# Synthesis and Mass Spectrometry of Bisconjugate Phase II Steroids

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## Author’s declaration

I declare that the work reported in this thesis was done by the author between September 2016 and May 2018 at the Research School of Chemistry at the Australian National University under the supervision of Associate Professor Malcolm McLeod, or otherwise has been appropriately referenced to the original publications. This thesis has not been previously submitted for any degree at any university and does not exceed 60,000 words.

Andy Pranata

23 August 2018

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Thank you to all RSC staff (technical officers, mass spectrometry, stores, and admin).

Last but not least, I would like to thank my parents and my brother for supporting me. I would not be able to go through this year without them.

## Abstract

The work described in this thesis details the efforts towards the synthesis of steroid bisconjugate materials. Specifically, a library of ten steroid sulfate glucuronide and ten bisglucuronide reference materials were prepared. In addition, a study of their mass spectrometry (MS) behaviour was conducted, with the goal of using these reference materials to identify new urinary markers that can then be used in anti-doping or diagnostic applications. Two extra steroid bis(sulfates) were also synthesised as reference materials for prenatal diagnosis.

Chapter 1 introduces steroids in general, their metabolism in mammalian systems, the strengths and limitations of the current steroid detection methods, and how they can be improved.

Chapter 2 discusses the synthetic steps that were taken to produce the steroid sulfate glucuronides and bisglucuronides, including stable isotope labelled derivatives. This includes a discussion of the sulfation, reduction, and glucuronylation reactions. This chapter also explains the MS study of the reference materials that were synthesised, especially using tandem mass spectrometry (MS/MS). It also details an LC-MS method developed to identify new steroid markers in human urine and efforts to confirm their identities against the prepared materials.

Chapter 3 discusses the synthetic steps that were taken to produce the steroid bis(sulfates) to be used as reference materials in prenatal diagnosis. Experimental procedures for the synthesis of these compounds are also presented.

Chapter 4 concludes the work presented in this thesis followed by suggestions for future work in this field.

## Abbreviations

| | |
|---|---|
| AAS | androgenic anabolic steroids |
| ABP | Athlete Biological Passport |
| ATP | adenosine triphosphate |
| br | broad |
| CID | collision induced dissociation |
| CIL | constant ion loss |
| conv. | conversion |
| δ | chemical shift |
| d | doublet |
| DHEA | dehydroepiandrosterone |
| DMF | *N*,*N*-dimethylformamide |
| EA | epiandrosterone |
| *E. coli* | *Escherichia coli* |
| (epi)DHT | (epi)dihydrotestosterone |
| (epi)T | (epi)testosterone |
| eq. | equivalent(s) |
| ESI | electrospray ionisation (mass spectrometry) |
| GC-MS | gas chromatography-mass spectrometry |
| h | hour(s) |
| HRMS | high-resolution mass spectrometry |
| IR | infrared |
| *J* | coupling constant (Hz) |
| LC-MS | liquid chromatography-mass spectrometry |
| LRMS | low-resolution mass spectrometry |
| m | multiplet |
| MS | mass spectrometry |

| | |
|---|---|
| MS/MS | tandem mass spectrometry |
| *m/z* | mass-to-charge ratio |
| NL | neutral loss |
| NMR | nuclear magnetic resonance |
| PaS | *Pseudomonas aeruginosa* arylsulfatase |
| PORD | cytochrome P450 Oxido-Reductase Deficiency |
| ppm | parts per million |
| q | quartet |
| $R_f$ | retention factor |
| s | singlet |
| SIM | single ion monitoring |
| SLOS | Smith-Lemli-Opitz Syndrome |
| $SO_3$·py | sulfur trioxide-pyridine complex |
| SPE | solid phase extraction |
| SRM | selected reaction monitoring |
| STSD | Steroid Sulfatase Deficiency |
| SULT(s) | sulfotransferase(s) |
| t | triplet |
| TLC | thin layer chromatography |
| TMS | trimethylsilyl |
| UDPGA | uridine diphosphate glucuronic acid |
| UGT(s) | uridine 5’-diphosphoglucuronosyltransferase(s) |
| UPLC-MS/MS | ultra-performance liquid chromatography–tandem mass spectrometry |
| v/v | unit volume per unit volume (ratio) |
| WADA | World Anti-Doping Agency |
| WAX | weak anion exchange |

## Steroid nomenclature

In Figure i, the numbering of steroid core is shown as well as the numbering of the extra carbons from the conjugate groups [1]. When both conjugate groups at the two ends of the steroid structure contain carbons, numbering continues from the conjugate at the lowest numbered steroid carbon. Figure i shows four possible doubly conjugated steroid metabolites with their names shown. The naming system used in this thesis is not fully systematic and always the steroid name (following the IUPAC rules) followed by the conjugate position (if required) and name. It is important to note that compounds that have two glucuronide units are called bisglucuronides where the two glucuronic acid units are attached at two different sites. This is not to be confused with diglucuronide conjugates that have two glucuronic acid units connected at a single site [2,3].

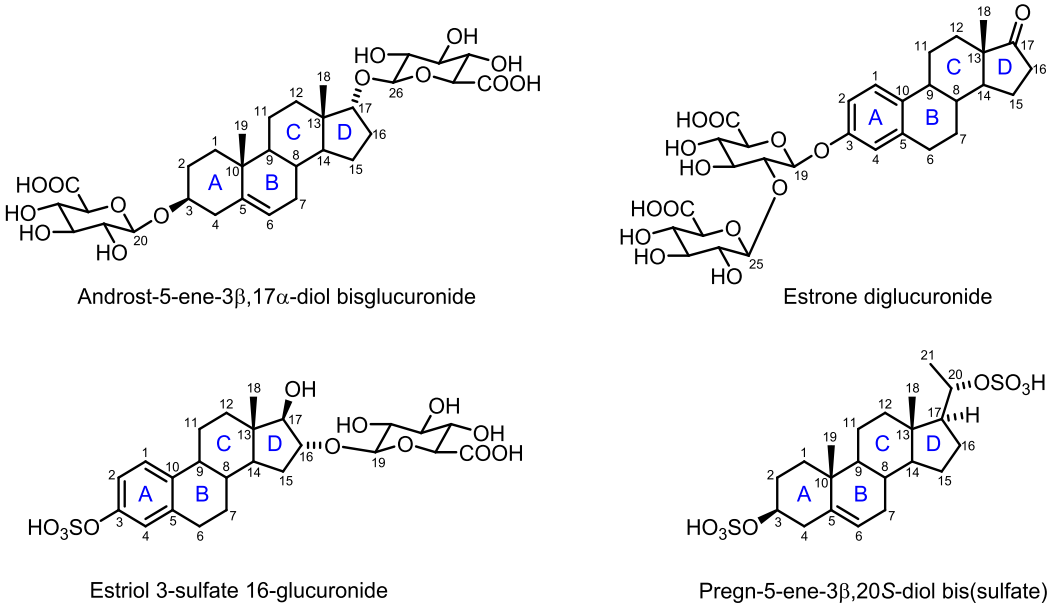



Figure i. Examples of bisconjugate steroids with numbering of steroid core and conjugates

According to their structures, the steroids presented in this thesis can be classed as one of four types: androstane, estrane, pregnane, and cholane (Figure ii).

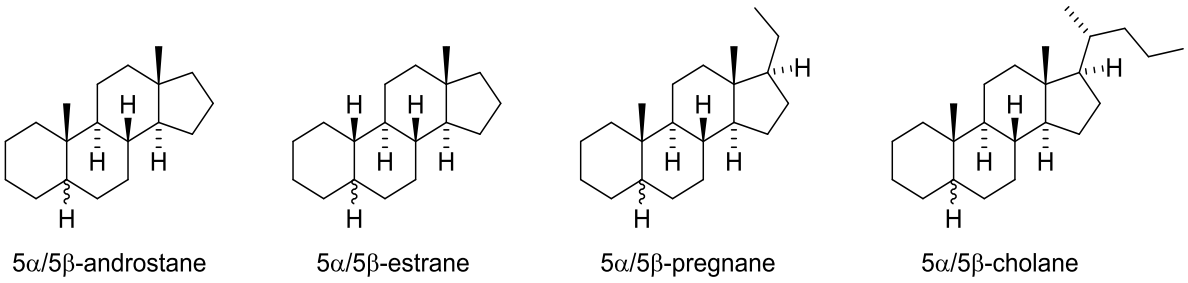



Figure ii. Different structure of steroids

# Contents

# Chapter 1 – Introduction

## 1.1. Steroids

Steroids are a large family of compounds, either synthetic or naturally occurring, that contain four fused carbocyclic rings as the core structure, which three six-membered rings (rings A, B, and C), and one five-membered ring (ring D). By their functionality, steroids can be grouped as corticosteroids or sex hormones. Corticosteroids can be divided further into two groups such as the glucocorticoids and mineralocorticoids, which can help regulate blood pressure. On the other hand, the sex hormones can be divided into three groups, progestogens, androgens, and estrogens. Sex hormones are important for both growth (anabolic) and sexual development (androgenic). Thus, often the use of sex hormones that have androgenic anabolic effect (androgenic anabolic steroids (AAS)) is abused in the world of sport. The AAS can range from estranes (nandrolone), androstanes (testosterone), or pregnanes (tetrahydrogestrinone). The use of synthetic AAS was banned by the International Olympic Committee in 1974 due to their performance enhancing effects. According to the World Anti-Doping Agency (WADA) annual statistics, around 300,000 samples were analysed in 2016, with around 4,800 leading to the detection of prohibited substances, metabolites or markers. Of these, 43% of them arose from AAS abuse [4].

## 1.2. Steroid metabolism

While steroids are naturally hydrophobic, they rapidly undergo metabolism to increase their water solubility for excretion in biological fluids [5]. In mammalian systems, steroids undergo two phases of metabolism [6]. Phase I metabolism mainly consists of oxidation, reduction, and hydroxylation reactions that introduce polar functionality to the steroid framework. This phase is responsible for the drug activation or inactivation and frequently, the introduction of the necessary functional groups for phase II metabolism. Phase II metabolism conjugates the steroid with the highly polar or charged groups to facilitate excretion. The two most important polar groups for phase II metabolism are sulfate and glucuronic acid. This conjugation process is catalysed by enzymes, including the sulfotransferases (SULTs) for sulfate conjugation [6,7], and the uridine 5'-diphosphoglucuronosyltransferases (UGTs) for the glucuronide conjugation [6,8]. Phase II metabolism is the main step that increases steroid hydrophilicity, so that they can be rapidly and efficiently excreted from the body in biological fluids such as urine and bile. In the drug testing laboratory, steroid metabolites are typically assayed from urine or sometimes in blood samples.

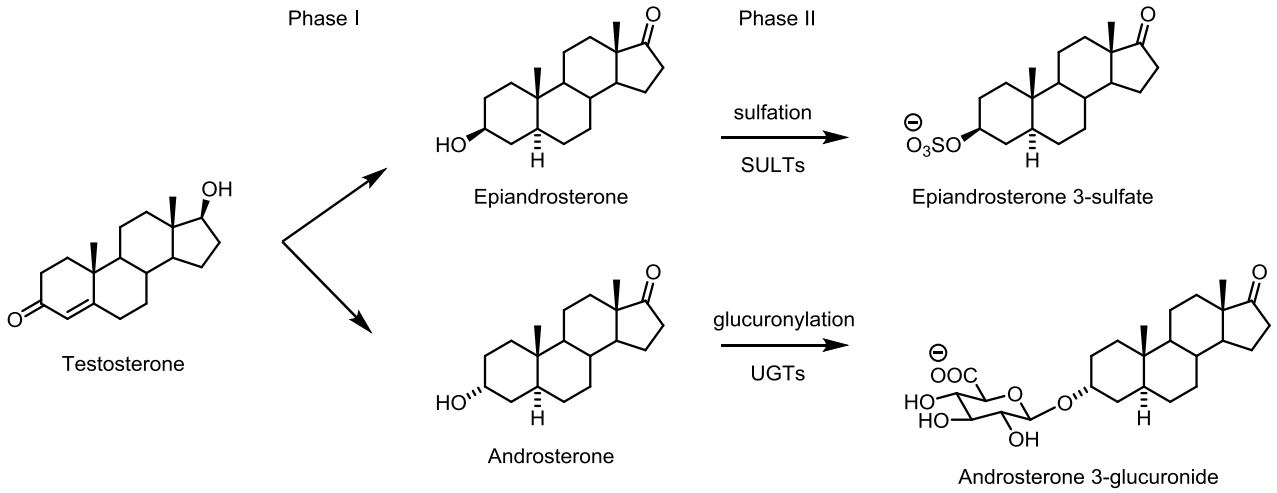



Figure 1. Steroid metabolism

### 1.3. Steroid detection (LC-MS vs GC-MS)

Since the steroids appear in a metabolised form in the urine sample, detecting steroids is not as straightforward as looking for the parent steroid. Instead, analytical data consistent with the downstream steroid metabolites must be obtained as a marker of steroid administration. The current approach for steroid metabolite detection in most analytical laboratories uses gas chromatography-mass spectrometry (GC-MS). However, steroid sulfates and steroid glucuronides are not volatile or thermally stable enough for GC-MS analysis. Thus, the laboratory must hydrolyse the phase II metabolites back to the phase I metabolites, and then chemically derivatise them, typically as the trimethylsilyl (TMS) derivative, for optimal GC performance [6,9]. This process takes time and the hydrolysis is not universally applicable to all steroids. The main enzyme that is used for the hydrolysis of steroid glucuronides is the *Escherichia coli* (*E. coli*) β-glucuronidase, but no enzyme is routinely used for the hydrolysis of steroid sulfates. The use of the β-glucuronidase enzyme is also mandated by WADA for the steroid module of the athlete biological passport (ABP). This means, urinary steroid sulfate metabolites are not routinely targeted for analysis. In order to improve the current detection methods, research is underway to try and find an enzyme that can effectively hydrolyse any steroid sulfate back to the parent steroid [10]. Another problem with the current approach is that not all steroid glucuronides can be hydrolysed by the β-glucuronidase enzyme. For example, 6β-hydroxyandrosterone 3-glucuronide and 6β-hydroxyetiocholanolone 3-glucuronide are resistant to hydrolysis and remain conjugated even after the treatment with the β-glucuronidase enzyme [11]. Chemical hydrolysis such as acid solvolysis can also be used. The drawbacks of this technique is that it cannot differentiate between sulfate and glucuronide conjugates, and it is known to

degrade sensitive analytes [9]. Overall, removing the conjugates also destroys any information that might be derived from the study of conjugation sites or levels.

Another approach is to detect the intact phase II conjugates in the urine sample using the liquid chromatography-mass spectrometry (LC-MS) methods, since phase II conjugates ionise well by electrospray ionisation (ESI), and no hydrolysis or derivatisation is required. This LC-MS technique can detect both sulfates and glucuronides more rapidly. However, sulfate and glucuronide reference materials are not commonly available. As a result, hydrolysis is often required prior to confirmation.

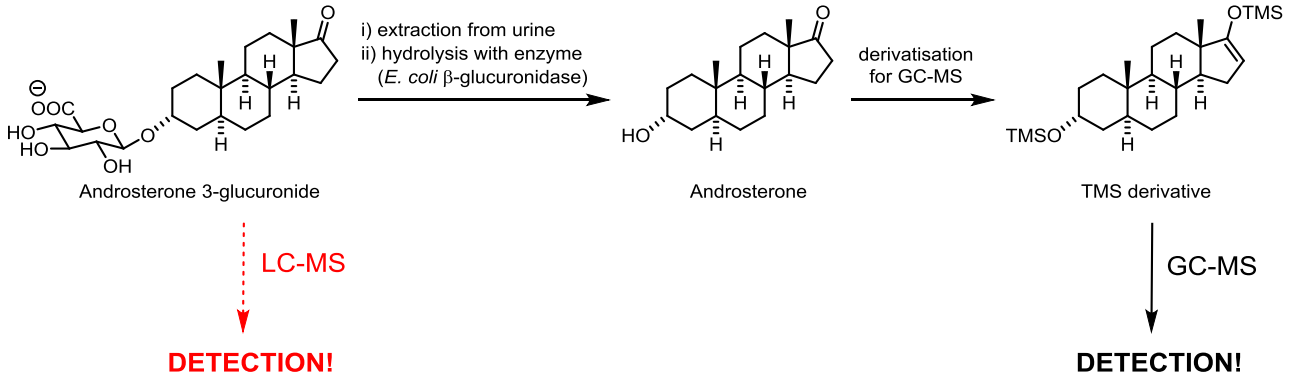


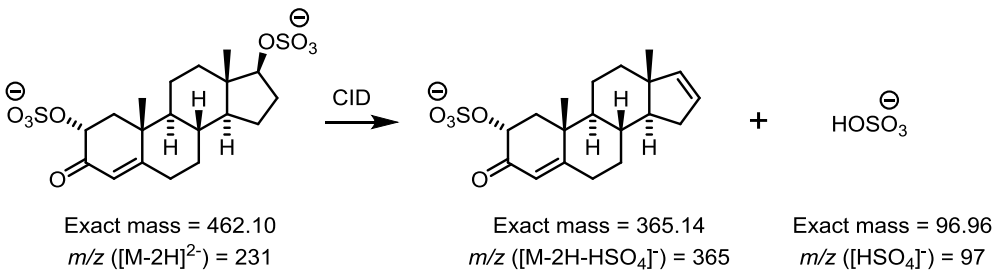

Figure 2. Methods of steroid detection in the analytical laboratory

### 1.4. Importance of bisconjugate steroid metabolites

Almost uniformly, analytical laboratories have targeted monoconjugate steroid sulfates and glucuronides as markers of steroid abuse however steroid bisconjugates could also potentially serve as markers of steroid administration. Previously, steroid bis(sulfates), bisglucuronides, and sulfate glucuronides were detected naturally in human plasma [12,13] and urine [14,15]. The similarities between these studies were that the steroid metabolites were extracted from either plasma or urine, which was then separated into fractions, solvolysed or hydrolysed using β-glucuronidase enzyme, derivatised, and finally detected using GC-MS. Fractionation took a great deal of effort, however it could be done either using electrophoresis (sometimes followed by paper chromatography) or ion exchange chromatography. In the studies of Miyabo & Kornel (1974) [13] and Kornel & Saito (1975) [14], radioactive steroid was administered to healthy volunteers, thus a radioactivity test was also used to detect the metabolites. At the end, the ability to detect these metabolites allowed them to study different levels of metabolites such as in patients with myocardial infarction and abnormal plasma lipid concentrations [12], patients with essential hypertension [13], or Cushing's syndrome [15].

These markers can also be found using an open screening method. This method starts by synthesising a representative library of putative target molecules including steroid bis(sulfates), bisglucuronides, or sulfate glucuronides, and studying their LC-MS ionisation and fragmentation to uncover patterns or trends common to all library members, and also those specific to different structure types. Once selected, common modes of fragmentation can be used to perform an open screen across a broad mass range including theoretical transitions for bisconjugates not contained in the original library. Once open screening is completed, the identity of newly identified bisconjugates must be assessed by evaluation of more complete MS fragmentation and where possible by comparison to the synthetically-derived reference materials. For compound confirmation, both chromatographic and mass spectrometric criteria are compared with the reference material, and they should match within tolerated range according to rules, such as those developed by WADA [16].

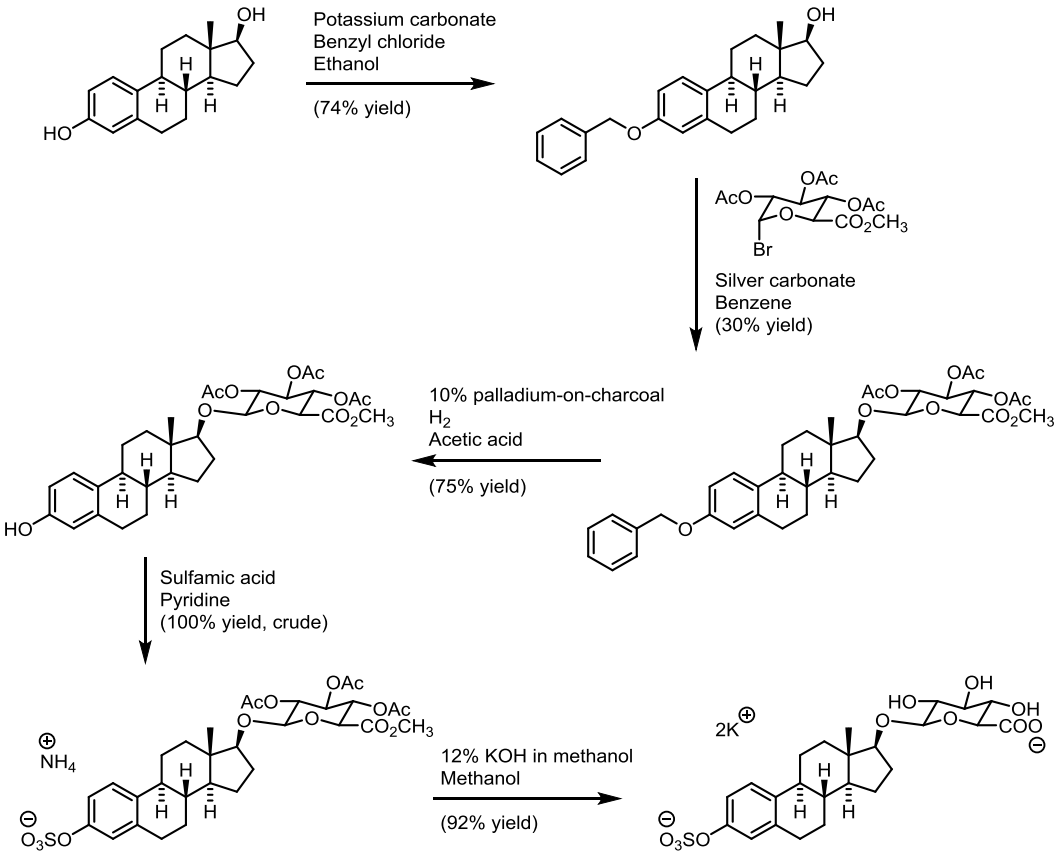
Promising results have come from the direct detection using LC-MS of bisconjugate steroid metabolites, especially steroid bis(sulfates). McLeod *et al*. (2017) synthesised 23 steroid bis(sulfates) and studied their characteristic MS fragmentation [17]. Using LC-MS/MS analysis, it was found that the di-anionic steroid bis(sulfates) fragment into a singly charged steroid mono-sulfate by loss of $[HSO_4]^-$, resulting in a mass decrease of 97 Da but an overall increase in mass-to-charge ratio (*m/z*) due to the loss of a negative charge. This fragmentation behaviour is specific to di-anionic steroid bis(sulfates) and can rapidly detect steroid bis(sulfates) in urine samples after simple extraction, without the hydrolysis and derivatisation steps. The method was used to study the endogenous steroid profile and was also applied to detect tibolone metabolites in drug administration trial samples. Recently, this technique was used to detect steroid bis(sulfates) that were important for the prenatal diagnosis of steroid metabolism pathologies such as: Steroid Sulfatase Deficiency (STSD), Smith-Lemli-Opitz Syndrome (SLOS) or cytochrome P450 Oxido-Reductase Deficiency (PORD) [18].




Scheme 1. Typical fragmentation of steroid bis(sulfates)

## 1.5. Previous syntheses of steroid bisglucuronides and sulfate glucuronides

Analytical studies of bisconjugated phase II steroid metabolites have been done in the past, but the access to reference materials for confirmation was very limited. In the 1960s, the focus of the steroid bisglucuronides and sulfate glucuronides was mainly directed to the estranes. Levitz *et al*. (1965) synthesised estriol 3-sulfate 16-glucuronide qualitatively from estriol using adenosine triphosphate (ATP) and guinea pig liver extract for sulfation, and uridine diphosphate glucuronic acid (UDPGA) and human liver extract for glucuronylation [19]. They found that conjugating estriol with the glucuronide first followed by sulfate worked, but the sequence did not work in the reverse order. Soon after, Cantrall *et al*. (1966) made the first sulfate glucuronide quantitatively and chemically using a Koenigs-Knorr reaction for the glucuronylation [20]. They also started by attaching the glucuronide first followed by the sulfate, as the sulfate group is quite labile in acidic conditions. After a process involving protection and deprotection, they could prepare estradiol 3-sulfate 17-glucuronide in 5 steps. This same strategy, adding the protected glucuronide group followed by sulfate group, was then used by Joseph *et al*. (1969) to synthesise estriol 3-sulfate 16-glucuronide in approximately 15% overall yield [21].




Scheme 2. Synthesis of estradiol 3-sulfate 17-glucuronide by Cantrall *et al*. (1966) [20]

For steroid bisglucuronides, estriol 3,16-bisglucuronide was first synthesised qualitatively by Kirdani *et al*. (1968) [22]. They synthesised the compound stepwise starting from the 3-glucuronide, followed by addition of the 16-glucuronide using either guinea pig liver or mouse liver extracts and UDPGA. They found that the reverse order of glucuronylation, adding 3-glucuronide after the 16-glucuronide, did not work. Mattox *et al*. (1983) then made bisglucuronide metabolites of deoxycorticosterone and corticosterone quantitatively and chemically using the Koenigs-Knorr reaction [23]. They were able to synthesise their target compounds from the dihydroxy steroids. Ma *et al*. (2014) prepared estradiol bisglucuronide qualitatively using the glucuronylsynthase enzyme and fluoride sugar in one step, but separation of the mixture of mono and bisglucuronide product was not performed [24]. Most recent examples came from Esquivel *et al*. (2017) [25]. They synthesized a library of 19 bisglucuronides qualitatively using the Koenigs-Knorr reaction. This was the first library made, but the products were only characterised by LC-MS. The mixture of steroidal products from the reaction was extracted and analysed by LC-MS.

