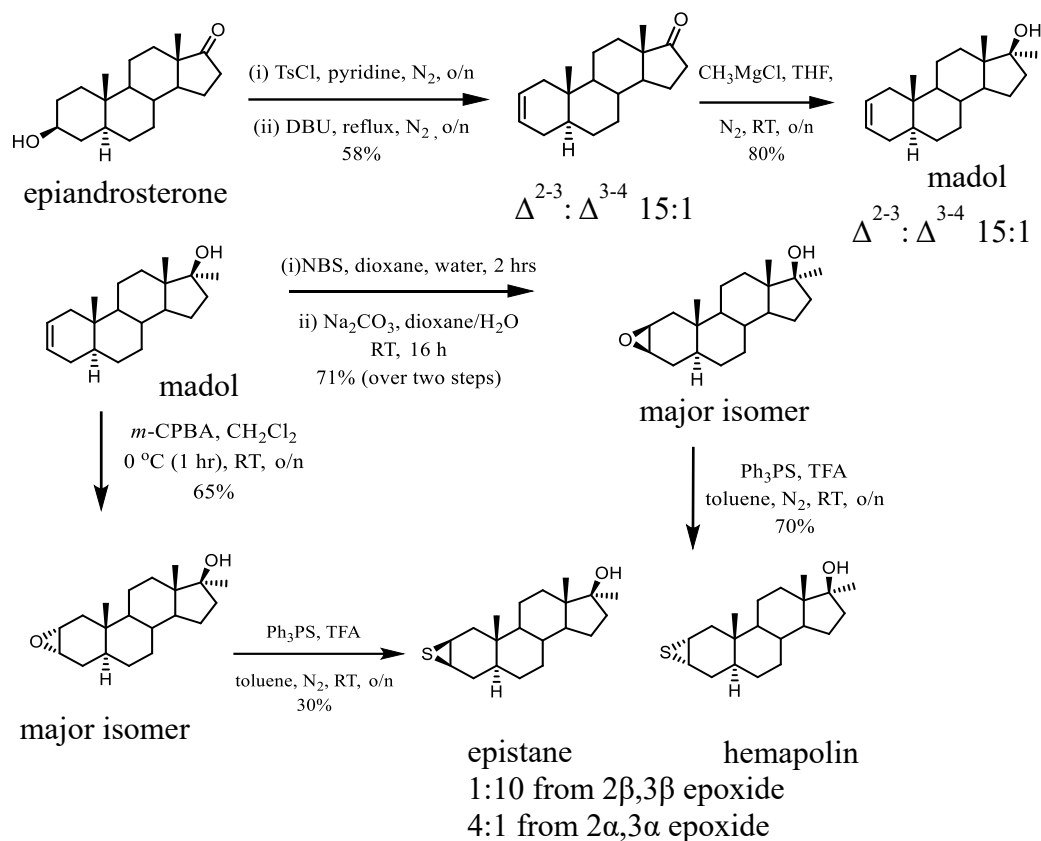
The synthesis of epistane was similar to hemapolin except for the epoxide starting material. Epistane contains a $2\beta,3\beta$ -epithio ring. Therefore, $2\alpha,3\alpha$ -epoxide was first synthesised starting from madol.

Peroxy acids are popular oxidizing reagents for epoxidation and stereoselectivity typically depends on steric effects.^[17] Epoxidation of madol was performed using *meta*-chloroperbenzoic acid (*m*-CPBA). As the β -face of the steroid is crowded with 19-methyl group, *m*-CPBA favours oxidation of α -face. Madol, employed here was a mixture of $\Delta^{2,3}$ and $\Delta^{3,4}$ steroids in a 15:1 mixture. This epoxidation gave $2\alpha,3\alpha$ epoxide as the major product and $3\alpha,4\alpha$ - and $2\beta,3\beta$ -epoxides as minor products with the ratio of 10:1:1 according to $^1\text{H-NMR}$.

The epithio group was introduced with Ph_3PS in a similar manner to the synthesis of hemapolin. Nucleophilic substitution with inversion followed by elimination of triphenylphosphine oxide yields the $2\beta,3\beta$ -epithio steroid. Even though the epoxide employed as the starting material is relatively pure, at the end of the reaction a mixture of epistane and hemapolin was obtained in 4:1 ratio and moderate yield.



Scheme 4.4: Synthesis of the steroidal $2\alpha,3\alpha$ -episulfide from $2\beta,3\beta$ -epoxide



Scheme 4.5: Synthesis of hemapolin and epistane

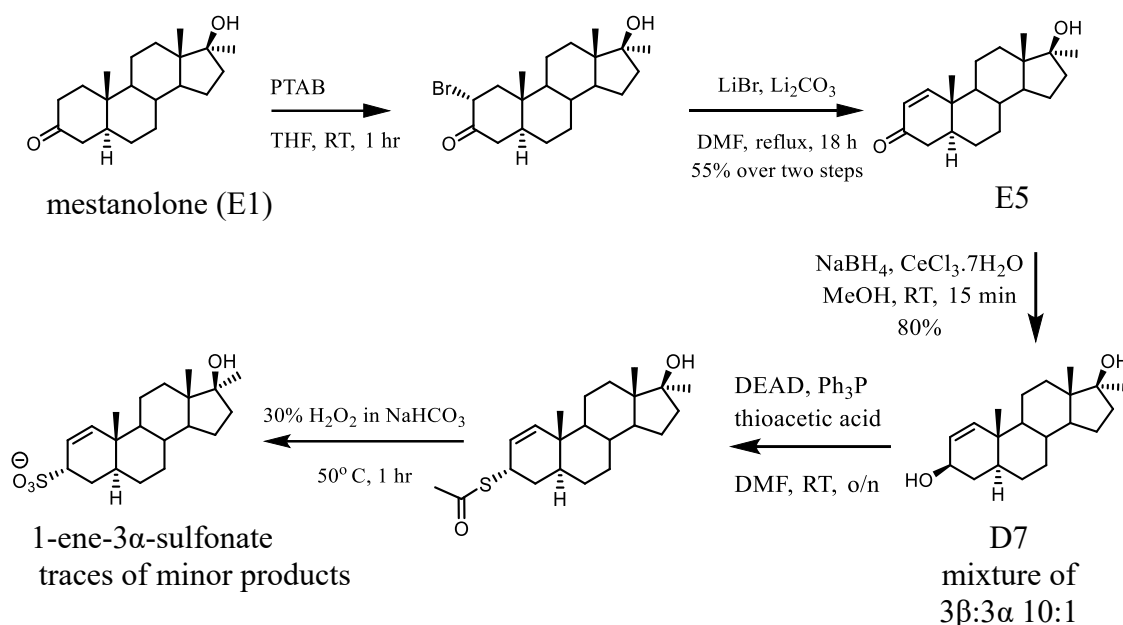
Extensive NMR studies were performed to confirm the structure and assign the peaks of hemapolin and epistane. Tori *et al.* in 1963^[3] and Tori and Lukas in 1974^[2] carried out thorough NMR studies on steroidal episulfides to demonstrate how structural features related to ¹³C chemical shifts of steroidal A ring epimeric pairs. Therefore, the NMR data generated for the synthesized steroidal episulfides were compared with the previously published data by Okano *et al.*^[11] and Tori *et al.*^[2] (table 4.1).

4.4.2 Synthesis of steroid sulfonates

4.4.2.1 Synthesis of 17 β -hydroxy-17 α -methyl-5 α -androst-1-ene-3 α -sulfonate (1-ene-3 α -sulfonate)

In order to prepare the 1-ene-3 α -sulfonate reference material, it was necessary to introduce a double bond at the 1-position. Therefore, as the starting material, an α,β unsaturated ketone was required for the synthesis (scheme 4.6). Bromo ketones are more often used to generate α,β unsaturated ketones by dehydrohalogenation.

The addition of bromide to the A ring depends on the enolization of the steroid ketone, as enolization is the rate determining step of this reaction. The preferred direction of enolization depends on the substituents and stereochemistry of the steroidal A-ring (scheme 4.7).

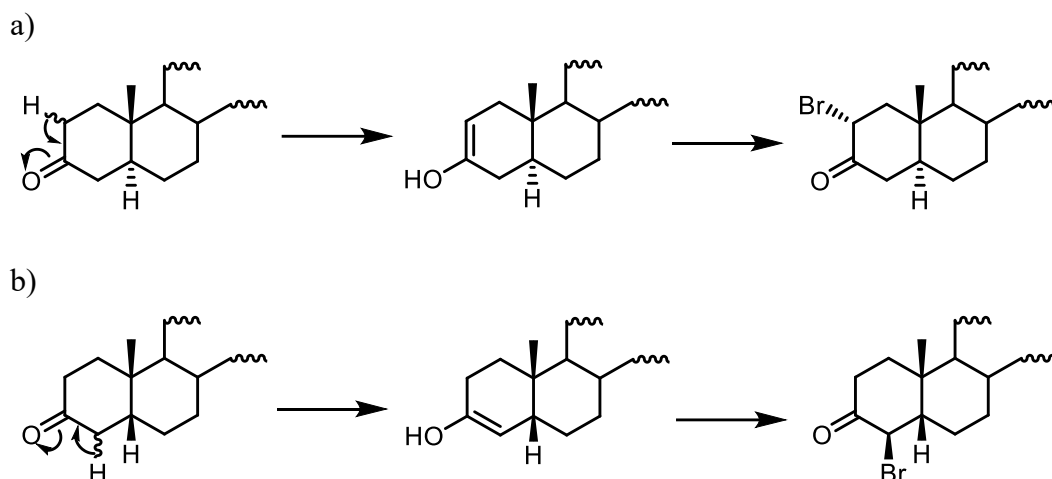


Scheme 4.6: Synthesis of reference material 1-ene-3 α -sulfonate.

Depending upon the A, B ring junction, there are two steroid types. 5 α -Steroids have trans-fused A,B ring junctions whereas 5 β -steroids have cis-fused A,B ring junctions. According to molecular geometry calculations, it has been shown that 3-keto-5 α -steroids favour the formation of Δ^2 -enol rather than Δ^3 -enol^[18], and further studies have demonstrated that 3-keto-5 α steroids form C2 substituted products rather than C4 substituted products.^{[19],[20]} Hence, bromination was expected to behave in the same manner.

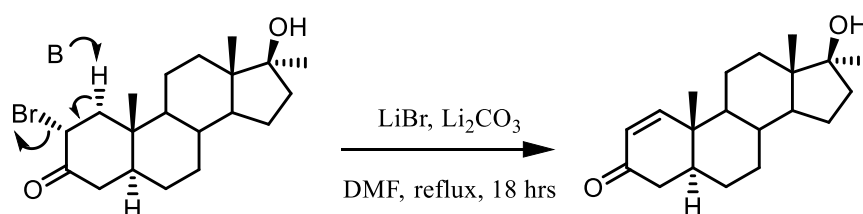
Carbon number	Hemapolin Current study	Okano <i>et al.</i> study	$\Delta\delta$ +/-	Epistane Current study	Okano <i>et al.</i> study	$\Delta\delta$ +/-
C1	40.5	40.3	-0.2	39.6	39.3	-0.3
C2	35.3	34.6	-0.7	37.2	36.8	-0.4
C3	38.3	37.6	-0.7	35.6	35.2	-0.4
C4	30.7	30.5	-0.2	30.3	30.1	-0.2
C5	35.5	35.4	-0.1	43.4	43.0	-0.4
C6	28.4	28.1	-0.3	28.6	28.3	-0.3
C7	31.5	31.0	-0.5	31.8	31.3	-0.5
C8	36.7	35.0	-1.7	35.8	34.6	-1.2
C9	53.9	53.6	-0.3	56.4	56.1	-0.3
C10	35.3	35.2	-0.1	34.8	34.6	-0.2
C11	20.5	20.2	-0.3	20.5	20.3	-0.2
C12	31.7	-	-	31.8	-	-
C13	45.5	-	-	45.6	-	-
C14	50.7	-	-	50.7	-	-
C15	23.4	-	-	23.4	-	-
C16	39.1	-	-	39.1	-	-
C17	81.9	-	-	81.8	-	-
C18	14.0	-	-	14.0	-	-
C19	12.9	12.6	-0.3	15.0	14.9	-0.1
C20	25.9	-	-	25.9	-	-

Table 4.1: Comparison of ^{13}C chemical shifts of synthesised hemapolin and epistane with previously published data.^[11]



Scheme 4.7: Enol formation of a) 5 α steroids and b) 5 β steroids

After the bromination step, the dehydrobromination was performed to yield the 1-en-3-one steroid. Previous studies have suggested that basic conditions are appropriate for the dehydrobromination.^[21] Zhang and Qiu^[22] have shown that lithium carbonate, in the presence of lithium bromide gives the best selectivity towards the 1-en-3-one product via the deprotonation shown in scheme 4.8. Once purified, the ^1H -NMR showed the presence of pure **E5**.



Scheme 4.8: Synthesis of 17 β -hydroxy-17 α -methyl-5 α -androst-1-en-3-one (E5**) via dehydrobromination, B; base**

Ketone **E5** was reduced with NaBH_4 , in the presence of cerium chloride heptahydrate ($\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$) and yielded a mixture of 3 β and 3 α -hydroxy steroids in a 10:1 ratio. This mixture was taken to the next step. The steroid **E5**, is an α - β -unsaturated ketone and the double bond must remain unaffected during the reduction. Therefore, Luche reduction was employed, with the addition of $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$ giving selective 1,2-reduction. Mechanistically, $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$ catalyses the methanolysis of NaBH_4 to create a harder reducing agent and it is known that alkoxyborohydrides are more reactive than BH_4^- for 1,2 addition.^[24]

Attempts to tosylate the secondary alcohol and then subject this product to substitution by a suitable sulfur nucleophile failed. Therefore, the Mitsunobu reaction was employed to obtain the desired thio-ester (scheme 4.9).

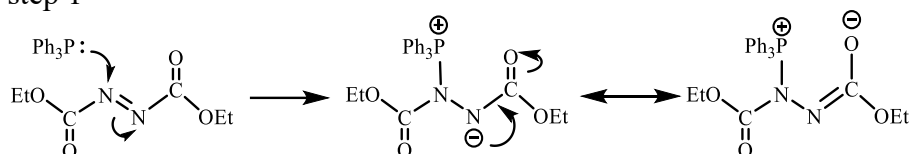
As the first step of the addition, diethyl azodicarboxylate (DEAD) activates Ph_3P , which is transferred to the oxygen atom of the alcohol, creating a good electrophile. Then substitution by thioacetic acid as a good nucleophile occurs with inversion of stereochemistry.

Several precautions were taken during the Mitsunobu reaction. The temperature and the order of addition played an important role in this reaction. At the beginning of the reaction, the temperature was maintained at 0 °C as was shown that mild conditions favour ester formation. DEAD was added drop-wise to a solution of Ph_3P in THF at 0 °C and stirred for 30 minutes. After that, the solution was treated with thioacetic acid followed by steroid, was allowed to warm to room temperature and stirred overnight. When the order of addition was changed, unreacted starting material was observed at the end of the reaction. The purification of the thio-ester proved challenging. Therefore, the thio-ester was directly taken to the next step.

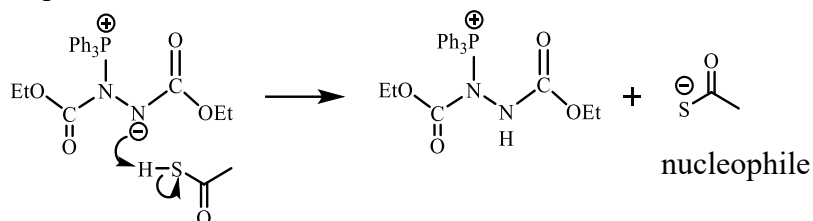
As the final step, the thio-ester formed was oxidized with hydrogen peroxide in the presence of sodium bicarbonate at elevated temperatures. The obtained 3 α -steroid sulfonate was purified with preparative HPLC and the stereochemistry was studied by NMR methods. ^1H -NMR showed traces of the minor isomer (3 β -sulfonate) which could not be separated with HPLC purification. Nuclear Overhauser Effect (NOE) experiments of the sulfonate provided a correlation between C3-H and C19- CH_3 and another strong correlation was observed between C3-H and the axial C4- H. Further to this, C3-H of diol **D7** showed two large couplings and two smaller couplings (figure 4.8). C3-H of diol **D7** is pseudo axial and can couple to the adjacent C4 protons and the alkene protons in C2. The large coupling ($J=9.2$ Hz) was due to the C4-axial hydrogen, as the dihedral angle between C4-axial hydrogen and C3-pseudo axial H ($\text{H}_{4\text{Ax}}\text{-C4-C3-H}_{3\text{Ax}}$) is 156.5° where the other coupling was 7.1 Hz from the C4- H_{Eq} , where the dihedral angle was 39.9°. The smallest coupling was caused by the alkene proton C2-H, which exhibits a dihedral angle of 65.7°. The couplings of the C3-proton of the sulfonate were not possible to resolve. As the dihedral angles were smaller, the coupling constants must be small, thus the peak was a cluster of several unresolved couplings. However, the C3-H peak width of 3 α -sulfonate was smaller

than that of 3 β -hydroxy steroid diol (**D7**) suggesting that the C3-H has changed orientation from pseudo-axial to pseudo equatorial, consistent with smaller couplings.

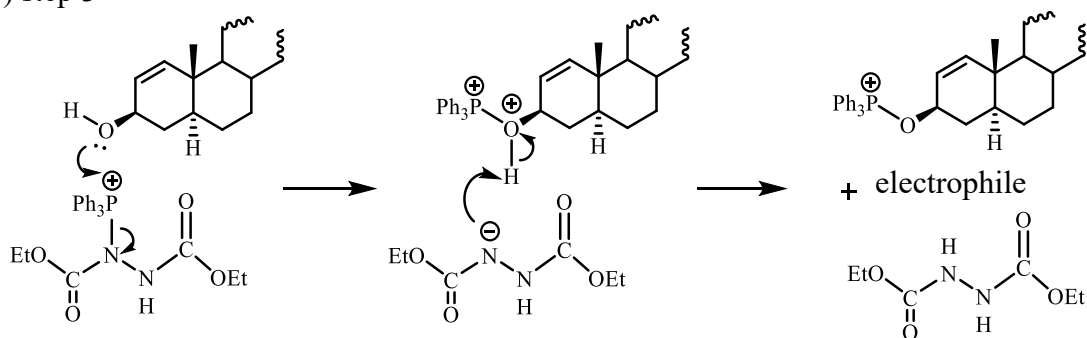
a) step 1



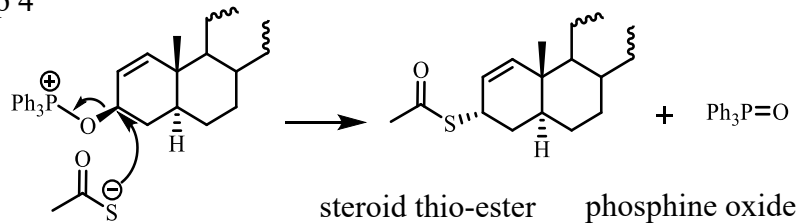
b) step 2



c) step 3



d) step 4



Scheme 4.9: Introduction of thioacetate to the steroid back-bone

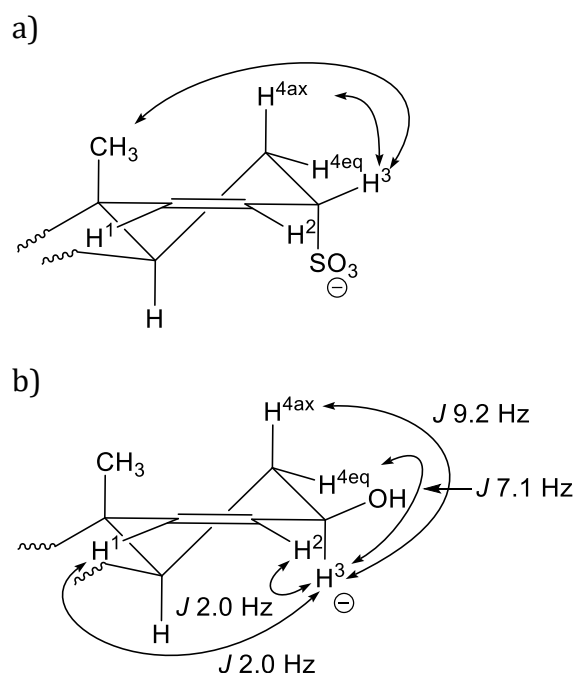


Figure 4.8: a) ChemDraw structure showing the NOE correlations of 1-ene-3 α -sulfonate. b) ChemDraw structure showing the couplings of C3-H of 1-ene-3 β -hydroxy steroid (**D7**).

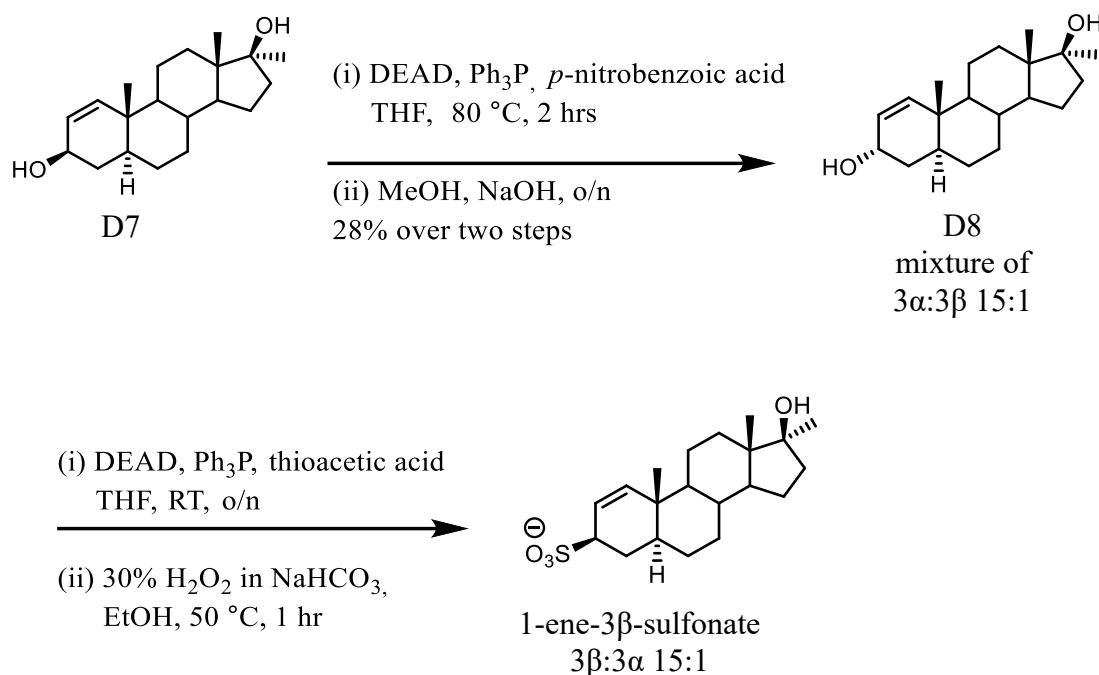
4.4.2.2 Synthesis of 17 β -hydroxy-17 α -methyl-5 α -androst-1-ene-3 β -sulfonate (1-ene-3 β -sulfonate)

Since the Mitsunobu reaction gives inversion of configuration, the 3 α -hydroxy steroid would be required to generate the 3 β -steroid thio-ester. Therefore, attempts were made to synthesise the 3 α -hydroxy steroid from **E5**. L- Selectride[®] as well as a mixture of LiAlH₄ and *tert*-butanol were employed with the expectation that they would act as large nucleophiles to generate the axial hydroxyl group. However, those attempts failed, leading to the 3 β -hydroxy group as the major product.