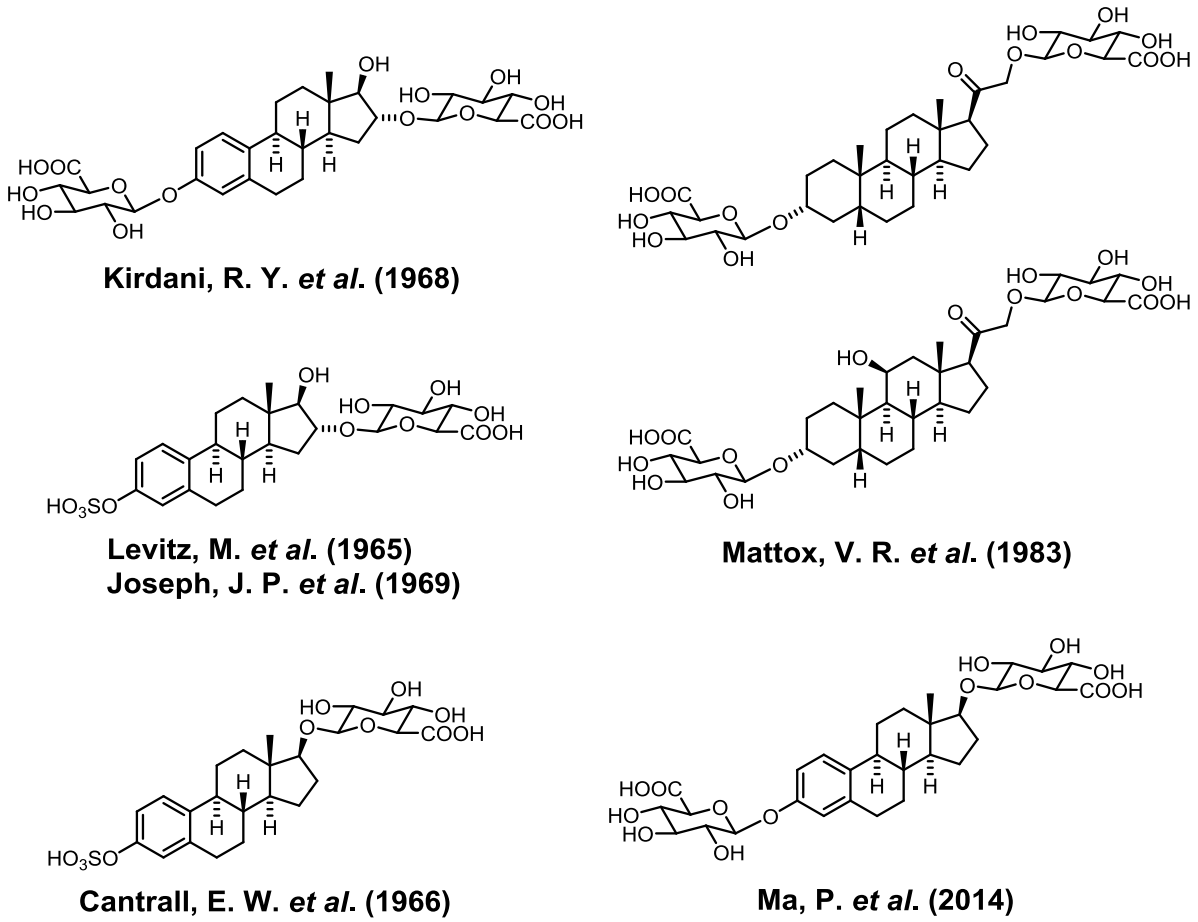



Figure 3. Previous syntheses of steroid bisglucuronides and sulfate glucuronides

There are a limited number of reference materials previously reported, and most of them have not been studied by modern LC-MS methods. Thus, the goal of this work was to prepare a library of steroid bisglucuronide and sulfate glucuronide reference materials. This paves the way for the development of new MS methods targeting bisconjugates to detect AAS doping in sports or the study of steroid metabolism associated with human disease.

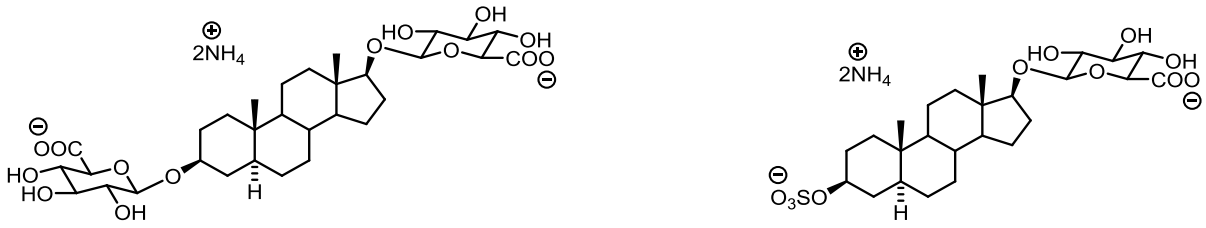



Figure 4. 5α-androstane-3β,17β-diol bisglucuronide, ammonium salt (left), 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide, ammonium salt (right)

# Chapter 2 – Steroid Bisglucuronides and Sulfate Glucuronides

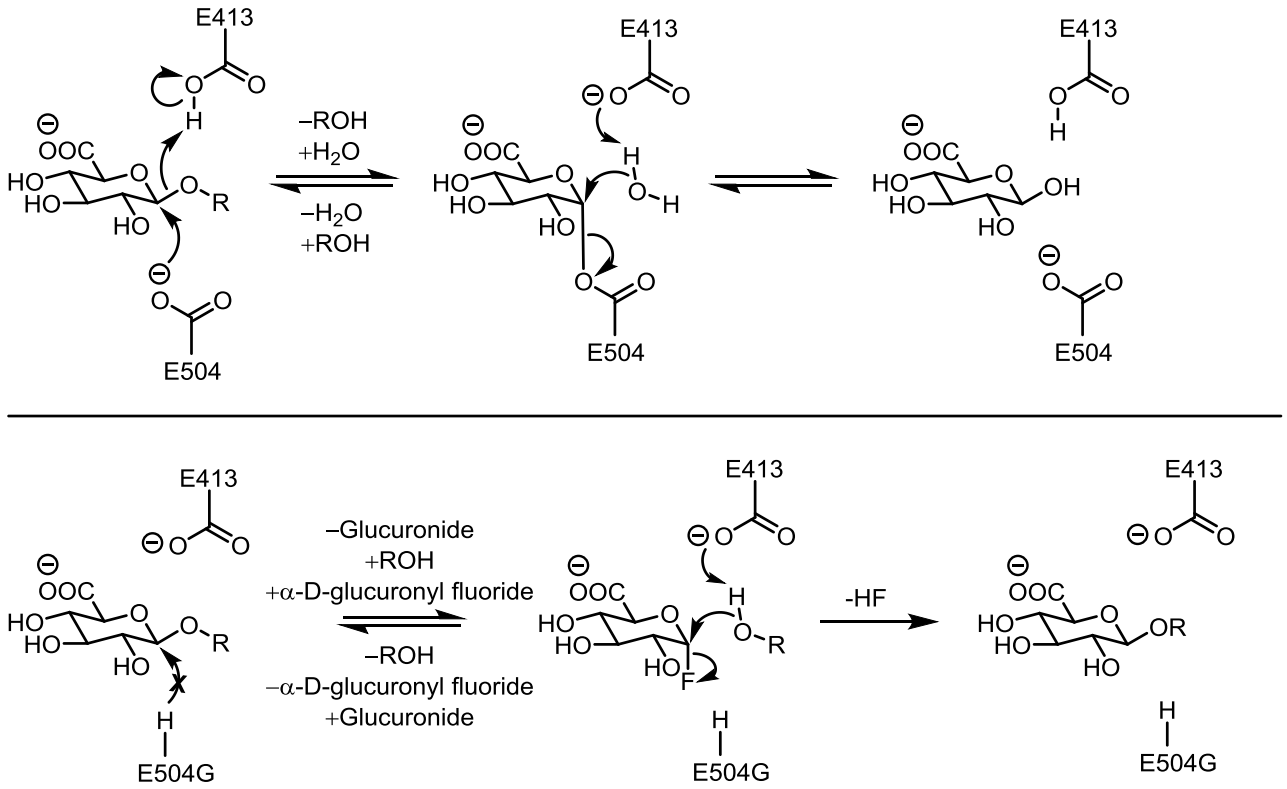
## 2.1. Foreword

At the time of writing this thesis, the following manuscript had been prepared for submission to “Journal of Steroid Biochemistry and Molecular Biology”. This publication describes the synthesis of bisconjugate steroids, especially steroid bisglucuronides and sulfate glucuronides. This publication was authored by Mr. Andy Pranata, Mr. Christopher C. Fitzgerald, Ms. Erin Westley, Ms. Natasha J. Anderson, Dr. Paul Ma, Dr. Oscar J. Pozo, and Associate Professor Malcolm D. McLeod. A full draft of the manuscript was prepared by Mr. Andy Pranata with the assistance of other co-authors and was coordinated by Associate Professor Malcolm D. McLeod.

In the past there were two main pathways to synthesise steroid glucuronides. Chemically, steroid glucuronide can be synthesised using the Koenigs-Knorr reaction [26] or its more modern variants including non-halide glycosyl donors. One of the first examples of steroid glucuronide synthesis using this reaction was dehydroepiandrosterone (DHEA) 3-glucuronide by Schapiro in 1939 [27]. This synthesis used methyl 2,3,4-tri-*O*-acetyl-α-D-glucuronyl bromide and silver carbonate to afford the protected glucuronide, which was then deprotected. Even though this reaction was successful, it suffers from low yield, unwanted side products, and the need of one or more deprotection steps to afford the free glucuronide. Another way to synthesise steroid glucuronides is to use the uridine 5’-diphosphoglucuronosyltransferases (UGTs) enzyme that is responsible for glucuronide conjugation in mammalian systems [6,8]. This enzymatic reaction removed the need of deprotection since the enzyme can transfer the free glucuronic acid. This one step reaction is also stereospecific, however it can be substrate-specific. Furthermore, the UGTs enzyme is often obtained from animals such as rats and dogs, this limits the reaction to only small scale.

In this thesis, glucuronylation reaction was performed using the *Eschericia coli* (*E. coli*) glucuronylsynthase enzyme. This enzyme is a mutant (E504G) of the *E. coli* β-glucuronidase developed within the group by Wilkinson *et al*. (2008) [28]. The *E. coli* β-glucuronidase is primarily used in the analytical laboratories to deconjugate glucuronide metabolites. The active site of *E. coli* β-glucuronidase contains two key glutamate residues at position 413 and 504. Glutamic acid 413 (E413) functions as a general acid/base, while glutamic acid 504 (E504) functions as a nucleophile. Together, these two residues are responsible for the double displacement hydrolysis (see Scheme 3). In 1998, Mackenzie *et al*. [29] managed to

remove the hydrolytic activity of the β-glucosidase enzyme found in *Agrobacterium* sp. to generate a glycosynthase mutant by a single-point mutation. Inspired by this finding, Wilkinson *et al*. (2008) performed a single-point mutation on *E. coli* β-glucuronidase at position 504 from glutamic acid to glycine (E504G). This became the glucuronylsynthase enzyme that can conjugate α-D-glucuronyl fluoride sugar donor to an alcohol acceptor such as a steroid alcohol (see Scheme 3).

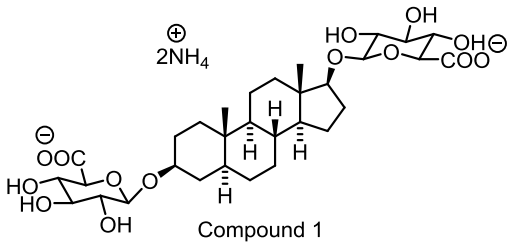
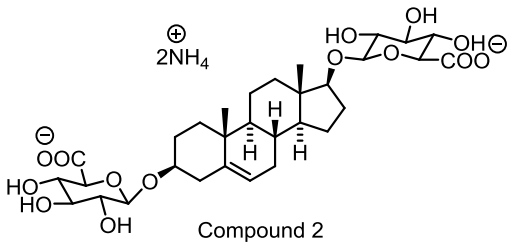
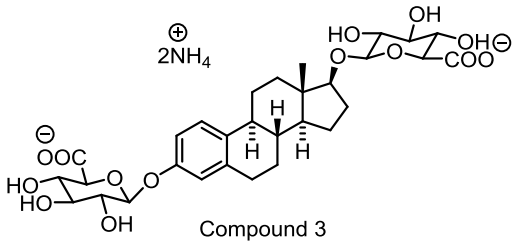
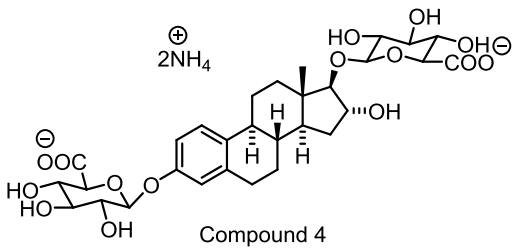



Scheme 3. Comparison of *E. coli* β-glucuronidase (top) and glucuronylsynthase (E504G) (bottom) mechanisms

After the finding of the glucuronylsynthase enzyme, the glucuronylation reaction is regularly performed within the group using the *E. coli* glucuronylsynthase (E504G) enzyme as catalyst and excess α-D-glucuronyl fluoride sugar donor [24,28,30]. Furthermore, the reaction mixture could be rapidly purified using a Waters OASIS weak anion exchange (WAX) solid phase extraction (SPE) cartridge to remove unreacted steroid, excess fluoro sugar, protein, and salts. Results from Ma *et al.* [24] obtained as part of his PhD candidature showed 14 examples of steroid monoglucuronides that were synthesised and gave between 5-90% conversions. In the same publication, the glucuronylation reaction on estradiol was performed giving a mixture of the 3- and 17-glucuronides and the bisglucuronide in a 1.0:1.6:1.1 ratio, respectively, as determined by NMR. However, no further purification was attempted.

This project was then continued by Ms. Natasha J. Anderson as part of a BSc (Honours) project who managed to glucuronylate steroid diols and a triol (i.e. steroids that have more than one hydroxyl group) to produce four mixtures containing steroid bisglucuronides [31]. Due to time limitations, only two of the mixtures were successfully purified using C18 SPE cartridges (Compound 1 and Compound 2 in Table 1 below). Table 1 below shows that full conversion to steroid bisglucuronides did not occur, instead the reactions gave steroid monoglucuronide impurities that were difficult to separate. For the glucuronylation of estradiol given below (Compound 3), doubling the amount of α-D-glucuronyl fluoride donor from the usual five equivalents gave a modest increase in estradiol bisglucuronide formation. However, doubling both sugar donor and the *E. coli* glucuronylsynthase enzyme gave a more significant increase in the proportion of estradiol bisglucuronide produced, but still mixtures containing estradiol 3- and 17-glucuronides.

Table 1. Results from previous attempt on making steroid bisglucuronides [31]

| Bisglucuronide product [a] | Ratio |
|---|---|
|  Compound 1 | 2 : 1 [b]<br>(bisglucuronide : 3-glucuronide) |
|  Compound 2 | 1 : 1 [b]<br>(bisglucuronide : 3-glucuronide) |
|  Compound 3 | 2 : 1 : 3 [b]<br>3 : 1 : 3 [b, c]<br>13 : 1 : 5 [b, c, d]<br>(bisglucuronide : 3-glucuronide : 17-glucuronide) |
|  Compound 4 | Complex mixture of mono- and bis-glucuronides |

[a] Reaction conditions: steroid (1.0 mg, 1.0 eq.), α-D-glucuronyl fluoride (5.0 eq.), *E. coli* glucuronylsynthase (E504G) (0.2 mg $mL^{-1}$), *tert*-butanol (10% v/v), sodium phosphate buffer (50 mM, pH 7.5), 37 °C, 2 days. [b] Determined by 400 MHz $^1H$ NMR integration of the key protons following OASIS WAX SPE. [c] 10 eq. of sugar. [d] A second addition of enzyme after 24 h, then incubated for a further 24 h.

On the other hand, one example of steroid sulfate glucuronide, 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide, was also successfully prepared using the glucuronylsynthase enzyme [10]. The synthesis started with a sodium borohydride reduction of epiandrosterone (EA) sulfate, followed by the glucuronylation reaction using the glucuronylsynthase enzyme and α-D-glucuronyl fluoride sugar. This steroid sulfate glucuronide was only prepared to then show the selectivity of *Pseudomonas aeruginosa* arylsulfatase (PaS) enzyme in hydrolysing the sulfate group and not the glucuronide [10]. A library of nine steroid sulfate glucuronides was then synthesised using the same method by Mr. Andy Pranata as part of a BSc (Honours) project. However, only four of them were successfully purified (> 95%) and characterised by NMR and MS. As part of subsequent MPhil studies, these examples were all subject to larger scale synthesis, purification and characterisation.

All these promising results were then continued by Mr. Andy Pranata to complete a library of ten steroid bisglucuronides as well as the other bisconjugate metabolite family, steroid sulfate glucuronides. Specific contributions of Mr. Andy Pranata are listed below:

- The synthesis, purification, and characterisation of ten steroid bisglucuronides through a one-step or step-wise enzymatic glucuronylation reaction.
- An investigation of the stepwise glucuronylation reaction was performed with the help from undergraduate project student, Ms. Erin Westley to investigate which order of glucuronylation was preferred to form steroid bisglucuronides.
- A simple inhibition study was conducted to give evidence that 5α-androstane-3β,17α-diol 3-glucuronide could inhibit the glucuronylation reaction of DHEA to form DHEA glucuronide without producing any 5α-androstane-3β,17α-diol bisglucuronide.
- The synthesis and characterisation of {$^{18}$O}-α-D-glucuronyl fluoride and epiandrosterone {$^{18}$O}-glucuronide.
- The synthesis, purification, and characterisation of ten steroid sulfate glucuronides through a sulfation, reduction, and glucuronylation reactions.
- LC-MS/MS study of all compounds described in the paper (some {$^{13}$C} labelled compounds were synthesised by Mr. Christopher C. Fitzgerald).
- Comparing synthesised materials with one urine sample by LC-MS/MS to detect two endogenous steroid sulfate glucuronides.

This publication aimed to give easier access to the analytical laboratory for reference materials and internal standards either for anti-doping or any medical research purposes. Synthesised samples were sent to Dr. Oscar J. Pozo at the Integrative Pharmacology and

Systems Neuroscience Group, IMIM, Hospital del Mar, Doctor Aiguader 88, Barcelona, Spain for further MS study and also applying these types of metabolites in medical research. A copy of supporting information containing all the experimental data alongside with $^{1}H$ NMR, $^{13}C$ NMR, LRMS, and IR spectra is available electronically and will be published online.

## 2.2. Synthesis of Steroid Bisglucuronide and Sulfate Glucuronide Reference Materials: Unearthing Neglected Treasures of Steroid Metabolism

Compound and reference numbers contained within the manuscript and associated supplementary material are relevant within these documents and not elsewhere in this thesis.

# Synthesis of Steroid Bisglucuronide and Sulfate Glucuronide Reference Materials: Unearthing Neglected Treasures of Steroid Metabolism

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**Abstract**

Doubly or bisconjugated steroid metabolites have long been known as minor components of the steroid profile that have traditionally been studied by laborious and indirect fractionation, hydrolysis and gas chromatography-mass spectrometry (GC-MS) analysis. Recently, the synthesis and characterisation of steroid bis(sulfate) (aka disulfate or bis-sulfate) reference materials enabled the liquid chromatography-tandem mass spectrometry (LC-MS/MS) study of this metabolite class and the development of a constant ion loss (CIL) scan method for the direct and untargeted detection of steroid bis(sulfate) metabolites. The CIL scan method has been employed to study the endogenous steroid profile, for the detection of exogenous steroid administration in anti-doping analysis, and for the pre-natal diagnosis of inborn errors of steroid metabolism by the analysis of maternal urine. Methods for direct LC-MS/MS detection of other bisconjugated steroids, such as steroid bisglucuronide and mixed steroid sulfate glucuronide metabolites, have great potential to reveal a more complete picture of the steroid profile. However, access to steroid bisglucuronide or sulfate glucuronide reference materials necessary for LC-MS/MS method development, metabolite identification or quantification is severely limited. In this work, ten steroid bisglucuronide and ten steroid sulfate glucuronide reference materials were synthesised through an ordered combination of chemical sulfation and/or enzymatic glucuronylation reactions. All compounds were purified and characterised using NMR and MS methods. Chemistry for the preparation of stable isotope labelled steroid {$^{13}C_6$}-glucuronide internal standards has also been developed and applied to the preparation of two selectively mono-labelled steroid bisglucuronide reference materials used to characterise more completely MS fragmentation pathways. The electrospray ionisation and fragmentation of steroid bisglucuronide and sulfate glucuronide reference materials has been studied. Preliminary targeted LC-MS/MS analysis of the reference materials prepared revealed pregn-5-ene-3β,20*R/S*-diol 3-sulfate 20-glucuronide as endogenous human urinary metabolites.

**Keywords:**

Steroid bisglucuronide, steroid sulfate glucuronide, steroid conjugate, phase II metabolism, stable isotope labelled internal standard, mass spectrometry

## Abbreviations

CID = collision induced dissociation, CIL = constant ion loss, DHEA = dehydroepiandrosterone, EA = epiandrosterone, *E. coli* = *Escherichia coli*, GC-MS = gas chromatography-mass spectrometry, LC-MS = liquid chromatography-mass spectrometry, NL = neutral loss, PORD = cytochrome P450 Oxido-Reductase Deficiency, SIM = single ion monitoring, SLOS = Smith-Lemli-Opitz Syndrome, SPE = solid phase extraction, SRM = selected reaction monitoring, STSD = Steroid Sulfatase Deficiency, UPLC-MS/MS = ultra-performance liquid chromatography–tandem mass spectrometry, WAX = weak anion exchange.

## Highlights

- Ten steroid bisglucuronide reference materials synthesised and characterised
- Ten steroid sulfate glucuronide reference materials synthesised and characterised
- Stable isotope labelled internal standards using $^{18}O$ and $^{13}C$ prepared
- Electrospray ionisation and fragmentation of reference materials studied
- Pregn-5-ene-3β,20*R*/*S*-diol 3-sulfate 20-glucuronide confirmed in human urine

## Introduction

Steroids are a large family of compounds with diverse roles as lipids, hormones and secondary metabolites, and as a result, numerous functions in biology and medicine. Many current therapeutic interventions target steroid biosynthesis or signalling pathways and this knowledge is also exploited in steroid abuse that remains a major problem for world sport and wider society [1]. In mammalian systems, steroids undergo two phases of metabolism [2]. Phase I metabolism involves changes to the steroidal carbon skeleton including the oxidation and reduction of functional groups. This metabolic change intersects and interacts with phase II metabolism involving the conjugation of steroids with highly polar, charged groups, commonly sulfate [3],[4] and glucuronic acid [5],[6]. Phase II metabolism is the major step that increases steroid hydrophilicity, allowing them to be rapidly and efficiently excreted from the body in biological fluids. At least 97% of steroids excreted in urine are present as some form of phase II conjugate [7]. However, phase II conjugates also serve other important roles in steroid transport and regulation, with steroids such as dehydroepiandrosterone sulfate and estrone sulfate serving as an endogenous depot in steroid hormone metabolism [8],[9].

Traditionally, steroid analysis has been conducted using gas chromatography-mass spectrometry (GC-MS) [10]. However, phase II conjugates such as steroid sulfates and steroid glucuronides are not volatile or thermally stable enough for direct GC-MS analysis. For this reason, chemical or enzymatic deconjugation of these metabolites to liberate the phase I metabolites, prior to derivatisation and GC-MS analysis, is typically employed [7]. The routine deconjugation of phase II metabolites has several drawbacks. Although acid solvolysis provides a general method of deconjugation, it cannot discriminate between sulfate and glucuronide conjugates, and is also known to degrade sensitive analytes [2],[11]. Milder enzymatic hydrolysis with *Escherichia coli* (*E. coli*) β-glucuronidase neglects the contribution of steroid sulfate metabolites and can result in incomplete hydrolysis [12], while the use of crude enzyme preparations containing glucuronidase and sulfatase enzymes can also lead to undesired steroid conversions [7]. More recently, purified bacterial arylsulfatase enzymes have been developed [13] to selectively hydrolyse steroid sulfates under conditions compatible with those employed for *E. coli* β-glucuronidase hydrolysis, but further work is required to establish the scope of these methods for analytical applications [14]. More generally, the routine use of deconjugation is undesirable as it destroys any information available from the study of conjugation patterns or levels.

Rapid advances in liquid chromatography-mass spectrometry (LC-MS) technology provide an improved method for the direct detection of the intact phase II conjugates since they ionise well by electrospray ionisation (ESI), and time-consuming hydrolysis and derivatisation steps are not required [15]. Typically, mono-conjugated steroid sulfate and glucuronide conjugates have been studied using this approach [12],[16]. On the other hand, doubly conjugated steroids that are also present as a minor component of the steroid profile, have generally only been studied through a laborious process of chromatographic fractionation and solvolysis [17],[18], typically coupled to GC-MS analysis [19],[20]. These steroidal conjugates (Figure 1) including bis(sulfates), sulfate glucuronides, bisglucuronides (single conjugation at two sites), or diglucuronides (double conjugation at one site) have received little attention over past decades, in large part due to an absence of suitable reference materials to aid analytical method development.