This problem was overcome with the introduction of a second Mitsunobu reaction to the synthesis. As mentioned in section 4.4.2.1, diol **D7** was obtained by reducing enone **E5** with NaBH₄. Then the first Mitsunobu along this pathway towards 3 β -sulfonate was performed. *Para*-nitro benzoic acid was employed as the nucleophile. After completion of this step, the steroid ester was hydrolysed to generate the 3 α -epimeric diol **D8**. Following this, the same procedure was conducted as the previous synthesis to generate the steroid 3 β -sulfonate (scheme 4.10). The product was

purified by WAX SPE and ^1H -NMR showed the presence of major isomer in a 15:1 ratio. The yield was not reported as it started with the crude thio-ester.

Regarding the splitting pattern, a similar, but opposite trend was observed with 1-ene-3 β -sulfonate. The C3-H of 3 β -sulfonate, has shown a similar splitting pattern of C3-H to that of diol **D7**, where they both have pseudo-axial C3 protons, and C3 pseudo-equatorial hydroxyl or sulfonate groups. Further, NOE experiments revealed a correlation between C3-pseudo-axial H and C5-axial H for the 1-ene-3 β -sulfonate. Other than that, the axial C3-H has shown a correlation to the equatorial C4-H as expected.



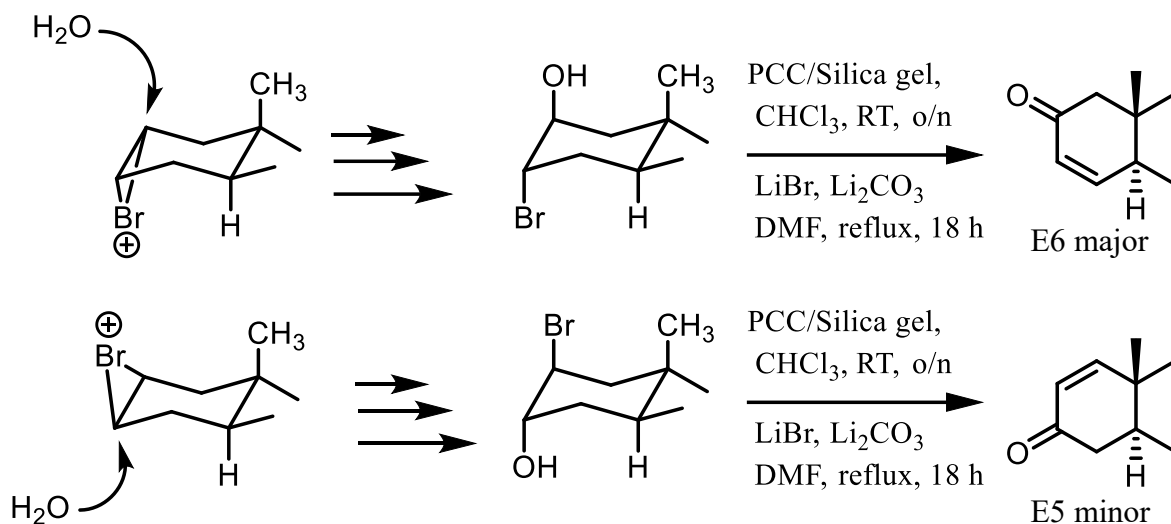
Scheme 4.10: Synthesis of 1-ene-3 β -sulfonate starting from diol **D7**

4.4.2.3 Synthesis of 17 β -hydroxy-17 α -methyl-5 α -androst-3-ene-2 α -sulfonate (3-ene-2 α -sulfonate)

Moving on to the C2-substituted sulfonates, the first requirement was the synthesis of a 3-en-2-one steroid.

First, bromination of madol was performed under aqueous conditions to obtain the bromohydrin (scheme 4.11). As it is less hindered, initial bromonium ion formation is favoured on the lower face. Axial addition with water then gives the 3 α -bromo-2 β -hydroxy compound. Oxidation followed by elimination finally gives the enone **E6** as the major product. However, the bromonium ion formation can occur on the

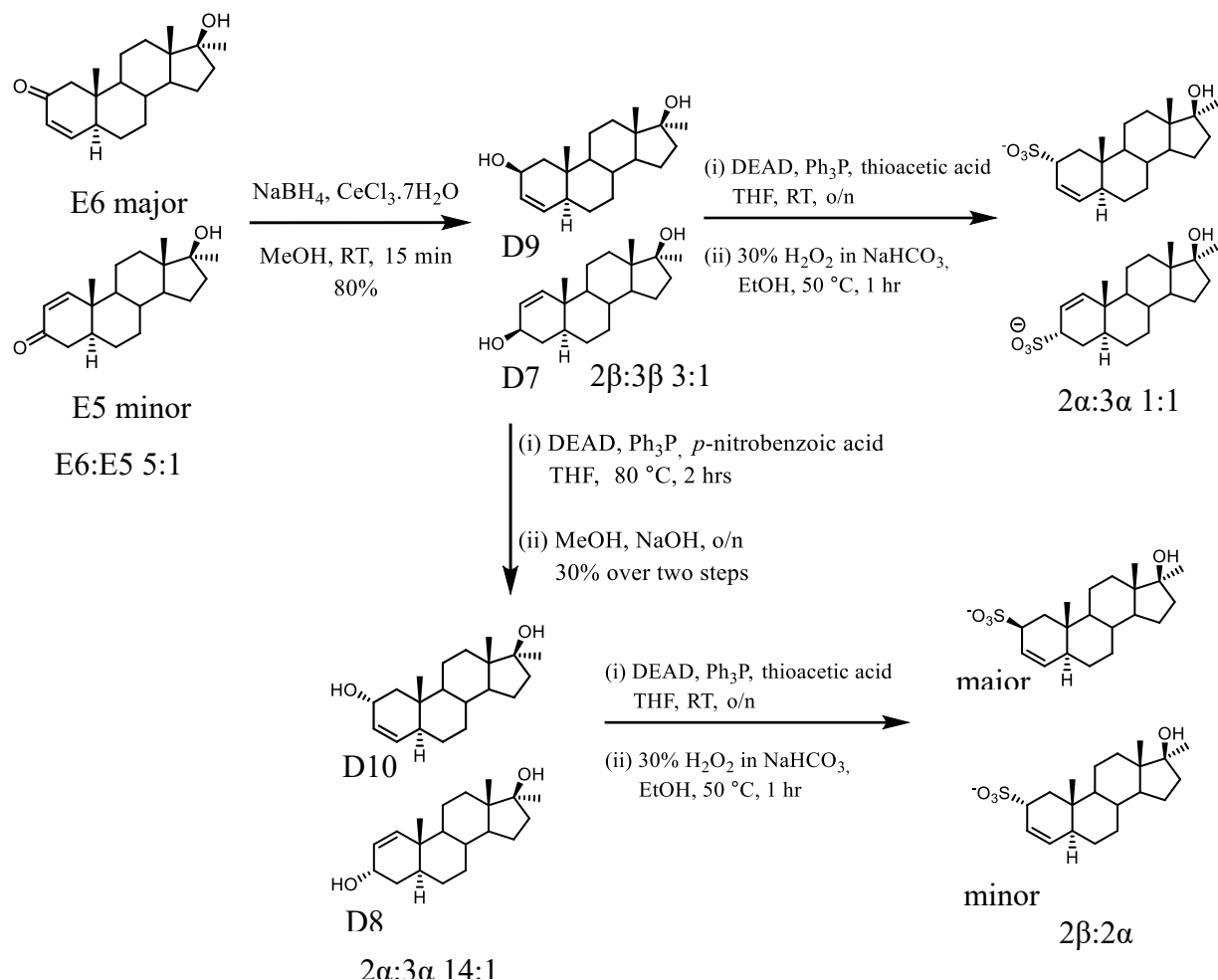
upper face despite being more sterically hindered. Axial addition of water to the minor bromonium ion gives the 2 β -bromo-3 α -hydroxy compound and finally enone **E5** as the minor isomer in a 1:5 ratio with the desired enone **E6** major product.



Scheme 4.11: Synthesis of E6 steroid enone

This mixture was taken through the remaining steps of the sequence with the 3-en-2-one expected to provide the 3-ene-2 α -sulfonate as shown in scheme 4.12. To apply the Mitsunobu reaction the ketone was first reduced with NaBH_4 to get the 2 β -hydroxy allylic alcohol **D9**. According to literature^[25], the NaBH_4 reduction of 5 α -steroids mainly afford the 2 β -hydroxy steroid. Therefore, according to NMR this reduction produced a mixture of 2 β :2 α :3 β hydroxy steroids in 3:1:1 ratio, with the 2 β -hydroxy steroid being the major isomer. Then Mitsunobu reaction with thioacetic acid followed by oxidation was performed. A mixture of 2 α and 3 α sulfonate isomers in 1:1 ratio were obtained at the end of this sequence. This mixture could not be separated by WAX SPE as they both are polar compounds. Nevertheless, in the previous synthesis, 1-ene-3 α -sulfonate was completely characterized. Therefore, with the ^1H -NMR it was easy to identify the new peaks related to the 3-ene-2 α -sulfonate. For further characterisation, high-resolution

mass spectrometry was also conducted on both isomers. Further purification by HPLC was possible. But has not been performed to date.



Scheme 4.12: Synthesis of 3-ene-2 α -sulfonate and 3-ene-2 β -sulfonate

4.4.2.4 Synthesis of 17 β -hydroxy-17 α -methyl-5 α -androst-3-ene-2 β -sulfonate (3-ene-2 β - sulfonate)

For the synthesis of 2 β - sulfonate, the 2 α -hydroxy steroid **D10** was synthesised using diol **D9**. First Mitsunobu was performed with *p*-nitrobenzoic acid followed by hydrolysis to obtain the 2 α -hydroxy-3-ene steroid. This was clearly visible as a mixture including 2 α :3 α steroid diols in a 14:1 ratio. However, 2 β -hydroxy-3-ene steroid must have been present in the mixture, as 3-ene-2 α -sulfonate was present at the end of the sequence. Alkene protons of 2 β -hydroxy-3-ene steroid were likely obscured below the signals of 3 α -hydroxy-1-ene steroid and 2 α -hydroxy-3-ene steroid as those signals were very close to each other (figure 4.9). Then the next Mitsunobu reaction yielded the steroid thio-ester as a mixture which was used for

the oxidation without further purification. With LC-MS studies, it was revealed that the oxidation gave a mixture of 3 compounds, 2 β and 2 α steroid sulfonates and another unidentified compound with the same mass (m/z 367) as the targeted sulfonate. The 1-ene-3 β -sulfonate derived from the minor 1-ene-3 α -ol was not observed at the end of the sequence.

The challenging nature of the 3-ene-2 β -sulfonate synthesis can be rationalised by the crowded β -face of the steroid with the 19-methyl group hindering approach of the nucleophile to the C2 position. The 3-ene-2 β -sulfonate was later purified by HPLC and the structure was assigned using NMR experiments.

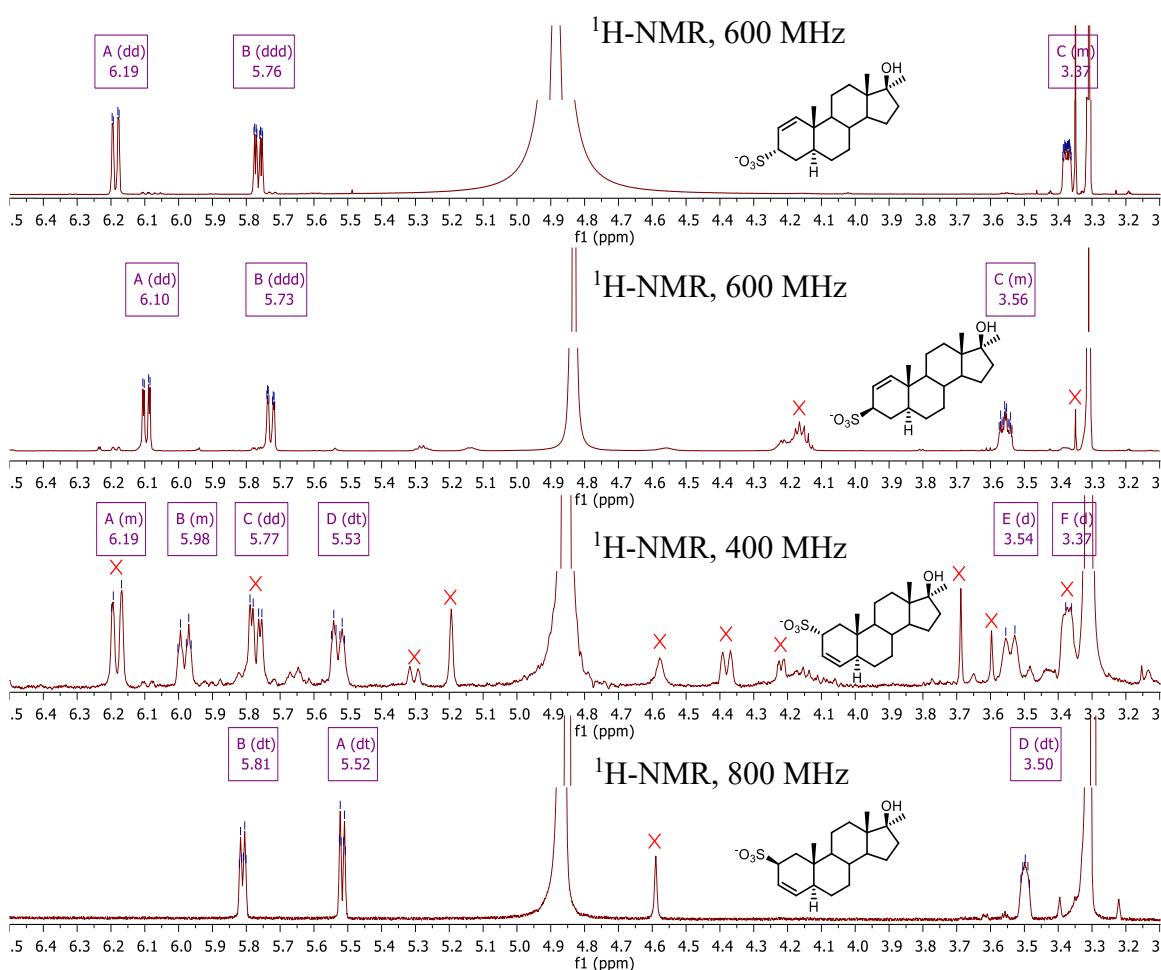


Figure 4.9: ^1H -NMR signals of alkene protons and C2-H or C3-H of the 4 sulfonates synthesised in deuterated methanol solvent. The 3-ene-2 α -sulfonate was prepared as a 1:1 mixture with the 1-ene-3 α -sulfonate.

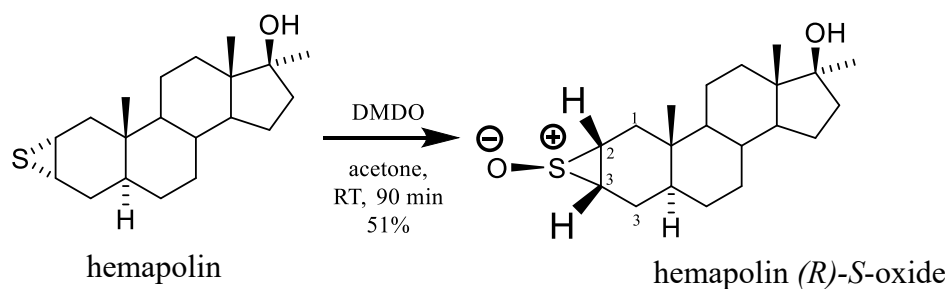
Steroid sulfonate	C1-H	C2-H	C3-H	C4-H	C18-CH ₃	C19-CH ₃	C18-CH ₃
1-ene-3 α -sulfonate	6.19	5.76	3.37	2.04, 1.82	0.86	0.86	1.18
1-ene-3 β -sulfonate	6.10	5.73	3.56	1.94, 1.79	0.86	0.95	1.18
3-ene-2 β -sulfonate	2.25, 1.52	3.50	5.81	5.52	0.88	0.86	1.22
3-ene-2 α -sulfonate	-	3.54	5.98	5.53	-	-	-

Table 4.2: Comparison of chemical shifts of selected protons of 1-ene-3 α -sulfonate, 1-ene-3 β -sulfonate 3-ene-2 β -sulfonate and 3-ene-2 α -sulfonate. Orange highlights indicate alkene protons. Yellow highlights indicate sulfonate methine protons.

4.4.3 Synthesis of hemapolin *S*-oxide

It was decided to synthesise hemapolin *S*-oxide as a reference material to confirm the products generated from chemical oxidation. As suggested in the mechanistic proposal, the proper orientation of the sulfoxide would lead through a series of steps to the formation of the sulfonate. However, the alternative sulfoxide isomer was predicted to be relatively stable as observed for epitiostanol (section 4.1.2)

To perform the synthesis of hemapolin *S*-oxide, dimethyldioxirane (DMDO) in acetone was used as the oxidising reagent (scheme 4.13). This produced the desired sulfoxide with negligible amounts of madol.



Scheme 4.13: Synthesis of hemapolin (*R*)-*S*-oxide

The oxygen of the sulfoxide is pointing away from the steroid molecule. Thus, the C1-H and C4-H are trans to the sulfoxide oxygen and there is no chance for an intramolecular proton transfer.

4.5 Identification of the *in vivo* metabolite from administered hemapolin (mixture with epistane)

As pure hemapolin was predicted to lead to the 1-ene-3 α -sulfonate (major) and 3-ene-2 α -sulfonate (minor) metabolites, these were considered as first targets. Soon after, the 1-ene-3 α -sulfonate was synthesised and “as the expected match” it was sent to Racing NSW laboratory to run alongside the urinary sample. However, the retention time of the 1-ene-3 α -sulfonate reference material did not match with the metabolite.

As noted previously this was something unexpected and it was necessary to find an answer to this puzzle. If the 1-ene-3 α -sulfonate did not match, it could also be the 3-ene-2 α -sulfonate, but this was considered less likely. Therefore, the original hemapolin synthesis data was analysed again and it was revealed that the administered hemapolin contained both hemapolin and the 2 β ,3 β -epithio steroid epistane in 10:1 ratio. It was then decided to prepare the 3-ene-2 α -sulfonate, 3-ene-2 β -sulfonate and 1-ene-3 β -sulfonate as mentioned at the beginning of this chapter.

Out of the four sulfonates, 3-substituted sulfonates were sent as pure samples after HPLC and WAX SPE purifications and 2-substituted sulfonates were sent as mixtures, in order to compare with the urinary metabolite. Surprisingly the retention time of the equine *in vivo* metabolite matched with 3-ene-2 β -sulfonate (figure 4.11).

The retention time of the urinary metabolite is 5.20 minutes. The retention time of the 3-ene-2 β -sulfonate reference material is 5.22 minutes. According to AORC criteria, for HPLC analysis, the acceptable range of retention time is +/- 50% of half-height peak width (0.033 min) or 3 seconds (0.05 min); whichever is greater. The retention time difference 0.02 minutes is within the acceptable limit of both requirements. Therefore, according to retention time, the urinary metabolite matches with 3-ene-2 β -sulfonate. By using the established ARFL LC conditions, the 1-ene-3 α -sulfonate and 1-ene-3 β -sulfonate were not separable. However, both were clearly distinguished from 3-ene-2 β -sulfonate.

However, the MS/MS data should also be compared prior to the confirmation. According to AORC MS criteria, 3 ions are required for comparison between the metabolite and the reference material. Further, the relative abundance of the selected product ions of the metabolite, should be within the maximum tolerance, 20% of absolute value or 40% of relative value of the reference material whichever is greater.