In 2017, an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) constant ion loss (CIL) scan method for the direct and untargeted detection of steroid bis(sulfate) metabolites was reported [21]. This method revealed a wide range of endogenous bis(sulfates) including examples from the estrane, androstane, and pregnane steroid families. The CIL scan method was applied to identify metabolites associated with sports doping and has also been employed in the analysis of maternal urine for the prenatal diagnosis of inborn errors of steroid biosynthesis associated with Smith-Lemli-Opitz Syndrome (SLOS), Steroid Sulfatase Deficiency (STSD), and cytochrome P450 Oxido-Reductase Deficiency (PORD) [22].

Access to reference materials is central in the development of MS methods to detect or quantify steroidal metabolites. In the study described above, a collection of 23 synthetically derived steroid bis(sulfates) was used to investigate the ionisation and fragmentation of this family, leading to the development of the selective, direct and untargeted CIL scan method [21]. Currently, well-characterised reference materials for the other bisconjugate families are not readily available. This limits analytical MS method development and precludes the unambiguous identification or quantification of these under-explored compounds. Few examples of the quantitative synthesis of steroid bisglucuronides [23],[24],[25], sulfate glucuronides [26],[27],[28] or diglucuronides [29] have been reported. Examples of small scale, qualitative chemical and biochemical synthesis of steroid bisglucuronides [30],[31],[29],[32],[33] and sulfate glucuronides [34] have also been reported where the products have generally not been fully purified or characterised spectroscopically.

In this work, the synthesis and characterisation of ten steroid bisglucuronide and ten steroid sulfate glucuronide reference materials is reported. The MS ionisation and fragmentation of these metabolites has been explored and two of these steroid sulfate glucuronides have been confirmed as endogenous human urinary metabolites by LC-MS/MS analysis. In addition, the development of stably labelled glucuronide reference materials is described, including selectively mono-labelled bisglucuronides, suitable for use as internal standards or as probes to interrogate the site selectivity of fragmentation processes. This chemistry will facilitate the development of new LC-MS methods for the direct detection of bisconjugates and open avenues in the study of this fascinating but neglected family of steroid metabolites in fields such as sports drug testing and medical science.

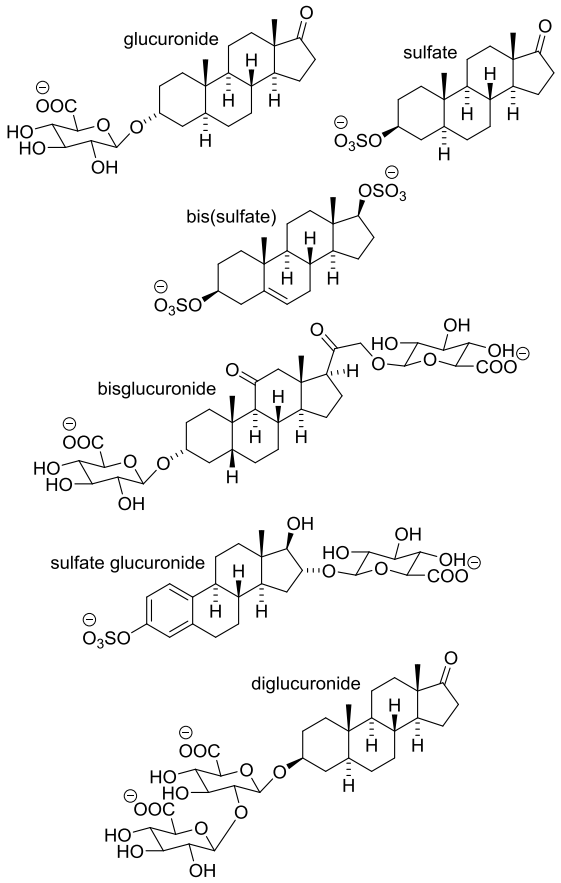



**Figure 1.** Examples of singly and doubly conjugated steroid metabolites

## Materials and methods

Materials and instruments associated with the chemical synthesis of bisconjugates are reported in the supplementary material (SI) section.

2.1. LC-MS/MS method for steroid bisconjugate analysis

Negative mode liquid chromatography-mass spectrometry (LC-MS) analysis was undertaken using a Waters 2695 Alliance Separations Module coupled to a Waters Acquity triple quadruple mass spectrometer and equipped with a Waters Symmetry C18 column (150 x 2.1 mm, 5 µm), eluting with a gradient consisting of the following mobile phases, A: methanol, B: water, both containing 0.01% formic acid and 10 mM ammonium formate, gradient: 0-9 min A-B (30:70 v/v) to A-B (90:10 v/v), 9-10 min A-B (90:10 v/v) to A-B (30:70 v/v), 5 min re-equilibration, flow rate 0.3 mL $min^{-1}$, and column temperature at 30 °C. Steroid sulfate glucuronides or steroid bisglucuronides were monitored for the mono-anion ($[M-H]^-$, cone voltage = 70 V) and di-anion ($[M-2H]^{2-}$, cone voltage = 26 V) using ESI in negative scan MS (*m/z* = 150-1000), targeted MS/MS mode (*m/z* = 50-700, collision energy = 10-50 eV) or selected reaction monitoring mode (collision energy = 10-20 eV, see Table S6 in SI) with 4000 V capillary voltage.

2.2. Urine sample preparation

The collection of human urine samples was conducted with approval of the Australian National University Human Research Ethics Committee (protocol 2013/654) and in accordance with the National Statement on Ethical Conduct in Human Research (2007) of the National Health and Medical Research Council. The procedure was adapted from the literature with minor modifications [35]. An aliquot of urine (2 mL) was fortified with nandrolone 17-sulfate (100 ng $mL^{-1}$) internal standard, treated with sodium phosphate buffer (50 mM, pH 7.5, 1 mL) and then centrifuged (2000 rpm, 5 min). The supernatant was then loaded onto a WAX SPE cartridge (3 cc) that was pre-conditioned with methanol (1 mL) and water (2 mL), and then washed with aqueous sodium hydroxide solution (0.1 M, 2 mL), sodium phosphate buffer (50 mM, pH 7.5, 2 mL), water (2 mL), and methanol (2 mL). The urinary steroid conjugates were then eluted with a solution of ethyl acetate:methanol:diethylamine (25:25:1 v/v/v, 2 mL). Concentration under a stream of nitrogen at 60 °C afforded a residue, which was reconstituted in water (200 µL) and transferred to a sealed vial for subsequent analysis by LC-MS/MS according to Section 2.1 above.

2.3. Glucuronylation inhibition study

To a tube containing sodium phosphate buffer (50 mM, pH 7.5, 60 µL) and dehydroepiandrosterone (DHEA) **1** solution in *tert*-butanol ($c_i$ = 500 µM, 10 µL, $c_f$ = 50 µM) was added diluted *E. coli* E504G glucuronylsynthase in sodium phosphate buffer ($c_i$ = 0.6 mg $mL^{-1}$, 10 µL, $c_f$ = 0.06 mg $mL^{-1}$). In another tube, α-D-glucuronyl fluoride **2** solution in

sodium phosphate buffer ($c_i$ = 500 µM, 10 µL, $c_f$ = 50 µM) was mixed with 5α-androstane-3β,17α-diol 3-glucuronide **3** solution in sodium phosphate buffer ($c_i$ = 200 µM, 10 µL, $c_f$ = 20 µM). The α-D-glucuronyl fluoride **2** and additive 5α-androstane-3β,17α-diol 3-glucuronide **3** mix was then added to the DHEA **1** and enzyme mix. Another four reactions were also set up by varying the final concentration of the additive to 0, 5, 10, and 15 µM. Negative controls were performed with only DHEA **1**, enzyme, and sodium phosphate buffer, while external standards were prepared with DHEA **1**, enzyme, sodium phosphate buffer, and DHEA glucuronide **4** ($c_i$ = 50 µM, 10 µL, $c_f$ = 5 µM). Reactions, negative controls, and external standards were then incubated in water bath at 37 °C for 10 min, and immediately quenched with methanol (100 µL) containing 50 µM etiocholanolone sulfate as internal standard. The quenched reaction mixture was centrifuged for 10 min (20000*g*), and then transferred to a 96-well plate ready for mass spectrometry analysis.

The production of DHEA glucuronide **4** was assayed by liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1290 Infinity LC injector, HTS sampler and 1260 Infinity UPLC system coupled to an Agilent 6120 quadrupole mass spectrometer. Chromatography was performed with an Agilent Poroshell 120 C18 column (30 mm x 2.1 mm, 2.7 µm) and a gradient of mobile phases, A: 10% v/v methanol:water, B: 90% v/v methanol:water, 10 mM ammonium acetate in both, gradient: 0-1 min A-B (58:42 v/v), 1-6 min A-B (58:42 v/v) to A-B (20:80 v/v), 6-7 min A-B (20:80 v/v) to B (100 v/v), 7-8 min held at B (100 v/v), 8-9 min B (100 v/v) to A-B (58:42 v/v), 5 min re-equilibration, flow rate 0.2 mL $min^{-1}$, and column temperature at 30 °C. The mono-anions ($[M-H]^-$) of DHEA glucuronide **4** (*m/z* = 463.2), 5α-androstane-3β,17α-diol bisglucuronide **5** (*m/z* = 643.3), and etiocholanolone sulfate (*m/z* = 369.2) were monitored using ESI in negative single ion monitoring (SIM) MS mode with 200 V fragmentor and 3000 V capillary voltage.

## 2.4. Synthesis

### 2.4.1. General procedure for small scale steroid conjugate purification by SPE

This step was performed to separate a steroid conjugate (such as a steroid sulfate, steroid glucuronide, or steroid bisglucuronide) from any unreacted starting steroid or steroid diol after a conjugation reaction (sulfation or glucuronylation). The procedure was adapted from literature methods [32],[36]. A WAX SPE cartridge (6 cc) was pre-conditioned with methanol (5 mL) followed by water (15 mL). The reaction mixture was then loaded onto the cartridge and eluted under a positive pressure of nitrogen at a flow rate of approximately 2 mL $min^{-1}$ with the following solutions: formic acid in water (2% v/v, 15 mL), water (15 mL), methanol (15 mL) and saturated aqueous ammonia solution in methanol (5% v/v, 15 mL). The

methanolic ammonia fraction was concentrated *in vacuo* to yield the desired steroid conjugate as the corresponding ammonium salt.

2.4.2. General procedure for determining conversion by $^{1}$H NMR analysis

This step was performed to calculate the ratio of steroid conjugate (steroid sulfate, steroid glucuronide, or steroid bisglucuronide) to steroid or steroid diol remaining after a conjugation reaction (sulfation or glucuronylation). The procedure employed a modified WAX SPE protocol (general procedure 2.4.1) eluting with only formic acid in water (2% v/v, 15 mL), water (15 mL) and saturated aqueous ammonia solution in methanol (5% v/v, 15 mL), followed by concentration of the methanolic ammonia fraction to yield a mixture containing both the starting steroid or steroid diol and the corresponding steroid conjugate as the ammonium salt. A $^{1}$H NMR spectrum was obtained and integration of a suitable signal (typically C3-H or C17-H) from both starting steroid or steroid diol and steroid conjugate was used to determine the percent conversion of the sulfation or glucuronylation reaction.

2.4.3. General procedure for C18 SPE purification of a steroid sulfate glucuronide or steroid bisglucuronide

A C18 SPE cartridge (3 cc) was pre-conditioned with methanol (2 mL) followed by water (6 mL). The solution of steroid sulfate glucuronide or steroid bisglucuronide mixture in water (1 mg mL$^{-1}$, 1 mL) was then loaded onto the cartridge and eluted under a positive pressure of nitrogen at a flow rate of approximately 2 mL mL$^{-1}$ with methanol:water (10-50% v/v, 3 mL), and methanol (3 mL). The methanol:water fraction was concentrated *in vacuo* to yield the desired steroid sulfate glucuronide or steroid bisglucuronide as the corresponding ammonium salt.

2.4.4. General procedure for the small scale reduction reaction of a steroid sulfate or steroid glucuronide containing a saturated ketone, with purification by SPE

The procedure was adapted from the literature [36]. A solution of steroid sulfate or steroid glucuronide (3.2-19 µmol) in methanol (100 µL) was treated by the addition of $NaBH_4$ over 1 minute (7.0 mg, 0.19 mmol) with cooling on ice. After the vigorous reaction had subsided, the reaction was capped, allowed to warm to room temperature and stirred for 16 h. The reaction was quenched by the slow addition of water (3 mL), adjusted to pH 7 (universal indicator strips) by the addition of aqueous hydrochloric acid (0.1 M, 2 mL) and subjected to SPE purification by general procedure 2.4.2 to afford the desired steroid diol monosulfate or monoglucuronide as the corresponding ammonium salt. A $^{1}$H NMR spectrum was obtained

and integration of a suitable signal (typically C19-$H_3$) from both the steroidal ketone and alcohol was used to determine the percent conversion of the reduction reaction.

2.4.5. General procedure for the small scale glucuronylation reaction of a steroid, steroid diol, steroid diol monosulfate, or steroid diol monoglucuronide with purification by SPE

The procedure was adapted from the literature [32]. The steroid, steroid diol, steroid diol monosulfate, or steroid diol monoglucuronide (2.1-20 µmol, 0.7 mM final concentration) in a tube was dissolved in *tert*-butanol (10% v/v), and sodium phosphate buffer (50 mM, pH 7.5, ~80% v/v), followed by *E. coli* E504G glucuronylsynthase (final concentration of 0.2 mg $mL^{-1}$). Finally, α-D-glucuronyl fluoride **2** (5.0 eq.) was dissolved in sodium phosphate buffer (50 mM, pH 7.5, ~10% v/v) and added to the reaction. The reaction was incubated without agitation at 37 °C for 2 days. The reaction mixture was then subjected to a series of SPE purification steps as detailed in the experimental method depending on which starting material was employed.

2.4.6. 5α-Androstane-3β,17β-diol bisglucuronide, ammonium salt **6**

2.4.6.1 Method A. The reaction was conducted with 5α-androstane-3β,17β-diol **7** [21] (5.0 mg, 17 µmol, see SI section) by general procedure 2.4.5. Purification of the reaction by general procedure 2.4.2 gave a mixture of the title compound **6** and 5α-androstane-3β,17β-diol 3-glucuronide **8** in a 2:1 ratio as determined by 400 MHz $^1$H NMR integration of the C20-H and C26-H protons (no starting steroid diol **7** observed). Performing the C18 purification procedure eluting with methanol:water (25% v/v) by general procedure 2.4.3 afforded the title compound **6** in pure form. **$R_f$** 0.15 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.41 (1H, d, *J* 7.7 Hz, C20-H), 4.35 (1H, d, *J* 7.8 Hz, C26-H), 3.80-3.72 (2H, m, C3-H and C17-H), 3.57 (1H, d, *J* 9.3 Hz, C24-H), 3.54 (1H, d, *J* 9.6 Hz, C30-H), 3.46-3.36 (4H, m, C23-H, C29-H, C22-H, C28-H), 3.21-3.15 (2H, m, C21-H and C27-H), 2.05 (1H, m), 1.98-1.89 (2H, m), 1.74-0.88 (18H, m), 0.85 (3H, s, C18-$H_3$), 0.83 (3H, s, C19-$H_3$), 0.67 (1H, m); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.4 (C25 or C31), 176.3 (C25 or C31), 104.6 (C26), 102.0 (C20), 89.7 (C17), 78.9 (C3), 77.9 (C22 or C28), 77.9 (C22 or C28), 76.5 (C24 or C30), 76.3 (C24 or C30), 75.3 (C27), 75.0 (C21), 73.8 (C23 or C29), 73.8 (C23 or C29), 55.9, 52.3, 46.0, 44.4, 38.9, 38.3, 36.8, 36.8, 35.3, 32.9, 30.3, 30.0, 29.6, 24.3, 22.0, 12.8 (C18), 12.1 (C19); **LRMS (-ESI)**: *m/z* 643 (90%, $[C_{31}H_{47}O_{14}]^-$), 467 (15%, $[C_{25}H_{39}O_8]^-$), 321 (100%, $[C_{31}H_{46}O_{14}]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{31}H_{47}O_{14}]^-$ 643.2966, found 643.2966

The steroid bisglucuronides **9**-**16** (Figure 2) were prepared using similar methods (see SI section)

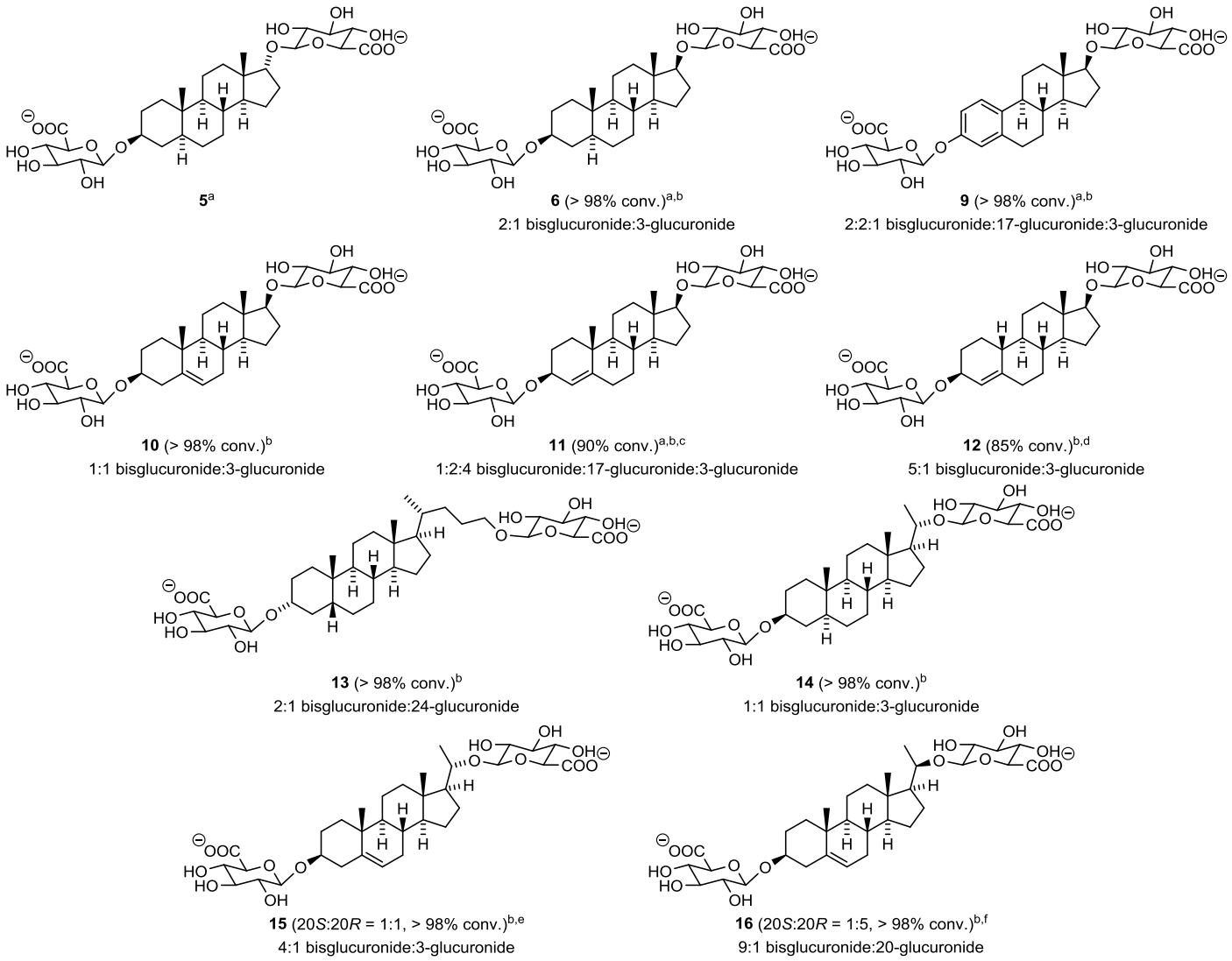



**Figure 2.** Steroid bisglucuronides **5**, **6**, **9**-**16** synthesised in this work. [a] Synthesis performed by sequential glucuronylation, [b] Synthesis performed in one step with % conversion from steroid diol to conjugated steroid diols shown, [c] One-step reaction started with a 1:9 ratio of the 3α:3β alcohol diastereomers, [d] One-step reaction started with a 1:6 ratio of the 3α:3β alcohol diastereomers, [e] One-step reaction started with a 2:1 ratio of the 20*S*:20*R* alcohol diastereomers, [f] One-step reaction started with a 1:6 ratio of the 20*S*:20*R* alcohol diastereomers.

2.4.6.2 Method B. The reaction was conducted with 5α-androstane-3β,17β-diol 17-glucuronide, ammonium salt [13] (derived in 19% conversion from dihydrotestosterone, assumed 3.2 μmol, a 1:9 ratio of the 3α:3β diastereomers, see SI section) by general procedure 2.4.5. Purification of the reaction by general procedure 2.4.2 gave the title compound **6** as a colourless solid with a 90% conversion overall (> 98% conversion from 3β-diol monoglucuronide to the bisglucuronide, with the 3α-diol monoglucuronide unreacted) as determined by 400 MHz $^{1}$H NMR integration of the C3-H protons. Performing

the C18 purification procedure eluting with methanol:water (25% v/v) by general procedure 2.4.3 afforded the title compound **6** in pure form.

2.4.7. 5α-Androstane-3β,17β-diol 3{$^{13}C_6$},17-bisglucuronide, ammonium salt {$^{13}C_6$}-**6**

The reaction was conducted with 5α-androstane-3β,17β-diol 17-glucuronide, ammonium salt [13] (1.0 mg, 2.1 μmol, a 1:7 ratio of the 3α:3β diastereomers, see SI section) and {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2** by general procedure 2.4.5. Purification of the reaction by general procedure 2.4.2 gave the title compound {$^{13}C_6$}-**6** as a colourless solid with 87% conversion overall (> 98% conversion from 3β-diol monoglucuronide to the bisglucuronide, with the 3α-diol monoglucuronide unreacted) as determined by 400 MHz $^1$H NMR integration of the C3-H protons. Performing the C18 purification procedure eluting with methanol:water (25% v/v) by general procedure 2.4.3 afforded the title compound {$^{13}C_6$}-**6** in pure form. **$^1$H NMR** (700 MHz, $CD_3OD$): δ 4.41 (1H, dd, *J* 158.1, 7.4 Hz, C20-H), 4.35 (1H, d, *J* 7.8 Hz, C26-H), 3.81-3.73 (2H, m, C3-H and C17-H), 3.69-3.05 (4H, m, C24-H, C23-H, C22-H, C21-H), 3.55 (1H, d, *J* 9.8 Hz, C30-H), 3.43 (1H, t, *J* 9.3 Hz, C29-H), 3.36 (1H, t, *J* 9.3 Hz, C28-H), 3.19 (1H, dd, *J* 9.1, 8.0 Hz, C27-H), 2.05 (1H, m), 2.00-1.87 (2H, m), 1.79-1.46 (7H, m), 1.46-1.07 (8H, m), 1.06-0.86 (3H, m), 0.85 (3H, s, C18-$H_3$), 0.83 (3H, s, C19-$H_3$), 0.67 (m, 1H); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 175.3 (m, C31), 104.7 (C20), 101.6 (m, C26), 89.8, 78.5-73.0 (4C, m, C27, C28, C29, C30), 55.9, 52.3, 46.0, 44.4, 38.9, 38.3, 36.8, 36.8, 35.3, 32.9, 30.8, 30.3, 30.0, 29.7, 24.3, 22.0, 12.8 (C18), 12.1 (C19), C21-25 obscured by C27-31 signals; **LRMS (-ESI)**: *m/z* 324 ([$C_{25}${$^{13}C_6$}$H_{46}O_{14}$]$^{2-}$); **HRMS (-ESI)**: *m/z* calcd. for [$C_{25}${$^{13}C_6$}$H_{47}O_{14}$]$^-$ 649.3173, found 649.3169.

The steroid {$^{13}C_6$}-bisglucuronide {$^{13}C_6$}-**9** and {$^{13}C_6$}-monoglucuronides {$^{13}C_6$}-**4** and {$^{13}C_6$}-**17**-{$^{13}C_6$}-**20** (Figure 3) were prepared using similar methods (see SI section).

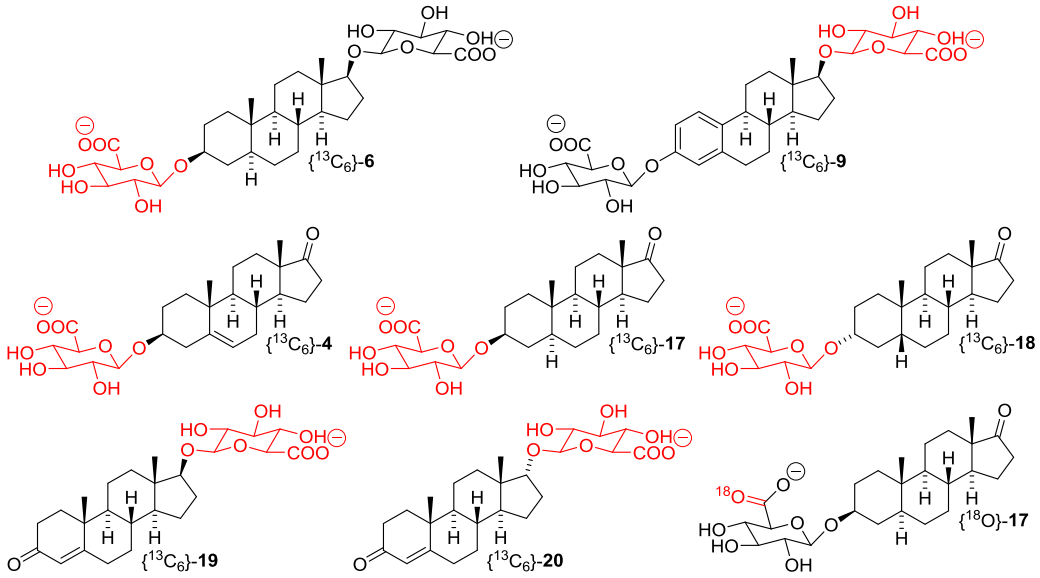


**Figure 3.** Synthesised $^{18}O$ and $^{13}C$ labelled steroid bisglucuronides {$^{13}C_6$}-**6** and {$^{13}C_6$}-**9** and monoglucuronides {$^{13}C_6$}-**4**, {$^{13}C_6$}-**17**-{$^{13}C_6$}-**20** and {$^{18}O$}-**17** with the $^{18}O$ or $^{13}C$ labelled glucuronide unit highlighted in red.

2.4.8. 5α-Androstane-3β,17β-diol 3-sulfate, ammonium salt **21**

The reaction was conducted with epiandrosterone (EA) sulfate, ammonium salt [36] (derived from EA **22**, 5.5 mg, 19 µmol, see SI section) by general procedure 2.4.4 to yield the title compound **21** as a colourless solid with > 98% conversion. $\mathbf{R_f}$ 0.42; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.25 (1H, tt, *J* 11.3, 4.9 Hz, C3-H), 3.56 (1H, t, *J* 8.6 Hz, C17-H), 2.05-0.88 (21H, m), 0.86 (3H, s, C19-$H_3$), 0.72 (3H, s, C18-$H_3$), 0.67 (1H, ddd, *J* 12.4, 10.4, 4.1 Hz); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 82.5 (C17), 79.7 (C3), 55.9, 52.3, 46.4, 44.1, 38.3, 38.1, 36.9, 36.6, 36.4, 32.8, 30.7, 29.8, 24.3, 21.9, 12.7 (C18), 11.7 (C19), one carbon peak obscured or overlapping; **LRMS (-ESI)**: *m/z* 371 (100%, $[C_{19}H_{31}O_5S]^-$); **HRMS (-ESI)**: calcd. for $[C_{19}H_{31}O_5S]^-$ 371.1892, found 371.1887.

2.4.9. 5α-Androstane-3β,17β-diol 3-sulfate 17-glucuronide, ammonium salt **23**

The reaction was conducted with 5α-androstane-3β,17β-diol 3-sulfate, ammonium salt **21** (derived from EA **22**, 5.5 mg, 19 µmol) by general procedure 2.4.5. Purification of the reaction by general procedure 2.4.2 gave the title compound **23** as a colourless solid with 93% conversion as determined by 400 MHz $^1$H NMR integration of the C18-$H_3$ protons. Performing the C18 purification procedure eluting with methanol:water (28% v/v) by general procedure 2.4.3 afforded the title compound **23** in pure form. $\mathbf{R_f}$ 0.29 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.35 (1H, d, *J* 7.8 Hz, C20-H), 4.24 (1H, tt, *J* 11.3, 4.7 Hz, C3-H), 3.79 (1H, t, *J* 8.6 Hz, C17-H), 3.53 (1H, d, *J* 9.4 Hz, C24-H), 3.56-3.37 (2H, m, C23-H and C22-H), 3.18 (1H, t, *J* 8.4 Hz, C21-H), 2.11-0.88 (21H, m), 0.85 (3H, s, C19-$H_3$), 0.83 (3H, s, C18-$H_3$), 0.69 (1H, ddd, *J* 12.4, 10.4, 4.1 Hz); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 104.6 (C20), 89.7 (C17), 79.7 (C3), 78.0 (C22), 75.3 (C21), 73.8 (C23), 55.8, 52.2, 46.3, 44.4, 38.8, 38.3, 36.8, 36.6, 36.4, 32.8, 29.8, 29.8, 29.6, 24.3, 22.0, 12.7, 12.1, C25 and C24 not observed; **LRMS (-ESI)**: *m/z* 569 (10%, $[C_{25}H_{38}O_{11}SNa]^-$), 547 (5%, $[C_{25}H_{39}O_{11}S]^-$), 471 (15%, $[C_{23}H_{35}O_8S]^-$), 371 (40%, $[C_{19}H_{31}O_5S]^-$), 273 (100%, $[C_{25}H_{38}O_{11}S]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{39}O_{11}S]^-$ 547.2213, found 547.2218.

The steroid sulfate glucuronides **24**-**32** (Figure 4) were prepared using similar methods (see SI section)

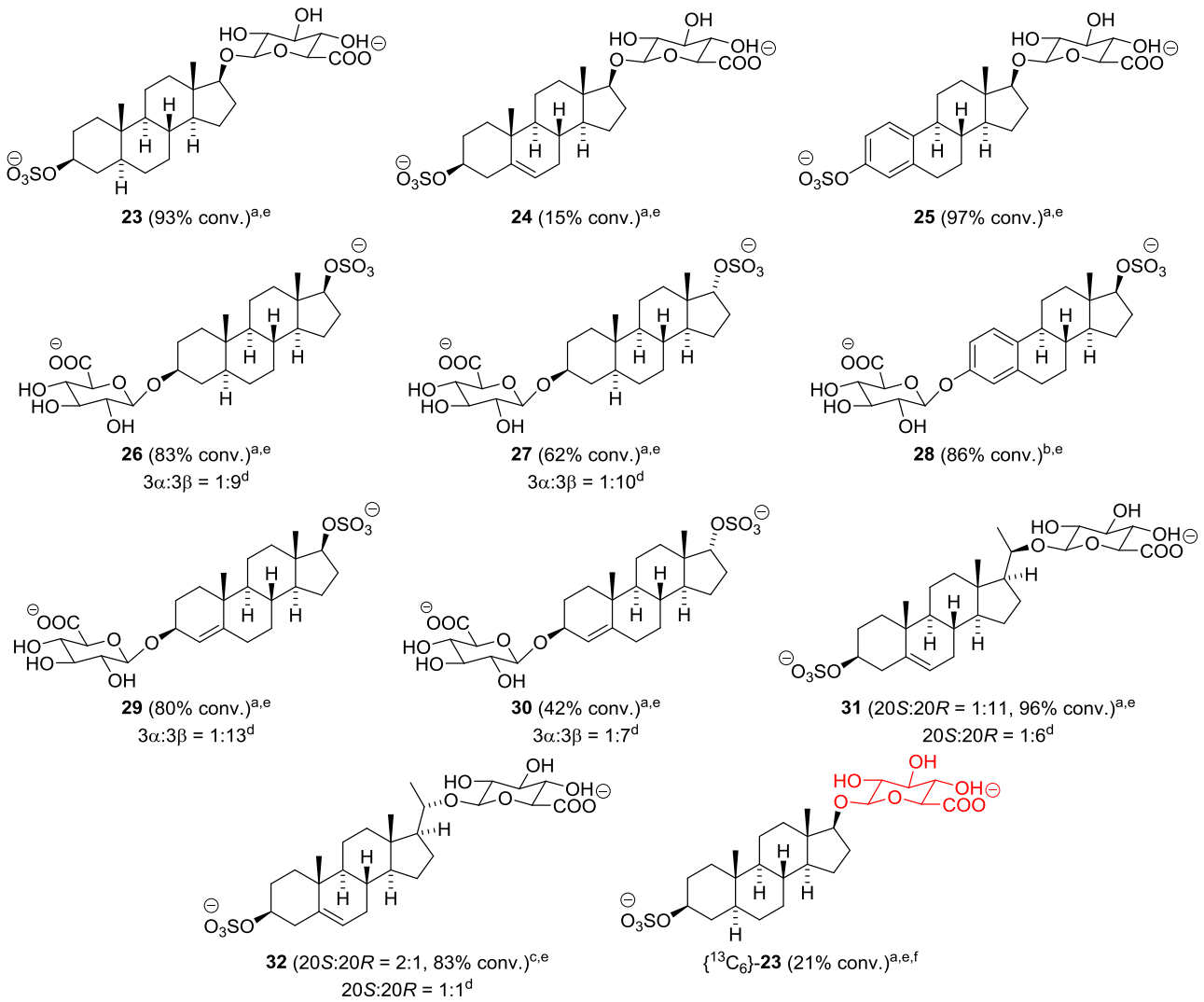



**Figure 4.** Steroid sulfate glucuronides **23**-**32** and {$^{13}C_6$}-**23** synthesised in this work. [a] Synthesis was performed in three steps by sulfation, reduction, and glucuronylation, [b] Synthesis was performed in two steps by selective sulfation followed by glucuronylation, [c] Synthesis was performed *via* tosylhydrazone formation, [d] Ratio of steroid diol monosulfate diastereomers after reduction, [e] The % conversion of the glucuronylation step is shown, [f] Glucuronylation was performed using {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2**.

2.4.10. 5α-Androstane-3β,17β-diol 3-sulfate 17{$^{13}C_6$}-glucuronide, ammonium salt {$^{13}C_6$}-**23**

The reaction was conducted with 5α-androstane-3β,17β-diol 3-sulfate, ammonium salt **21** (3.0 mg, 7.7 µmol) and {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2** by general procedure 2.4.5. Purification of the reaction by general procedure 2.4.2 gave the title compound {$^{13}C_6$}-**23** as a colourless solid with 21% conversion as determined by 400 MHz $^1$H NMR integration of the C18-$H_3$ protons. Performing the C18 purification procedure eluting with methanol:water (20% v/v) by general procedure 2.4.3 afforded the title compound {$^{13}C_6$}-**23** in pure form. **$^1$H NMR** (700 MHz, $CD_3OD$): δ 4.48-4.19 (2H, m, C17-H and C20-H), 3.79 (1H, m), 3.63-3.37 (3H, m), 3.15 (1H, m), 2.11-1.94 (3H, m), 1.83-1.74 (2H, m), 1.71-1.48 (4H, m), 1.45-1.13

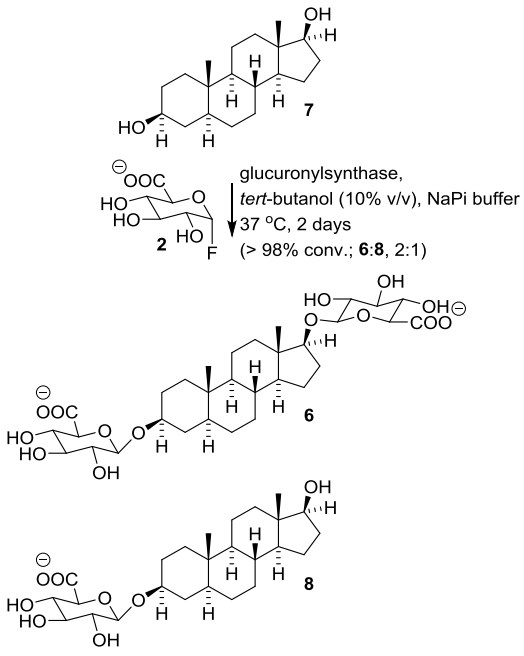
(9H, m), 1.05-0.85 (3H, m), 0.85 (3H, s, C18-$H_3$), 0.83 (3H, s, C19-$H_3$), 0.69 (1H, m); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.4 (m, C25), 104.6 (m, C20), 89.7, 79.7, 78.8-71.85 (4C, m, C21, C22, C23, C24), 55.8, 52.2, 46.3, 44.4, 38.9, 38.3, 36.8, 36.6, 36.4, 32.8, 29.8, 29.8, 29.6, 24.3, 22.0, 12.7 (C18), 12.1 (C19); **LRMS (-ESI)**: *m/z* 276 (100%, $[C_{19}\{^{13}C_6\}H_{38}O_{11}S]^{2-}$); **HRMS (-ESI)**: *m/z* calcd. for $[C_{19}\{^{13}C_6\}H_{39}O_{11}S]^-$ 553.2316, found 553.2309.

## Results

3.1. Synthesis of steroid bisglucuronide reference materials

Glucuronylation was performed enzymatically using α-D-glucuronyl fluoride **2** as the glucuronide donor and the *E. coli* glucuronylsynthase as catalyst [32]. In earlier work, this method was applied to hydroxylated keto-steroids with various structures and stereochemistries, and successfully produced a library of 14 steroid monoglucuronides with 5-90% conversion [32]. Also in earlier work, this method was applied to estradiol and gave a mixture of estradiol bisglucuronide **9**, estradiol 3-glucuronide, and estradiol 17-glucuronide in 1.1:1.0:1.6 ratio. Given the success of this earlier trial, we initially sought to access a library of steroid bisglucuronides through the direct enzymatic glucuronylation of steroid diols.

Steroid bisglucuronides in this study were generally synthesised in a single glucuronylation reaction of steroid diols using an excess α-D-glucuronyl fluoride **2** donor (5 eq.) and the glucuronylsynthase enzyme, as shown for 5α-androstane-3β,17β-diol **7** (Scheme 1) [32].



**Scheme 1.** One-step synthesis of 5α-androstane-3β,17β-diol bisglucuronide **6** from 5α-androstane-3β,17β-diol **7** promoted by the *E. coli* glucuronylsynthase enzyme.

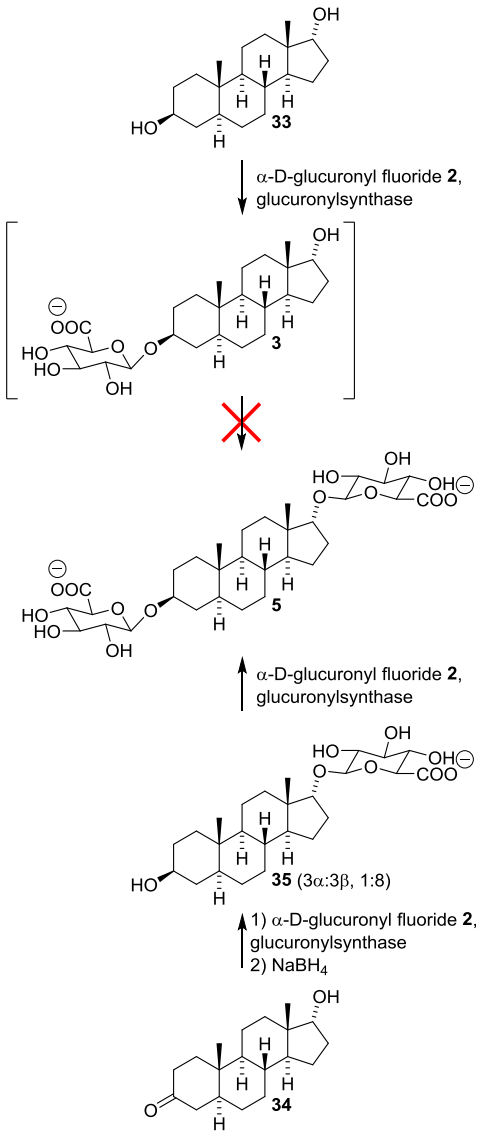
After the reaction, solid-phase extraction (SPE) using an Oasis weak-anion exchange (WAX) cartridge was performed as outlined in general procedure 2.4.2 and the product mixture analysed by $^{1}$H NMR spectroscopy. All steroid diols gave > 98% conversion to conjugated steroid mixtures, with the exception of reactions targeting bisglucuronides **11** and **12**. The starting diols for the synthesis of bisglucuronides **11** and **12** contained mixtures of 3α and 3β alcohols, and the 3α alcohol did not react [32], thus lower conversions of 90% and 85%, respectively, were observed. Where required, a second WAX SPE purification was performed as outlined in general procedure 2.4.1 to remove the unreacted steroid diols.

When the mixtures only contained conjugated steroid diols, $^{1}$H NMR integration could also provide a ratio of the steroid diol monoglucuronides and bisglucuronide formed. In the example shown in Scheme 1 above, bisglucuronide **6** and diol monoglucuronide **8** were produced in 2:1 ratio as determined by $^{1}$H NMR integration of the anomeric protons. Typically, the steroid bisglucuronide was the major product, except for bisglucuronide **11** where a mixture of steroid diol monoglucuronides were the major products formed (Figure 2). When 3β,17β- or 3β,20*S*-diols were available (bisglucuronides **6**, **10**, **11**, **12**, **14**, **15**), reactions produced bisglucuronide and diol 3-glucuronide, except for bisglucuronide **11** where diol 17-glucuronide was also observed in the mixture. In contrast, when 3α,24- or 3β,20*R*-diols were available (bisglucuronides **13** and **16**), diol 20- or 24-glucuronides were present at the end of the reaction. These data suggested relative reactivity for the *E. coli* glucuronylsynthase-promoted glucuronylation that paralleled that revealed earlier by Ma *et. al*. [32], with 3β(5α), 3β(5-ene), 3(phenolic), 20*R* and 24 hydroxyl groups showing the highest reactivity, while 3α(5β), 17β, 17α and 20*S* hydroxyl groups showed lower reactivity, and 3α(5α) hydroxyl groups proved unreactive.

To isolate pure steroid bisglucuronide from conjugated steroid diol mixtures, a C18 SPE method was used since it separated mixtures based on polarity, much like a reverse-phase chromatography. The more polar compound (steroid bisglucuronide) was eluted by lower concentrations of methanol in water. While the less polar compound in the mixture (steroid diol monoglucuronides) were subsequently eluted with 100% methanol. For steroid bisglucuronides with similar carbon skeletons such as 5α-androstanes (**5** and **6**), androst-4/5-enes (**10** and **11**), estr-4-ene (**12**), and pregnane (**14**) types, the bisglucuronide could be eluted selectively with 15-25% v/v methanol in water. A lower methanol concentration was

required to selectively elute estradiol bisglucuronide **9** (10% v/v methanol in water), while the less polar compounds based on cholane (**13**) and pregnene (**15** and **16**) skeletons needed 50% and 40% v/v methanol in water, respectively. At the end, nine pure steroid bisglucuronides **6** and **9**-**16** were obtained, with the exception of bisglucuronide **15** and **16**, which were isolated as 20*S*:20*R* diastereomeric mixtures as the bisglucuronides were not separable using the C18 method.

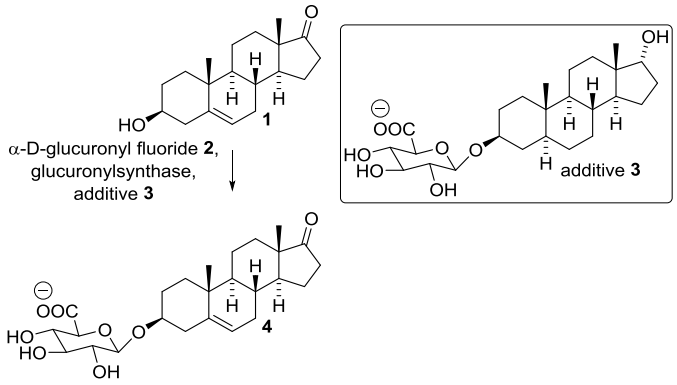
Although the enzymatic glucuronylation was successful in many cases, the bisglucuronide **5** could not be prepared by this approach. Direct glucuronylation of 5α-androstane-3β,17α-diol **33** afforded 5α-androstane-3β,17α-diol 3-glucuronide **3** as the sole conjugated product. This was despite earlier work [32], where several 17α-hydroxy steroids had been successfully subjected to enzymatic monoglucuronylation. A stepwise approach proved more productive. Glucuronylation of epidihydrotestosterone **34**, followed by a reduction reaction using sodium borohydride, gave a 1:8 mixture of 3α- and 3β- alcohol diastereomers **35**. A second glucuronylation reaction was then performed, and as desired, the bisglucuronide **5** was obtained completing a library of ten steroid bisglucuronides.




**Scheme 2.** Step-wise synthesis of 5α-androstane-3β,17α-diol 3,17-bisglucuronide **5** from epidihydrotestosterone **34**.

Two hypotheses were advanced to explain the requirement for stepwise synthesis: the intermediate steroid 3β,17α-diol 3-glucuronide **3** substrate provided a poor fit for the enzyme active site preventing further glucuronylation, or that the same intermediate **3** bound unproductively in the enzyme active site and so inhibited further reaction. To explore this, a simple inhibition experiment was conducted using DHEA **1** as a model substrate and 5α-androstane-3β,17α-diol 3-glucuronide **3** as a potential inhibitory additive (Scheme 3). The aim was to explore if increasing concentrations of 5α-androstane-3β,17α-diol 3-glucuronide **3** could decrease the production of DHEA glucuronide **4**. The additive **3** was investigated at final concentrations of 0, 5, 10, 15, 20 µM. The LC-MS analysis of these reactions showed that 15 and 20 µM of the additive **3** significantly reduced the production of DHEA glucuronide **4** ($p < 0.05$), and by approximately 50% at 20 µM (Figure 5). As a steroidal alcohol, the

additive **3** could potentially serve as substrate, leading to the steroid bisglucuronide **5**, but as expected based on attempted synthesis from steroidal diol **33**, this was not observed by LC-MS. The results show that 5α-androstane-3β,17α-diol 3-glucuronide **3** inhibits *E. coli* glucuronylsynthase promoted synthesis of DHEA glucuronide **4**, and implicates unproductive binding of this intermediate as the reason for the failed conversion of 5α-androstane-3β,17α-diol **33** to the bisglucuronide **5** (Scheme 2).




**Scheme 3.** Study of DHEA glucuronide **4** synthesis inhibition by additive 5α-androstane-3β,17α-diol 3-glucuronide **3**.

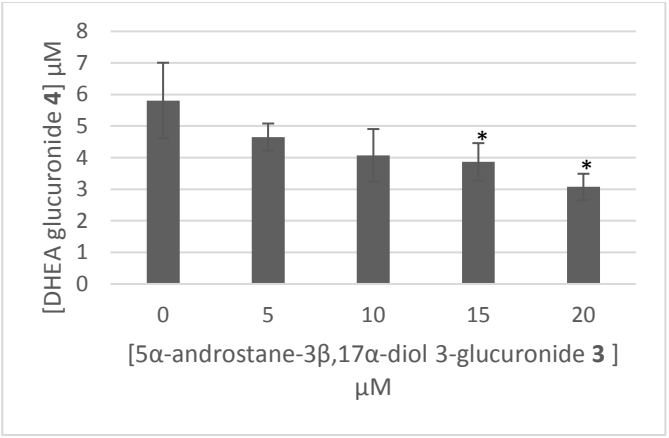



**Figure 5.** Concentration of DHEA glucuronide **4** produced vs 5α-androstane-3β,17α-diol 3-glucuronide **3** concentration. * $p < 0.05$ calculated using t-test (two-sample assuming unequal variances).

The stepwise synthesis described above (Scheme 2) showed that order of glucuronylation was important for the *E. coli* glucuronylsynthase promoted synthesis of bisglucuronides. The one-step glucuronylation also proved unsuccessful for a number of other steroid diols including 5β-androstane-3α,17β-diol, androst-4-ene-3β,17α-diol, and 16α-hydroxy-DHEA. The stepwise approach was not investigated for these examples, but could be pursued in future research. Further investigations (see SI section) revealed that 5α-androstane-3β,17β-

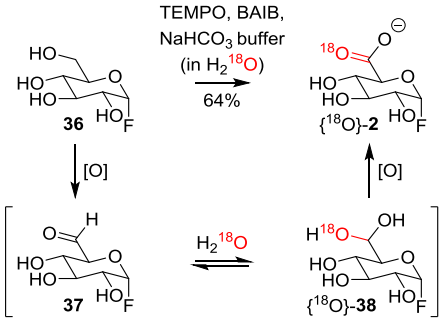
diol bisglucuronide **6** (Figure 2) was accessible from the 5α-androstane-3β,17β-diol 17-glucuronide (90% conversion, section 2.4.6.2) and not the corresponding 3-glucuronide **8**, estradiol bisglucuronide **9** was accessible from estradiol 3-glucuronide (> 98% conversion) and not the corresponding 17-glucuronide, and androst-4-ene-3β,17β-diol bisglucuronide **11** was accessible from both androst-4-ene-3β,17β-diol 3-glucuronide (42% conversion) and 17-glucuronide (60% conversion). Although longer, the stepwise method was observed to give cleaner product in the final step, and in one case, eliminated the need for C18 purification to remove diol monoglucuronide by-products. Another advantage of the capacity to make a bisglucuronide from a specific steroid diol monoglucuronide intermediate was the potential to isotopically label one of the two glucuronide units of the bisglucuronide selectively to generate MS probes or internal standards.