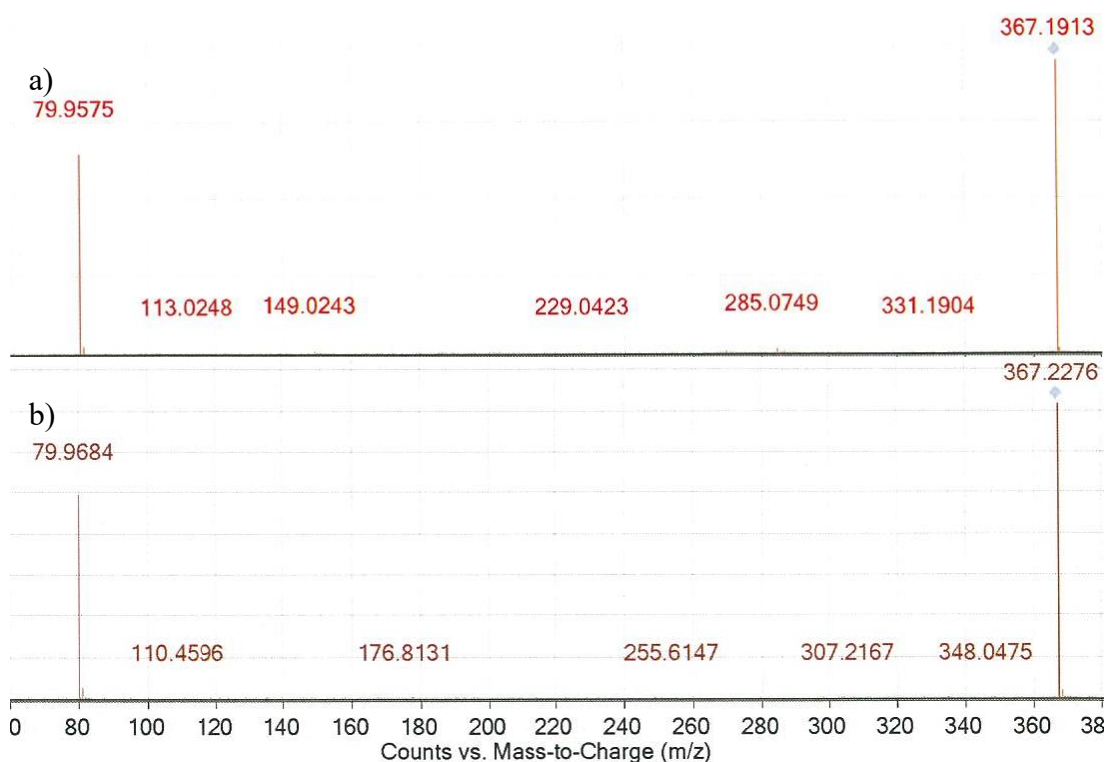


Figure 4.10: LC-MS/MS spectra at 25 eV of the a) urinary metabolite, b) 3-ene-2β-sulfonate reference material

The concern here was that the metabolite and the reference materials were run in negative mode as they contained acidic functionality and were negatively charged. In the negative mode ESI MS/MS fragmentation, only the precursor ion and the m/z 80 fragment could be observed in the mass spectrum (figure 4.10). Instead of comparing three ions at the same collision energy, two ions were compared over three collision energies.

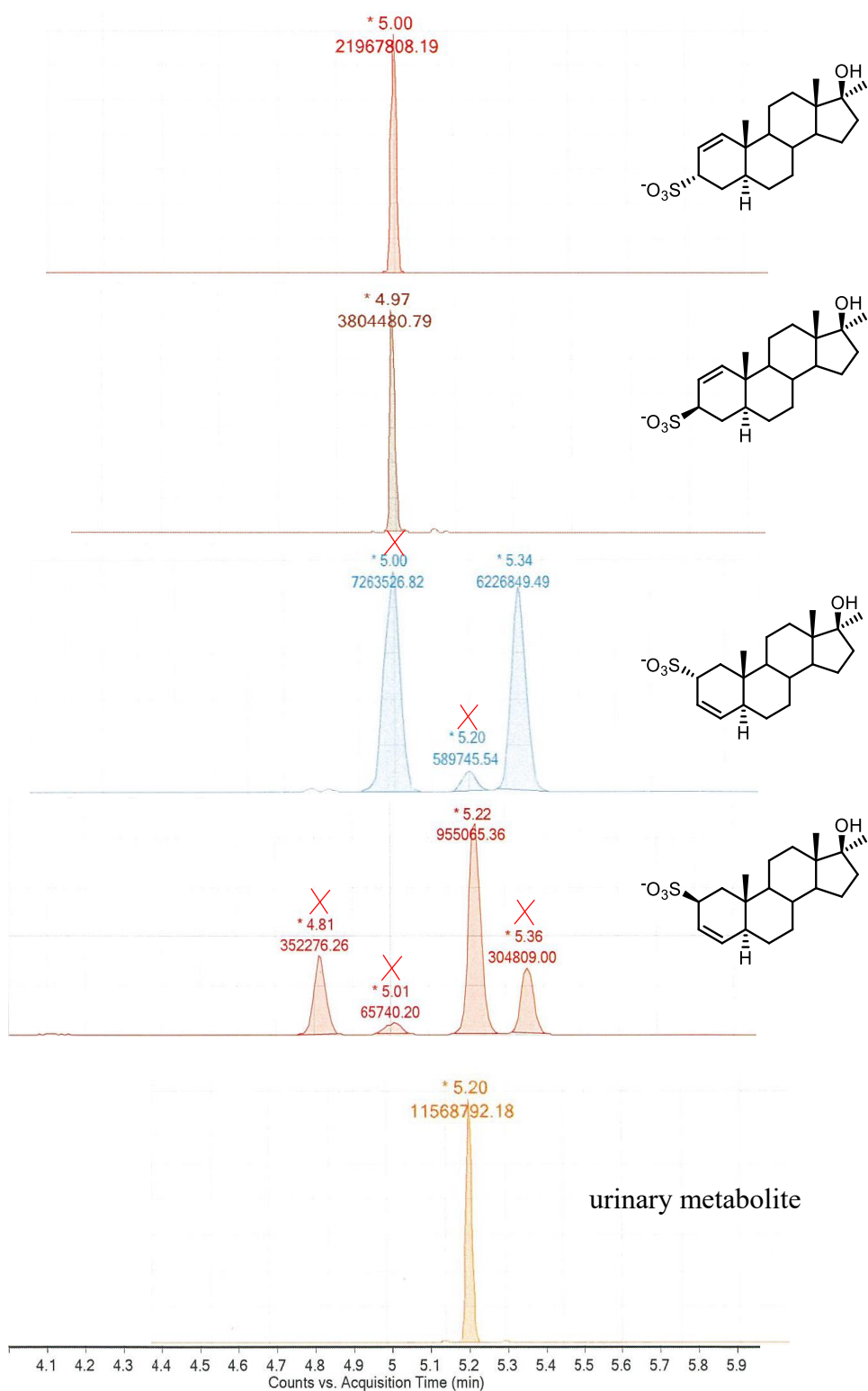


Figure 4.11: Extracted ion chromatograms showing (m/z 367.1949) four steroid sulfonate reference materials and the urinary metabolite.

When the comparison of two ions is performed at three collision energies, the values of the urinary metabolite are within tolerance according to AORC criteria as shown

in table 4.3. Although this approach gives an acceptable match, GC-MS or other methods leading to additional product ions are crucial for the complete confirmation of the *in vivo* metabolite.

Collision energy (eV)	RT reference material (min)	RT metabolite [tolerance] (min)	Relative abundance reference material (%)		Relative abundance urine sample (%) [tolerance]	
			<i>m/z</i> 367	<i>m/z</i> 80	<i>m/z</i> 367	<i>m/z</i> 80
20	5.22	5.20 [5.17-5.27]	100	22	100 [60-100]	18 [2-42]
25			100	69	100 [60-100]	69 [41-97]
30			39	100	42 [19-59]	100 [60-100]

Table 4.3: The comparison of 3-ene-2 β -sulfonate reference material with the urinary metabolite using two ions over three collision energies.

4.6 Chemical oxidation of steroid episulfides

In the section 4.3, the proposed biogenesis of the suspected sulfonate metabolite was discussed. The proposed metabolic pathway involved the oxidation of an epithio-steroid and ring-opening to form the relevant sulfenic acid and further oxidation leading to the sulfonate. Therefore, it was considered that chemical oxidation may provide support for this biogenesis proposal.

4.6.1 Chemical oxidation of hemapolin

The previous equine *in vivo* metabolism study of hemapolin did not report any sulfur-containing compounds during the GC-MS analysis. At the same time, previous studies have proven that episulfides and episulfoxides can decompose easily to form

the corresponding olefin. Therefore, the chemical oxidation was performed under mild conditions.

Hydrogen peroxide (H_2O_2) and sodium bicarbonate (NaHCO_3) were used as reagents for the oxidation of hemapolin, in a 10:1 ratio of hemapolin and epistane. The reaction was performed in ethanol and the reaction mixture was stirred for one hour at 50 °C. After that, the products were recovered by WAX SPE purification, and an NMR of the methanolic neutral fraction provided a spectrum matching to hemapolin (*R*)-*S*-oxide and this was confirmed with both low and high resolution ESI LC-MS. The $\text{NH}_4\text{OH}/\text{MeOH}$ anionic fraction showed traces of the 1-ene-3 α -sulfonate and this was confirmed by comparing the NMR with previously synthesised sulfonate (figure 4.12). Further, LC-MS data revealed the presence of the 1-ene-3 α -sulfonate:3-ene-2 β -sulfonate in 8:1 ratio (figure 4.13). This observation is satisfactory as hemapolin initially contained epistane as a minor component. Overall, chemical oxidation of hemapolin provided hemapolin (*R*)-*S*-oxide as the major product with the 1-ene-3 α -sulfonate provided as a minor product as expected based on the previously described biosynthesis proposal.

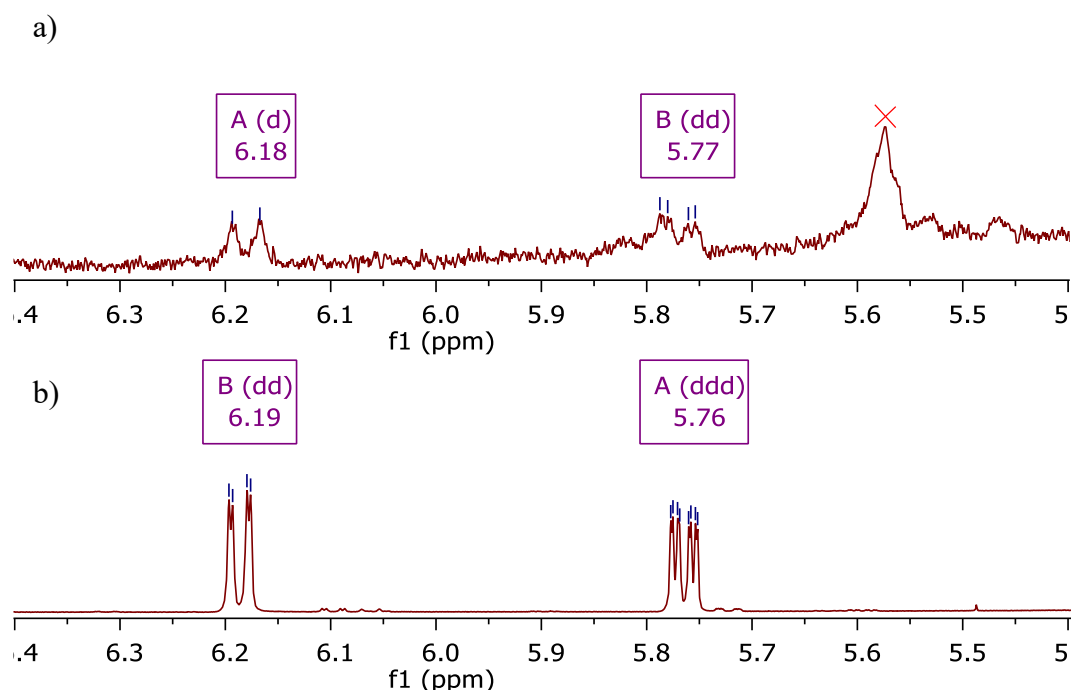


Figure 4.12: a) Traces of 1-ene-3 α -sulfonate observed in chemical oxidation of hemapolin. b) Pure 1-ene-3 α -sulfonate reference material.

4.6.2 Chemical oxidation of epistane

Chemical oxidation of epistane was performed using the similar protocol starting from the epistane:hemapolin 4:1 mixture prepared in section 4.4.1. After the purification, the $\text{NH}_4\text{OH}/\text{MeOH}$ anionic fraction did not show any traces of 3-ene-2 β -sulfonate by NMR. Therefore, the samples were analysed in LC-MS instrument. To run the samples, first it was necessary to develop a method which could separate all four sulfonate isomers. The separation of 1-ene-3 α -sulfonate and 1-ene-3 β -sulfonate was challenging. Therefore, a method was developed based on the ARFL UHPLC method but including a very shallow gradient. Using this new method, it was possible to separate 3 α -sulfonate and 3 β -sulfonate, having retention time difference with ~ 0.4 minutes.

Therefore, with the developed method it was possible to identify both 1-ene-3 α -sulfonate and 3-ene-2 β -sulfonate from the epistane oxidation. Compared to hemapolin oxidation, more of the 3-ene-2 β -sulfonate was present, although it remained as the minor product (figure 4.13 c). The peak area ratio between 3-ene-2 β -sulfonate and 1-ene-3 α -sulfonate was 1:3.6, where the production of 2 β -sulfonate was higher than that of hemapolin oxidation (1:8). Other than those two sulfonates, the 1-ene-2 α -sulfonate was also present at a very low level and there were a number of other peaks which could not be identified. The neutral methanolic fraction of this purification contained a compound, which has the mass of m/z 337 in positive mode LC-MS that is expected to be epistane (*S*)-*S*-oxide. However, for further confirmation, epistane (*S*)-*S*-oxide reference material must be synthesised for comparison.

In summary oxidation of hemapolin:epistane 10:1 gave hemapolin (*R*)-*S*-oxide as the major product observed by ^1H -NMR and LC-MS. Minor products included 1-ene-3 α -sulfonate (^1H -NMR, LC-MS) derived from hemapolin and the 3-ene-2 β -sulfonate derived from epistane. The 1-ene-3 α -sulfonate and the 3-ene-2 β -sulfonates were observed in a 8:1 ratio based on peak areas, together with the number of unidentified peaks. The chemical oxidation results reveal that the hemapolin-(*R*)-sulfoxide is relatively stable as an end product for the chemical oxidation.

Oxidation of epistane:hemapolin 4:1 gave a product consistent with epistane-(*S*)-*S*-oxide as major product by ^1H -NMR and LC-MS. Minor products included the 1-ene-

3 α -sulfonate and the 3-ene-2 β -sulfonate observed with LC-MS in 3.6:1 peak area ratio as mentioned above. Of the sulfonate products, the observation of 1-ene-3 α -sulfonate as the major isomer was unexpected given that the starting steroid mixture had epistane as the major isomer.

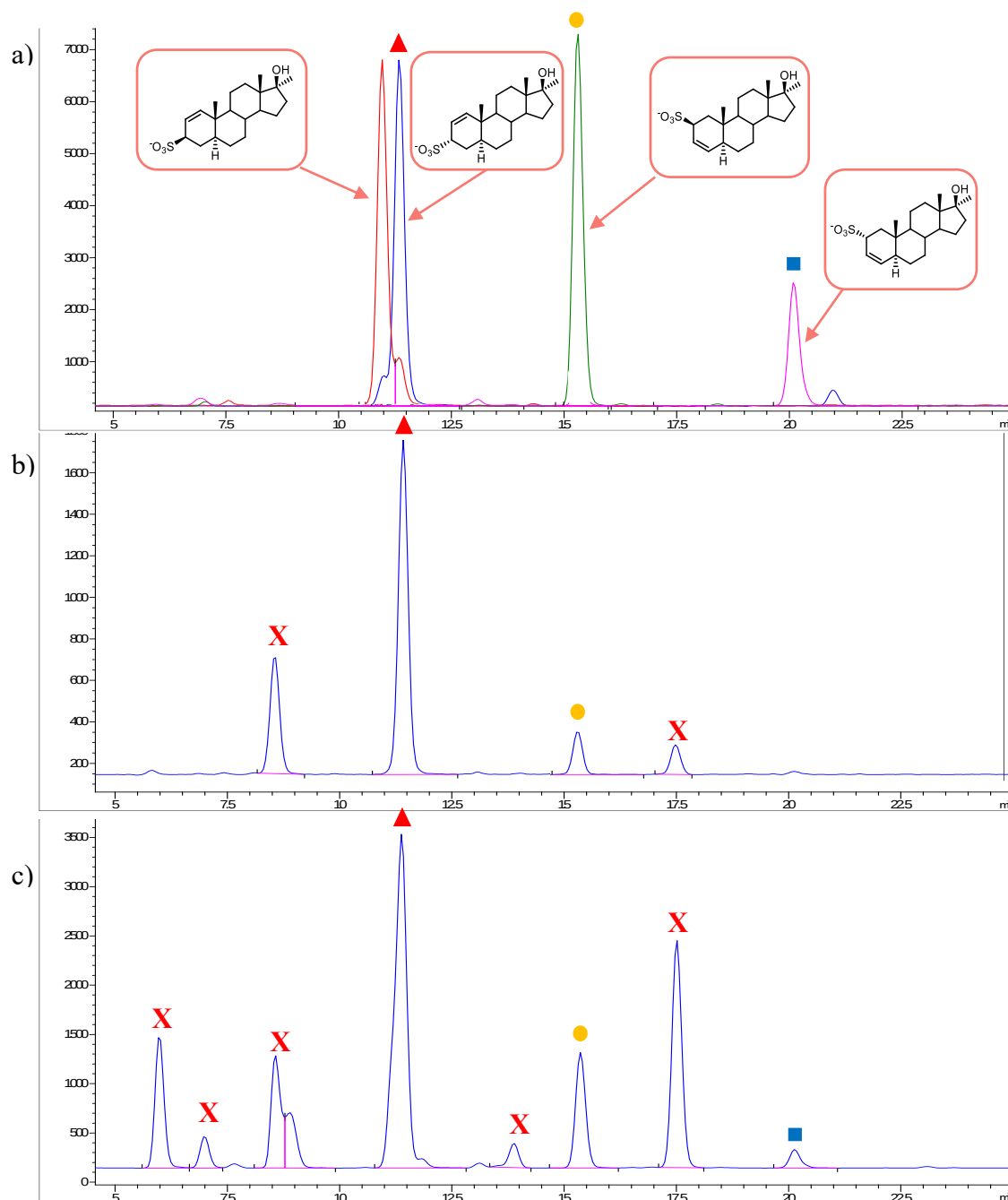


Figure 4.13: LC-MS extracted ions chromatograms (m/z 367) showing a) an overlay of the four sulfonates synthesised b) chemical oxidation of hemapolin c) chemical oxidation of epistane. Unidentified peaks denoted by 'X'

This observation reveals the steric hindrance disfavors the formation of 2 β -sulfonate product. In contrast to that, the oxidation and ring opening of 2 α ,3 α -episulfide towards 1-ene-3 α -sulfonate is more favourable.

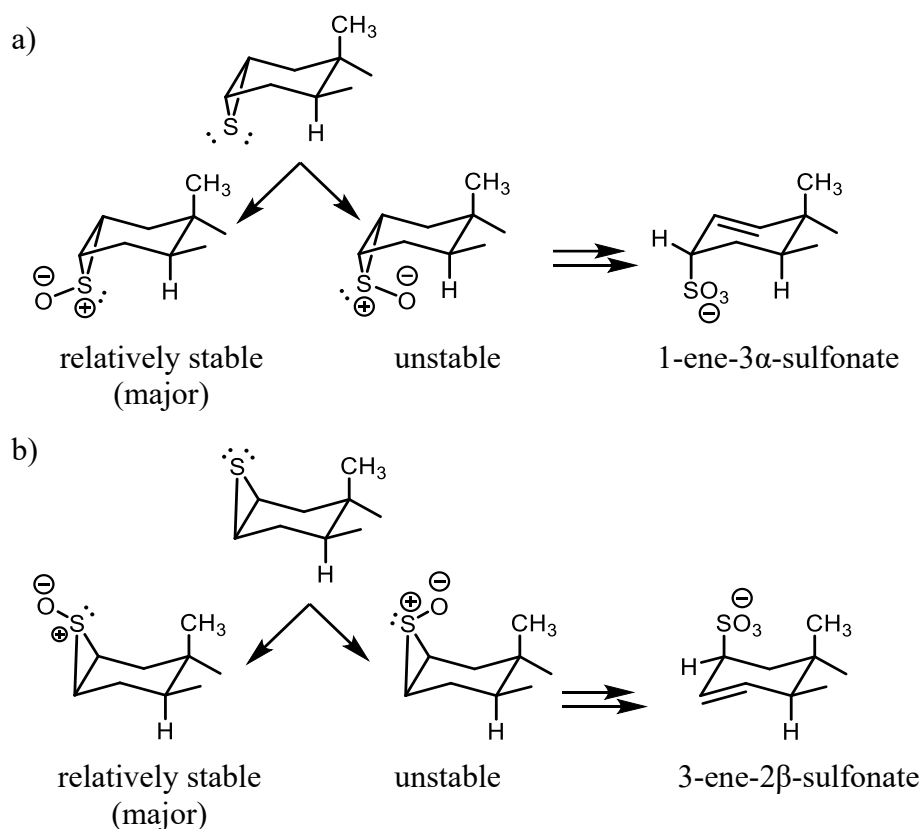


Figure 4.14: Two possible oxidation routes of a) hemapolin b) epistane

The geometrical positioning of the oxygen atom theoretically can be in two ways. The oxygen can either point towards the steroid ring or can point away from the steroid ring as shown in the figure 4.14. Hemapolin (*R*)-*S*-oxide has oxygen atom pointing away from the steroid ring system and this is the structure that is more favoured by the peroxide oxidizing at the less hindered sulfur lone pair. There are no β -hydrogens with correct geometry for elimination and that is the major reason for this becoming the major and stable sulfoxide isomer. Further to that, the published crystal structure of the 2 α ,3 α -epithio-5 α -androstan-17 β -ol (*R*)-*S*-oxide, oxygen atom is projecting away from the steroid ring system, which agrees with the above argument.^[10] For epistane, oxidation of less hindered sulfur lone pair gives epistane-(*S*)-*S*-oxide with no β hydrogens arranged for ring opening. Therefore, the steroid sulfoxides produced by oxidation of the least hindered lone pair on sulfur for hemapolin and epistane are expected to be stable and not to open the ring for further oxidation.

This discussed relates to the chemical oxidation of the two epithio steroids. However, inside the mamalian systems, enzymes govern metabolic oxidation. It could be that the spatial arrangement associated with enzyme substrate interactions allows the steroid to process through a selected metabolic pathway. This may mean that metabolic oxidation of hemapolin does not produce the (*S*)-sulfoxide leading to ring opening and 3 α -sulfonate formation.

This is conducted as a preliminary study and in future work we will conduct this study using pure hemapolin and epistane. The structure of the products should also be confirmed by a range of additional techniques, including ¹H-NMR, LC-MS/MS and GC-MS/MS.

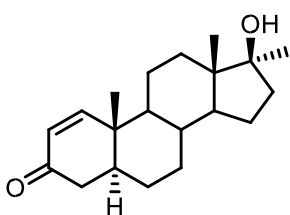
4.7 Conclusion and future directions

This chapter provides strong preliminary evidence for a novel phase I metabolic pathway leading to the formation of steroid sulfonate metabolites. However, the full details of the formation of the observed *in vivo* metabolite is yet to be revealed. When considering the urinary metabolite, as we could not obtain required number of transitions/ions, the confirmation is not yet complete according to AORC criteria. Therefore, it is necessary to develop a GC-MS method instead of LC-MS, as GC-MS could generate a range of fragments which can be used for comparison. To do this it may be necessary to form alkyl or silyl esters of those steroid sulfonates.