3.2. Synthesis of stable isotope labelled steroid monoglucuronides and bisglucuronides

Introduction of stable isotope labels to the glucuronide unit would allow differentiation of the two conjugated positions of a bisglucuronide and enable the development of internal standards for both steroidal glucuronides, bisglucuronides and sulfate glucuronides. Stable labelling of the glucuronide unit would also afford other advantages. A range of stable isotope labelled monoglucuronides are available that incorporate deuterium atoms within the steroid skeleton. New synthetic routes are required for each labelled glucuronide involving multiple chemical steps. Labelling of the glucuronide unit would provide a more general method for the introduction of the label in the final step of the synthesis. Given this, we sought methods to stably label the α-D-glucuronyl fluoride **2** donor employed in the *E. coli* glucuronylsynthase-promoted glucuronylation reaction.

Labelling was first attempted using $^{18}O$ derived from labelled water. The α-D-glucuronyl fluoride **2** is prepared through an oxidation of α-D-glucosyl fluoride **36** using bis(acetoxy)iodobenzene (BAIB) and TEMPO in acetonitrile and sodium bicarbonate buffer. These modified conditions avoided the use of aqueous bleach as stoichiometric oxidant [37] and permitted a simple substitution with labelled water. Under these conditions, oxidation afforded a mixture of labelled {$^{18}O_2$}-α-D-glucuronyl fluoride, {$^{18}O_1$}-α-D-glucuronyl fluoride {$^{18}O$}-**2**, and unlabelled α-D-glucuronyl fluoride **2** in approximately 20:12:1 ratio based on the LRMS (-ESI) analysis. Mechanistically, the formation of doubly labelled and unlabelled sugar in the reaction was consistent with the depicted equilibrium between aldehyde **37** and aldehyde hydrate {$^{18}O$}-**38** in the oxidation step (Scheme 4). The resulting $^{18}O$ labelled α-D-glucuronyl fluoride {$^{18}O$}-**2** mixture was reacted with EA **22** to form $^{18}O$ labelled EA

glucuronide {$^{18}$O}-**17**. Based on LRMS (-ESI), the ratio between EA {$^{18}O_2$}-glucuronide, EA {$^{18}O_1$}-glucuronide {$^{18}$O}-**17**, and EA glucuronide **17** was again 20:12:1.



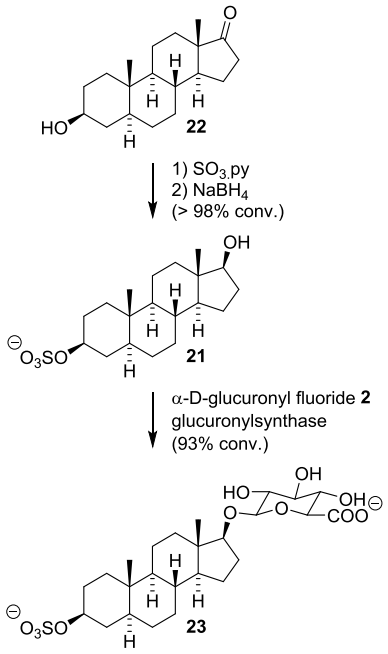

**Scheme 4.** Proposed pathway for TEMPO-promoted oxidation of α-D-glucosyl fluoride **36** to afford $^{18}$O labelled α-D-glucuronyl fluoride {$^{18}$O}-**2** with the $^{18}$O label highlighted in red.

The $^{18}$O labelled α-D-glucuronyl fluoride {$^{18}$O}-**2** would be suitable for the preparation of a mass spectrometry probe to distinguish between the glucuronide units in a bisglucuronide, but could not serve as a stable isotope labelled internal standard due to the presence of unlabelled material. This prompted a second approach to label the α-D-glucuronyl fluoride **2** by using a $^{13}$C label. A four-step synthesis of α-D-glucuronyl fluoride {$^{13}C_6$}-**2** was employed, starting from {$^{13}C_6$}-D-glucose [37]. This method successfully produced the sugar with six $^{13}$C isotopes fully incorporated based on LRMS (-ESI). This was higher than the theoretically expected labelling based on a lower threshold of at least 99 atom % $^{13}$C specified by the supplier (94.2% hexa-labelled {$^{13}C_6$}, 5.7% penta-labelled {$^{13}C_5$}, 0.1% tetra-labelled {$^{13}C_4$}, and 0.0% tri-labelled {$^{13}C_3$}). Two steroid bisglucuronides with one selectively labelled glucuronide unit were prepared: 5α-androstane-3β,17β-diol 3{$^{13}C_6$},17-bisglucuronide {$^{13}C_6$}-**6** and estradiol 3,17{$^{13}C_6$}-bisglucuronide {$^{13}C_6$}-**9**. These were synthesised using the stepwise approach described above (Scheme 2) with the final glucuronylation step performed using {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2**. In addition, five $^{13}$C labelled steroid monoglucuronides were also synthesised, including DHEA {$^{13}C_6$}-glucuronide {$^{13}C_6$}-**4**, EA {$^{13}C_6$}-glucuronide {$^{13}C_6$}-**17**, etiocholanolone {$^{13}C_6$}-glucuronide {$^{13}C_6$}-**18**, testosterone {$^{13}C_6$}-glucuronide {$^{13}C_6$}-**19** and epitestosterone {$^{13}C_6$}-glucuronide {$^{13}C_6$}-**20**. The steroid monoglucuronides {$^{13}C_6$}-**4**, {$^{13}C_6$}-**17**-{$^{13}C_6$}-**20**, and bisglucuronides {$^{13}C_6$}-**6** and {$^{13}C_6$}-**9** were also shown to have full incorporation of the {$^{13}C_6$}-glucuronide moiety based on LRMS (-ESI). The presence of the {$^{13}C_6$}-glucuronide moiety gave distinctive couplings in $^{1}$H and $^{13}$C NMR spectra. In the $^{1}$H NMR spectrum, additional coupling was observed, when compared to the unlabelled compounds. This was caused by both short (one bond) and long-range (two or three bond) $^{13}$C-$^{1}$H coupling, making the $^{1}$H NMR spectrum complex.

However, characterisation was more straightforward in the broadband decoupled $^{13}C$ NMR spectrum, due to the characteristic splitting caused by $^{13}C$-$^{13}C$ couplings. For example, testosterone {$^{13}C_6$}-glucuronide {$^{13}C_6$}-**19** had an apparent doublet of triplets (δ 176.6, *J* 58.8, 4.9 Hz) observed for the carbonyl carbon, while a second doublet of triplets (δ 104.5, *J* 47.1, 4.9 Hz) was observed for the anomeric carbon. Another four glucuronide carbons were observed as a multiplet (δ 71.9-79.0). As expected, these labelled glucuronide $^{13}C$ NMR signals had significantly greater signal intensity than the non-enriched carbons of the steroidal backbone. In summary, the $^{13}C$ labelling provided fully labelled bisglucuronide ({$^{13}C_6$}-**6** and {$^{13}C_6$}-**9**) and monoglucuronide ({$^{13}C_6$}-**4**, {$^{13}C_6$}-**17**-{$^{13}C_6$}-**20**) conjugates suitable for use a stable isotope labelled internal standards and mass spectrometry probes.

3.3. Synthesis of steroid sulfate glucuronide reference materials

A second family of steroid bisconjugates produced in this study was the steroid sulfate glucuronides. Ten steroid sulfate glucuronides were synthesised on a preparative scale, generally by a three-step sequence involving sulfation, ketone reduction, and glucuronylation as shown below for the conversion of EA **22** to 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** (Scheme 5). This order of synthesis was chosen as initial glucuronylation would introduce three additional hydroxyl groups on the sugar ring, and subsequent sulfation could then occur unselectively on any of the available hydroxyl groups.




**Scheme 5.** Three-step synthesis of 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** from EA **22**.

The sulfation reaction was performed using sulfur trioxide-pyridine complex according to a literature method [36]. Ketone reduction was performed using sodium borohydride [36], or with additional cerium (III) chloride heptahydrate (Luche conditions) for α,β-unsaturated ketones like testosterone and epitestosterone sulfate [38]. Reduction of the C17 ketones afforded the 17β-hydroxy steroid as the sole diastereomer. However, reduction of the C3 or C20 ketones gave diastereomeric mixtures. The C3 ketone reduction afforded 1:7-13 mixtures of 3α:3β diastereomers [10]. The C20 ketone reduction proceeded under Felkin-Anh control to favour the 20*R* diastereomer (1:6 *S*:*R*) as observed in the literature for the reduction of pregnenolone [39],[40]. Reduction of the toluenesulfonylhydrazone derivative of pregnenolone sulfate under conditions similar to those reported by Tada *et al*. [41], afforded a 1:1 ratio of the 20*S*:20*R* diastereomers.

In the final step, steroid diol monosulfates were glucuronylated using the *E. coli* glucuronylsynthase and the α-D-glucuronyl fluoride **2** donor [32] to give steroid diol sulfate glucuronides **23**-**32**. Glucuronylation was observed for 3β(5α), 3β(5-ene), 3(phenolic), 17β, 20*S* and 20*R* hydroxyl groups. The starting diol monosulfates for the synthesis of sulfate glucuronides **26**, **27**, **29** and **30** contained mixtures of 3α and 3β alcohols, and the 3α alcohol did not react [32], observed by $^{1}$H NMR analysis of the product mixture. The starting diol monosulfates for the synthesis of sulfate glucuronides **31** and **32** contained mixtures of 20*S* and 20*R* alcohols, and both diastereomers were observed to react. For glucuronylation at the C3 position to form 17-sulfate 3-glucuronides, higher conversions (83-86%) were observed for steroid 3β,17β-diols **26** and **29** or estradiol **28**, than the steroid 3β,17α-diols **27** and **30** (42-62%). Glucuronylation at the C17 position to form steroid 3-sulfate 17-glucuronides **23** and **25**, typically proceeded in high conversion (93-97%). Surprisingly, glucuronylation to afford androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24** consistently gave low conversion (15%). Purification was achieved by SPE (WAX and C18) in a manner similar to that described for the bisglucuronides above (Section 3.1) to afford the products with > 95% purity and these were characterised by $^{1}$H NMR, $^{13}$C NMR, LRMS, and HRMS. In total, ten pure steroid sulfate glucuronides **23**-**32** were obtained, including diastereomeric mixtures **31** and **32** favouring the 20*R*- and 20*S*- diastereomers respectively, which were not separable using the C18 method.

One $^{13}$C labelled steroid sulfate glucuronide was synthesised, 5α-androstane-3β,17β-diol 3-sulfate 17{$^{13}C_6$}-glucuronide {$^{13}C_6$}-**23** using the same three-step synthesis, but using {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2** in the final glucuronylation step (Figure 4). Full incorporation of six $^{13}$C isotopes from the glucuronide moiety was observed based on LRMS (-ESI). The

presence of the {$^{13}C_6$}-glucuronide moiety gave distinctive splitting in $^{1}H$ and $^{13}C$ NMR spectra in a manner similar to that observed for selectively labelled bisglucuronides {$^{13}C_6$}-**6** and {$^{13}C_6$}-**9** (Figure 3).

3.4. NMR analysis of steroid bisglucuronide and sulfate glucuronide reference materials

All reference materials prepared by this study were characterised by $^{1}H$ and $^{13}C$ NMR spectroscopy. In addition to providing important evidence of compound identity and purity, the application of NMR chemical shift and multiplicity data within emerging NMR metabolomics workflows may aid in the rapid assignment of metabolite structure [42]. Several diagnostic proton signals were observed in the $^{1}H$ NMR spectra of the synthetically derived steroid bisglucuronides **5**, **6**, **9**-**16** and sulfate glucuronides **23**-**32**. On glucuronylation, adjacent C3-H and C17-H protons shifted downfield by 0.20-0.35 ppm. For example, the C17-H signal in diol monosulfate **21** (δ 3.56, t, *J* 8.6 Hz) shifted downfield by 0.23 ppm (δ 3.79, t, *J* 8.6 Hz) on glucuronylation to afford sulfate glucuronide **23** (Scheme 5). In contrast, the C20-H protons displayed different behaviour. The 20*S* diastereomer (more clearly seen with bisglucuronide **14**), showed a downfield shift of 0.08 ppm for the C20-H signal. On the other hand, the C20-H signal for the 20*R* diastereomer shifted 0.35 ppm in bisglucuronide **16**. For the aromatic protons in estradiol bisglucuronide **9**, the C1-H proton moved 0.11 ppm (*meta*-position to the reacting site), and the C2-H and C4-H protons moved 0.34 ppm (*ortho*-position to the reacting site). Other than these steroidal proton shifts, new peaks in $^{1}H$ NMR that were typical of bisglucuronide and sulfate glucuronide compounds included the anomeric protons from each glucuronide unit. For bisglucuronide compounds (**5**, **6**, **9**-**16**), these were typically resolved, with each appearing as a doublet. For bisglucuronide **6**, the 3-glucuronide anomeric proton signal (δ 4.41, d, *J* 7.7 Hz) was well resolved from that of the 17-glucuronide (δ 4.35, d, *J* 7.8 Hz). These assignments were readily made by comparisons to previously reported $^{1}H$ NMR data for EA glucuronide **17** and dihydrotestosterone glucuronide [32]. Estradiol bisglucuronide **9** displayed one anomeric proton signal for the 17-glucuronide (δ 4.40, d, *J* 7.8 Hz) with the second anomeric proton from the 3-glucuronide obscured by the water peak from the deuterated methanol solvent (δ 4.85). Eight additional protons from the two glucuronide units in bisglucuronides and four additional protons in sulfate glucuronides appeared between δ 3.1-3.7.

Similarly, diagnostic protons for steroid sulfate glucuronides (**23**, **24**, **26**-**32**) were the oxymethine protons that were shifted downfield after sulfation reaction. After sulfation reaction, C3-H or C17-H shifted downfield by 0.65-0.76 ppm as expected from the previously

reported data [36]. For estradiol 3-sulfate 17-glucuronide **25**, the aromatic protons were also shifted, C1-H proton moved 0.18 ppm (*meta*-position to the reacting site), and the C2-H and C4-H protons moved 0.54 ppm (*ortho*-position to the reacting site). After glucuronylation reaction, the oxymethine protons shifts were smaller than after sulfation reaction as mentioned above and as previously reported for monoglucuronides [32]. In summary, the protons associated with sulfation and glucuronylation reaction sites were typically resolved (δ 3.0 to 5.0) from the rest of steroidal backbone protons, and so the chemical shift and multiplicity of these signals is likely some diagnostic value. Characteristic $^{1}$H NMR signals all steroid bisglucuronide and steroid sulfate glucuronide reference materials are tabulated in the supplementary material (Table S1).

### 3.5. MS analysis of unlabelled and labelled steroid bisglucuronides

#### 3.5.1. Ionisation

In the full MS with 70 V cone voltage, the mono-anion $[M\text{-}H]^-$ was the major ion observed with some minor $[M\text{-}H\text{-}gluc]^-$ in-source fragmentation also found (where "gluc" was the dehydrated glucuronic acid moiety ($C_6H_8O_6$) 176 Da). The highest relative abundance for the $[M\text{-}H\text{-}gluc]^-$ ion appeared for estradiol bisglucuronide **9** and 5β-cholane-3α,24-diol bisglucuronide **13** with 30% and 25% respectively, while only between 5-10% was observed for the other compounds (**5**, **6**, **10**-**12**, **14**-**16**). In addition, estradiol bisglucuronide **9** also showed another in-source fragment $[M\text{-}H\text{-}2gluc]^-$ *m/z* 271 in the scan MS. In contrast, scan MS with 26 V cone voltage formed the di-anion $[M\text{-}2H]^{2-}$ as the major ion, while still forming mono-anion $[M\text{-}H]^-$ with 50-100% relative abundance. In-source fragmentation was only observed for estradiol bisglucuronide **9**, giving 5% $[M\text{-}H\text{-}gluc]^-$. A recent MS study on a library of crude chromatographically resolved steroid bisglucuronides reported similar findings [33]. The current study highlights potential to favour the formation of either mono- or di-anion precursors of the bisglucuronides for subsequent MS/MS studies.

#### 3.5.2. Fragmentation

Collision Induced Dissociation (CID) was then applied to mono- and di-anionic precursor ions to study their MS fragmentation. With the mono-anion $[M\text{-}H]^-$, the major fragments at 50 eV collision energy that retained the steroid backbone were $[M\text{-}H\text{-}gluc]^-$ and $[M\text{-}H\text{-}C_2H_4O_3]^-$, corresponding to neutral loss (NL) of 176 Da and 76 Da respectively. The NL of 76 Da was not observed for estradiol bisglucuronide **9** and 5β-cholane-3α,24-diol bisglucuronide **13**. Instead, estradiol bisglucuronide **9** showed $[M\text{-}H\text{-}2gluc]^-$ (*m/z* 271) or a

combined NL of 352 Da. Another minor fragment containing the steroid backbone involved the combined NL of the glucuronide unit and water ([M-H-gluc-$H_2O$]$^-$), and this was more prominent for the unsaturated steroid bisglucuronides **11** and **12**. Fragments from the glucuronide moiety itself (*m/z* 175, 157, 129, 113, 85, 75) were also observed as earlier reported by for monoglucuronides [12] and bisglucuronides [33].

For the di-anionic precursor ions [M-2H]$^{2-}$, fragmentation with 20 eV collision energy, the common fragments formed were derived from ion loss of *m/z* 175 ([gluc-H]$^-$) and 75 ([$C_2H_3O_3$]$^-$), to give [M-2H-(gluc-H)]$^-$ (equivalent to [M-H-gluc]$^-$ above) and [M-2H-($C_2H_3O_3$)]$^-$ (equivalent to [M-H-$C_2H_4O_3$]$^-$ above) respectively. These fragments were noteworthy because of the increase in *m/z* caused by an ion loss from the precursor ion during fragmentation. A similar pattern of ion loss from di-anionic precursor ions was previously identified in steroid bis(sulfates) leading to the development of the constant ion loss (CIL) scan method [21]. Estradiol bisglucuronide **9** showed a fragment ion at *m/z* 271 ([M-2H-(gluc-H)-gluc]$^-$) resulting from the combined ion loss of *m/z* 175 and NL of 176 Da. Interestingly, 5β-cholane-3α,24-diol bisglucuronide **13** gave an ion loss of *m/z* 75 giving a fragment ion at *m/z* 637 that was not formed by NL 76 from the corresponding mono-anion. The NL of water after ion loss of *m/z* 175 ([M-2H-(gluc-H)-$H_2O$]$^-$) and fragments of the glucuronide moiety (*m/z* 175, 157, 129, 113, 85, 75) were also typically observed as described earlier for the mono-anion fragmentation.

Collision-induced dissociation was also performed on the stably-labelled steroid monoglucuronides and selectively mono-labelled steroid bisglucuronides. For the monoglucuronides, three labelled EA glucuronide reference materials were available, unlabelled **17**, $^{18}O$ labelled {$^{18}O$}-**17**, and $^{13}C$ labelled {$^{13}C_6$}-**17** (Figure 3), and for each CID was performed targeting the [M-H]$^-$ precursor ion at 50 eV collision energy (Table 1). For the $^{18}O$ labelled EA glucuronide {$^{18}O$}-**17** the fragmentation study targeted the doubly labelled precursor ion (increase of *m/z* 4). The major fragments in the unlabelled EA glucuronide **17** were the glucuronide moiety fragments at *m/z* 113, 85, and 75. The $^{18}O$ labelled EA glucuronide {$^{18}O$}-**17** confirmed that fragments at *m/z* 113 ([gluc-H-$H_2O$-$CO_2$]$^-$) and 85 ([gluc-H-$H_2O$-$CO_2$-CO]$^-$) formed with loss of the C6 carboxylate since no $^{18}O$ labelling of the fragments was observed. However, the fragment at *m/z* 75 ([$C_2H_3O_3$]$^-$) was observed at *m/z* 79 due to the inclusion of two $^{18}O$ isotopes (increase of *m/z* 4) indicating that this fragment contained the C6 carboxylate unit. Furthermore, for fragmentation of the $^{13}C$ labelled {$^{13}C_6$}-**17** precursor ion this glucuronide [$C_2H_3O_3$]$^-$ fragment became *m/z* 77 due to the inclusion of two $^{13}C$ isotopes (increase of *m/z* 2) consistent with the inclusion of two glucuronide carbons,

likely C5 and C6. The $^{13}C$ labelled EA glucuronide {$^{13}C_6$}-**17** also showed increased in *m/z* to 118 and 89 for the other glucuronide fragments due to the inclusion of five and four $^{13}C$ atoms respectively (Table 1).

**Table 1.** Fragmentation of selected monoglucuronide precursor ions $[M-H]^-$ (70 V cone voltage, 50 eV collision energy): [a] indicates $^{18}O$ labelled precursor or fragment; [b] indicates $^{13}C$ labelled precursor or fragment.

| | Precursor ion, *m/z* (relative abundance) | Fragment ions, *m/z* (relative abundance) | | | | |
|---|---|---|---|---|---|---|
| Compound | $[M-H]^-$ | $[gluc-H]^-$ | $[gluc-H-H_2O]^-$ | $[gluc-H-H_2O-CO_2]^-$ | $[gluc-H-H_2O-CO_2-CO]^-$ | $[HOCH_2COO]^-$ |
| EA glucuronide **17** | 465 (100) | 175 (1) | 157 (3) | 113 (30) | 85 (50) | 75 (50) |
| EA {$^{18}O_2$}-glucuronide {$^{18}O$}-**17** | 469 (100)[a] | 179 (1)[a] | 161 (2)[a] | 113 (25) | 85 (50) | 79 (50)[a] |
| EA {$^{13}C_6$}-glucuronide {$^{13}C_6$}-**17** | 471 (100)[b] | 181 (1)[b] | 163 (2)[b] | 118 (30)[b] | 89 (60)[b] | 77 (60)[b] |

The fragmentation behaviour of two selectively mono-labelled steroid bisglucuronide compounds, 5α-androstane-3β,17β-diol 3{$^{13}C_6$},17-bisglucuronide {$^{13}C_6$}-**6** and estradiol 3,17{$^{13}C_6$}-bisglucuronide {$^{13}C_6$}-**9** were also studied by CID. When the mono anion ($[M-H]^-$) of 5α-androstane-3β,17β-diol 3{$^{13}C_6$},17-bisglucuronide {$^{13}C_6$}-**6** was fragmented, two ions *m/z* 467 ($[M-H-(\{^{13}C_6\}\text{-gluc})]^-$) and 473 ($[M-H-gluc]^-$) were observed with similar intensity (Table 2). This suggested that similar energies were required for fragmentation of each end

of the bisglucuronide. On the other hand, estradiol 3,17{$^{13}C_6$}-bisglucuronide {$^{13}C_6$}-**9** only showed one fragment at *m/z* 453 ([M-H-gluc]$^-$) where the unlabelled glucuronide at the 3-position had been lost, indicating that cleavage of the phenolic glucuronide was preferred due to the conjugated nature of the linking glycosidic oxygen atom. Both labelled and unlabelled glucuronide fragments (*m/z* 175, 157, 113, 85, 75) appeared for both selectively labelled bisglucuronides. Fragmentation of the di-anions ([M-2H]$^{2-}$) showed similar behaviour (Figure 6, Table 3). In conclusion, selectively mono-labelled bisglucuronide reference materials provide a means to study the fragmentation associated with both glucuronide units of a bisglucuronide compound.

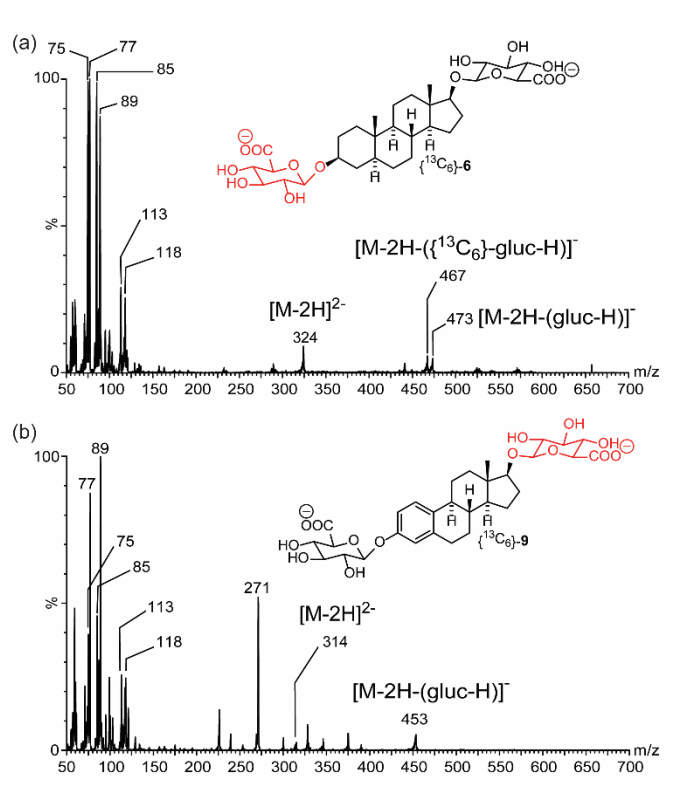



**Figure 6.** Fragmentation of di-anion precursor ions [M-2H]$^{2-}$ (26 V cone voltage, 30 eV collision energy): (a) 5α-androstane-3β,17β-diol 3{$^{13}C_6$},17-bisglucuronide {$^{13}C_6$}-**6**, (b) estradiol 3,17{$^{13}C_6$}-bisglucuronide {$^{13}C_6$}-**9**, with the $^{13}C$ labelled glucuronide unit highlighted in red

**Table 2.** Fragmentation of selected bisglucuronide mono-anion precursor ions $[M-H]^-$ (70 V cone voltage, 50 eV collision energy): [a] indicates $^{13}C$ labelled precursor or fragment.

| Compound | $[M-H]^-$ | $[M-H-C_2H_4O_3]^-$ | $[M-H-gluc]^-$ | $[M-H-2gluc]^-$ | $[gluc-H]^-$ | $[gluc-H-H_2O]^-$ | $[gluc-H-H_2O-CO_2]^-$ | $[gluc-H-H_2O-CO_2-CO]^-$ | $[C_2H_3O_3]^-$ |
|---|---|---|---|---|---|---|---|---|---|
| EA 3,17-bisglucuronide **6** | 643 (20) | 567 (5) | 467 (10) | - | - | 157 (5) | 113 (50) | 85 (100) | 75 (60) |
| EA 3{$^{13}C_6$},17-bisglucuronide {$^{13}C_6$}-**6** | 649 (20)[a] | 573 (3)[a] | 467 (6)<br>473 (10)[a] | - | - | 157 (5)<br>163 (5)[a] | 113 (50)<br>118 (40)[a] | 85 (100)<br>89 (80)[a] | 75 (60)<br>77 (60)[a] |
| Estradiol 3,17-bisglucuronide **9** | 623 (5) | - | 447 (35) | 271 (40) | 175 (10) | 157 (5) | 113 (85) | 85 (100) | 75 (50) |
| Estradiol 3,17{$^{13}C_6$}-bis(glucuronide {$^{13}C_6$}-**9** | 629 (5)[a] | - | 453 (50)[a] | 271 (50) | 175 (10) | 157 (5)<br>163 (3)[a] | 113 (100)<br>118 (30)[a] | 85 (75)<br>89 (75)[a] | 75 (30)<br>77 (35)[a] |

**Table 3.** Fragmentation of selected bisglucuronide di-anion precursor ions $[M-2H]^{2-}$ (26 V cone voltage, 30 eV collision energy): [a] indicates $^{13}C$ labelled precursor or fragment.

| Compound | $[M-2H]^{2-}$ | $[M-2H-(gluc-H)]^{-}$ | $[M-2H-(gluc-H)-gluc]^{-}$ | $[gluc-H-H_2O-CO_2]^{-}$ | $[gluc-H-H_2O-CO_2-CO]^{-}$ | $[C_2H_3O_3]^{-}$ |
|---|---|---|---|---|---|---|
| EA 3,17-bisglucuronide **6** | 321 (5) | 467 (5) | - | 113 (25) | 85 (90) | 75 (100) |
| EA 3{$^{13}C_6$},17-bisglucuronide {$^{13}C_6$}-**6** | 324 (10)[a] | 467 (5)<br>473 (5)[a] | - | 113 (30)<br>118 (25)[a] | 85 (100)<br>89 (85)[a] | 75 (100)<br>77 (100)[a] |
| Estradiol 3,17-bisglucuronide **9** | 311 (5) | 447 (5) | 271 (40) | 113 (40) | 85 (100) | 75 (95) |
| Estradiol 3,17{$^{13}C_6$}-bis(glucuronide {$^{13}C_6$}-**9** | 314 (3)[a] | 453 (5)[a] | 271 (50) | 113 (25)<br>118 (25)[a] | 85 (45)<br>89 (100)[a] | 75 (40)<br>77 (90)[a] |

## 3.6. MS analysis of steroid sulfate glucuronide reference materials

### 3.6.1. Ionisation

During the scan MS analysis, 70 V and 26 V cone voltages were used to maximise the response of the mono-anion ($[M-H]^-$) and di-anion ($[M-2H]^{2-}$) respectively. When using 70 V cone voltage, mono-anion ($[M-H]^-$) but no di-anion was formed. An in-source fragment was typically observed corresponding to loss of the dehydrated glucuronic acid ($[M-H-gluc]^-$, 25-100%). Other in-source fragments such as $[M-H-C_4H_6O_5]^-$ were observed for the androstane type sulfate glucuronides **23**, **26**, **27**, and both $[M-H-C_7H_{12}O_7]^-$ and $[M-H-gluc-H_2O]^-$ were observed for sulfate glucuronides **23**, **26**, **29, 30**. The estradiol 3-sulfate 17-glucuronide **25** formed an in-source fragment *m/z* 271, corresponding to ($[M-H-gluc-SO_3]^-$).

When the 26 V cone voltage was used, the formation of the di-anion ($[M-2H]^{2-}$) was favoured, with the mono-anion ($[M-H]^-$) still observed at lower relative abundance (15-55%). Similar to above, the in-source fragment $[M-2H-(gluc-H)]^-$ (corresponding to $[M-H-gluc]^-$ above) was also observed but of lower intensity (5-50%). In-source fragmentation of estradiol 3-glucuronide 17-sulfate **28** also afforded the ion derived from dehydrated glucuronic acid *m/z* 175 ($[gluc-H]^-$).

### 3.6.2. Fragmentation

Collision Induced Dissociation (CID) experiments were performed for all steroid sulfate glucuronides from the mono- and di-anion precursor ions. For the mono-anion ($[M-H]^-$), the most intense fragment at 50 eV collision energy retaining the steroid backbone was $[M-H-gluc]^-$, except for the unsaturated steroid sulfate glucuronides **29** and **30** where $[M-H-gluc-H_2O]^-$ fragment was more intense. In addition, minor $[M-H-gluc-H_2O]^-$ fragment was also observed for steroid sulfate glucuronides **26**, **27**, and **31**. Another common fragmentation was loss of *m/z* 97 corresponding to hydrogen sulfate ion ($[HSO_4]^-$). The loss of $[HSO_4]^-$ was a typically intense fragment throughout the library except for estradiol 3-sulfate 17-glucuronide **25**, where neutral loss of 80 Da corresponding to sulfur trioxide ($SO_3$) was observed instead. The neutral loss of $SO_3$ rather than loss of the ion $[HSO_4]^-$ arose as the A ring was aromatic and fragmentation of neutral $SO_3$ generates a stabilised phenolate anion. Due to this, fragments such as *m/z* 447 and *m/z* 271 corresponding to $[M-H-SO_3]^-$ and $[M-H-gluc-SO_3]^-$, respectively, were only seen for estradiol 3-sulfate 17-glucuronide **25**. The *m/z* 80 fragment corresponding to sulfur trioxide radical anion ($[^{\bullet}SO_3]^-$) was also formed from this compound.

Fragmentation of the di-anion precursor $[M-2H]^{2-}$ at 20 eV collision energy gave a greater number of fragments than the mono-anion $[M-H]^-$. Similar to the mono-anion, loss of the glucuronide derived anion ($[M-2H-(gluc-H)]^-$, corresponding to $[M-H-gluc]^-$ above) or hydrogen sulfate ($[HSO_4]^-$, except estradiol 3-sulfate 17-glucuronide **25**) were typically the two most intense fragments throughout the library. In addition, $[M-2H-(gluc-H)-H_2O]^-$ (corresponding to $[M-H-gluc-H_2O]^-$ above) was more commonly observed in the di-anion fragmentation compared to the mono-anion. Another common fragment formed in the di-anion fragmentation was derived from ion loss of *m/z* 75 ($[C_2H_3O_3]^-$), to give $[M-2H-(C_2H_3O_3)]^-$ (corresponding to $[M-H-C_2H_4O_3]^-$ above) similar to the steroid bisglucuronide di-anion fragmentation (Section 3.5.2). Once again, the ion loss fragmentation from $[M-2H]^{2-}$ to $[M-2H-(C_2H_3O_3)]^-$, $[M-2H-(gluc-H)]^-$, and $[M-2H-(gluc-H)-H_2O]^-$ lead to an increase in *m/z* ratio. Other fragments were also observed including $[M-2H-(C_3H_2O_5)]^-$ (typically for A ring glucuronides **26** and **27**) and $[M-2H-(C_3H_4O_5)]^-$ (typically for D ring glucuronides **23**, **24**, **25**). As described in the mono-anion, the di-anion derived from estradiol 3-sulfate 17-glucuronide **25** underwent neutral loss of 80 Da ($SO_3$), giving rise to fragments *m/z* 271 and *m/z* 239 that corresponded to $[M-2H-(gluc-H)-SO_3]^-$ and $[M-2H-(C_7H_{11}O_7)-SO_3]^-$. On the other hand, the aromatic glucuronide estradiol 3-glucuronide 17-sulfate **28** fragmented to give $[gluc-H]^-$ (*m/z* 175), while only glucuronide fragments (*m/z* 113, 85, 75) were usually observed in the other library members.

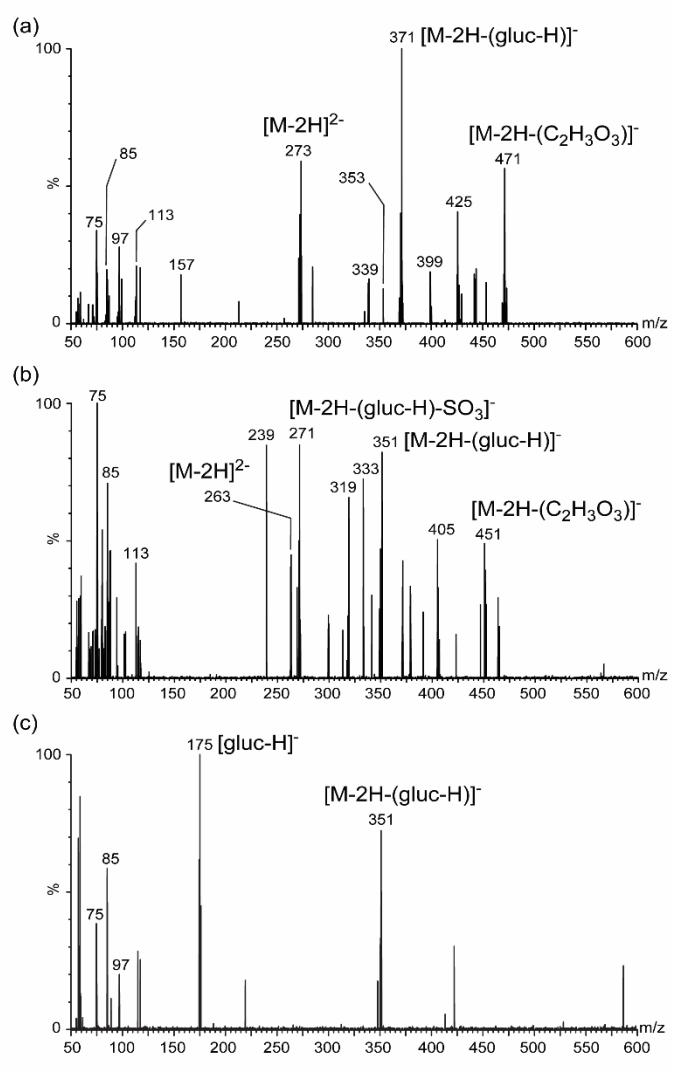



**Figure 7.** Fragmentation of di-anion precursor ions $[M-2H]^{2-}$ (26 V cone voltage, 20 eV collision energy): (a) 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide **23**, (b) estradiol 3-sulfate 17-glucuronide **25**, (c) estradiol 3-glucuronide 17-sulfate **28**.

### 3.6.3. LC-MS analysis of a male urine sample

The library of ten steroid sulfate glucuronide reference materials was developed according a range of design criteria including structural diversity and synthetic accessibility, but without specifically targeting putative metabolites. Despite this, the potential existed for library members to occur as endogenous metabolites. To explore this, a selected reaction monitoring (SRM) method for the detection of sulfate glucuronide library members as endogenous metabolites was developed. To increase analytical sensitivity, the di-anion ($[M-2H]^{2-}$) was selected as precursor as MS conditions necessary to favour the mono-anion ($[M-H]^{-}$) typically resulted in lower ion counts. Further, the SRM method was developed for each library member using as small number of diagnostic transitions only. The diagnostic transitions were not necessarily the most intense transitions observed for the library but

retained the steroid backbone in the fragment and so contained information on ion structure (summarised in Table 4 for pregn-5-ene-3β,20*R/S*-diol 3-sulfate 20-glucuronide **31** and **32**). Using this SRM method on a single male urine sample resulted in matches to urinary metabolites for both pregn-5-ene-3β,20*R*-diol 3-sulfate 20-glucuronide **31** and pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32** reference materials (Figure 8). Matches were confirmed using the World Anti-Doping Agency (WADA) MS criteria for retention time and the relative abundance of three diagnostic transitions (see SI section) [43]. This confirmed the existence of pregn-5-ene-3β,20*R/S*-diol 3-sulfate 20-glucuronide **31** and **32** as endogenous steroid sulfate glucuronide metabolites that have not previously been reported, as endogenous steroid metabolites. Although brief, this study provides the motivation for more detailed studies on steroid sulfate glucuronides as endogenous urinary metabolites. Access to a range of steroid sulfate reference materials provides avenues to develop sensitive and selective LC-MS methods for the direct and untargeted detection of this metabolite family.

**Table 4.** Diagnostic transitions (1-3) for pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32** (20*S*:20*R* = 2:1).

| Precuror ion (*m/z*) | Product ion (*m/z*) | Cone Voltage (V) | Collision Energy (eV) | Proposed product ion |
|---|---|---|---|---|
| 286.1 | 397.2 | 26 | 20 | $[M\text{-}2H\text{-}(gluc\text{-}H)]^-$ |
| 286.1 | 379.2 | 26 | 20 | $[M\text{-}2H\text{-}(gluc\text{-}H)\text{-}H_2O]^-$ |
| 286.1 | 277.1 | 26 | 10 | $[M\text{-}2H\text{-}H_2O]^{2-}$ |

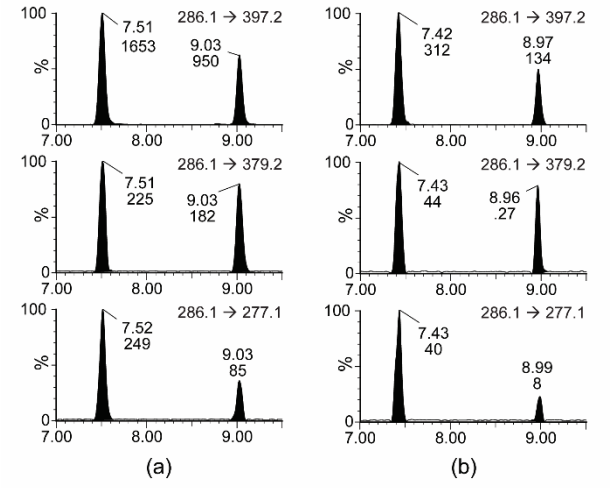



**Figure 8.** Chromatogram of SRM transitions for (a) pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32** (20*S*:20*R* = 2:1) and (b) male urine sample.

## Discussion

Steroidal bisconjugates (Figure 1) have long been known as minor components of the steroid profile. In the past, these were typically analysed using laborious chromatographic fractionation and hydrolysis [17],[18], followed by GC-MS detection [19],[20]. Recent developments in both chemical synthesis and LC-MS technology have created avenues for the direct detection of these minor metabolites. These advances are exemplified by the CIL scan method for the direct and untargeted detection of urinary steroid bis(sulfate) metabolites [21]. The CIL scan method has been employed to study the endogenous steroid bis(sulfate) profile, including during pregnancy, to identify markers associated with sports doping [21], and for the analysis of maternal urine to provide discriminating prenatal diagnosis of inborn errors of steroid biosynthesis associated with SLOS, STSD, and PORD [22]. Integral to the development of the CIL scan method was the interplay between chemical synthesis and MS method development. Synthetic access to a wide range of steroidal bis(sulfate) reference materials revealed ion loss fragmentation as a common feature of this compound class and enabled the development of a UPLC-MS CIL scan method with high selectivity and good levels of detection for the targeted analytes [21].

In this work, the synthesis of other neglected steroidal bisconjugate families, steroid bisglucuronides and steroid sulfate glucuronides, was achieved using the *E. coli* glucuronylsynthase enzyme [32],[37],[44]. The glucuronylsynthase is an engineered glycosynthase variant [45],[46] of the *E. coli* β-glucuronidase enzyme that is widely employed in chemical analysis for steroid glucuronide hydrolysis in sample preparation [7]. The glucuronylsynthase incorporates an active mutation that disables glucuronide hydrolysis, but using the enzyme in concert with the synthetically derived α-D-glucuronyl fluoride **2** substrate promotes the single-step chemical synthesis of glucuronides under mild conditions. The synthesis of 14 steroid monoglucuronides using the glucuronylsynthase was the subject of earlier research [32]. This study extends the glucuronylsynthase approach to the synthesis of ten steroid bisglucuronide, and ten steroid sulfate glucuronide, reference materials on a scale suitable for purification and characterisation by MS and NMR to confirm compound identity.

As an enzymatic method of glucuronylation, the *E. coli* glucuronylsynthase is mechanistically distinct from the UGT-promoted biosynthesis of steroid glucuronides [47],[48] but shares several key attributes, including mild and single-step conjugation. One notable feature of the glucuronylsynthase approach is the ability to adjust reaction scale using standard laboratory

methods [37]. The glucuronylsynthase method is also distinct from chemical methods of glucuronylation that employ protected and activated glucuronide donors and require multiple protection and deprotection steps and more forcing reaction conditions [49],[50]. Of the reference materials targeted by this work, estradiol 3-sulfate 17 glucuronide **25** (Figure 4) has previously been prepared by a five step chemical synthesis from estradiol [26]. Using the glucuronylsynthase approach, sulfation, reduction and glucuronylation of estrone afforded the estradiol 3-sulfate 17 glucuronide **25** in three steps. Further, selective sulfation of estradiol followed by glucuronylation afforded the regioisomeric estradiol 17-sulfate 3-glucuronide **28** in just two steps. The synthesis described herein significantly expands the range of steroid bisconjugate reference materials accessible, providing for the first time access to steroid sulfate glucuronides where the sulfate is conjugated to saturated rather than phenolic hydroxyl groups. The study delineates the scope and some of the limitations of the glucuronylsynthase promoted synthesis but clearly establishes the method as a valuable complement to biochemical or chemical synthesis approaches for the preparation of steroid bisconjugate reference materials.

The *E. coli* glucuronylsynthase also provides a general approach to prepare stable isotope labelled steroid bisconjugates through the late stage introduction of a labelled glucuronic acid unit (Figure 3). The fully labelled {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2** was prepared in four steps by established routes [37] from the relatively inexpensive and commercially available {$^{13}C_6$}-D-glucose. Using a sequential glucuronylation pathway provided for the selective labelling of steroid bisglucuronides **6** and **9** suitable for use as internal standards or mass spectrometry probes. In this work, the MS study of the mono- and di-anions showed no significant preference for fragmentation of the glucuronide units appended to the saturated A- and D-rings in 5α-androstane-3β,17β-diol 3{$^{13}C_6$},17-bisglucuronide, ammonium salt {$^{13}C_6$}-**6** (Figure 6). In contrast, fragmentation of estradiol 3,17{$^{13}C_6$}-bisglucuronide {$^{13}C_6$}-**9** revealed fragmentation preferentially occurred at the phenolic A-ring. Similar methods provided access to five stable isotope labelled steroid mono-glucuronides {$^{13}C_6$}-**4**, {$^{13}C_6$}-**17**-{$^{13}C_6$}-**20** and one steroid sulfate glucuronide {$^{13}C_6$}-**23**.

By design, this study has not targeted the preparation of particular steroid bisconjugate reference materials, instead exploring the scope and limitations of the glucuronylsynthase method and providing a diverse range of derivatives for MS method development. Despite this, preliminary investigations have employed the reference materials to study MS ionisation and fragmentation patterns, so confirming the presence of the steroid bisconjugates pregn-5-ene-3β,20*R*-diol 3-sulfate 20-glucuronide **31** and pregn-5-ene-

3β,20*S*-diol 3-sulfate 20-glucuronide **32** as endogenous human urinary metabolites by LC-MS analysis. In this respect, the current work is only at an early stage. Future studies using the steroidal bisconjugates prepared in this work will target the development of selective MS-based methods for the direct and untargeted detection of these metabolite families using modern MS instrumentation. Such studies promise to reveal in rich detail the role of steroidal bisconjugates in the steroid profile, unearthing these neglected treasures of steroidal metabolism.

## Conclusions

A library of ten steroid bisglucuronides and ten steroid sulfate glucuronides was synthesised, purified and characterised by MS and NMR methods. The synthesis of stable isotope labelled internal standards by late-stage introduction of labelled glucuronide units is also reported, and applied to study the MS fragmentation of selectively labelled steroid bisglucuronides. Access to steroidal bisconjugate reference materials promised to expand the MS methods available to detect these minor steroid metabolites in fields such as sports drug testing or medical research.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version ……

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Supplementary Material

# Synthesis of Steroid Bisglucuronides and Sulfate Glucuronides Reference Materials: Unearthing Neglected Treasures of Steroid Metabolism

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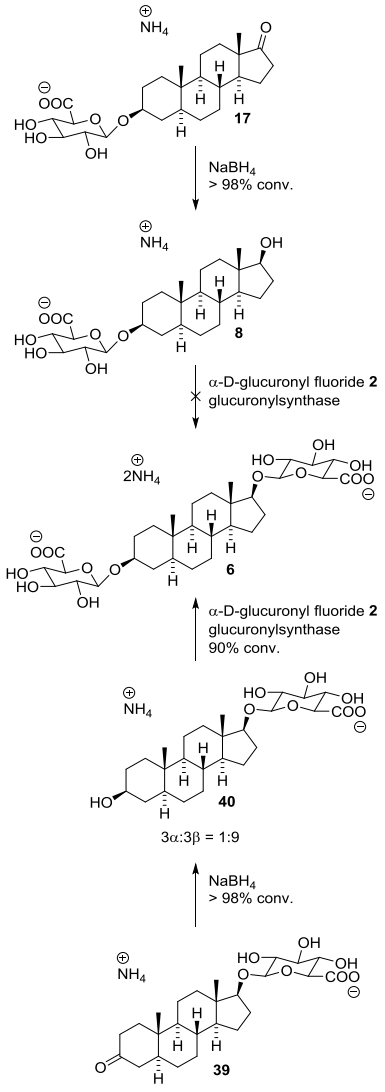
# Contents

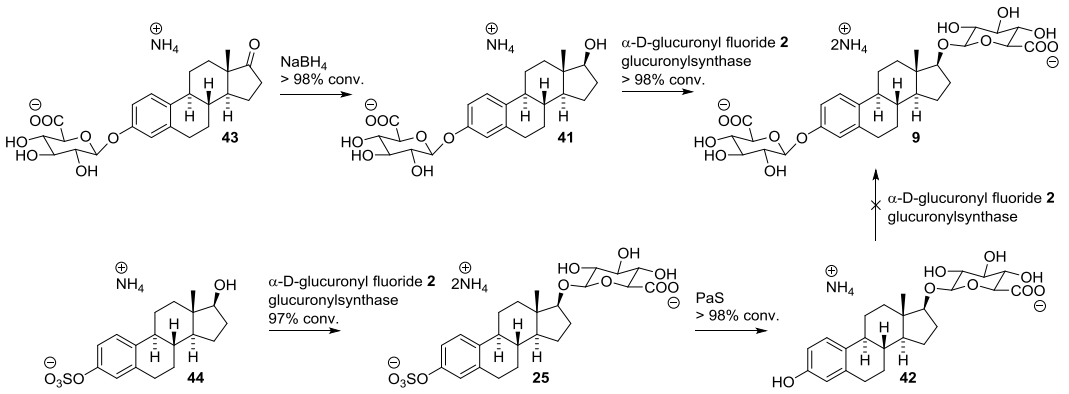
## Chapter 1: Steroid bisglucuronides stepwise examples

The first example was the formation of bisglucuronide **6**. Epiandrosterone (EA) glucuronide **17** and dihydrotestosterone (DHT) glucuronide **39** were synthesised from EA **22** and DHT, respectively, using the same glucuronylation method (see Section 2.4.5) [1]. These two compounds were then reacted with sodium borohydride [2] to form 5α-androstane-3β,17β-diol 3-glucuronide **8** and 5α-androstane-3β,17β-diol 17-glucuronide **40**, the two possible intermediates. When the ketone at 3-position was reduced, a 1:9 mixture of 3α and 3β alcohols was formed, with 3β alcohol preferred [3]. On the other hand, 17-ketone reduction only gave 17β alcohol. The second glucuronylation reaction was then performed on these steroid diol monoglucuronides **8** and **40**. Attempted reaction of the steroid diol 3-glucuronide **8** resulted in no conversion to the bisglucuronide **6** with the starting material **8** recovered unchanged. In contrast, the steroid diol 17-glucuronide **40** derived from DHT reacted to form the bisglucuronide **6** with 90% conversion overall (> 98% conversion from the 3β,17β-diol 17-glucuronide to the bisglucuronide, with the 3α,17β-diol 17-glucuronide unreacted). The unreacted 3α,17β-diol 17-glucuronide could then be removed using C18 cartridge to obtain the pure bisglucuronide **6**. In conclusion, this showed that the diol 17-glucuronide **40** reacted faster than the diol 3-glucuronide **8** to form the bisglucuronide **6**, thus no diol 17-glucuronide **40** was observed at the end of the one-step glucuronylation reaction.