One of our main future goals is to perform chemical oxidation on pure hemapolin and epistane. For pure hemapolin, only 1-ene-3 α -sulfonate (major) and 3-ene-2 α -sulfonate (minor) are expected. Similar to this, for pure epistane, only 3-ene-2 β -sulfonate (major) and 1-ene-3 β -sulfonate (minor) are expected. Therefore, further confirmation of the products of chemical oxidation are required. For that, it will also be necessary to ensure that the peaks we observe are exactly the peaks what we think, by applying LC-MS/MS or other techniques.

4.8 Experimental

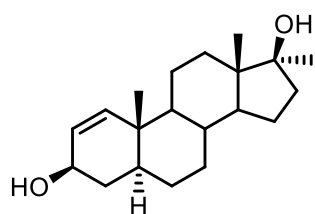
4.8.1 17 β -Hydroxy-17 α -methyl-5 α -androst-1-en-3-one (E5)^[26]



A solution of 17 β -hydroxy-17 α -methyl-5 α -androst-3-one (311 mg, 1.02 mmol) in THF (15 mL) was treated with phenyltrimethylammonium tribromide (435 mg, 1.16 mmol) and stirred at room temperature for 60 min. The reaction was diluted with ethyl acetate (100 mL) and the organic extract washed with saturated aqueous sodium bicarbonate solution (100 mL), water (100 mL), saturated aqueous sodium chloride solution (100 mL), dried with anhydrous magnesium sulfate and concentrated under reduced pressure to afford 2 α -bromo-17 β -hydroxy-17 α -methyl-5 α -androst-3-one which was used without further purification. R_f 0.46 (40% EtOAc/hexanes); ^1H NMR (400 MHz, CDCl_3): 4.75 (dd, J 6.3 Hz, 13.3 Hz, 1H, C2-H), 2.65 (dd, J 6.3 Hz, 12.8 Hz, 1H, C4/C1-H), 2.42 (m, 2H), 1.90-0.70 (m, 17H), 1.21 (s, 3H, C20-H₃), 1.10 (s, 3H, C19-H₃), 0.87 (s, 3H, C18-H₃), OH not observed. The crude product was then dissolved in DMF (35 mL) and the solution was transferred to a dry flask containing lithium bromide (145 mg, 1.67 mmol) and lithium carbonate (139 mg, 1.89 mmol) under a nitrogen atmosphere. The solution was then brought to reflux and stirred for 16 h. After cooling to room temperature, the reaction was diluted with ethyl acetate (100 mL) and the organic extract was washed with aqueous hydrochloric acid solution (2 M, 2 x 100 mL), water (2 x 100 mL), saturated aqueous sodium chloride solution (100 mL). Finally, the organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound (171 mg, 55% over two steps) as a pale yellow solid. R_f 0.40 (40% EtOAc/hexanes); mp 145-148 °C (lit^[26] 158-160 °C); $[\alpha]^{25}_{\text{D}} +36$ (c 1.0, CHCl_3) (lit^[26] $[\alpha]^{25}_{\text{D}} +30$ [CHCl_3]); ^[26] ^1H NMR (400 MHz, CDCl_3): 7.15 (d, J 10.2 Hz, 1H, C1-H), 5.85 (d, J 10.2 Hz, 1H, C2H), 2.37 (dd, J = 17.7, 14.1 Hz, 1H, C4-H), 2.22 (dd, J = 17.7, 4.1 Hz, 1H, C4-H), 1.91 (m, 1H, C5-H), 1.86-1.70 (m, 4H), 1.62-1.20 (m, 9H), 1.22 (s, 3H, C20-H₃), 1.03 (s, 3H, C19-H₃), 1.01-0.90 (m, 2H), 0.89 (s, 3H, C18-H₃), OH not observed; ^{13}C NMR (150 MHz, CDCl_3): 200.4 (C3), 158.6 (C1), 127.6 (C2), 81.7 (C17), 50.7, 50.1, 45.8, 44.5, 41.1, 39.2, 39.1, 36.6, 31.7, 31.1, 27.7, 26.0, 23.3, 21.0, 14.3 (C18), 13.2 (C19); LRMS (+EI): m/z 302 (50%, $[\text{C}_{20}\text{H}_{30}\text{O}_2]^+$), 269 (28%), 260 (23%), 245 (50%), 242 (35%), 200 (23%), 163 (40%), 134 (30%),

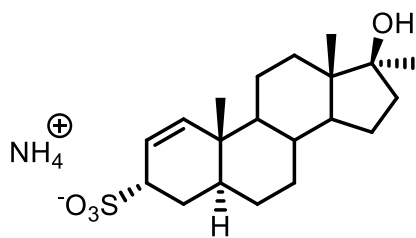
122 (100%), 107 (50%), 91 (35%), 79 (35%), HRMS (+EI): found 302.2245, $[C_{20}H_{30}O_2]^+ \bullet$ requires 302.2246.

4.8.2 17 α -Methyl-5 α -androst-1-ene-3 β ,17 β -diol (D7)^[27]



A solution of 17 β -hydroxy-17 α -methyl-5 α -androst-1-ene-3-one (470 mg, 1.56 mmol) and cerium chloride ($CeCl_3 \cdot 7H_2O$, 469 mg, 12.3 mmol) in methanol (30 mL) was slowly treated over 10 minutes with sodium borohydride (478 mg, 12.5 mmol) at 0 °C. The resulting white slurry was stirred at room temperature for 15 minutes. The reaction was diluted with water (50 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic extract was washed with brine (100 mL), dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound as a 10:1 mixture of 3 β and 3 α alcohols (383 mg, 81%). Data is reported for the major isomer where appropriate. R_f 0.36 (40% EtOAc/hexanes); mp 201-205 °C (lit^[27] 213.5-214.5); $[\alpha]^{25}_D +24$ (C 1.0 $CHCl_3$) (lit^[27] +17); ^1H NMR (600 MHz, $CDCl_3$): 5.92 (dd, J = 10.2, 1.9 Hz, 1H, C1-H), 5.49 (dt, J = 10.2, 1.8 Hz, 1H, C2-H), 4.30 (m, 1H, C3-H), 1.83-1.68 (m, 5H), 1.58-1.46 (m, 4H), 1.45-1.32 (m, 4H), 1.30-1.21 (m, 3H), 1.20 (s, 3H, C20- H_3), 0.93 (s, 3H, C19- H_3), 0.92-0.88 (m, 1H), 0.86 (s, 3H, C18- H_3), 0.85-0.80 (m, 1H), 2 x OH not observed; ^{13}C NMR (150 MHz, $CDCl_3$): 138.1 (C1), 128.9 (C2), 81.8 (C17), 69.0 (C3), 51.7 (C9), 50.9 (C14), 45.8 (C13), 43.8 (C5), 39.2 (C16), 38.3 (C10), 36.6 (C8), 36.0 (C4), 31.8 (C7), 31.8 (C12), 28.2 (C6), 26.0 (C20), 23.4 (C15), 21.0 (C11), 16.0 (C19), 14.3 (C18); LRMS (+EI): m/z 304 (35%, $[C_{20}H_{32}O_2]^+ \bullet$), 286 (30%), 268 (27%), 253 (35%), 246 (33%), 234 (33%), 228 (30%), 216 (45%), 215 (33%), 213 (25%), 176 (50%), 178 (30%), 163 (28%), 161 (50%), 160 (37%), 159 (27%), 149 (28%), 147 (35%), 145 (25%), 135 (27%), 133 (25%), 131 (25%), 123 (25%), 122 (25%), 121 (33%), 119 (37%), 118 (30%), 116 (25%), 109 (37%), 108 (30%), 107 (51%), 106 (57%), 105 (80%), 95 (43%), 93 (47%), 91 (100%), 81 (40%), 79 (47%), 77 (25%), 71 (26%), 79 (47%), 67 (28%), 55 (25%), 43 (33%),; HRMS (+EI): found 304.2403, $[C_{20}H_{32}O_2]^+ \bullet$ requires 304.2402.

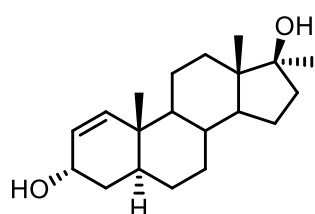
4.8.3 17 β -Hydroxy-17 α -methyl-5 α -androst-1-ene-3 α -sulfonate, ammonium salt



Triphenylphosphine (880 mg, 3.36 mmol) dissolved in THF (15 mL) at 0 °C was treated with diethyl azodicarboxylate (DEAD, 590 μ L, 3.20 mmol) and stirred at 0 °C for 30 minutes. Then the mixture was treated with thioacetic acid (250 μ L, 3.69 mmol) followed 17 α -methyl-5 α -androst-1-ene-3 β ,17 β -diol (475 mg, 1.56 mmol) in THF (18 mL) and stirred 16 hours at room temperature. The resulting yellow colour solution was diluted with water (50 mL) and extracted with EtOAc (3x100 mL). The combined organic extract was washed with brine (100 mL), dried over anhydrous magnesium sulfate and concentrated under reduced pressure to afford 17 β -hydroxy-17 α -methyl-5 α -androst-1-ene-3 α -yl thioacetate (525 mg). R_f 0.63 (5% EtOH/DCM); ^1H NMR (400 MHz, CDCl_3): 6.01 (dd, J = 9.9, 1.4 Hz, 1H, C1/C2-H), 5.47 (ddd, J = 10.0, 4.7, 1.3 Hz, 1H, C1/C2-H), 4.18 (m, 1H, C3-H), 2.3 (s, 3H, C21-H₃), 2.09 (ddd, J = 14.9, 12.9, 5.5 Hz, 1H), 1.84-1.66 (m, 4H), 1.60-1.16 (m, 9H), 1.201 (s, 3H, C20-H₃), 1.0-0.9 (m, 1H), 0.86 (s, 3H, C19-H₃), 0.86 (s, 3H, C20-H₃), OH not observed. A portion of the crude product (20 mg) was dissolved in ethanol (1 mL) and treated with sodium bicarbonate (2 M, 35 μ L) and heated to 50 °C. This mixture was treated with H_2O_2 (28 μ L, 30%), in sodium bicarbonate (2 M, 35 μ L) and stirred at 50 °C for one hour. Then the reaction mixture was evaporated to dryness and SPE WAX purification was performed to afford the title compound (13 mg). Further purification was done with preparative HPLC. An isocratic method with 50% solvent A (10 mM ammonium acetate in methanol + 0.1% formic acid) and 50% solvent B (10 mM ammonium acetate in water + 0.1% formic acid) was set up using Waters Alliance 2695 instrument to separate the desired product. A reverse-phase Agilent Zorbax SB-C18 (4.6 x 150 mm) column was used with 15 minutes run time and 50 μ L injection volume. Desired product was detected with a mass detector and the fractions were collected separately. R_f 0.69 (7:2:1 EtOAc/MeOH/ H_2O); ^1H NMR (600 MHz, CD_3OD): 6.19 (dd, J = 10.2, 2.1 Hz, 1H, C1-H), 5.76 (ddd, J = 10.2, 3.9, 1.3 Hz, 1H, C2-H), 3.37 (m, 1H, C3-H), 2.04 (m, 1H, C4-H), 1.90 (m, 1H, C5-H), 1.87-1.77 (m, 3H, C4-H, C11-H, C16-H) 1.71 (m, 1H, C7-H), 1.64 (m, 1H, C16-H), 1.59-1.52 (m, 2H, C12-H, C15-H), 1.48 (1H, m, C8-H), 1.44-1.38 (m, 2H, C6-H, C11-H), 1.33-1.23 (m, 4H, C6-H, C12-H, C14-H, C15-H), 1.18 (s, 3H, C20-H₃), 0.97 (m, 1H, C7-H), 0.91-0.87

(m, 1H, C9-H), 0.86 (s, 6H, C18-H₃, C19-H₃); ¹³C NMR (150 MHz, CD₃OD): 141.5 (C1), 121.8 (C2), 82.24 (C17), 57.6 (C3), 52.2 (C14), 52.1 (C9), 47.0 (C13), 40.8 (C5), 39.3 (C16), 38.4 (C10), 37.9 (C8), 33.0 (C12), 32.8 (C7), 29.0 (C6), 27.9 (C4), 26.1 (C20), 24.3 (C15), 21.9 (C11), 15.1 (C18), 14.8 (C19); LRMS (-ESI): *m/z* 367 (100%), ([C₂₀H₃₁O₄S]⁻), HRMS (-ESI): found 367.1949, [C₂₀H₃₁O₄S]⁻ requires 367.1936.

4.8.4 17 α -Methyl-5 α -androst-1-ene-3 α ,17 β -diol (D8)

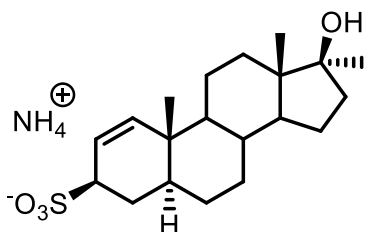


17 α -Methyl-5 α -androst-1-ene-3 β ,17 β -diol (200 mg, 0.66 mmol, Ph₃P (440 mg, 1.67 mmol), and 4-nitrobenzoic acid (290 mg, 1.73 mmol), in a flame dried flask were dissolved in dry toluene (10 mL) and the mixture was brought to 80 °C. Then DEAD (350 μ L, 1.92 mmol) was added dropwise.

After 2 hours the reaction was allowed to cool down, diluted with water (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic extract was washed with brine (100 mL) and dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (25% EtOAc/hexanes) to afford 17 α -methyl-5 α -androst-1-ene-3 β -*p*-nitrobenzoate. This compound was then hydrolyzed with aqueous sodium hydroxide in methanol. The crude residue was purified by column chromatography (30% EtOAc/hexanes) to afford the title compound (58 mg, 28% yield over two steps) as a 10:1 mixture of 3 α and 3 β alcohols. Data is reported for the major isomer where appropriate. *R_f* 0.35 (40% EtOAc/hexanes); mp 169-173 °C; ¹H NMR (600 MHz, CDCl₃): 6.05 (d, *J* = 10.0 Hz, 1H, C1-H), 5.63 (ddd, *J* = 10.0, 4.4, 1.5 Hz, 1H, C2-H), 4.07 (m, 1H, C3-H), 1.81-1.67 (m, 5H), 1.58-1.42 (m, 5H), 1.40-1.18 (m, 6H), 1.19 (s, 3H, C20-H₃), 0.97-0.81 (m, 2H), 0.85 (s, 3H, C18-H₃), 0.80 (s, 3H, C19-H₃), 2 x OH not observed; ¹³C NMR (150 MHz, CDCl₃): 140.4 (C1), 126.3 (C2), 81.7 (C17), 64.4 (C3), 51.0 (C9), 51.0 (C14), 45.8 (C13), 43.8 (C5), 39.1 (C5/C16), 39.1 (C5/C16), 38.2 (C10), 36.7 (C8), 35.0 (C4), 31.8 (C7), 31.8 (C12), 28.0 (C6), 26.0 (C20), 23.4 (C15), 21.0 (C11), 14.3 (C18), 14.0 (C19); LRMS (+EI): *m/z* 304 (30%, [C₂₀H₃₂O₂]⁺•), 286 (25%), 268 (30%), 253 (37%), 246 (30%), 228 (40%), 216 (45%), 215 (30%), 176 (50%), 178 (30%), 163 (28%), 161 (50%), 160 (37%), 159 (27%), 149 (28%), 147 (35%), 145 (25%), 135 (27%), 133 (25%), 131 (25%), 122 (25%), 121 (33%), 119 (37%), 116 (25%), 109 (37%), 108 (30%), 107 (51%), 105 (80%), 93 (47%), 91

(100%), 81 (40%), 79 (47%), 71 (26%), 79 (47%), 67 (28%), 55 (25%); HRMS (+EI): found 304.2403, $[C_{20}H_{32}O_2]^+$ requires 304.2402

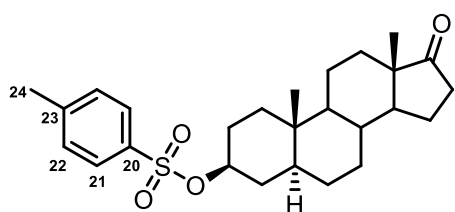
4.8.5 17 β -Hydroxy-17 α -methyl-5 α -androst-1-ene-3 β -sulfonate, ammonium salt



Triphenylphosphine (105 mg, 400 μ mol) dissolved in THF (2 mL) at 0 $^{\circ}$ C was treated with diethyl azodicarboxylate (DEAD, 70 μ L, 384 μ mol) and stirred at 0 $^{\circ}$ C for 30 minutes. Then the mixture was treated with thioacetic acid (25 μ L, 369 μ mol) followed by 17 α -methyl-5 α -androst-1-ene-3 α ,17 β -diol (51.2 mg, 167 μ mol) in THF (1 mL) and stirred 16 hours at room temperature. The resulting yellow colour solution was diluted with water (50 mL), and extracted with EtOAc (3x50 mL). The combined organic extract was washed with brine (100 mL), dried over anhydrous magnesium sulfate and concentrated under reduced pressure to afford 17 β -hydroxy-17 α -methyl-5 α -androst-1-ene-3 β -yl thioacetate. R_f 0.78 (5% EtOH/DCM); ^1H NMR (400 MHz, $CDCl_3$): 5.97 (dd, J = 10.1, 2.4 Hz, 1H, C1/C2-H), 5.37 (ddd, J = 10.0, 2.6, 1.2 Hz, 1H, C1/C2-H), 4.19 (ddt, J = 9.9, 7.2, 2.4 Hz, 1H, C3-H) 2.3 (s, 3H, C21-H₃), 1.8-1.65 (m, 5H), 1.62-1.22 (m, 11H), 1.20 (s, 3H, C20-H₃), 0.94 (m, 1H), 0.88 (s, 3H, C19-H₃), 0.86 (s, 3H, C18-H₃), 0.80 (m, 1H), OH not observed. Then 35 mg from the crude product was dissolved in ethanol (2 mL) and treated with sodium bicarbonate (2 M, 60 μ L) and allowed to heat up to 50 $^{\circ}$ C. This mixture was treated with H_2O_2 (30%, 45 μ L), in sodium bicarbonate (2 M, 60 μ L) and stirred at 50 $^{\circ}$ C for an hour. Then the reaction mixture was evaporated to dryness and SPE WAX purification was done to afford the title compound (18 mg). R_f 0.65 (7:2:1 EtOAc/MeOH/ H_2O); ^1H NMR (600 MHz, CD_3OD): 6.10 (dd, J = 10.3, 2.5 Hz, 1H, C1-H), 5.73 (ddd, J = 10.3, 2.6, 1.2 Hz, 1H, C2-H), 3.55 (m, 1H, C3-H), 1.94 (m, 1H, C4-H), 1.88-1.72 (m, 4H, C4-H, C7-H, C11-H, C16-H) 1.70 (m, 1H, C16-H), 1.60-1.47 (m, 3H, C8-H, C12-H, C15-H), 1.45-1.38 (m, 4H, C5-H, 2 x C6-H, C11-H), 1.33-1.23 (m, 3H, C12-H, C14-H, C15-H), 1.18 (s, 3H, C20-H₃), 0.95 (s, 3H, C19-H₃), 0.94 (m, 1H, C7-H), 0.86 (s, 3H, C18-H₃), 0.82 (m, 1H, C9-H); ^{13}C NMR (150 MHz, CD_3OD): 140.5 (C1), 122.3 (C2), 82.2 (C17), 60.5 (C3), 52.9 (C9), 52.2 (C14), 47.0 (C13), 45.2 (C5), 39.3 (C16), 38.8 (C10), 37.8 (C8), 33.0 (C7), 33.0 (C12), 29.3 (C6), 28.7 (C4), 26.2 (C20), 24.3 (C15), 21.9 (C11), 16.0

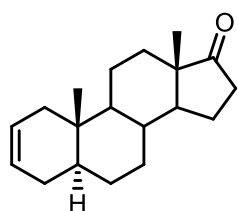
(C19), 14.8 (C18); LRMS (-ESI): m/z 367 (100%), $[\text{C}_{20}\text{H}_{31}\text{O}_4\text{S}]^-$, , HRMS (-ESI): found 367.1936, $[\text{C}_{20}\text{H}_{31}\text{O}_4\text{S}]^-$ requires 367.1949.

4.8.6 Epiandrosterone 3-tosylate^{[28],[29]}



A solution of epiandrosterone (10.0 g, 34.5 mmol) in pyridine (100 mL) was added to a flask containing TsCl (10.0 g, 52.4 mmol) and was stirred at room temperature for 18 h. The reaction was then diluted with water (500 mL) and filtered off the solid. The crude tosylate was then purified by recrystallisation from acetone to afford Epiandrosterone 3-tosylate (13.92 g, 90%) as small white needles. R_f 0.78 (40% EtOAc/hexanes); mp 168-172 °C (lit^[28] 164-165 °C); $[\alpha]^{25}_D$ +58 (c 1.0, CHCl_3) (lit^[29] +46 [CHCl_3]); ^1H NMR (400 MHz, CDCl_3): 7.79 (dd, J = 8.2, 2H, C21-H), 7.32 (d, J = 8.0 Hz, 2H, C22-H), 4.41 (m, 1H, C3-H), 2.44 (s, 3H, C24-H₃), 2.43 (1H, C16-H), 2.1-0.60 (m, 21H), 0.84 (s, 3H, C18-H₃), 0.80 (s, 3H, C19-H₃); ^{13}C NMR (100 MHz, CDCl_3): 221.2 (C17), 144.5 (C20), 134.9 (C23), 129.9 (C21), 127.7 (C22), 82.3 (C3), 54.3, 51.5, 47.9, 44.9, 36.9, 36.0, 35.5, 35.1, 35.0, 31.6, 30.9, 28.5, 28.3, 21.9, 21.8, 20.6, 14.0 (C18), 12.2 (C19); LRMS (+EI): m/z 444 (15%, $[\text{C}_{26}\text{H}_{36}\text{O}_4\text{S}]^{+\bullet}$), 273 (25%), 272 (100%), 257 (30%), 218 (90%), 190 (35%), 172 (50%), 161 (25%), 147 (20%), 108 (45%), 107 (70%), 93 (35%), 91 (100%), 79 (40%), 67 (25%), 55 (25%); HRMS (+EI): found 444.2340, $[\text{C}_{26}\text{H}_{36}\text{O}_4\text{S}]^{+\bullet}$ requires 444.2334.

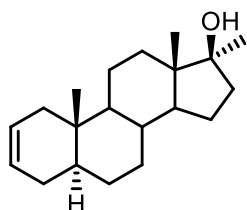
4.8.7 5 α -Androst-2-en-17-one^{[30],[31]}



A solution of epiandrosterone 3-tosylate (5.0 g, 11 mmol) in DBU (25 mL, 167 mmol) under nitrogen was brought to reflux and stirred for 18 h. The reaction was diluted with ethyl acetate (200 mL) and washed with a solution of aqueous hydrochloric acid (2 M, 2 x 200 mL). The aqueous phase was then re-extracted with additional ethyl acetate (2 x 200 mL). The combined organic extract was then washed with water (200 mL), saturated aqueous sodium chloride solution (200 mL), dried over anhydrous magnesium sulfate and concentrated under reduced pressure. Purification of the crude residue by column chromatography (10% EtOAc/hexanes)

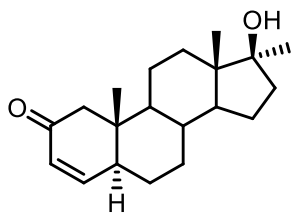
afforded the title compound (1.77 g, 59%) as a 92:8 inseparable mixture of Δ^{2-3} and Δ^{3-4} alkene isomers. Data is reported for the major isomer where appropriate. R_f 0.80 (40% EtOAc/hexanes); mp 101-105 °C (lit^[30] 92-96 °C); $[\alpha]^{25}_D +171$ (c 1.0, CHCl₃) (lit^[31] +140); ¹H NMR (400 MHz, CDCl₃): 5.59 (m, 2H, C2-H and C3-H), 2.44 (dd, J = 8.6 Hz, 19.1 Hz, 1H, C16-H), 2.11-0.73 (m, 19H), 0.87 (s, 3H, C18-H₃), 0.78 (s, 3H, C19-H₃); ¹³C NMR (100 MHz, CDCl₃): 221.7 (C17), 125.9 (2 peaks, C2 and C3), 54.3, 51.6, 47.9, 41.6, 39.8, 36.0, 35.3, 34.9, 31.7, 30.8, 30.4, 28.6, 21.9, 20.3, 13.9 (C18), 11.8 (C19); LRMS (+EI): 272 (92%, [C₁₉H₂₈O]⁺•), 218 (100%), 190 (32%), 161 (37%), 147 (15%), 122 (20%), 106 (20%), 93 (20%), 91 (20%), 79 (11%); HRMS (+EI): found 272.2143, [C₁₉H₂₈O]⁺• requires 272.2140.

4.8.8 17 α -Methyl-5 α -androst-2-en-17 β -ol (madol, **M**)^[32]



A solution of A solution of 5 α -androst-2-en-17-one (2.05 g, 7.52 mmol, 92:8 inseparable mixture of Δ^{2-3} and Δ^{3-4} alkene isomers) in dry THF (30 mL) under an atmosphere of nitrogen was treated with a solution of methylmagnesium bromide in diethyl ether (3.0 M, 13 mL, 39 mmol) and stirred at room temperature 16 h. The reaction was diluted with ethyl acetate (100 mL) and washed with saturated aqueous ammonium chloride solution (100 mL), water (100 mL), saturated aqueous sodium chloride solution (100 mL), dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (30% EtOAc/hexanes) to afford the title compound **M** (1.72 g, 80%) as a 92:8 inseparable mixture of Δ^{2-3} and Δ^{3-4} alkene isomers. Data is reported for the major isomer where appropriate. R_f 0.64 (40% EtOAc/hexanes); mp 145-150 °C (lit^[32] 154-155 °C); $[\alpha]^{25}_D +72$ (c 1.0, CHCl₃); ¹H NMR (400 MHz, CDCl₃): 5.59 (m, 2H, C2-H, C3-H), 2.0-0.6 (m, 20 H), 1.21 (s, 3H, C20-H₃), 0.86 (s, 3H, C18-H₃), 0.77 (s, 3H, C19-H₃), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 126.0 (2 peaks, C2 and C3), 81.9 (C17), 54.3, 50.8, 45.6, 41.8, 40.0, 39.1, 36.7, 34.9, 31.8, 31.7, 30.5, 28.8, 25.9, 23.4, 20.7, 14.1, 11.9; LRMS (+EI): 288 (60%, [C₂₀H₃₂O]⁺•), 270 (51%), 255 (95%), 238 (60%), 230 (84%), 216 (100%), 215 (64%), 201 (40%), 176 (66%), 161 (54%), 159 (27%), 147 (45%), 119 (27%), 107 (40%), 105 (48%), 95 (26%), 93 (55%), 91 (72%), 81 (32%), 79 (59%), 77 (34%), 67 (36%), 55 (25%), 43 (39%); HRMS (+EI): found 288.2455, [C₂₀H₃₂O]⁺• requires 288.2453.

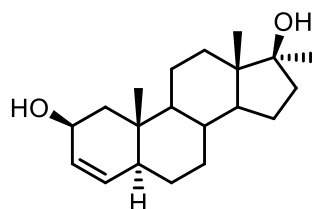
4.8.9 17 β -Hydroxy-17 α -methyl-5 α -androst-3-en-2-one (E6)



A solution of 17 α -methyl-5 α -androst-2-en-17 β -ol **M** (2.02 g, 7.00 mmol) in dioxane (100 mL) was treated with a solution of NBS (1.62 g, 9.10 mmol) in water (40 mL) and the reaction was stirred at room temperature for 2 h. The reaction was diluted with water (100 mL), extracted with chloroform (3 x 200 mL) and the organic extract was then dried with anhydrous magnesium sulfate and concentrated under reduced pressure to afford 3-bromo-17 α -methyl-5 α -androstane-2,17 β -diol which was used without further purification. R_f 0.64 (40% EtOAc/hexanes); ^1H NMR (400 MHz, CDCl_3): 4.31 (br s, 1H, C3-H), 4.19 (br s, 1H, C2-H), 2.30-0.60 (m, 20 H), 1.19 (s, 3H, C20-H₃), 1.00 (s, 3H, C19-H₃), 0.83 (s, 3H, C18-H₃), 2 x OH not observed. The crude product was then dissolved in chloroform (70 mL), treated with PCC (7.35 g, 34.1 mmol) and silica (7 g) and stirred at room temperature for 18 h. The reaction was then filtered and the solid residue was washed with diethyl ether (10 x 25 mL). The combined organic extract was then washed with aqueous hydrochloric acid solution (2 M, 2 x 100 mL), water (2 x 200 mL), saturated aqueous sodium chloride solution (200 mL), dried with anhydrous magnesium sulfate and concentrated under reduced pressure to afford 3-bromo-17 β -hydroxy-17 α -methyl-5 α -androstan-2-one, which was used without further purification. R_f 0.50 (40% EtOAc/hexanes); ^1H NMR (400 MHz, CDCl_3): 4.29 (br s, 1H, C3-H), 2.83 (d, J 13.5 Hz, 1H, C1-H), 2.31 (d, J 13.5 Hz, 1H, C1-H), 2.2-0.6 (m, 18 H), 1.22 (s, 3H, C20-H₃), 0.84 (s, 3H, C19-H₃), 0.75 (s, 3H, C18-H₃), OH not observed. The crude product was then dissolved in DMF (50 mL) and the solution was transferred to a dry flask containing lithium bromide (915 mg, 10.5 mmol) and lithium carbonate (780 mg, 10.5 mmol) under a nitrogen atmosphere. The solution was then brought to reflux and stirred for 18 h. After cooling to room temperature, the reaction was diluted with ethyl acetate (400 mL) and the organic extract was washed with aqueous hydrochloric acid solution (2 M, 2 x 200 mL), water (2 x 200 mL), saturated aqueous sodium chloride solution (200 mL), dried with anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford an 85:15 mixture containing the title compound and **E5** (683 mg, 32% over three steps) as a pale yellow solid. Data is reported for the major isomer where appropriate. R_f 0.37 (40% EtOAc/hexanes); ^1H NMR (400 MHz, CDCl_3): 6.57 (dd, J = 10.0, 2.0 Hz, 1H, C4-

H), 5.97 (dd, $J = 9.8$, 1H, C3-H), 2.57 (d, $J = 15.9$ Hz, 1H, C1-H), 2.07 (d, $J = 16.0$ Hz, 1H, C1-H), 2.4-0.8 (m, 16H), 1.22 (s, 3H, C20-H₃), 0.90 (s, 3H, C19-H₃), 0.86 (s, 3H, C18-H₃), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 200.3 (C2), 154.3 (C4), 128.7 (C3), 81.7 (C17), 53.6, 52.2, 50.6, 47.2, 45.8, 41.1, 39.0, 35.7, 31.8, 31.6, 26.6, 26.0, 23.3, 20.7, 14.2 (C18), 12.9 (C19); LRMS (+EI): m/z 302 (65%, [C₂₀H₃₀O₂]⁺), 284 (45%), 269 (100%), 245 (45%), 229 (45%), 161 (35%), 147 (25%), 121 (55%), 107 (55%), 91 (45%), 79 (45%), 55 (30%); HRMS (+EI): found 302.2242, [C₂₀H₃₀O₂]⁺ requires 302.2246.

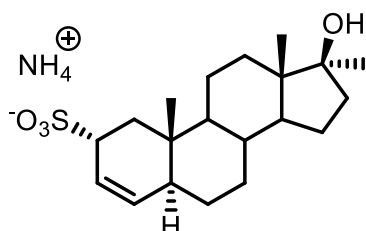
4.8.10 17 α -Methyl-5 α -androst-3-ene-2 β ,17 β -diol (D9)



A solution of 17 β -hydroxy-17 α -methyl-5 α -androst-3-en-2-one (mixture E5:E6 15:85; 600 mg, 1.98 mmol) and CeCl₃·7H₂O (3.1 g, 8.3 mmol) in methanol (20 mL) was slowly treated over 10 minutes with sodium borohydride (300 mg, 7.93 mmol) at 0 °C. The resulting white slurry was stirred at room temperature for 15 minutes. The reaction was diluted with water (100 mL) and extracted with ethylacetate (3 x 100 mL). The combined organic extract was washed with brine and dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (40% EtOAc/hexanes) to afford the title compound, (360 mg, 60%) a colourless solid as a 3:1:1 mixture of 3-en-2 β -ol, 1-en-3 β -ol and 3-en-2 α -ol enol isomers. Data is reported for the major isomer 17 α -methyl-5 α -androst-3-ene-2 β ,17 β -diol. R_f 0.35 (40% EtOAc/hexanes); ¹H NMR (400 MHz, CDCl₃): 5.60 (d, $J = 9.9$ Hz, 1H, C3-H), 5.42 (dt, $J = 9.9, 1.8$ Hz, 1H, C4-H), 4.30 (m, 1H, C2-H), 2.23 (dd, $J = 12.2, 6.6$ Hz, 1H, C1-H), 2.10-0.70 (m, 17H), 1.21 (s, 3H, C20-H₃), 0.85 (s, 3H, C19-H₃), 0.83 (s, 3H, C18-H₃) 2 x OH not observed; ¹³C NMR (100 MHz, CDCl₃): 134.1 (C4), 128.4 (C3), 81.9 (C17), 67.7 (C2), 53.2, 50.7, 46.7, 45.8, 45.0, 39.1 (2 peaks), 35.8, 31.8 (2 peaks), 26.8, 26.0, 23.3, 20.9, 14.2, 12.8; LRMS (+EI): m/z 304 (35%, [C₂₀H₃₂O₂]⁺), 286 (30%), 268 (27%), 253 (35%), 246 (33%), 234 (33%), 228 (30%), 216 (45%), 215 (33%), 213 (25%), 176 (50%), 178 (30%), 163 (28%), 161 (50%), 160 (37%), 159 (27%), 149 (28%), 147 (35%), 145 (25%), 135 (27%), 133 (25%), 131 (25%), 123 (25%), 122 (25%), 121 (33%), 119 (37%), 118 (30%), 116 (25%), 109 (37%), 108 (30%), 107 (51%), 106 (57%), 105 (80%), 95 (43%), 93

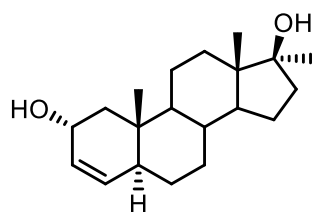
(47%), 91 (100%), 81 (40%), 79 (47%), 77 (25%), 71 (26%), 79 (47%), 67 (28%), 55 (25%), 43 (33%); HRMS (+EI): found 304.2403, $[C_{20}H_{32}O_2]^+$ requires 304.2402

4.8.11 17 β -Hydroxy-17 α -methyl-5 α -androst-1-ene-3 α -sulfonate, ammonium salt



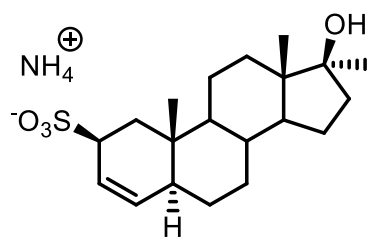
Triphenylphosphine (100 mg, 381 μ mol) dissolved in THF (1 mL) at 0 $^{\circ}$ C was treated with DEAD (70 μ L, 384 μ mol) and stirred at 0 $^{\circ}$ C for 30 minutes. Then the mixture was treated with thioacetic acid (24 μ L, 354 μ mol) followed by 17 α -methyl-5 α -androst-3-ene-2 β ,17 β -diol (steroid diol mixture, 56.0 mg, 183 μ mol) in THF (1 mL) and stirred 16 hours at room temperature. The resulting yellow colour solution was diluted with water (20 mL) and extracted with EtOAc (3x20 mL). The combined organic extract was washed with brine (20 mL) and dried over anhydrous magnesium sulfate and concentrated under reduced pressure to afford 17 β -hydroxy-17 α -methyl-5 α -androst-3-ene-2 α -yl thio acetate as a mixture. R_f 0.78 (5% EtOH/DCM). The crude product was used without purification. A portion of the crude product (20 mg) from was dissolved in ethanol (1 mL) and treated with sodium bicarbonate (2 M, 35 μ L) and heated to 50 $^{\circ}$ C. This mixture was treated with H_2O_2 (28 μ L, 30%), in sodium bicarbonate (2 M, 35 μ L) and stirred at 50 $^{\circ}$ C for one hour. Then the reaction mixture was evaporated to dryness and SPE WAX purification was done to afford the title compound mainly as 1:1 mixture of 2 α and 3 α sulfonates. Data is reported for 3-ene-2 α sulfonate. R_f 0.68 (7:2:1 EtOAc/MeOH/ H_2O); ^1H NMR (400 MHz, CD_3OD): 5.98 (d, J = 9.8 Hz, 1H, C3/C4- H), 5.53 (dt, J = 10.1, 2.7 Hz, 1H, C3/ C4- H), 3.54 (m, 1H, C2- H), 2.50-0.60 (m, 19 H), 1.19 (s, 3H, C20- H_3), 0.86 (s, 6H, C18- H_3 , C19- H_3); LRMS (-ESI): m/z 367 (100%), $[C_{20}H_{31}O_4S]^-$, HRMS (-ESI): found 367.1949, $[C_{20}H_{31}O_4S]^-$ requires 367.1905.

4.8.12 17 α -Methyl-5 α -androst-3-ene-2 α ,17 β -diol



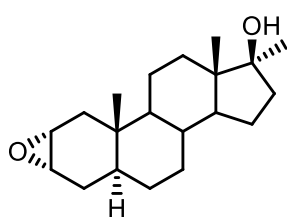
17 α -methyl-5 α -androst-3-ene-2 β ,17 β -diol (mixture of isomeric alcohols) 200 mg, 0.66 mmol, Ph₃P (440 mg, 1.67 mmol), and 4-nitrobenzoic acid (290 mg, 1.73 mmol), in a flame dried flask were dissolved in dry toluene (10 mL) and the mixture was brought to 80 °C. Then DEAD (350 μ L, 1.92 mmol) was added dropwise over 5 minutes. After 2 hours, the reaction was allowed to cool down, diluted with water (50 mL) and extracted with ethylacetate (3 x 50 mL). The combined organic extract was washed with brine (50 mL) and dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude 17 α -methyl-5 α -androst-3-ene-2 β -*p*-nitrobenzoate was used without further purification. This compound was then hydrolyzed with aqueous sodium hydroxide in methanol. The crude residue was purified by column chromatography (30% EtOAc/hexanes) to afford the title compound (49 mg) a colourless solid as a mixture of 3-ene-2 α -ol, 2-ene-3 α isomers in 14:1 ratio. Data is reported for the major isomer where appropriate. R_f 0.35 (40% EtOAc/hexanes); ¹H NMR (400 MHz, CDCl₃): 5.72 (dd, J = 8.7, 4.7 Hz, 1H, C1/ C2-H), 5.47 (dd, J = 9.8, 1.7 Hz, 1H C1/ C2-H), 4.22 (m, 1H, C3-H), 2.05 (d, J = 15.6 Hz, 1H, C1-H), 1.85-0.8 (m, 16H), 1.20 (s, 3H, C20-H₃), 0.92 (s, 3H, C18-H₃), 0.85 (s, 3H, C19-H₃), 0.68 (m, 1H). 2 x OH not observed; ¹³C NMR (100 MHz, CDCl₃): 134.2 (C4), 127.6 (C3), 81.7 (C17), 65.2 (C2), 53.9, 50.7, 46.0, 45.8, 44.3, 39.1, 36.0, 33.9, 31.9, 31.8, 27.4, 26.0, 23.4, 20.9, 14.2 (C18), 14.0 (C19); LRMS (+EI): *m/z* 304 (45%), [C₂₀H₃₂O₂]⁺•, 286 (50%), 276 (30%), 271 (27%), 269 (25%), 253 (48%), 245 (30%), 246 (90%), 231 (30%), 227 (55%), 228 (100%), 216 (30%), 215 (55%), 213 (82%), 200 (48%), 189 (45%), 188 (76%), 189 (27%), 176 (45%), 177 (38%), 176 (36%), 161 (63%), 159 (48%), 147 (50%), 145 (43%), 135 (35%), 133 (45%), 131 (33%), 123 (55%), 121 (50%), 119 (52%), 109 (27%), 107 (65%), 105 (85%), 95 (45%), 93 (55%), 91 (95%), 81 (38%), 79 (25%), 77 (30%), 67 (28%); HRMS (+EI): found 304.2411, [C₂₀H₃₂O₂]⁺• requires 304.2402.

4.8.13 17 β -Hydroxy-17 α -methyl-5 α -androst-3-ene-2 β -sulfonate, ammonium salt



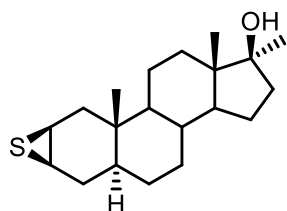
Triphenylphosphine (60.1 mg, 105 μ mol) dissolved in THF (1 mL) at 0 $^{\circ}$ C was treated with DEAD (50.0 μ L, 274 μ mol) and stirred at 0 $^{\circ}$ C for 30 minutes. Then the mixture was treated with thioacetic acid (15.0 μ L, 221 μ mol) followed 17 α -methyl-5 α -androst-3-ene-2 α ,17 β -diol (mixture of isomers, 32.0 mg, 105 μ mol) in THF (1 mL) and stirred 16 hours at room temperature. The resulting yellow colour solution was diluted with water (20 mL) and extracted with EtOAc (3x20 mL). The combined organic extract was washed with brine and dried over anhydrous magnesium sulfate and concentrated under reduced pressure to afford 17 β -hydroxy-17 α -methyl-5 α -androst-3-ene-2 β -yl thio acetate. Then the crude product was dissolved in ethanol (1 mL) and treated with sodium bicarbonate (2 M, 35 μ L) and heated to 50 $^{\circ}$ C. This mixture was treated with H₂O₂ (28 μ L, 30%), in sodium bicarbonate (2 M, 35 μ L) and stirred at 50 $^{\circ}$ C for an hour. Then the reaction mixture was evaporated to dryness and SPE WAX purification was done to afford the title compound (5 mg) as a mixture of 3-ene-2 β , 3-ene-2 α and 1-ene-3 α sulfonate isomers. Further purification was done with preparative HPLC as mentioned in section 4.7.1.3. Data is reported for 3-ene-2 β sulfonate. R_f 0.70 (7:2:1 EtOAc/MeOH/H₂O); ¹H NMR (600 MHz, CD₃OD): 5.83 (m, 1H, C3-H), 5.54 (m, 1H, C4-H), 3.52 (m, 1H, C2-H), 2.25 (dd, J = 12.9, 6.3 Hz, 1H, C1-H), 1.95 (m, 1H, C5-H), 1.85 (m, 1H, C16-H), 1.77 (m, 1H, C7-H), 1.73 (m, 1H, C12-H), 1.67 (m, 1H, C16-H), 1.62-1.49 (m, 5H, C1-H, C6-H, C8-H, C12-H, C15-H), 1.41-1.26 (m, 5H, C6-H, C11-H, C12-H, C14-H, C15-H), 1.22 (s, 3H, C20-H₃), 1.00 (m, 1H, C7-H), 0.88 (s, 3H, C18-H₃), 0.86 (s, 3H, C19-H₃), 0.90-0.82 (m, 1H, C9-H); ¹³C NMR (150 MHz, CD₃OD): 1136.1 (C4), 122.8 (C3), 82.3 (C17), 58.9 (C3), 54.8 (C9), 52.1 (C14), 47.0 (C5/C13), 47.0 (C5/C13), 39.2 (C16), 37.9 (C1), 37.7 (C8), 37.6 (C10), 32.9 (C7/C12), 32.9 (C7/C12), 28.2 (C6), 26.2 (C20), 24.3 (C15), 21.9 (C11), 14.8 (C18), 12.9 (C19); LRMS (-ESI): *m/z* 367 (100%), [(C₂₀H₃₁O₄S)]⁻, , HRMS (-ESI): found 367.1952, [C₂₀H₃₁O₄S]⁻ requires 367.1949.