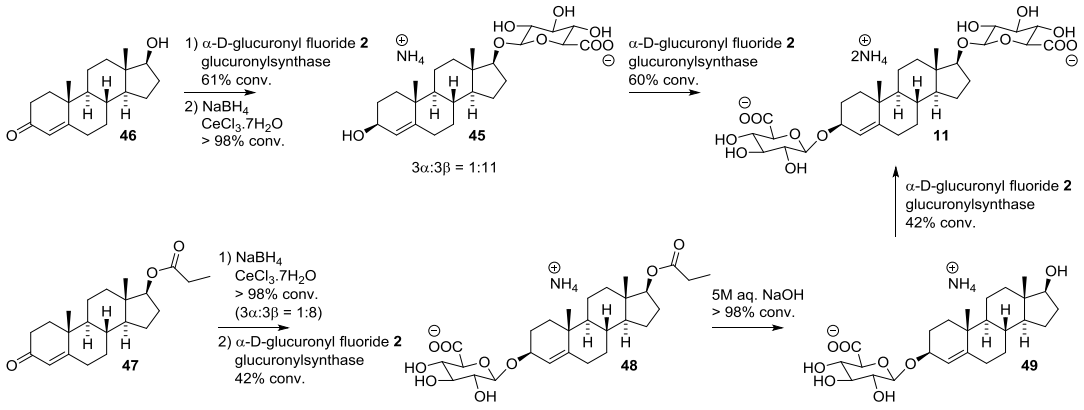

**Scheme S1.** Stepwise synthesis of 5α-androstane-3β,17β-diol bisglucuronide **6**

The second example was the formation of estradiol bisglucuronide **9**. The possible intermediates in this case were estradiol 3-glucuronide **41** and estradiol 17-glucuronide **42**. Estradiol 3-glucuronide **41** was synthesised by reducing estrone 3-glucuronide **43** using sodium borohydride. On the other hand, estradiol 17-glucuronide **42** was synthesised by a longer synthetic route. This started from estradiol 3-sulfate **44** that was synthesised in two steps from estrone [2]. This was then glucuronylated to produce estradiol 3-sulfate 17-glucuronide **25**, and the sulfate group selectively cleaved by the *Pseudomonas aeruginosa* arylsulfatase (PaS) enzyme to afford estradiol 17-glucuronide **42** [4]. Now that two intermediates **41** and **42** had been obtained, the second glucuronylation reaction was performed. In contrast with previous example, bisglucuronide **9** could only be formed from estradiol 3-glucuronide **41** with > 98% conversion, eliminating the need of C18 purification. Performing the second glucuronylation on estradiol 17-glucuronide **42** gave no conversion.




**Scheme S2.** Stepwise synthesis of estradiol bisglucuronide **9**

The third example of stepwise glucuronylation was the bisglucuronide **11**. The androst-4-ene-3β,17β-diol 17-glucuronide **45** derivative was obtained from testosterone **46** which was glucuronylated, followed by reduction at the 3-ketone under Luche conditions to afford androst-4-ene-3β,17β-diol 17-glucuronide **45** [5]. This afforded a 1:11 mixture of 3α and 3β alcohols. The longer route for the other intermediate started from a Luche reduction of testosterone propionate **47**, which again gave diastereomeric mixture (1:8 ratio of 3α and 3β alcohols). Glucuronylation at the 3-position gave androst-4-ene-3β,17β-diol 3-glucuronide 17-propionate **48** as a single diastereomer as the 3α hydroxyl group did not react and was subsequently separated by WAX SPE. The propionate ester protecting group was then hydrolysed using sodium hydroxide to afford the steroid diol 3-glucuronide **49**. In the final glucuronylation, both steroid diol 3-glucuronide **49** and 17-glucuronide **45** derivatives reacted further to form the bisglucuronide **11**, with 42% and 60% conversion respectively. Again, the 3α,17β-diol 17-glucuronide did not react. In both cases, purification of the bisglucuronide **11** was afforded by C18 SPE.




**Scheme S3.** Stepwise synthesis of androst-4-ene-3β,17β-diol bisglucuronide **11**

## Chapter 2: NMR analysis of steroid bisglucuronides and sulfate glucuronides

**Table S1.** $^{1}$H NMR diagnostic peaks of steroid bisglucuronides and sulfate glucuronides

| Compound | C3-H or aromatic | C17-, C20-, or C24-H | Glucuronide anomeric |
|---|---|---|---|
| 5α-Androstane-3β,17α-diol bisglucuronide **5** | 3.77 (m, C3-H)[a] | 3.93 (d, *J* 5.5 Hz, C17-H)[a] | 4.41 (d, *J* 7.8 Hz, C20-H)[a], 4.24 (d, *J* 7.8 Hz, C26-H)[a] |
| 5α-Androstane-3β,17β-diol bisglucuronide **6** | 3.80-3.72 (m, C3-H and C17-H)[a] | 3.80-3.72 (m, C3-H and C17-H)[a] | 4.41 (d, *J* 7.7 Hz, C20-H)[a], 4.35 (d, *J* 7.8 Hz, C26-H)[a] |
| Estradiol bisglucuronide **9** | 7.18 (d, *J* 8.6 Hz, C1-H), 6.87 (dd, *J* 8.6, 2.6 Hz, C2-H), 6.81 (d, *J* 2.5 Hz, C4-H)[a] | 3.89 (t, *J* 8.6 Hz, C17-H)[a] | 4.40 (d, *J* 7.8 Hz, C25-H)[a], C19-H obscured[b] |
| Androst-5-ene-3β,17β-diol bisglucuronide **10** | 3.65 (m, C3-H) | 3.81 (t, *J* 8.5 Hz, C17-H)[c] | 4.40 (d, *J* 7.8 Hz, C20-H), 4.36 (d, *J* 7.8 Hz, C26-H)[c] |
| Androst-4-ene-3β,17β-diol bisglucuronide **11** | 4.26 (m, C3-H)[a,d] | 3.77 (t, *J* 8.6 Hz, C17-H)[a] | 4.43 (d, *J* 7.8 Hz, C20-H)[a,d], 4.35 (d, *J* 7.8 Hz, C26-H)[a] |
| 19-Norandrost-4-ene-3β,17β-diol bisglucuronide **12** | 4.28 (m, C3-H)[e] | 3.80 (t, *J* 8.6 Hz, C17-H)[e] | 4.43 (d, *J* 7.8 Hz, C19-H)[e], 4.35 (d, *J* 7.9 Hz, C25-H)[e] |
| 5β-Cholane-3α,24-diol bisglucuronide **13** | 3.82 (m, C3-H)[f] | 3.96 (m, C24-$H_A$), 3.49-3.36 (m, C24-$H_B$, C27-H, C28-H, C33-H, C34-H)[g] | 4.41 (d, *J* 7.7 Hz, C25-H)[f], 4.25 (d, *J* 7.7 Hz, C31-H) |
| 5α-Pregnane-3β,20*S*-diol bisglucuronide **14** | 3.78 (m, C3-H)[h] | 3.64 (m, C20-H) | 4.40 (d, *J* 7.8 Hz, C22-H)[h], 4.35 (d, *J* 7.8 Hz, C28-H) |
| Pregn-5-ene-3β,20*S*/*R*-diol bisglucuronide **15** or **16** | 3.63 (m, C3-H)[i] | 3.98 (m, C20-H)[j] | 4.40 (d, *J* 7.8 Hz, C22-H)[i], 4.36 (d, *J* 7.7 Hz, C28-H)[j] |

| | | | |
|---|---|---|---|
| 5α-Androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** | 4.24 (tt, *J* 11.3, 4.7 Hz, C3-H)[a] | 3.79 (t, *J* 8.6 Hz, C17-H)[a] | 4.35 (d, *J* 7.8 Hz, C20-H)[a] |
| Androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24** | 4.14 (tt, *J* 11.0, 4.5 Hz, C3-H)[a] | 3.78 (t, *J* 8.5 Hz, C17-H)[a] | 4.37 (d, *J* 7.8 Hz, C20-H)[a] |
| Estradiol 3-sulfate 17-glucuronide **25** | 7.23 (d, *J* 8.5 Hz, C1-H), 7.03 (dd, *J* 8.5, 2.6 Hz, C2-H), 7.00 (d, *J* 2.5 Hz, C4-H)[a] | 3.88 (t, *J* 8.6 Hz, C17-H)[a] | 4.41 (d, *J* 7.8 Hz, C19-H)[a] |
| 5α-Androstane-3β,17β-diol 3-glucuronide 17-sulfate **26** | 3.77 (tt, *J* 10.8, 5.1 Hz, C3-H)[a] | 4.21 (t, *J* 8.0 Hz, C17-H)[a] | 4.41 (d, *J* 7.7 Hz, C20-H)[a] |
| 5α-Androstane-3β,17α-diol 3-glucuronide 17-sulfate **27** | 3.76 (tt, *J* 9.3, 5.2 Hz, C3-H)[a] | 4.31 (d, *J* 5.7 Hz, C17-H)[a] | 4.42 (d, *J* 7.8 Hz, C20-H)[a] |
| Estradiol 3-glucuronide 17-sulfate **28** | 7.19 (d, *J* 8.6 Hz, C1-H), 6.87 (dd, *J* 8.5, 2.7 Hz, C2-H), 6.81 (d, *J* 2.6 Hz, C4-H)[a] | 4.31 (t, *J* 8.6 Hz, C17-H)[a] | 4.85 (C19-H)[k] |
| Androst-4-ene-3β,17β-diol 3-glucuronide 17-sulfate **29** | 4.27 (m, C3-H)[a] | 4.21 (t, *J* 8.5 Hz, C17-H)[a] | 4.43 (d, *J* 7.8 Hz, C20-H)[a] |
| Androst-4-ene-3β,17α-diol 3-glucuronide 17-sulfate **30** | 4.27 (m, C3-H)[a] | 4.32 (d, *J* 5.7 Hz, C17-H)[a] | 4.44 (d, *J* 7.8 Hz, C20-H)[a] |
| Pregn-5-ene-3β,20*R*/*S*-diol 3-sulfate 20-glucuronide **31** or **32** | 4.13 (tt, *J* 11.0, 4.8 Hz, C3-H)[a] | 3.99 (m, C20-H)[a] | 4.36 (d, *J* 7.7 Hz, C22-H)[a] |

[a] Assigned by stepwise synthesis, [b] Assigned by corresponding sulfate glucuronide **28**, [c] Assigned by corresponding sulfate glucuronide **24**, [d] Assigned by corresponding sulfate glucuronide **29**, [e] Assigned by similar bisglucuronide **11**, [f] Assigned by similar glucuronide (etiocholanolone glucuronide) [1], [g] Assigned by similar glucuronide (butyl glucuronide) [6], [h] Assigned by similar bisglucuronide **5**, [i] Assigned by similar bisglucuronide **10**, [j] Assigned by corresponding sulfate glucuronide **31**/**32**, [k] Assigned by COSY cross peak analysis.

## Chapter 3: MS analysis of steroid bisglucuronides and sulfate glucuronides

**Table S2.** Fragmentation of bisglucuronides mono-anion precursor ions $[M-H]^-$ (70 V cone voltage, 50 eV collision energy)

| Compound | $[M-H]^-$ | $[M-H-C_2H_4O_3]^-$ | $[M-H-gluc]^-$ | $[M-H-gluc-H_2O]^-$ | $[M-H-2gluc]^-$ | $[gluc-H]^-$ | $[gluc-H-H_2O]^-$ | $[gluc-H-H_2O-CO]^-$ | $[gluc-H-H_2O-CO_2]^-$ | $[gluc-H-H_2O-CO_2-CO]^-$ | $[C_2H_3O_3]^-$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 5α-Androstane-3β,17α-diol bisglucuronide **5** | 643 (70) | 567 (20) | 467 (20) | 449 (2) | - | 175 (10) | 157 (10) | 129 (10) | 113 (60) | 85 (80) | 75 (100) |
| 5α-Androstane-3β,17β-diol bisglucuronide **6** | 643 (85) | 567 (5) | 467 (25) | 449 (1) | - | 175 (5) | 157 (15) | 129 (10) | 113 (70) | 85 (100) | 75 (65) |
| Estradiol bisglucuronide **9** | 623 (20) | - | 447 (35) | 429 (5) | 271 (30) | 175 (20) | 157 (10) | 129 (15) | 113 (100) | 85 (75) | 75 (40) |
| Androst-5-ene-3β,17β-diol bisglucuronide **10** | 641 (55) | 565 (10) | 465 (20) | - | - | 175 (10) | 157 (30) | 129 (20) | 113 (60) | 85 (100) | 75 (45) |
| Androst-4-ene-3β,17β-diol bisglucuronide **11** | 641 (65) | 565 (10) | 465 (30) | 447 (20) | - | 175 (10) | 157 (5) | 129 (15) | 113 (65) | 85 (100) | 75 (55) |
| 19-Norandrost-4-ene-3β,17β-diol bisglucuronide **12** | 627 (45) | 551 (10) | 451 (15) | 433 (15) | - | 175 (5) | 157 (20) | 129 (5) | 113 (50) | 85 (100) | 75 (70) |
| 5β-Cholane-3α,24-diol bisglucuronide **13** | 713 (65) | - | 537 (100) | 519 (2) | - | 175 (10) | 157 (1) | 129 (2) | 113 (35) | 85 (30) | 75 (25) |
| 5α-Pregnane-3β,20*S*-diol bisglucuronide **14** | 671 (80) | 595 (15) | 495 (30) | - | - | 175 (10) | 157 (10) | 129 (10) | 113 (70) | 85 (100) | 75 (75) |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pregn-5-ene-3β,20*S*-diol bisglucuronide **15** | 669 (65) | 593 (5) | 493 (25) | 475 (5) | - | 175 (10) | 157 (10) | 129 (10) | 113 (65) | 85 (100) | 75 (60) |
| Pregn-5-ene-3β,20*R*-diol bisglucuronide **16** | 669 (100) | 593 (10) | 493 (20) | 475 (5) | - | 175 (5) | 157 (20) | 129 (5) | 113 (55) | 85 (90) | 75 (65) |

**Table S3.** Fragmentation of bisglucuronides di-anion precursor ions $[M-2H]^{2-}$ (26 V cone voltage, 20 eV collision energy)

| Compound | $[M-H-C_2H_4O_3]^-$ | $[M-H-gluc]^-$ | $[M-H-gluc-H_2O]^-$ | $[M-2H]^{2-}$ | $[M-H-2gluc]^-$ | $[gluc-H]^-$ | $[gluc-H-H_2O]^-$ | $[gluc-H-H_2O-CO]^-$ | $[gluc-H-H_2O-CO_2]^-$ | $[gluc-H-H_2O-CO_2-CO]^-$ | $[C_2H_3O_3]^-$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 5α-Androstane-3β,17α-diol bisglucuronide **5** | 567 (20) | 467 (30) | - | 321 (100) | - | - | 157 (10) | 129 (10) | 113 (10) | 85 (25) | 75 (40) |
| 5α-Androstane-3β,17β-diol bisglucuronide **6** | 567 (15) | 467 (25) | 449 (2) | 321 (100) | - | - | - | 129 (5) | 113 (20) | 85 (10) | 75 (20) |
| Estradiol bisglucuronide **9** | - | 447 (55) | - | 311 (70) | 271 (25) | 175 (20) | 157 (5) | 129 (20) | 113 (75) | 85 (100) | 75 (40) |
| Androst-5-ene-3β,17β-diol bisglucuronide **10** | 565 (10) | 465 (10) | - | 320 (100) | - | 175 (2) | 157 (10) | 129 (10) | 113 (10) | 85 (20) | 75 (40) |
| Androst-4-ene-3β,17β-diol bisglucuronide **11** | 565 (10) | 465 (50) | 447 (30) | 320 (100) | - | 175 (5) | 157 (2) | 129 (10) | 113 (10) | 85 (20) | 75 (40) |
| 19-Norandrost-4-ene-3β,17β-diol bisglucuronide **12** | 551 (5) | 451 (30) | 433 (25) | 313 (100) | - | - | 157 (10) | 129 (15) | 113 (10) | 85 (30) | 75 (40) |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 5β-Cholane-3α,24-diol bisglucuronide **13** | 637 (5) | 537 (15) | - | 356 (100) | - | - | - | 129 (5) | 113 (3) | 85 (10) | 75 (15) |
| 5α-Pregnane-3β,20*S*-diol bisglucuronide **14** | 595 (10) | 495 (20) | - | 335 (100) | - | 175 (1) | 157 (10) | 129 (5) | 113 (10) | 85 (20) | 75 (40) |
| Pregn-5-ene-3β,20*S*-diol bisglucuronide **15** | 593 (15) | 493 (20) | 475 (10) | 334 (100) | - | 175 (2) | 157 (10) | 129 (5) | 113 (10) | 85 (40) | 75 (20) |
| Pregn-5-ene-3β,20*R*-diol bisglucuronide **16** | 593 (30) | 493 (40) | 475 (10) | 334 (100) | - | - | 157 (1) | 129 (15) | 113 (30) | 85 (10) | 75 (45) |

**Table S4.** Fragmentation of sulfate glucuronides mono-anion precursor ions $[M-H]^-$ (70 V cone voltage, 50 eV collision energy)

| Compound | $[M-H]^-$ | $[M-H-SO_3]^-$ | $[M-H-gluc]^-$ | $[M-H-gluc-H_2O]^-$ | $[M-H-gluc-SO_3]^-$ | $[gluc-H-H_2O-CO_2]^-$ | $[HSO_4]^-$ | $[^{\bullet}SO3]^-$ | $[gluc-H-H_2O-CO_2-CO]^-$ | $[C_2H_3O_3]^-$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 5α-Androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** | 547 (50) | - | 371 (100) | - | - | - | 97 (80) | - | 85 (5) | - |
| Androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24** | 545 (20) | - | 369 (40) | - | - | 113 (20) | 97 (100) | - | 85 (10) | 75 (15) |
| Estradiol 3-sulfate 17-glucuronide **25** | 527 (30) | 447 (40) | 351 (100) | - | 271 (85) | 113 (35) | - | 80 (85) | 85 (15) | - |
| 5α-Androstane-3β,17β-diol 3-glucuronide 17-sulfate **26** | 547 (25) | - | 371 (100) | 353 (10) | - | - | 97 (50) | - | - | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 5α-Androstane-3β,17α-diol 3-glucuronide 17-sulfate **27** | 547 (30) | - | 371 (100) | 353 (5) | - | - | 97 (75) | - | 85 (20) | 75 (15) |
| Estradiol 3-glucuronide 17-sulfate **28** | 527 (30) | - | 351 (100) | - | - | - | 97 (75) | - | - | - |
| Androst-4-ene-3β,17β-diol 3-glucuronide 17-sulfate **29** | 545 (50) | - | 369 (50) | 351 (100) | - | - | 97 (20) | - | - | - |
| Androst-4-ene-3β,17α-diol 3-glucuronide 17-sulfate **30** | 545 (15) | - | 369 (20) | 351 (45) | - | - | 97 (100) | - | 85 (30) | 75 (10) |
| Pregn-5-ene-3β,20*R*-diol 3-sulfate 20-glucuronide **31** | 573 (25) | - | 397 (50) | 379 (5) | - | 113 (10) | 97 (100) | - | 85 (40) | 75 (25) |
| Pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32** | 573 (20) | - | 397 (15) | - | - | 113 (10) | 97 (100) | - | 85 (30) | 75 (15) |

**Table S5**. Fragmentation of sulfate glucuronides di-anion precursor ions $[M-2H]^{2-}$ (26 V cone voltage, 20 eV collision energy)

| Compound | $[M-H-C_2H_4O_3]^-$ | $M-H-C_3H_3O_5]^-$ | $[M-H-C_3H_5O_5]^-$ | $[M-H-C_5H_7O_5]^-$ | [M-H-gluc]$^-$ | [M-H-gluc-$H_2O$]$^-$ | $[M-H-C_7H_{12}O_7]^-$ | $[M-2H]^{2-}$ | [gluc-H-$H_2O$-$CO_2$]$^-$ | $[HSO_4]^-$ | [gluc-H-$H_2O$-$CO_2$-CO]$^-$ | $[C_2H_3O_3]^-$ | Others |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5α-Androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** | 471 (60) | - | 425 (40) | 399 (20) | 371 (100) | 353 (15) | 339 (20) | 273 (60) | 113 (20) | 97 (30) | 85 (20) | 75 (30) | 157 (20) [gluc-H-$H_2O$]$^-$ |
| Androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24** | 469 (40) | - | 423 (25) | 397 (10) | 369 (80) | - | 337 (35) | 272 (40) | 113 (15) | 97 (100) | 85 (65) | 75 (85) | 129 (40) [gluc-H-$H_2O$-CO]$^-$ |
| Estradiol 3-sulfate 17-glucuronide **25** | 451 (50) | - | 405 (50) | - | 351 (85) | 333 (75) | 319 (70) | 263 (50) | 113 (40) | - | 85 (70) | 75 (100) | 271 (85) [M-H-gluc-$SO_3$]$^-$ |
| | | | | | | | | | | | | | 239 (85) [M-H-$C_7H_{12}O_7$-$SO_3$]$^-$ |
| 5α-Androstane-3β,17β-diol 3-glucuronide 17-sulfate **26** | 471 (50) | 427 (30) | - | 399 (45) | 371 (70) | 353 (100) | 339 (20) | 273 (70) | 113 (40) | 97 (45) | 85 (70) | 75 (60) | 264 (15) [M-2H-$H_2O$]$^{2-}$ |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5α-Androstane-3β,17α-diol 3-glucuronide 17-sulfate **27** | 471 (70) | 427 (40) | - | - | 371 (60) | 353 (40) | - | 273 (90) | 113 (85) | 97 (60) | 85 (90) | 75 (100) | - |
| Estradiol 3-glucuronide 17-sulfate **28**[a] | - | - | - | - | 351 (70) | - | - | - | - | 97 (20) | 85 (60) | 75 (40) | 175 (100) $[gluc\text{-}H]^-$ |
| Androst-4-ene-3β,17β-diol 3-glucuronide 17-sulfate **29** | 469 (25) | - | 423 (10) | 397 (20) | 369 (90) | 351 (50) | 337 (35) | 272 (25) | 113 (25) | 97 (10) | 85 (20) | 75 (100) | - |
| Androst-4-ene-3β,17α-diol 3-glucuronide 17-sulfate **30** | - | - | 423 (20) | - | 369 (100) | 351 (55) | 337 (25) | 272 (40) | - | 97 (35) | 85 (25) | 75 (60) | - |
| Pregn-5-ene-3β,20*R*-diol 3-sulfate 20-glucuronide **31** | 497 (15) | - | 451 (20) | 425 (10) | 397 (50) | 379 (15) | - | 286 (40) | 113 (20) | 97 (95) | 85 (65) | 75 (100) | - |
| Pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32** | 497 (10) | - | 451 (30) | 425 (10) | 397 (60) | 379 (15) | - | 286 (100) | 113 (10) | 97 (65) | 85 (40) | 75 (55) | 277 (20) $[M\text{-}2H\text{-}H_2O]^{2-}$ |

[a] Di-anion precursor ion $[M\text{-}2H]^{2-}$ was not observed.

## Chapter 4: Confirmation of male urine sample

**Table S6.** SRM confirmation of pregn-5-ene-3β,20*R*-diol 3-sulfate 20-glucuronide **31** and pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32** according to WADA criteria [7]

| Analyte | Retention time (min) (maximum tolerance) | | Ion transition | Relative abundance (%) (maximum tolerance) | | Cone voltage (V) | Collision energy (eV) | Proposed product ion |
|---|---|---|---|---|---|---|---|---|
| | Standard | Sample | | Sample | Standard | | | |
| Pregn-5-ene-3β,20*R*-diol 3-sulfate 20-glucuronide | 7.51 | 7.43 (7.41-7.61) | 286.1 → 397.2 | 100 | 100 (90-100) | 26 | 20 | $[M-2H-(gluc-H)]^-$ |
| | | | 286.1 → 379.2 | 14 | 14 (9-19) | 26 | 20 | $[M-2H-(gluc-H)-H_2O]^-$ |
| | | | 286.1 → 277.1 | 15 | 13 (10-20) | 26 | 10 | $[M-2H-H_2O]^{2-}$ |
| Pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide | 9.03 | 8.97 (8.93-9.13) | 286.1 → 397.2 | 100 | 100 (90-100) | 26 | 20 | $[M-2H-(gluc-H)]^-$ |
| | | | 286.1 → 379.2 | 19 | 20 (14-24) | 26 | 20 | $[M-2H-(gluc-H)-H_2O]^-$ |
| | | | 286.1 → 277.1 | 9 | 6 (4-14) | 26 | 10 | $[M-2H-H_2O]^{2-}$ |

# Chapter 5: Experimental

## Materials

Chemicals and solvents including sulfur trioxide-pyridine complex ($SO_3$·py), sodium borohydride ($NaBH_4$), cerium(III) chloride heptahydrate ($CeCl_3.7H_2O$), *para*-toluenesulfonyl hydrazide, hydrogen fluoride-pyridine (~70% hydrogen fluoride, ~30% pyridine), bis(acetoxy)iodobenzene (BAIB), 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), dihydrotestosterone (DHT, 17β-hydroxy-5α-androstan-3-one), estrone (3-hydroxyestra-1,3,5(10)-trien-17-one), estradiol (estra-1,3,5(10)-triene-3,17β-diol), testosterone propionate **47** (17β-hydroxyandrost-4-en-3-one 17-propionate), and pregnenolone (3β-hydroxypregn-5-en-20-one) were purchased from Sigma–Aldrich (Castle Hill, Australia). Epiandrosterone **22** (EA, 3β-hydroxy-5α-androstan-17-one), etiocholanolone (3α-hydroxy-5β-androstan-17-one), testosterone **46** (17β-hydroxyandrost-4-en-3-one), nandrolone (17β-hydroxyestr-4-en-3-one), androst-5-ene-3β,17β-diol, 5β-cholane-3α,24-diol, and 5α-pregnane-3β,20*S*-diol were purchased from Steraloids (Newport RI, USA). Dehydroepiandrosterone **1** (DHEA, 3β-hydroxyandrost-5-en-17-one) was obtained from BDH (Poole, UK). $^{13}$C-Labelled glucose (99 atom %) was purchased from Omicron Biochemicals Incorporated (South Bend, IN, USA). $^{18}$O-Enriched water ($D_2{}^{18}O$: 99.5+ atom % $^{18}$O and 99.9+ atom % D) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Epitestosterone (epiT, 17α-hydroxyandrost-4-en-3-one) and epidihydrotestosterone **34** (epiDHT, 17α-hydroxy-5α-androstan-3-one) were synthesised from testosterone **46** and DHT respectively according to literature methods [8]. Nandrolone sulfate and etiocholanolone sulfate were synthesised from nandrolone and etiocholanolone, respectively, according to literature methods [2]. DHEA 3-glucuronide **4** was synthesised from DHEA **1** according to literature methods [1]. The *Escherichia coli* (*E. coli*) E504G glucuronylsynthase mutant (typically 5.4-16.5 mg $mL^{-1}$) and α-D-glucuronyl fluoride **2** were prepared according to literature methods [9]. The *Pseudomonas aeruginosa* arylsulfatase (PaS) wild type enzyme (60 mg $mL^{-1}$) was prepared according to literature methods [4]. Acetic anhydride was freshly distilled and sodium methoxide was freshly prepared using literature methods [10]. MilliQ water was used in all aqueous solutions and in the liquid chromatography mobile phase. Liquid chromatography (gradient) grade methanol was obtained from Merck (Kilsyth, Australia). *N*,*N*-Dimethylformamide (DMF), sodium acetate, and aqueous ammonia solution were obtained from Chem-Supply (Gillman, Australia). Formic acid was obtained from Ajax Chemicals (Auburn, Australia). Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis weak anion exchange

(WAX) 6cc cartridges (PN 186004647), Oasis WAX 3 cc cartridges (PN 186002492), or Sep-Pak Vac C18 3cc cartridges (PN 186004619).