4.8.14 2 α ,3 α -epoxy-17 α -methyl-5 α -androstan-17 β -ol^{[32],[33]}



A solution of 17 α -methyl-5 α -androst-2-en-17 β -ol (mixture of Δ^{2-3} and Δ^{3-4} alkene isomers, 270 mg, 940 μ mol) in ethylacetate (100 mL) was treated with *m*-CPBA (306 mg, 1.77 mmol,) and stirred at room temperature for 16 h. The reaction was diluted with ethylacetate (100mL) and washed with aqueous sodium sulfite (10% w/v, 100 mL), saturated aqueous sodium bicarbonate (100 mL), water (100 mL) and brine (100 mL). The organic extract was dried with anhydrous magnesium sulfate and concentrated under reduced pressure. Crude residue was purified by column chromatography (30% EtOAc/hexanes) to afford the title compound (white solid, 190 mg, 66%) as a mixture of 2 α ,3 α and 3 α ,4 α -epoxy isomers. Data is reported for the major isomer where appropriate. R_f 0.61 (40% EtOAc/hexanes); mp 190-195 °C (lit^[34] 205-207 °C); [α]_D²⁵ +21 (c 1.0, CHCl₃) (lit^[34] +0.5, CHCl₃); ¹H NMR (400 MHz, CDCl₃): 3.15 (m, 1H, C3-H), 3.11 (m, 1H, C2-H), 1.92 (dd, J = 15.1, 6.0 Hz, 1H, C1-H), 1.88 (m, 1H, C4-H), 1.78 (m, 1H, C16-H), 1.71 (m, 1H, C16-H), 1.64 (m, 1H, C7-H), 1.59-1.50 (m, 3H, C4-H, C11-H, C15-H), 1.48 (m, 1H, C12-H), 1.42 (d, J = 15.1 Hz, 1H, C1-H), 1.35-1.18 (m, 6H, C5-H, C6-H, C8-H, C11-H, C12-H, C15-H), 1.17-1.09 (m, 2H, H14, H16), 1.20 (s, 3H, C20-H₃), 0.83 (s, 3H, C18-H₃), 0.82-0.78 (m, 1H, C7-H), 0.77 (s, 3H, C19-H₃), 0.60 (m, 1H, C9-H); ¹³C NMR (100 MHz, CDCl₃): 81.9 (C17), 53.9 (C9), 52.5 (C3), 51.2 (C2), 50.7 (C14), 45.5 (C13), 39.1 (C16), 38.5 (C1), 36.7 (C8), 36.5 (C5), , 33.9 (C10), 31.7 (C7/C12), 31.6(C7/C12), 29.2 (C4), 28.5 (C6), 26.0 (C20), 23.4 (C15), 20.7 (C11), 14.0 (C18), 13.1 (C19); LRMS (+EI): *m/z* 304 (25%, [C₂₀H₃₂O₂]⁺•), 289 (55%), 286 (40%), 271 (40%), 247 (25%), 246 (60%), 231 (70%), 229 (100%), 215 (70%), 213 (50%), 176 (30%), 163 (80%), 147 (60%), 133 (75%), 108 (65%), 106 (95%); HRMS (+EI): found 304.2404, [C₂₀H₃₂O₂]⁺• requires 304.2402.

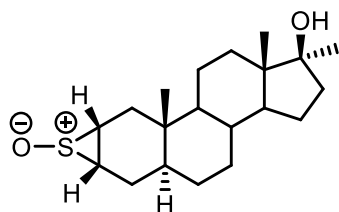
4.8.15 2 β ,3 β -Epithio-17 α -methyl-5 α -androstan-17 β -ol (epistane)



A solution of 2 α ,3 α -epoxy-17 α -methyl-5 α -androstan-17 β -ol (a mixture of 2 α ,3 α and 2 β ,3 β epoxides, 40.1 mg, 131 μ mol) in dry toluene (2 mL) under an atmosphere of nitrogen was treated with a solution of trifluoroacetic acid (20 μ L, 270

μmol) and Ph_3PS (43.2 mg, 131 μmol) was added in 6 portions over a period of 30 minutes and the reaction stirred at room temperature for 18 h. Then the reaction was diluted with ethyl acetate (50 mL), washed with water (2 x 50 mL), saturated aqueous sodium chloride solution (50 mL), dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by column chromatography (5% EtOAc/hexanes, silica pre-treated with Et_3N (5% v/v) to afford a 4:1 mixture of the title compound and 2 α ,3 α -epithio-17 α -methyl-5 α -androstan-17 β -ol (12.5 mg, 30%) as a colourless solid. Data is reported for the major isomer where appropriate. R_f 0.80 (30% EtOAc/hexanes); mp 160-165 $^\circ\text{C}$ (lit^[35] 151-153 $^\circ\text{C}$); ^1H NMR (600 MHz, CDCl_3): 3.24 (m, 1H, C3-H), 3.20 (m, 1H, C2-H), 2.43 (d, J = 15.4 Hz, 1H, C1-H), 2.10 (ddd, J = 15.5, 8.6, 4.8 Hz, 1H, C4-H), 1.86 (ddd, J = 15.7, 13.1, 2.7 Hz, 1H, C4-H), 1.82-1.75 (m, 2H, C1-H and C16-H), 1.71 (m, 1H, C16-H), 1.67-1.62 (m, 1H, C7-H), 1.65 (m, 2H, C11-H, C15-H), 1.49 (m, 1H, C12-H), 1.38-1.17 (m, 6H, C5-H, C6-H, C8-H, C11-H, C7/12-H, C15-H) 1.20 (s, 3H, C20-H₃), 1.17-1.06 (m, 2H, C6-H, C14-H), 0.92 (s, 3H, C19-H₃), 0.86-0.77 (m, 1H, C7/12-H), 0.83 (s, 3H, C18-H₃), 0.56 (ddd, J = 12.1, 10.5, 4.2 Hz, 1H, C9-H), OH not observed; ^{13}C NMR (150 MHz, CDCl_3): 81.8 (C17), 56.4 (C9), 50.7 (C14), 45.6 (C13), 43.4 (C5), 39.6 (C1), 39.1 (C16), 37.2 (C2), 35.8 (C8), 35.6 (C3), 34.8 (C10), 31.8 (C7/12), 31.8 (C7/12), 30.3 (C4), 28.6 (C6), 25.9 (C20), 23.5 (C15), 20.5 (C11), 15.0 (C19), 14.1 (C18); LRMS (-ESI): m/z 321 (6%, [$\text{C}_{20}\text{H}_{33}\text{O}_4\text{S}$]⁺), m/z 303 (100%, [$\text{C}_{20}\text{H}_{31}\text{O}_3\text{S}$]⁺), HRMS (-ESI): found 321.2210 [$\text{C}_{20}\text{H}_{33}\text{O}_4\text{S}$]⁺ requires 321.2207

4.8.16 2 α ,3 α -episulfinyl-17 α -methyl-5 α -androstan-17 β -ol (hemapolin S-oxide)



A dry flask containing 2 α ,3 α -epithio-17 α -methyl-5 α -androstan-17 β -ol (5.2 mg, 16 μmol) in acetone 500 μL under a nitrogen atmosphere was treated with a solution of DMDO in acetone (0.098 M, 320 μL , 31.4 μmol) and stirred at room temperature for 60 min. The reaction was diluted with ethyl acetate (10 mL), washed with aqueous sodium metabisulfite solution (10% w/v, 10 mL), water (10 mL), saturated aqueous sodium chloride solution (10 mL), dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The crude residue was purified by SPE WAX to afford the title

compound (2.8 mg, 51%) as a white solid. R_f 0.2 (40% EtOAc/hexanes); ¹H NMR (400 MHz, CDCl₃): 3.25 (td, J = 10.6, 3.5 Hz, 1H, C2-H), 3.11 (dd, J = 10.8, 5.8 Hz, 1H, C3-H), 2.45 (dd, J = 15.9, 10.4 Hz, 1H, C1-H), 2.18 (ddd, J = 15.7, 4.6, 1.2 Hz, 1H, C4-H), 1.85-1.15 (m, 13H, C1-H, C4-H, C6-H, C7-H, C8-H, C11-H₂, C12-H₂, C15-H₂, C16-H₂), 1.18 (s, 3H, C20-H₃), 1.13-1.00 (m, 2 H, C6-H, C14-H) 0.81 (s, 3H, C18-H₃), 0.78 (s, 3H, C19-H₃), 0.78-0.62 (m, 1H, C7-H), 0.46 (m, 1H, C9-H), 0.40 (m, 1H, C5-H), OH not observed; ¹³C NMR (100 MHz, CDCl₃): 81.7 (C17), 53.8 (C3/C9), 53.6 (C3/C9), 51.3 (C2), 50.6 (C14), 45.4 (C13), 40.6 (C15), 39.0 (C16), 36.6 (C8), 35.7 (C1), 33.3 (C10), 31.6 (C12), 31.1 (C7), 28.9 (C6), 25.9 (C20), 25.7 (C4), 23.3 (C15), 20.5 (C11), 14.0 (C18), 12.7 (C19); LRMS (+ESI): *m/z* 359 (100%, [C₂₀H₃₂O₂SNa]⁺); HRMS (+ESI): found 359.2009, [C₂₀H₃₂O₂SNa]⁺ requires 359.2015.

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CHAPTER 5

Conclusions and future directions

The work presented in this thesis has addressed different aspects of anti-doping analysis. This final chapter provides a summary of major achievements and suggests several avenues for future investigation.

The second chapter addresses one of the common limitations associated with drug metabolism research that *in vitro* studies mainly focus on glucuronylation rather than sulfation. The co-factor, or the universal sulfate donor, PAPS is extremely expensive and as a result limited attention is typically provided towards *in vitro* sulfation. In metabolism, the sulfate metabolites of some steroids, have been observed as important long term markers or are implicated in the generation of important metabolite classes such as C17 epimers. Therefore, methods to generate those sulfates in an affordable and a reliable way to better mimic *in vivo* metabolism are important.

The method developed in this thesis is inexpensive and it could mimic sulfation patterns observed in *in vivo* studies involving the direct use of the native cofactor PAPS. Comparative studies showed that ATP and sodium sulfate was capable of generating the same sulfate metabolites as generated with PAPS. Even though, the comparison was made between 16 mM ATP and 200 μ M PAPS, it is still significantly cheaper than using PAPS as the sulfate donor.

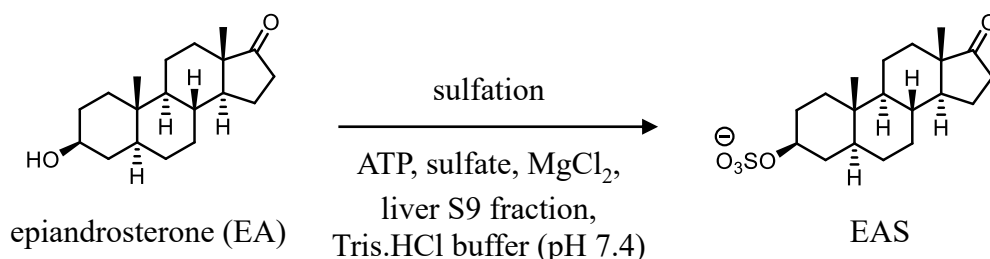


Figure 5.1: *In vitro* sulfation method established

The *in vitro* sulfation method developed, can be used to conduct *in vitro* metabolism to mimic *in vivo* metabolism studies in a more successful manner, which can be an answer to the ethical issues arise with *in vivo* studies.

Despite of all the rules and regulations established against doping, new designer steroids are still emerging as performance enhancing drugs with the potential to evade detection. $\Delta 6$ -Methyltestosterone is one of those that was identified in a black market product named “junglewarrfare”. A plan was set to chemically synthesise the above mentioned steroid, and to study the *in vitro* metabolism. To identify the metabolites formed, it was decided to synthesise reference materials targeting reduction pathways similar to those previously observed in an *in vivo* human study of $\Delta 6$ -MT metabolism. The synthesised reference materials were sent to three external institutes to carry out the *in vivo* studies in to the metabolism of this steroid with results expected over the months ahead.

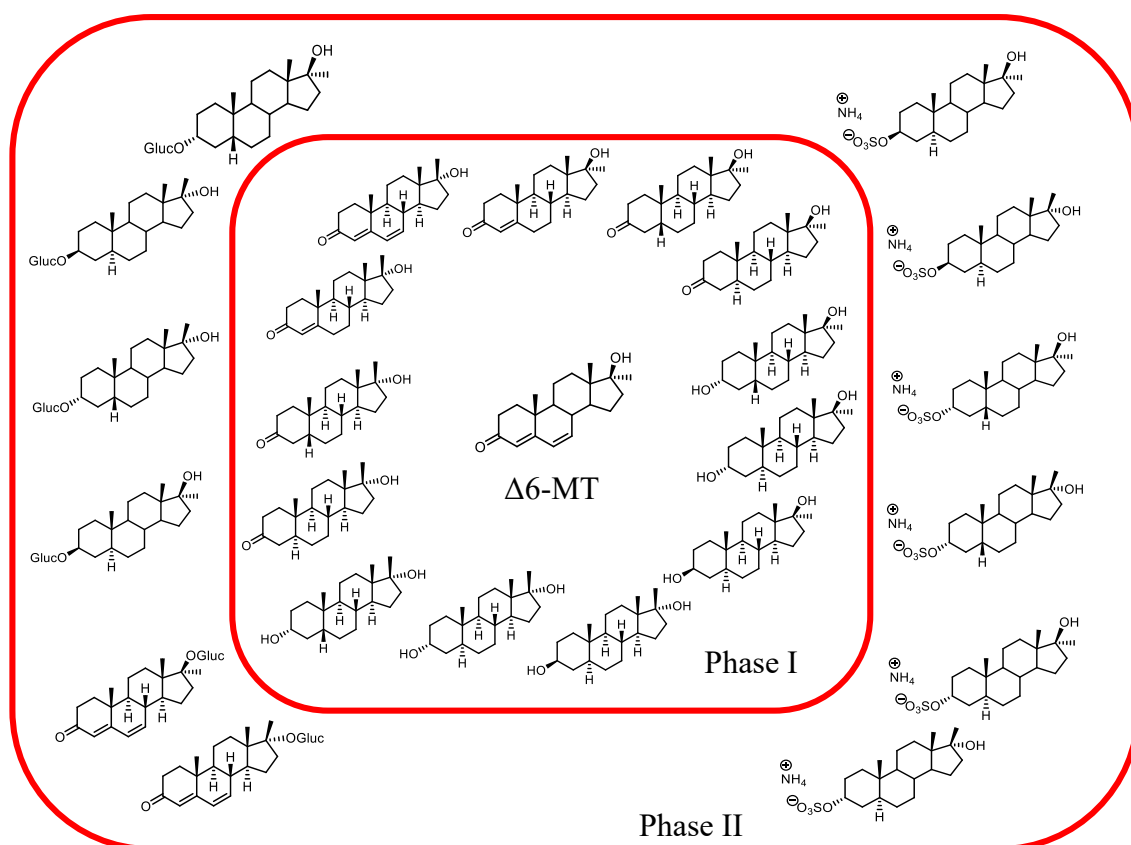


Figure 5.2: Synthesised reference materials of $\Delta 6$ -methyl testosterone

For this thesis a pilot *in vitro* metabolism study was carried out using three different liver fractions to predict the likely *in vivo* metabolites. The study generated a large number of phase I and phase II glucuronide metabolites including the parent

glucuronide. However, phase II sulfation failed in this *in vitro* study. Notably, a positive control reacting EA, without phase I co-factors gave EAS after 24 hours incubation. So, it may be that phase I metabolism or phase II glucuronylation have affected phase II sulfation of Δ^6 -MT and out competed this metabolism pathway. Once the *in vivo* data is available it will be timely to revisit the *in vitro* study once again in an effort to resolve this issue, with the goal of closely matching *in vivo* with *in vitro* metabolic profiles.

The fourth chapter investigated an unidentified metabolite observed during an equine *in vivo* metabolism study of the unusual sulfur containing steroid hemapolin. The steroid involved in this chapter contained an epithio group, 2 α ,3 α -epithio-17 α -methyl-5 α -androstan-17 β -ol. During the *in vivo* study of hemapolin, which was previously reported by Dr. Chris Waller in his PhD thesis, he observed an unusual metabolite, which had a molecular formula consistent with a proposed metabolite, madol sulfate. However, it did not exhibit the most common fragment ion, m/z 97 (HSO_4^-), observed for saturated sulfates. Therefore, more attention was turned towards this metabolite with the goal of unveiling the true structure.

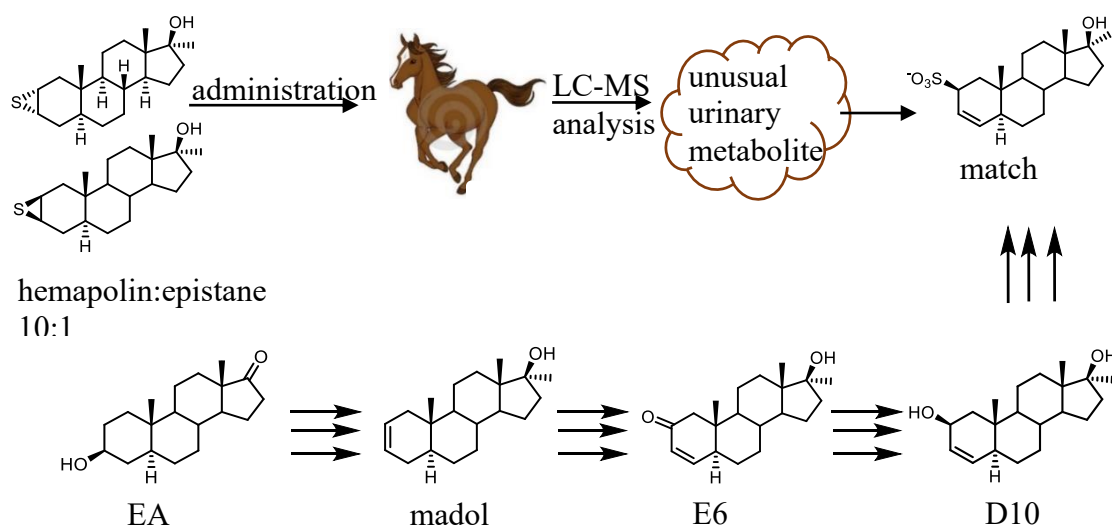


Figure 5.3: Synthesised reference material matched with the urinary metabolite

Based on mechanistic proposal for an unprecedented metabolic pathway the possible reference materials were designed, synthesised and finally compared with the actual urinary metabolite. One out of the four synthesised reference materials,

17 β -hydroxy-17 α -methyl-5 α -androst-3-en-2 β -sulfonate matched with the retention time of the metabolite. However, the confirmation was not possible due to the lack of diagnostic fragment ions during negative mode ESI tandem mass spectrometry. Therefore, further experiments must be carried out in order to confirm the preliminary results presented in this thesis.

To provide additional evidence in favour of the proposed metabolic pathway, the parent drug was chemically oxidized to examine whether it produces any steroid sulfonates. This experiment produced steroid sulfoxides as the major products and traces of the desired steroid sulfonates. Chemical oxidation of both hemapolin and epistane produce the expected oxidation products. Oxidation of the less hindered episulfide lone pair leads to a stable sulfoxide as major product. However, oxidation of the more hindered episulfide lone pair leads to ring opening and further oxidation to generate the expected steroid sulfonate products.

These experiments were complicated by the use of mixtures of starting steroids. Future work will verify the observed reactivity starting from purified epithio steroids. Future work will also aim to obtain additional data confirming the products of chemical oxidation match the synthesised reference materials by LC-MS/MS or other approaches.

Appendix

RESEARCH ARTICLE

WILEY

In vivo metabolism of the designer anabolic steroid hemapolin in the thoroughbred horse

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Abstract

Hemapolin (2 α ,3 α -epithio-17 α -methyl-5 α -androstane-17 β -ol) is a designer steroid that is an ingredient in several “dietary” and “nutritional” supplements available online. As an unusual chemical modification to the steroid A-ring could allow this compound to pass through antidoping screens undetected, the metabolism of hemapolin was investigated by an *in vivo* equine drug administration study coupled with GC-MS analysis. Following administration of synthetically prepared hemapolin to a thoroughbred horse, madol (17 α -methyl-5 α -androst-2-en-17 β -ol), reduced and dihydroxylated madol (17 α -methyl-5 α -androstane-2 β ,3 α ,17 β -triol), and the isomeric enone metabolites 17 β -hydroxy-17 α -methyl-5 α -androst-3-en-2-one and 17 β -hydroxy-17 α -methyl-5 α -androst-2-en-4-one, were detected and confirmed in equine urine extracts by comparison with a library of synthetically derived reference materials. A number of additional madol derivatives derived from hydroxylation, dihydroxylation, and trihydroxylation were also detected but not fully identified by this approach. A yeast cell-based androgen receptor bioassay of available reference materials showed that hemapolin and many of the metabolites identified by this study were potent activators of the equine androgen receptor. This study reveals the metabolites resulting from the equine administration of the androgen hemapolin that can be incorporated into routine GC-MS antidoping screening and confirmation protocols to detect the illicit use of this agent in equine sports.

KEYWORDS

androgen bioassay, antidoping, designer steroid, 2 α ,3 α -epithio-17 α -methyl-5 α -androstane-17 β -ol, hemapolin, metabolism

1 | INTRODUCTION

In recent years the potential for anabolic androgenic steroid (AAS) abuse in equine sports has increased due to the growing availability of synthetic or so-called “designer” steroids, which are common components of “dietary” or “nutritional” supplements and can be purchased through online vendors. Typically marketed towards human bodybuilders, these preparations contain steroidal

compounds that have never been tested or approved as therapeutic agents for medical or veterinary purposes. The compounds are chemically modified derivatives of the endogenous AAS testosterone (17 β -hydroxyandrost-4-en-3-one), with unusual structures or substitution patterns that may pass undetected through current antidoping screens. Despite receiving considerable attention in human sports,^{1–5} until recently there has been only limited investigation of these compounds in equine systems.⁶ In order to effectively

respond to the threat of these synthetic steroids, a detailed understanding of their metabolism is needed to identify markers and metabolites arising from their misuse.

The synthetic steroid hemapolin (2 α ,3 α -epithio-17 α -methyl-5 α -androst-17 β -ol, **H**, Figure 1) is exceptional in that it possesses a 2,3-episulfide; a motif that is not frequently encountered by chemists due to its rarity in nature, chemical instability, as well as a general lack of reliable methodology for its synthesis.⁷ Hemapolin has been observed in “dietary” supplements^{8,9} and also marketed as a component of supplements that contained instead the related compounds: epistane (2 β ,3 β -epithio-17 α -methyl-5 α -androst-17 β -ol), madol (17 α -methyl-5 α -androst-2-en-17 β -ol, **M**), and 17 α -methyl-5 α -androst-3-en-17 β -ol.¹⁰ Hemapolin has been reported to exert significant activity in yeast cell and human androgen receptor bioassays,⁹ and has a predicted activity in computational QSAR models.¹¹ Hemapolin and related epithio-containing steroids are banned in competition by the World Anti-Doping Agency (WADA), and the International Federation of Horseracing Authorities (IFHA) as they have similar chemical structures and biological effects to other banned AAS.^{12,13}

The goal of this work was to investigate the *in vivo* equine metabolism of **H** to identify suitable metabolite markers and to prepare the corresponding reference materials required for screening and confirmation of this agent in equine sport. The equine metabolism of **H** has not been previously reported, highlighting the need for this work. The study also sought to evaluate the potential threat posed by **H** as a performance enhancing substance by testing this agent and its metabolites in a yeast cell-based equine androgen receptor bioassay. This metabolic profiling study provides antidoping laboratories with the information required to establish routine screening methods for the detection of **H** misuse in horses.

2 | MATERIALS AND METHODS

2.1 | Materials

Chemicals and solvents including acetyl chloride, *N*-bromosuccinimide (NBS), *m*-chloroperbenzoic acid, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), hydrazine hydrate, lithium bromide, methylmagnesium bromide, 4-toluenesulfonyl chloride (TsCl), pyridinium chlorochromate (PCC), anhydrous *N,N*-dimethylformamide (DMF), phenyltrimethylammonium tribromide (PTAB), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), dithiothreitol (DTT), and *n*-

dodecane were purchased from Sigma-Aldrich (Castle Hill, Australia), and were used as supplied unless otherwise stated. Aqueous perchloric acid solution, formic acid, and pyridine were purchased from Ajax Chemicals (Auburn, Australia). Acetone, dichloromethane, diethyl ether, 1,4-dioxane (dioxane), ethyl acetate (EtOAc), lithium carbonate, methanol (MeOH), petroleum spirit (bp 40–60°C, hexanes), and diisopropyl ether (DIPE), were purchased from Merck (Darmstadt, Germany). Chloroform and aqueous hydrogen peroxide solution was purchased from Chem-Supply (Gillman, Australia). Epiandrosterone (3 β -hydroxy-5 α -androst-17-one), mestanolone (17 β -hydroxy-17 α -methyl-5 α -androst-3-one), and 17 α -methyltestosterone (17 β -hydroxy-17 α -methyl-5 α -androst-4-en-3-one) were purchased from Steraloids (Newport RI, USA). 16,16,17- d_3 -Testosterone was purchased from the National Measurement Institute (North Ryde, Australia). *N*-bromosuccinimide was recrystallized from water and dried thoroughly under vacuum before use. Tetrahydrofuran (THF) was distilled from sodium wire before use. TsCl was purified before use according to literature methods.¹⁴ 3,3-Dimethyldioxirane (DMDO) was prepared as a solution in acetone according to literature methods and titrated immediately before use.^{15,16} MilliQ water was used in all aqueous solutions. Solid-phase extraction (SPE) was performed using Agilent (Santa Clara, USA) Bond-Elute NEXUS cartridges (3 mL, 60 mg, polymeric non-polar sorbent, PN 12103101).

2.2 | Reference material characterization

Melting points were determined using a SRS (Sunnyvale CA, USA) Optimelt MPA 100 melting point apparatus and are uncorrected. Optical rotations were determined using a Perkin-Elmer (Waltham, USA) Model 343 polarimeter (sodium D line, 298 K) in the indicated solvents. ¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded using either Bruker Ascend 400 MHz or Bruker Ascend 800 MHz spectrometers at 298 K using deuterated chloroform or deuterated methanol solvent. Data are reported in parts per million (ppm), referenced to residual protons or ¹³C in deuterated chloroform (CDCl₃: ¹H 7.26 ppm, ¹³C 77.16 ppm) or deuterated methanol (CD₃OD: ¹H 3.31 ppm, ¹³C 49.0 ppm) unless otherwise specified, with multiplicity assigned as follows: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Coupling constants *J* are reported in Hertz. Low-resolution (LR) and high-resolution (HR) mass spectrometry for reference material characterization was performed using positive electron ionization (+EI) on a Hewlett Packard 6890 GC interfaced to a 5973 mass selective detector (LR) or Micromass VG Autospec mass spectrometer (HR), or using negative electrospray ionization (–ESI) on a Micromass ZMD ESI-Quad (LR), or a Waters LCT Premier XE mass spectrometer (HR). Reactions were monitored by analytical thin layer chromatography (TLC) using Merck Silica gel 60 TLC plates and the solvents as specified, and were visualized by staining with a solution of concentrated sulfuric acid in methanol (5% v/v), with heating as required.

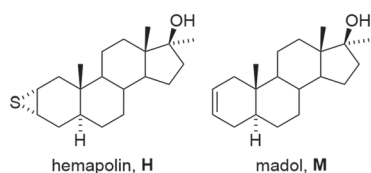


FIGURE 1 Structures of synthetic steroids hemapolin and madol

2.3 | Hemapolin reference materials

A range of synthetically derived reference materials was employed to aid the identification of phase I metabolites. These were hemapolin **H**, madol **M**, 17 β -hydroxy-17 α -methyl-5 α -androst-2-en-1-one **E1**, 17 β -hydroxy-17 α -methyl-5 α -androst-3-en-2-one **E2**, 17 β -hydroxy-17 α -methyl-5 α -androst-1-en-3-one **E3**, 17 β -hydroxy-17 α -methyl-5 α -androst-2-en-4-one **E4**, 17 α -methyltestosterone **E5**, 17 α -methyl-5 α -androst-2-ene-1 α ,17 β -diol **D1**, 17 α -methyl-5 α -androst-2-ene-4 α ,17 β -diol **D2**, 17 α -methyl-5 α -androstane-2 β ,3 α ,17 β -triol **T1**, 17 α -methyl-5 α -androstane-3 α ,4 β ,17 β -triol **T2**, and hemapolin *S*-oxide **HS**. The structures of these compounds are outlined in the supporting information, together with experimental procedures, characterization data, and copies of the ^1H NMR, ^{13}C NMR, and +EI LRMS or -ESI LRMS where appropriate.

2.4 | GC-MS/MS analysis of equine urine samples

Gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis was undertaken on an Agilent 7890A GC system coupled to an Agilent 7000B GC/MS Triple Quadrupole mass spectrometer equipped with an Agilent HP-5MS Ultra Inert column (30 m $\times$ 250 μm $\times$ 0.25 μm). Helium was used as a carrier gas with a constant flow rate of 1.2 mL/min. Injection volumes of 2 μL (quantitative selected reaction monitoring [SRM] analysis) or 3 μL (qualitative product ion scan analysis) were performed in pulsed splitless mode. The injector temperature was set to 260°C, and the MSD transfer line was set to 300°C. The oven temperature commenced at 180°C with a hold time of 0.2 min, followed by a 5°C/min ramp to 235°C, followed by a 15°C/min ramp to 265°C, followed by a 25°C/min ramp to 300°C, and a final hold time of 10 min (total run time 24.6 min). The solvent delay was set to 6.5 min. Steroids were derivatized as the enol trimethylsilyl (TMS) derivative using MSTFA/ NH_4I /DTT (1000:2:4 v/w/w, 50 μL) at 80°C for 60 min,¹⁷ then dried under a stream of dry nitrogen gas at 80°C, and reconstituted in *n*-dodecane (50 μL). Analysis was performed using positive electron ionization (+EI, 70 eV) in product ion scan or MRM mode using ultra-high purity nitrogen and helium as the collision gas and quench gas, respectively. Additional MS parameters are provided in Table 1 and Table 3 below.

2.5 | In vivo equine metabolism study

2.5.1 | Animal administration

The study (RP75) was approved by the Racing NSW Animal Care and Ethics Committee (ARA 62). Animal administration was conducted on a single horse on ethical grounds, to limit exposure to an agent that was not approved for veterinary use, and as it was expected that the metabolites produced from this synthetically derived steroid would be distinct from endogenous metabolites such that the determination of threshold concentrations would not be required. A sample of

synthetic **H** (200 mg) was administered orally as a suspension in water by way of a nasogastric tube to a thoroughbred gelding (21 years old, 580 kg), with urine samples collected at 0, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h post-administration by conditioned spontaneous voiding. The samples were stored at -20°C until required for analysis. Food and water were freely available before and during the drug administration trial.

2.5.2 | Extraction of the urine samples for GC-MS/MS analysis

An aliquot of urine (3 mL) was adjusted manually to pH 5.0–5.5 with aqueous hydrochloric acid solution (10% w/v), and spiked with d_3 -testosterone (final concentration 50 ng/mL) as an internal standard. An Agilent NEXUS SPE cartridge was pre-conditioned with methanol (3 mL) and water (3 mL). The sample was loaded and washed with water (3 mL), a solution of aqueous sodium hydroxide (0.1 M, 3 mL), additional water (3 mL), and eluted with methanol (3 mL). Concentration under a stream of nitrogen at 60°C afforded a residue, which was reconstituted in a solution of acetyl chloride in methanol (1.0 M, 500 μL) and heated in a capped tube at 60°C for 10 min. The hydrolysis reaction was quenched with a solution of aqueous sodium hydroxide (2 M, 3 mL), and extracted with DIPE (3.5 mL) for 30 min. Concentration of the ether layer under a stream of nitrogen at 60°C afforded a residue which was derivatized as the TMS enol ether, and subjected to GC-MS/MS analysis as in section 2.4.

2.5.3 | Excretion profiling of urinary hemapolin metabolites by GC-MS/MS analysis

Aliquots of blank equine urine (3 mL) were adjusted manually to pH 5.0–5.5 with aqueous hydrochloric acid solution (10% w/v), spiked with d_3 -testosterone (final concentration 10 ng/mL) as an internal standard and with the reference material **E4** to generate a series of duplicate calibrators at concentrations of 0, 5, 10, 20, 50, 100, 150, and 200 ng/mL, respectively. Quality control (QC) equine urine samples at concentrations of 10 ng/mL and 100 ng/mL were prepared in duplicate from independent standard solutions in the same manner. Calibrators and QC samples were subjected to SPE as per section 2.5.2 above, and subsequent GC-MS/MS analysis of these together with equine urine samples prepared in quadruplicate was performed using MRM as described in section 2.4. Data analysis was performed using MassHunter[®] software (Agilent Technologies) and Microsoft Excel. The lower and upper limits of quantification were estimated from the lowest and highest concentration duplicate calibrators, respectively, with acceptable accuracy (85–115% of calculated) and precision ($\pm 15\%$ CV). All quality control samples returned a value within acceptable accuracy (85–115% of calculated) and precision ($\pm 15\%$ CV). The limit of detection (LOD) was estimated separately from equine urine spikes at 0.5, 1, 2, 5 ng/mL, achieving a response with signal-to-noise (S/N) ratio greater than 3. Selectivity

TABLE 1 In vivo equine H metabolites detected by GC-MS/MS analysis

Metabolite	Retention time (min) ^a	Theoretical m/z ^b	Precursor ion ^c and MS/MS fragments (% of base peak), [collision energy] ^{a,b}
M	11.24 ^c	360.3	360 ⁺ (30), 345 (35), 291 (15), 275 (100), 261 (15), 197 (15), 143 (15), 73 (20), [10 eV]
E2	14.16 ^e	446.3	446 ⁺ (25), 431 (15), 356 (15), 341 (15), 207 (50), 194 (40), 179 (100), 143 (20), [10 eV]
E4	13.96 ^d	446.3	446 ⁺ (5), 431 (100), 341 (20), 301 (10), 195 (45), 179 (55), 149 (35), 143 (50), 109 (15), 73 (25) [10 eV]
T1	14.52 ^f	538.4	538 ⁺ (100), 523 (5), 453 (45), 439 (50), 349 (90), 259 (35), 144 (20), 73 (40) [10 eV]
Hydroxylated madol M1	11.42 ^g	448.3	448 ⁺ (100), 433 (25), 358 (50), 343 (50), 297 (50), 284 (75), 268 (60), 253 (70), 246 (45), 215 (90), 195 (60), 162 (30), 147 (25), [10 eV]
Hydroxylated madol M2	13.61 ^g	448.3	448 ⁺ (10), 231 (100), 143 (10), [10 eV]
Hydroxylated madol M3	13.79 ^g	448.3	448 ⁺ (25), 231 (100), 172 (5), [10 eV]
Hydroxylated madol M4	13.96 ^g	448.3	448 ⁺ (15), 433 (55), 358 (40), 343 (25), 268 (25), 226 (25), 182 (20), 149 (15), 143 (100), 117 (15), 73 (25), [10 eV]
Hydroxylated madol M5	14.09 ^g	448.3	448 ⁺ (35), 433 (10), 143 (100), [10 eV]
Hydroxylated madol M6	14.52 ^g	448.3	448 ⁺ (100), 433 (30), 358 (70), 343 (45), 301 (30), 268 (60), 253 (75), 240 (30), 226 (40), 215 (60), 183 (45), 159 (30), 143 (70), 123 (15), 106 (15), [10 eV]
Dihydroxylated madol M7	15.29	536.4	536 ⁺ (100), 231 (45), 202 (15), 143 (20), [5 eV]
Dihydroxylated madol M8	15.75	536.4	536 ⁺ (100), 341 (20), 251 (20), 196 (50), 156 (50), [5 eV]
Dihydroxylated madol M9	16.71	536.4	536 ⁺ (100), 341 (25), 266 (15), 231 (20), 212 (10), 196 (60), 181 (25), 156 (30), 143 (15), 117 (70), [10 eV]
Trihydroxylated and reduced madol M10	15.75	626.4	626 ⁺ (15), 231 (100), 143 (10), [10 eV]
Trihydroxylated and reduced madol M11	16.71	626.4	626 ⁺ (15), 231 (100), 143 (10), [10 eV]

^aRetention time from GC-MS/MS acquisition (2.4).^bPrecursor ion ([M]⁺) for corresponding enol-TMS derivative.^cConfirmed against **M** reference material.^dConfirmed against **E4** reference material.^eConfirmed against **E2** reference material.^fConfirmed against **T1** reference material.^gNo match to **D1** or **D2** reference materials.

was demonstrated with no detectable presence of target analytes in blank control urine or in pre-administration samples. No carryover was evident from analysis of solvent blanks injected immediately after the highest calibrator (200 ng/mL).

2.6 | Equine androgen receptor bioassay

The yeast cell-based androgen receptor bioassay was based on the human androgen receptor assay described previously by Death et al.¹⁸ except that the human androgen receptor expression plasmid was replaced with an equine androgen receptor cDNA expression plasmid. Briefly, a DNA fragment that contained the metallothionein promoter (CUP-1)-ubiquitin gene-linker followed by unique EagI and BssHI sites and the iso-1-cytochrome C was inserted into YEp351 (ATCC37672) and the equine androgen receptor cDNA

(NM_001163891.1) was cloned from pcDNA3.1 (GenScript OHb00135) into YEp351 using the EagI and BssHI sites. The ARE/GRE/PRE-B-galactosidase reporter plasmid was kindly provided by Professor D. P. McDonnell (Duke University Medical Centre, Durham, NC). Yeast strain YPH500 (MAT α , ura3-52, lys2-801, ade2-101, trp1- Δ 63, his3- Δ 200, leu2- Δ 1) was co-transformed with both plasmids using the Quick and Easy Transformation Mix (Takara Bio USA, CA USA). Transformants were selected by leucine and uracil auxotrophy.

To perform the assay, early to mid-log growth phase yeast cells were sub-cultured into 24-well plates (500 μ L/well) and androgen receptor expression activated with 1 μ M CuSO₄. The cells were then treated with steroids (5 μ L/well) dissolved in ethanol over a concentration range of 1.95×10^{-5} M to 4.72×10^{-11} M for 24 h to generate a sigmoidal dose curve used to calculate the EC₅₀ of the steroid response. To harvest, the plates were cooled on ice for 30 min and

the optical density (600 nm) was measured before the cells were lysed and assayed for β -galactosidase activity using a standard assay.¹⁸ The EC₅₀ was determined for each steroid from a sigmoidal curve fit using GraphPad Prism v8.1.1.

3 | RESULTS

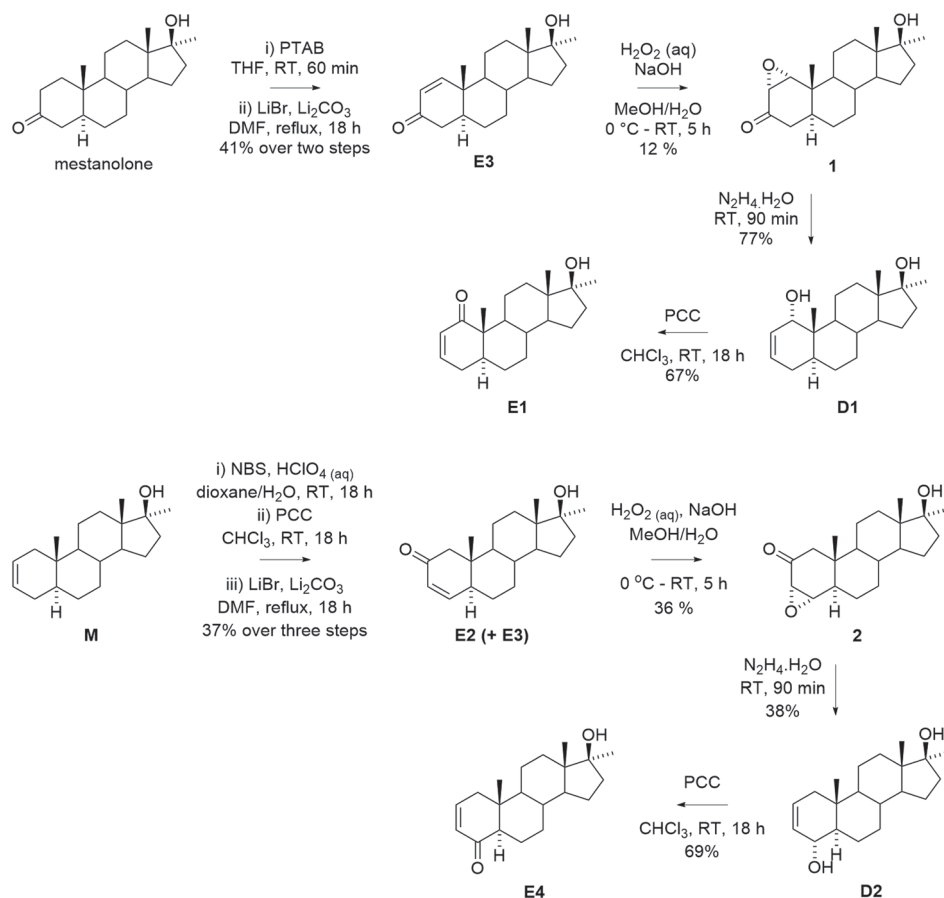
3.1 | Synthesis of hemapolin and reference materials

As **H** was required on large scale (200 mg) and in high purity, a laboratory synthesis was undertaken. Synthetic **H** and its episulfide isomer epistane were prepared in a 10:1 ratio from epiandrosterone in six synthetic steps in 6% overall yield through a novel synthetic strategy. The experimental procedures, characterization data, and copies of the ¹H NMR, ¹³C NMR, and +EI LRMS or -ESI LRMS are available for this synthesis, but have been excluded from the final version of this manuscript due to concerns over the potential for misuse of this information in the manufacture, supply, and illicit use of new synthetic steroids. Requests for this information from legitimate parties can be made to the corresponding author. Enones **E1–E4** and diols **D1** and **D2** were prepared from **M** or mestanolone as described below. Briefly, bromination of mestanolone with PTAB cleanly afforded the 2-bromomestanolone, which was eliminated by treatment with lithium bromide and lithium carbonate in anhydrous DMF to afford **E3**

(Scheme 1).^{19,20} Subsequent treatment of **E3** with hydrogen peroxide under basic conditions afforded epoxide **1**, which was subjected to Wharton conditions to selectively generate diol **D1**.^{19,21} This diol was oxidized with PCC to afford **E1**.¹⁹

Enone **E2** (Scheme 2) was prepared from **M** by formation of the 3-bromohydrin, followed by oxidation to the 3-bromoketone, and subsequent elimination using the conditions outlined for the **E3** synthesis.²⁰ A small amount (~15%) of **E3** formed alongside **E2**, presumably resulting from the formation of the undesired 2-bromohydrin, which was carried forward into subsequent steps. Subsequent treatment of **E2** with hydrogen peroxide afforded epoxide **2** in pure form after column chromatography, which was converted to diol **D2** under Wharton conditions.²¹ Oxidation of **D2** with PCC afforded **E4**.¹⁹

The ¹H NMR analysis of the alkene protons in deuterated chloroform agreed with the proposed structures of the enone reference materials: enone **E1** produced a pair of doublet of triplets at 6.65 ppm (C3-H, *J* = 10.1 Hz, *J* = 3.9 Hz) and 5.80 ppm (C2-H, *J* = 10.1 Hz, *J* = 2.1 Hz); **E2** produced a pair of doublets at 6.56 ppm (C4-H, *J* = 9.8 Hz) and 5.96 ppm (C3-H, *J* = 9.8 Hz); **E3** produced a pair of doublets at 7.14 ppm (C1-H, *J* = 10.2 Hz) and 5.85 ppm (C2-H, *J* = 10.2 Hz); **E4** produced a doublet of doublet of doublets at 6.79 ppm (C2-H, *J* = 10.1 Hz, *J* = 6.0 Hz, *J* = 2.3 Hz), and a doublet of doublets at 5.98 ppm (C3-H, *J* = 10.1 Hz, *J* = 3.0 Hz) and; commercially sourced **E5** displayed a singlet at 5.73 ppm (C4-H). These signals distinguish between the different chemical environments of the steroid A-ring, and matched the expected splitting patterns.

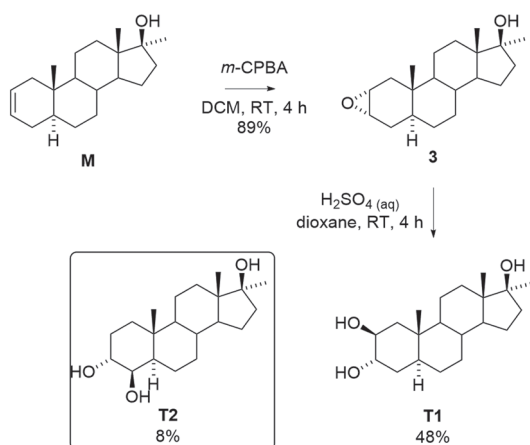


Based on a human *in vitro* study of **M** reported by Gauthier et al.,¹⁹ the triol reference material **T1** was also prepared. Formation of the major epoxide **3** from **M**, followed by acid-catalyzed ring opening with water to afford **T1**¹⁹ and an isomeric triol **T2** in a 6:1 ratio (Scheme 3). The ¹H NMR shifts and coupling constants for C2-H and C3-H of the major isomer **T1** matched those reported previously,¹⁹ consistent with an axial orientation of the A-ring hydroxyl groups. The minor isomer also displayed coupling constants for the oxymethine protons consistent with an axial orientation of the A-ring hydroxyl groups and the compound was assigned as 17 α -methyl-5 α -androstane-3 α ,4 β ,17 β -triol **T2** arising from the minor quantities (5%) of 17 α -methyl-5 α -androst-3-en-17 β -ol present in the precursor **M**.

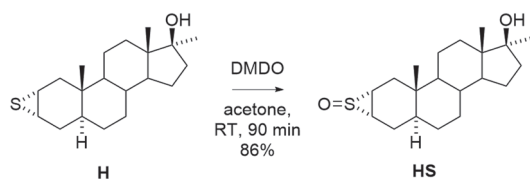
Additionally, based on observations reported by Okano et al. on the metabolism of a related epithio-steroid mepitiostane,²² **HS** was also prepared in low yield by treatment of **H** with hydrogen peroxide in acetic acid. In our hands, **M** was observed as the major product of this reaction, so milder conditions for the synthesis of **HS** were investigated. Treatment of **H** with DMDO^{15,16} effected the generation of **HS** as the major product (Scheme 4). This compound proved unstable at room temperature, decomposing slowly to generate **M** over the course of several days. However, storage at lower temperatures (–20°C) resulted in minimal decomposition over several weeks.

3.2 | Analysis of phase I urinary metabolites

The phase I urinary metabolism of **H** was investigated by a method involving SPE, acid solvolysis, and enol-TMS derivatization (2.5.2), prior to GC-MS/MS analysis using product-ion scan mode (2.4). Post-



SCHEME 3 Synthesis of triol **T1** reference material



SCHEME 4 Synthesis of hemapolin *S*-oxide reference material

administration urinary metabolites were detected by comparison with pre-administration urine extracts, and by evaluation of the extracted ion chromatograms for precursor ions theoretically derived from pathways composed of up to four metabolic transformations, including elimination of sulfur, hydroxylation, oxidation, and reduction. Detected metabolites were confirmed against synthesized reference materials where available (Table 1).²³

The metabolites identified indicated that **H** was modified extensively in the A-ring. Compounds **M** (minor), **E2** (minor), **E4** (major), and **T1** (minor) were detected in post administration equine urine extracts, with relative levels assessed by a comparison of normalized peak areas. All four compounds were confirmed against available reference materials according to the Association of Official Racing Chemists (AORC) MS criteria involving a comparison of GC retention times and relative abundance derived from the peak area of extracted ion chromatograms for three MS/MS product ions at unit resolution (Table 2).²³ The product ion spectrum of the **E4** metabolite and the corresponding reference material is also shown in Figure 2.

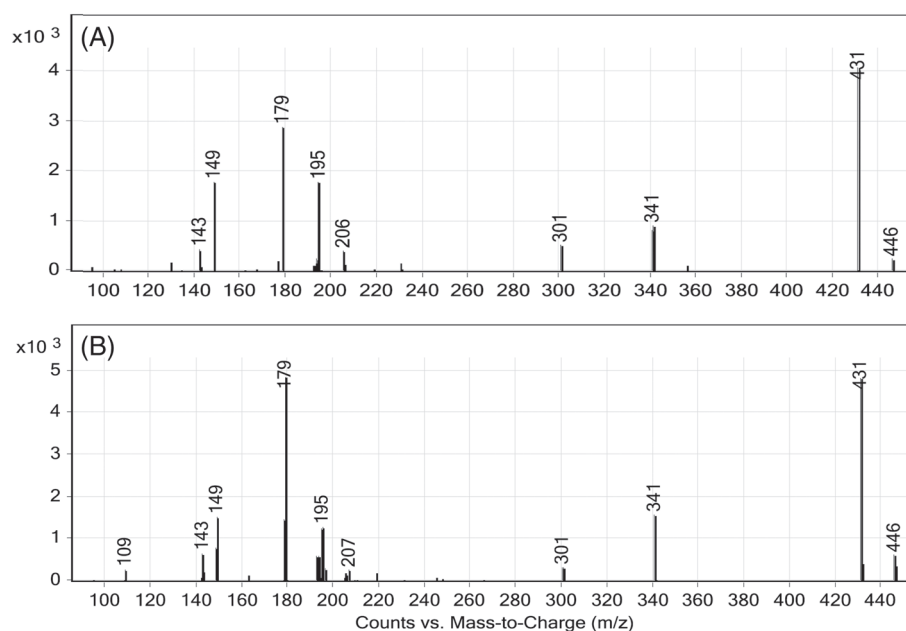
Analysis by GC-MS/MS also detected a number of additional metabolites for which reference materials were not available, such that the structures remain only partly defined. Major metabolites included four hydroxylated madol metabolites (**M1–M3**, **M6**), while minor metabolites included two hydroxylated madol metabolites (**M4**, **M5**), three dihydroxylated madol metabolites (**M7–M9**), and two trihydroxylated and reduced madol metabolites (**M10**, **M11**) (Table 1). The major metabolites **M2** and **M3**, were tentatively assigned as a pair of 15 α / β - or 16 α / β -hydroxylated madol metabolites, based on the observed *m/z* 231 fragment ion, which has been previously reported to be characteristic of 15- and 16-hydroxylated-17-methylated steroids.^{24,25} This fragment ion was also observed for minor metabolites **M7**, **M9**, **M10**, and **M11**, which were also tentatively assigned to contain 15- or 16-hydroxylation. A comparison of available reference materials **E1**, **E3**, **E5**, **D1**, or **D2** by GC-MS/MS against pre- and post-administration urine extracts did not reveal corresponding urinary metabolites. In behavior similar to that reported for the metabolism of the related epithio-steroid mepitiostane,²² subjecting **H** or **HS** reference materials to sample preparation (2.5.2) and GC-MS/MS analysis (2.4) resulted in decomposition of the epithio or epithionyl functionality, respectively, to generate **M**, also identified above as a minor component during the analysis of post-administration urine extracts. Given this, the current GC-MS/MS study was unable to distinguish the sulfur-containing compounds **H** and **HS** from **M**, a situation that could be addressed in future by the detection of **H** and **HS** by LC-MS in a manner similar to that pioneered by Okano et al.²² for the mepitiostane metabolites epitiostanol and epitiostanol *S*-oxide.

3.3 | Excretion profiling of equine **H** metabolites by GC-MS/MS

The goal of this study was to identify metabolites of **H** and to prepare the corresponding reference materials required for the development of antidoping screening and confirmation protocols. This had been

TABLE 2 Confirmation of metabolites using GC-MS/MS product ions according to AORC criteria

Reference material				Metabolite	
	Retention time (min)	Product ion (m/z)	Relative abundance ^a (%)	Retention time [tolerance] (min)	Relative abundance ^a [tolerance] (%)
M	11.26	291	16	11.24 [11.15-11.37]	16 [0-36]
		275	100		100 [60-100]
		143	24		17 [4-44]
E2	14.16	194	48	14.16 [14.02-14.30]	35 [28-68]
		179	100		100 [60-100]
		143	39		37 [19-59]
E4	13.95	431	100	13.96 [13.81-14.09]	100 [60-100]
		179	69		71 [41-96]
		149	35		46 [15-55]
T1	14.47	453	25	14.52 [14.33-14.61]	25 [5-45]
		433	56		40 [33-78]
		349	100		100 [60-100]

^aDerived from the peak area of extracted ion chromatograms.**FIGURE 2** GC-MS/MS product ion spectra (10 eV) of (A) E4 metabolite identified in the 2 h urine, (B) E4 reference material

achieved for the metabolites **M**, **E2**, **E4**, and **T1**. To enhance sensitivity and assist with the translation to a routine screening protocol, a series of MRM transitions (Table 3) were optimized from the most intense ions observed in the product ion spectra. The use of three MRM transitions at unit mass resolution could also be used to fulfil the AORC MS criteria for compound confirmation, and this is reported in the supporting information for metabolite **E4**.²³ Limits of detection ($S/N > 3$) were assessed (2.5.3) and urine samples taken between 1 h and 168 h were evaluated for the presence of metabolites to estimate detection windows for screening: **M** 2–24 h, **E2** 2–4 h, **E4** 1–72 h, and **T1** 1–12 h. In the absence of reference materials, a qualitative evaluation of detection windows for the hydroxylated madol

metabolites **M1–M6** was also undertaken. The metabolites **M2** and **M3** displayed 448 231 as the most sensitive transition, which was clearly observed ($S/N > 3$) from 1–48 h post-administration. The remaining metabolites **M1**, **M4–M6** were detected by monitoring for the transition 448 143, and were all observed ($S/N > 3$) from 1–8 h post-administration. Based on the low experimentally determined LOD (Table 3), the relatively long excretion profile (1–72 h), and the availability of a synthetic reference material, the major metabolite **E4** appeared to be the best candidate for routine screening and confirmation purposes.

Although the detection and confirmation of exogenous substances or their metabolites is sufficient grounds for prosecution in

TABLE 3 Excretion profiling of urinary H metabolites by GC-MS/MS

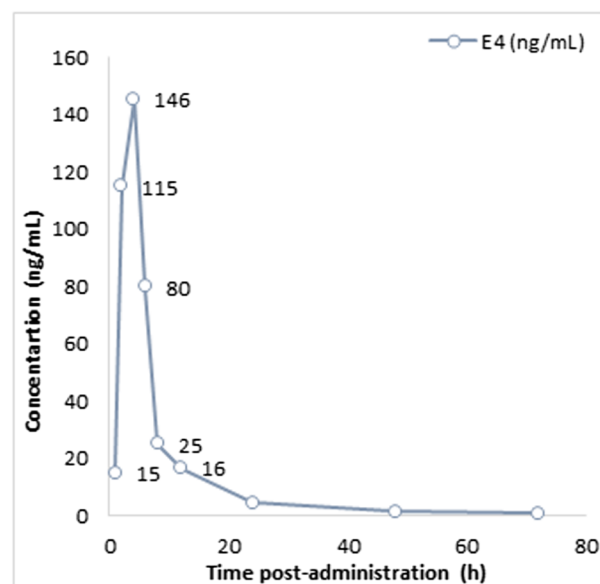
	Transition (m/z)	Collision energy (eV)	Relative abundance (%)	LOD (ng/mL)
M	360→275 ^a	10	100	5
	360→345	10	60	
	360→143	15	30	
E2	446→179 ^a	10	100	2
	431→341	5	80	
	446→431	10	40	
E4	431→179 ^a	10	100	1
	431→341	5	11	
	446→431	10	10	
T1	538→349 ^a	10	100	2
	538→433	10	40	
	538→259	10	30	
d₃-testosterone^b	435→420 ^a	10	-	-

^aTransition employed for LOD or quantification;^bInternal standard.

the racing industry, and all metabolites identified by this study are clearly classified as exogenous steroids due to the presence of C17 methylation, the excretion profile of the major metabolite **E4** was briefly studied. A partially validated analytical method was developed for the analysis of **E4** in equine urine. Calibrators were generated from spiking blank equine urine with the **E4** reference material (2.5.3), before sample preparation (2.5.2) and GC-MS/MS analysis (2.4). The response ratio of the most intense MRM transition for **E4** (431→179) provided linear calibration over the range (0–200 ng/mL, $R^2 > 0.99$, LLOQ 5 ng/mL, ULOQ 200 ng/mL) and acceptable accuracy and precision. The enone metabolite **E4** was quantified from 1–12 h with peak excretion (146 ± 10 ng/mL) observed at 4 h (Figure 3) reflective of rapid **H** uptake and metabolism.

3.4 | Androgen receptor bioactivity of H and metabolites

The results from the yeast cell-based equine androgen receptor bioassay are summarized in Table 4. The parent compound **H** showed strong androgen receptor bioactivity, with similar potency to testosterone. Eight of the nine reference materials tested were active in the androgen receptor bioassay. Enones **E3**, **E4**, and **E5** (17 α -methyltestosterone) showed strong bioactivity, with relative potencies (compared with testosterone) of 172%, 76%, and 96%, respectively. The reference materials **M**, **E1**, **E2** and **D1** showed EC₅₀ values that were within one order of magnitude less potent than testosterone. Compound **D2** was a weaker androgen, with an EC₅₀ more than 10 times weaker than that of testosterone and triol **T1** had no activity in the androgen receptor bioassay. Of direct relevance to this study, the results show that in addition to **H**, major metabolites including **M**, **E2**, and **E4** are potent activators of the androgen receptor.

**FIGURE 3** Urinary excretion profile of equine **H** metabolite **E4** [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Epithio-steroids such as **H** and the related steroid mepitiostane provide particular challenges for antidoping analysis. As demonstrated for mepitiostane, analysis by LC-MS provides avenues to detect the epithio-steroid metabolites or their sulfoxide derivatives.¹⁰ However, LC-MS is less well suited to the detection of many of the non-polar downstream products of metabolism derived from loss of the sulfur substituent, typified in the current study by the metabolite **M**, due to poor ionization efficiencies under electrospray ionization conditions.

TABLE 4 Equine androgen receptor bioactivity of H and related reference materials

Reference material	EC ₅₀ ± SD ^a (nM)	RP ^b (%)
Testosterone	5.5 ± 3.2	100
H	12.9 ± 2.2	43
M	21.7 ± 5.7	25
E1	34.7 ± 4.4	16
E2	26 ± 5.2	21
E3	3.2 ± 1.5	172
E4	7.6 ± 2.1	76
E5	5.7 ± 3.9	96
D1	29.3 ± 4.2	19
D2	71.5 ± 5.6	8
T1	Not active	0

^aEC₅₀ value as determined from the sigmoidal dose response curve for each reference material.

^bPercent relative potency (RP) [EC₅₀ reference material]/[EC₅₀ testosterone].

Analysis by GC-MS is the method of choice to detect such non-polar metabolites, but in this case the low stability of the epithio and epithionyl functionality results in the loss of sulfur during sample preparation or GC-MS analysis.¹⁰ Metabolism profiling of these epithio-steroids is also complicated by previously observed pathways of biotransformation, described in detail for mepitiostane, that involve oxidation at sulfur and dethionylation following oral administration.^{26,27} This study adopted GC-MS analysis, after sample preparation and enol-TMS derivatization, leading to the detection of a range

of compounds in post administration urine including **M**, **E2**, **E4**, **T1**, and 11 additional madol derivatives **M1–M11** with varying patterns and levels of hydroxylation and reduction (Table 1). As noted above, the presence of parent **H** or **HS** as urinary metabolites could not be ascertained by this GC-MS approach as analysis of the corresponding reference materials led to a loss of sulfur and the formation of **M**, leaving the origin of this minor compound in urinary extracts a topic for future research.

The metabolic oxidation of sulfur and dethionylation of epithio-steroids^{26,27} suggests that the current study into **H** metabolism may provide some guidance on the likely metabolites arising from **M** administration to horses. A previous study of the equine in vitro metabolism of **M** (used as a 3:1 mixture of Δ^{2-3} and Δ^{3-4} alkene isomers) identified nine equine metabolites after TMS derivatization and GC-MS/MS analysis, resulting from hydroxylation (2), hydroxylation, and oxidation (enone, 2), dihydroxylation and reduction (2) and trihydroxylation and reduction (3). One of the enone metabolites was confirmed as **E3** by comparison with a reference material.²⁸ In this study, the two enone metabolites **E2** and **E4** were confirmed by comparison with reference materials, and **E3** was not observed. This difference could reflect the different starting steroids for two studies, the 3:1 mixture of Δ^{2-3} and Δ^{3-4} alkene isomers of **M** used in the in vitro study, or differences between in vitro and in vivo metabolic profiles. The human in vivo and in vitro metabolism of **M** has also been the subject of extensive study with the results detailed in a publication by Gauthier et al.¹⁹ The enone **E4** was identified from in vitro studies using the human liver S9 fraction. Post administration urine samples revealed a wide range of putative metabolites, of which the dihydroxylated and reduced madol **T1** was suggested as the major human urinary

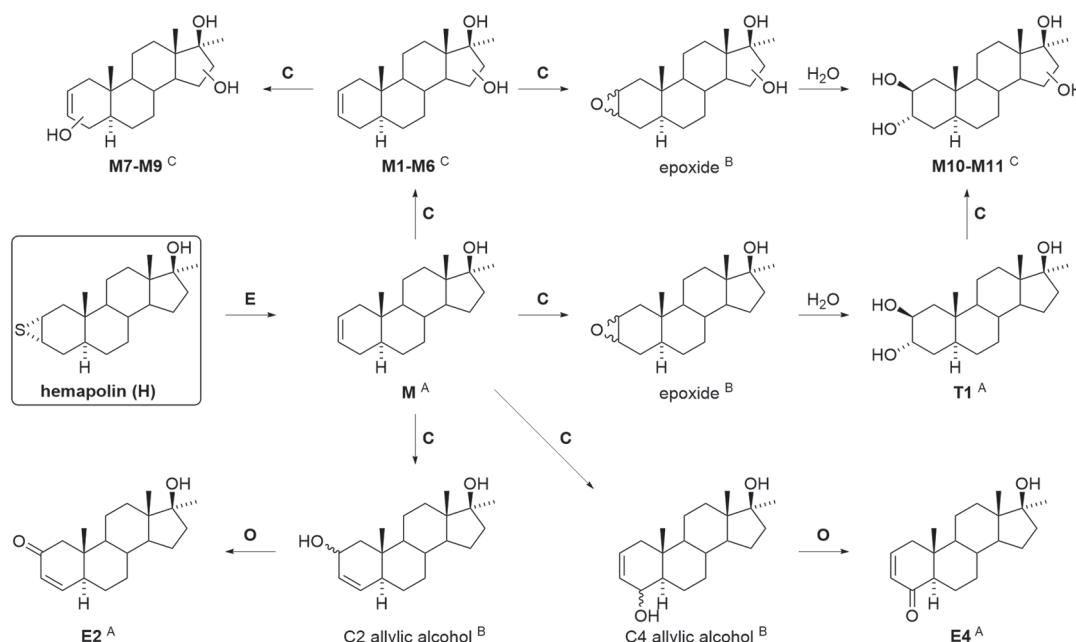


FIGURE 4 Proposed pathways for urinary phase I metabolites of hemapolin in the horse. ^Amatched to reference material, ^Bnot observed, ^Cstructure undefined. E, elimination; C, cytochrome P450 oxidation; O, oxidation

metabolite.^{3,19,29} However, the clear identification of other urinary metabolites could not be achieved with confidence, as it was observed that a seized sample of **M** contained a wide range of steroidal compounds at low levels, including enones **E1**, **E4**, and **E5**, believed to be by-products of synthesis or to arise from sample degradation.¹⁹

The observation of metabolites **E2**, **E4**, and **T1** identified by this study led to speculation as to their metabolic origins (Figure 4). Mechanistically, cytochrome P450 enzymes perform hydroxylation through a sequence involving hydrogen atom abstraction to form a carbon-centered radical, followed by rapid hydroxyl radical recombination. Hydroxylation adjacent to alkenes is often accompanied by a formal allylic rearrangement, leading to regioisomeric mixtures of alcohol products.^{30,31} Abstraction of a hydrogen atom at C4 of **M** would result in a stabilized allylic radical that would then undergo radical recombination at C2 or C4, resulting in the corresponding allylic alcohols which would be subsequently oxidized to the corresponding enones **E2** and **E4**, respectively.^{30–33} Alternatively, abstraction of a hydrogen atom at C1 would result in C1 or C3 allylic alcohols and enones **E1**, and **E3**, respectively. The exclusive observation of enones **E2** and **E4** as equine metabolites is likely controlled through cytochrome P450 enzyme–substrate interactions and steric effects. The C1 position is more hindered due to the adjacent C19 methyl group. Notably, the α -configured diol reference materials **D1** and **D2**, potential intermediates on the conversion of **M** to **E2** and **E4**, were not detected as equine urinary metabolites. The formation of the formally reduced and dihydroxylated madol metabolite **T1** was proposed from the cytochrome P450 mediated epoxidation and then hydrolysis leading to a *trans*-configured vicinal diol within the A-ring.

With regard to androgenic potential, **H**⁹ and **M**^{9,34} have previously been reported to display potent human androgenic activity. In this study, **H** and the equine metabolites **M**, **E2**, and **E4** have also been shown to be potent androgens in the equine androgen receptor bioassay, and in the case of **E4** of greater activity than the parent steroid **H**.

5 | CONCLUSIONS

Synthetic steroids such as **H** have the potential to harm the integrity of sporting competition if left unchecked. The metabolism of **H** was investigated in the horse for the first time by an *in vivo* drug administration study and GC-MS/MS analysis. The compounds 17 α -methyl-5 α -androst-2-en-17 β -ol **M**, 17 β -hydroxy-17 α -methyl-5 α -androst-3-en-2-one **E2**, 17 β -hydroxy-17 α -methyl-5 α -androst-2-en-4-one **E4**, and 17 α -methyl-5 α -androstane-2 β ,3 α ,17 β -triol **T1**, were identified in urine extracts and confirmed against a library of synthetically derived reference materials. A range of additional metabolites was detected corresponding to derivatives of **M** arising from hydroxylation, dihydroxylation, and trihydroxylation and reduction that remain incompletely identified. The enone metabolite **E4** derived by elimination of sulfur from **H**, followed by cytochrome P450-mediated hydroxylation and oxidation was identified as the preferred target for

routine screening and confirmation protocols and was detected up to 72 h post-administration. Hemapolin and a number of the compounds identified in this study including **M** and the enones **E2** and **E4** were potent androgens in an equine androgen receptor bioassay. These investigations delineate the metabolic fate and androgenic potential of the synthetic steroid **H** and have identified metabolite **E4** as the preferred target for antidoping analysis aimed at detecting illicit administration of this agent in equine sport.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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