## Instruments

The $^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were recorded using either a Bruker Avance 400 MHz, 600 MHz, 700 MHz, or 800 MHz spectrometer at 298 K using deuterated methanol ($CD_3OD$) or other deuterated solvent specified. Data is reported in parts per million (ppm), referenced to residual protons or $^{13}C$ in the deuterated solvent specified (for $CD_3OD$: $^{1}H$ 3.31 ppm, $^{13}C$ 49.00 ppm), with multiplicity assigned as follows: br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, or combinations of the above. Coupling constants *J* are reported in Hertz (Hz). Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) for compound characterisation were performed using negative or positive electrospray ionisation (-ESI or +ESI) on a Micromass ZMD ESI-Quad or a Waters LCT Premier XE mass spectrometer. Infrared spectra were recorded on a Perkin-Elmer 1800 Series FTIR spectrometer. Melting points were measured on an SRS Opti-melt MPA 100 automated melting point system and are uncorrected. Optical rotations were recorded in $CHCl_3$ or $H_2O$ using a Rudolph Research Analytical Autopol I Automatic Polarimeter (sodium D line, 298 K). Reactions were monitored by analytical thin layer chromatography (TLC) using Merck (Bayswater, Australia) Silica gel 60 TLC plates with 7:2:1 ethyl acetate:methanol:water eluant, unless otherwise specified and were visualised by staining with a solution of concentrated sulfuric acid:methanol (5% v/v), with heating as required. Anion exchange column chromatography was performed using Dowex 1x8, 200-400, Mesh Cl resin.

## General procedures

### GP1. General procedure for the small scale reduction reaction of a steroid sulfate or steroid glucuronide containing an α,β-unsaturated ketone, with purification by SPE

The procedure followed the literature with minor modifications [2]. A solution of steroid sulfate or glucuronide (10-19 µmol) in methanol (100 µL) was treated with cerium(III) chloride heptahydride (5.0 equiv.), and then a portionwise addition of solid $NaBH_4$ over 1 minute (5.0 equiv.) with cooling on ice. After the vigorous reaction had subsided, the reaction was capped, allowed to warm to room temperature and stirred for 15 minutes. The reaction was quenched by the slow addition of water (4 mL), and then purified by SPE as per Section 2.4.2 to afford the desired steroid diol monosulfate or monoglucuronide as the corresponding ammonium salt. A $^{1}H$ NMR spectrum was obtained and integration of a suitable signal

(typically C19-$H_3$) of both the steroidal ketone and alcohol provided a ratio of the two compounds which was used to determine the percent conversion of the reduction reaction.

**GP2. General procedure for the small scale sulfation reaction of a steroid with purification by SPE**

The procedure followed the literature with minor modifications [2]. A solution of $SO_3$·py (50.0 mg, 314 µmol) in DMF (500 µL) was added to a solution of steroid (5.0 mg) in 1,4-dioxane (500 µL) and the resulting solution was then stirred in a capped vial at room temperature for 16 h. The reaction was then quenched with water (7.5 mL) and subjected to purification by SPE as per Section 2.4.2 then Section 2.4.1 to afford the desired steroid sulfate as the corresponding ammonium salt.

## Reduction reaction

### Reduction of steroid

5α-Androstane-3β,17β-diol **7** [11]

The reaction was conducted with epiandrosterone **22** (EA, 5.0 mg, 17 µmol) as per the general procedure 2.4.4 to yield the title compound **7** as a colourless solid with > 98% conversion. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 3.55 (1H, t, *J* 8.6 Hz, C17-H), 3.50 (1H, m, C3-H), 2.01-0.62 (22H, m), 0.85 (3H, s, C18-$H_3$), 0.72 (3H, s, C19-$H_3$). The $R_f$, $^1$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [11].

Androst-4-ene-3β,17β-diol **50** [12],[13]

The reaction was conducted with testosterone **46** (5.0 mg, 17 µmol) as per the general procedure GP1 to yield the title compound **50** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C3-H protons showed 1:9 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.25 (1H, s, C4-H), 4.06 (1H, m, C3-H), 3.55 (1H, t, *J* 8.7 Hz, C17-H), 2.23 (1H, m), 2.04-1.94 (2H, m), 1.90-1.81 (2H, m), 1.79-1.71 (2H, m), 1.63-1.22 (8H, m), 1.09 (3H, s, C19-$H_3$), 1.04-0.70 (4H, m), 0.76 (3H, s, C18-$H_3$). The $^1$H NMR and $^{13}$C NMR matched the literature [12],[13].

19-Norandrost-4-ene-3β,17β-diol **51** [14]

The reaction was conducted with nandrolone (5.0 mg, 18 µmol) as per the general procedure GP1 to yield the title compound **51** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C4-H protons showed 1:6 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$R_f$** 0.68; **$^1$H NMR** (400 MHz,

$CD_3OD$): δ 5.35 (1H, s, C4-H), 4.07 (1H, m, C3-H), 3.57 (1H, t, *J* 8.6 Hz, C17-H), 2.26-2.21 (1H, m), 2.09-1.93 (4H, m), 1.85-1.70 (4H, m), 1.59 (1H, m), 1.47 (1H, m), 1.33-0.80 (8H, m), 0.77 (3H, s, C18-$H_3$), 0.59 (1H, m); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 143.4 (C5), 125.7 (C4), 82.5 (C17), 68.1 (C3), 52.0, 51.3, 44.2, 43.2, 42.3, 37.9, 36.1, 32.9, 32.7, 30.6, 27.3, 26.9, 24.2, 11.6 (C18); **LRMS (+ESI)**: *m/z* 299 (100%, $[C_{18}H_{28}O_2Na]^+$); **HRMS (+ESI)**: calcd. for $[C_{18}H_{28}O_2Na]^+$ 299.1987, found 299.1986.

Pregn-5-ene-3β,20*R*-diol (20*S*:20*R* = 1:6) **52** [15]

The reaction was conducted with pregnenolone (5.0 mg, 16 µmol) as per the general procedure 2.4.4 to yield the title compound **52** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C18-$H_3$ protons showed 1:6 ratio of the 20*S*:20*R* diastereomers. Data is reported for the major diastereomer where relevant. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.34 (1H, d, *J* 5.2 Hz, C6-H), 3.64 (1H, m, C20-H), 3.40 (1H, m, C3-H), 2.27-2.14 (3H, m), 2.00-1.77 (3H, m), 1.71-1.44 (7H, m), 1.33 (1H, m), 1.24-0.92 (6H, m), 1.11 (3H, d, *J* 6.2 Hz, C21-$H_3$), 1.03 (3H, s, C19-$H_3$), 0.77 (3H, s, C18-$H_3$). The $^1$H NMR and $^{13}$C NMR matched the literature [15].

Pregn-5-ene-3β,20*S*-diol (20*S*:20*R* = 2:1) **53**

The reaction was conducted according to literature method with minor modifications [16]. Pregnenolone tosylhydrazone **54** (derived from pregnenolone, 5.0 mg, 16 µmol) was dissolved in 10:1 2-propanol:water (480 µL). Sodium borohydride (4.2 mg, 0.11 mmol, 6.9 equiv.) was added to the solution, and the reaction mixture was stirred for 16 h at room temperature. The solution was then quenched with water (5 mL) and subjected to purification by WAX SPE as per Section 2.4.2 to yield the title compound as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C18-$H_3$ protons showed a 2:1 ratio of the 20*S*:20*R* diastereomers. **20*S***: δ 1.21 (3H, d, *J* 6.2 Hz, C21-$H_3$), 0.70 (3H, s, C18-$H_3$), **20*R***: δ 1.11 (3H, d, *J* 6.1 Hz, C21-$H_3$), 0.78 (3H, s, C18-$H_3$); **LRMS (+ESI)**: *m/z* 341 (100%, $[C_{21}H_{34}O_2Na]^+$).

Androst-4-ene-3β,17β-diol 17-propionate **55**

The reaction was conducted with testosterone propionate **47** (5.0 mg, 15 µmol) as per the general procedure GP1 to yield the title compound **55** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C3-H protons showed 1:8 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$R_f$** 0.44; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.27 (1H, s, C4-H), 4.59 (1H, t, *J* 8.4 Hz, C17-H), 4.06 (1H, m, C3-H), 2.32 (2H, q, *J* 7.7 Hz, C21-H), 2.28-2.09 (2H, m), 2.02 (1H, m), 1.87 (1H, m), 1.80-

1.63 (4H, m), 1.60-1.24 (7H, m), 1.20-1.04 (2H, m), 1.11 (3H, t, *J* 8.0 Hz, C22-$H_3$), 1.09 (3H, s, C19-$H_3$), 0.91 (1H, m), 0.85 (3H, s, C18-$H_3$), 0.77 (1H, m); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 176.2 (C20), 147.6 (C5), 125.1 (C4), 84.0 (C17), 68.4 (C3), 56.1, 51.8, 43.8, 38.5, 38.1, 37.1, 36.8, 33.9, 33.2, 29.9, 28.5, 28.5, 24.5, 21.7, 19.4 (C18), 12.5 (C19), 9.6 (C22); **LRMS (+ESI)**: *m/z* 369 (100%, $[C_{22}H_{34}O_3Na]^+$); **HRMS (+ESI)**: calcd. for $[C_{22}H_{34}O_3Na]^+$ 369.2406, found 369.2413.

**Reduction of steroid mono-glucuronide**

5α-Androstane-3β,17β-diol 3-glucuronide, ammonium salt **8**

The reaction was conducted with EA 3-glucuronide, ammonium salt **17** [1] (derived from EA **22**, 5.0 mg, 17 µmol) as per the general procedure 2.4.4 to yield the title compound **8** as a colourless solid with > 98% conversion. **$R_f$** 0.29; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.40 (1H, d, *J* 7.7 Hz, C20-H), 3.78 (1H, m, C3-H), 3.58-3.53 (2H, m, C17-H and C24-H), 3.46-3.34 (2H, m, C23-H and C22-H), 3.17 (1H, t, *J* 8.2 Hz, C21-H), 2.01-0.87 (21H, m), 0.85 (3H, s, C18-$H_3$), 0.72 (3H, s, C19-$H_3$), 0.66 (1H, m); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 102.0 (C20), 82.5 (C17), 79.0 (C3), 77.9 (C22), 76.4 (C24), 75.0 (C21), 73.8 (C23), 56.0, 52.4, 46.1, 44.1, 38.4, 38.1, 37.0, 36.8, 35.3, 32.9, 30.7, 30.3, 29.9, 24.3, 22.0, 12.8 (C18), 11.7 (C19), C25 not observed; **LRMS (-ESI)**: *m/z* 467 (100%, $[C_{25}H_{39}O_8]^-$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{39}O_8]^-$ 467.2645, found 467.2639.

5α-Androstane-3β,17β-diol 17-glucuronide, ammonium salt **40**

The reaction was conducted with dihydrotestosterone (DHT) 17-glucuronide, ammonium salt **39** [1] (derived from 19% conversion of DHT, assumed 3.2 µmol) as per the general procedure 2.4.4 to yield the title compound **40** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C3-H protons showed 1:9 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$R_f$** 0.25; **$^1$H NMR** (700 MHz, $CD_3OD$): δ 4.35 (1H, d, *J* 7.8 Hz, C20-H), 3.80 (1H, t, *J* 8.7 Hz, C17-H), 3.52 (1H, d, *J* 9.8 Hz, C24-H), 3.42 (1H, t, *J* 9.4 Hz, C23-H), 3.36 (1H, t, *J* 9.1 Hz, C22-H), 3.19 (1H, t, *J* 8.5 Hz, C21-H), 2.05 (1H, m), 1.97 (1H, m), 1.77-0.89 (20H, m), 0.84 (3H, s, C18-$H_3$), 0.83 (3H, s, C19-$H_3$), 0.66 (1H, m); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 104.5 (C20), 89.5 (C17), 78.0 (C22), 76.5 (C24), 75.3 (C21), 73.8 (C23), 71.9 (C3), 56.0, 52.3, 46.3, 44.4, 39.0, 38.3, 36.8, 36.7, 32.8, 32.2, 29.9, 29.6, 24.3, 22.0, 12.8 (C18), 12.1 (C19), C25 not observed and one additional carbon peak overlapping or obscured; **LRMS (-ESI)**: *m/z* 467 (80%, $[C_{25}H_{39}O_8]^-$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{39}O_8]^-$ 467.2645, found 467.2643.

5α-Androstane-3β,17α-diol 17-glucuronide, ammonium salt **35**

The reaction was conducted with epidihydrotestosterone (epiDHT) 17-glucuronide, ammonium salt **56** (derived from 36% conversion of epiDHT **34**, assumed 6.1 µmol) as per the general procedure 2.4.4 to yield the title compound **35** as a colourless solid with > 98% conversion. The 400 MHz $^{1}$H NMR integration of the C3-H protons showed 1:8 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$R_f$** 0.30; **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 4.26 (1H, d, *J* 7.7 Hz, C20-H), 3.88 (1H, d, *J* 5.5 Hz, C17-H), 3.55 (1H, d, *J* 9.6 Hz, C24-H), 3.44 (1H, t, *J* 9.3 Hz, C23-H), 3.38 (1H, t, *J* 8.9 Hz, C22-H), 3.19 (1H, t, *J* 8.4 Hz, C21-H), 1.98 (1H, m), 1.76-0.66 (22H, m), 0.83 (3H, s, C19-H3), 0.71 (3H, s, C18-H3); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.1 (C25), 101.9 (C20), 86.4 (C17), 78.0 (C22), 76.9 (C24), 74.9 (C21), 73.7 (C23), 71.8 (C3), 55.7, 50.7, 46.2, 46.1, 38.4, 38.3, 37.3, 36.7, 33.7, 32.9, 32.1, 30.0, 29.9, 25.8, 21.9, 17.5 (C18), 12.8 (C19); **LRMS (-ESI)**: *m/z* 467 (100%, $[C_{25}H_{39}O_8]^-$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{39}O_8]^-$ 467.2645, found 467.2648.

Estradiol 3-glucuronide, ammonium salt **41** [1]

The reaction was conducted with estrone 3-glucuronide, ammonium salt **43** [1] (derived from 37% conversion of estrone, assumed 7.0 µmol) as per the general procedure 2.4.4 to yield the title compound **41** as a colourless solid with > 98% conversion. **$R_f$** 0.27; **$^{1}$H NMR** (700 MHz, $CD_3OD$): δ 7.19 (1H, d, *J* 8.6 Hz, C1-H), 6.87 (1H, dd, *J* 8.7, 2.6 Hz, C2-H), 6.81 (1H, d, *J* 2.6 Hz, C4-H), 4.87 (1H, d, *J* 7.1 Hz, C19-H), 3.73 (1H, d, *J* 9.3 Hz, C23-H), 3.66 (1H, t, *J* 8.6 Hz, C17-H), 3.52-3.46 (3H, m, C22-H, C21-H, and C20-H), 2.83-2.81 (2H, m, C6-$H_2$), 2.32 (1H, m), 2.17 (1H, td, *J* 11.3, 4.3 Hz), 2.04 (1H, m), 1.96 (1H, m), 1.89 (1H, m), 1.71 (1H, m), 1.54-1.26 (6H, m), 1.20 (1H, td, *J* 11.6, 7.3 Hz), 0.78 (3H, s, C18-$H_3$); **$^{13}$C NMR** (150 MHz, $CD_3OD$): δ 157.0 (C3), 139.0, 135.8, 127.2, 118.0, 115.4, 102.7 (C19), 82.5 (C17), 77.8 (C21), 74.8 (C20), 73.6 (C22), 51.4, 45.5, 44.4, 40.4, 38.0, 30.7, 30.7, 28.4, 27.6, 24.0, 11.7 (C18), C24 and C23 not observed; **LRMS (-ESI)**: *m/z* 447 (100%, $[C_{24}H_{31}O_8]^-$); **HRMS (-ESI)**: *m/z* calcd. for $[C_{24}H_{31}O_8]^-$ 447.2019, found 447.2015.

Androst-4-ene-3β,17β-diol 17-glucuronide, ammonium salt **45**

The reaction was conducted with testosterone 17-glucuronide, ammonium salt **19** [1] (derived from 61% conversion of testosterone **46**, assumed 10 µmol) as per the general procedure GP1 to yield the title compound **45** as a colourless solid with > 98% conversion. The 400 MHz $^{1}$H NMR integration of the C3-H protons showed 1:11 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$R_f$** 0.28; **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 5.25 (1H, s, C4-H), 4.36 (1H, d, *J* 7.8 Hz, C20-H), 4.06 (1H, m, C3-

H), 3.79 (1H, t, *J* 8.6 Hz, C17-H), 3.51 (1H, d, *J* 9.0 Hz, C24-H), 3.43-3.37 (2H, m, C23-H and C22-H), 3.19 (1H, t, *J* 8.1 Hz, C21-H), 2.22 (1H, m), 2.10-1.98 (3H, m), 1.87 (1H, m), 1.77-1.15 (10H, m), 1.08 (3H, s, C19-$H_3$), 1.02-0.80 (3H, m), 0.87 (3H, s, C18-$H_3$), 0.74 (1H, m); **$^{13}$C NMR** (150 MHz, $CD_3OD$): δ 147.8 (C5), 124.9 (C4), 104.7 (C20), 89.7 (C17), 77.9 (C22), 75.3 (C21), 73.7 (C23), 68.5 (C3), 56.2, 52.0, 44.3, 38.8, 38.5, 37.2, 36.8, 33.9, 33.2, 29.9, 29.6, 24.2, 21.8, 19.4 (C18), 12.0 (C19), C25 and C24 not observed; **LRMS (-ESI)**: *m/z* 465 (100%, $[C_{25}H_{37}O_8]^-$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{37}O_8]^-$ 465.2488, found 465.2485.

**Reduction of steroid mono-sulfate**

Androst-5-ene-3β,17β-diol 3-sulfate, ammonium salt **57** [17]

The reaction was conducted with dehydroepiandrosterone (DHEA) 3-sulfate, ammonium salt **58** [2] (derived from DHEA **1**, 5.5 mg, 19 µmol) as per the general procedure 2.4.4 to yield the title compound **57** as a colourless solid with > 98% conversion. **$R_f$** 0.52; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.39 (1H, d, *J* 5.3 Hz, C6-H), 4.13 (1H, tt, *J* 11.5, 4.7 Hz, C3-H), 3.58 (1H, t, *J* 8.6 Hz, C17-H), 2.54 (1H, ddd, *J* 13.1, 4.8, 2.0 Hz, C16-H), 2.35 (1H, m, C16-H), 2.09-0.94 (17H, m), 1.05 (3H, s, C19-$H_3$), 0.75 (3H, s, C18-$H_3$); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 141.7 (C5), 123.1 (C6), 82.5 (C17), 79.8 (C3), 52.7, 51.8, 43.9, 40.4, 38.5, 37.9, 37.8, 33.3, 32.7, 30.6, 30.0, 24.4, 21.8, 19.8 (C18), 11.5 (C19); **LRMS (-ESI)**: *m/z* 369 (100%, $[C_{19}H_{29}O_5S]^-$), 97 (20%, $[HSO_4]^-$); **HRMS (-ESI)**: calcd. for $[C_{19}H_{29}O_5S]^-$ 369.1736, found 369.1734.

Estradiol 3-sulfate, ammonium salt **44** [2]

The reaction was conducted with estrone 3-sulfate, ammonium salt **59** [2] (derived from 90% conversion of estrone, assumed 20 µmol) as per the general procedure 2.4.4 to yield the title compound **44** as a colourless solid with > 98% conversion. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 7.24 (1H, d, *J* 8.5 Hz, C1-H), 7.03 (1H, dd, *J* 8.6, 2.5 Hz, C2-H), 6.99 (1H, d, *J* 2.5 Hz, C4-H), 3.67 (1H, t, *J* 8.6 Hz, C17-H), 2.86-2.83 (2H, m, C6-$H_2$), 2.37-1.17 (13H, m), 0.77 (3H, s, C18-$H_3$). The $R_f$, $^1$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [2].

5α-Androstane-3β,17β-diol 17-sulfate, ammonium salt **60**

The reaction was conducted with DHT 17-sulfate, ammonium salt **61** [2] (derived from DHT, 5.5 mg, 19 µmol) as per the general procedure 2.4.4 to yield the title compound **60** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C3-H protons showed 1:9 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$R_f$** 0.48; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.23 (1H, t, *J* 8.5 Hz, C17-H), 3.51 (1H, tt, *J* 10.8, 4.5 Hz, C3-H), 2.15 (1H, ddt, *J* 15.4, 9.4, 4.7 Hz), 1.93 (1H, dt,

*J* 12.5, 2.9 Hz), 1.79-0.89 (19H, m), 0.86 (3H, s, C18-$H_3$), 0.81 (3H, s, C19-$H_3$), 0.67 (1H, dt, *J* 12.4, 4.2 Hz); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 88.3 (C17), 71.8 (C3), 55.9, 51.9, 46.3, 44.0, 38.9, 38.3, 38.1, 36.8, 36.7, 32.8, 32.1, 29.9, 29.2, 24.4, 21.8, 12.8 (C18), 12.2 (C19); **LRMS (-ESI)**: *m/z* 371 (100%, $[C_{19}H_{31}O_5S]^-$), 97 (10%, $[HSO_4]^-$); **HRMS (-ESI)**: calcd. for $[C_{19}H_{31}O_5S]^-$ 371.1892, found 371.1895.

5α-Androstane-3β,17α-diol 17-sulfate, ammonium salt **62**

The reaction was conducted with epiDHT 17-sulfate, ammonium salt **63** (derived from epiDHT **34**, 5.0 mg, 17 µmol) as per the general procedure 2.4.4 to yield the title compound **62** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C3-H protons showed 1:10 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$R_f$** 0.48; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.31 (1H, d, *J* 5.8 Hz, C17-H), 3.51 (1H, tt, *J* 9.1, 4.6 Hz, C3-H), 2.15 (1H, m), 1.94 (1H, m), 1.75-0.65 (20H, m), 0.84 (3H, s, C19-$H_3$), 0.74 (3H, s, C18-$H_3$); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 88.1 (C17), 71.9 (C3), 55.6, 51.0, 46.2, 46.2, 38.9, 38.3, 37.2, 36.7, 33.7, 32.9, 32.2, 31.2, 30.0, 25.6, 21.8, 17.3 (C18), 12.8 (C19); **LRMS (-ESI)**: *m/z* 371 (100%, $[C_{19}H_{31}O_5S]^-$), 97 (15%, $[HSO_4]^-$); **HRMS (-ESI)**: calcd. for $[C_{19}H_{31}O_5S]^-$ 371.1892, found 371.1892.

Androst-4-ene-3β,17β-diol 17-sulfate, ammonium salt **64**

The reaction was conducted with testosterone 17-sulfate, ammonium salt **65** [2] (derived from testosterone **46**, 5.5 mg, 19 µmol) as per the general procedure GP1 to yield the title compound **64** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C3-H protons showed 1:13 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$R_f$** 0.46; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.26 (1H, s, C4-H), 4.21 (1H, t, *J* 8.5 Hz, C17-H), 4.07 (1H, m, C3-H), 2.26-0.85 (18H, m), 1.08 (3H, s, C19-$H_3$), 0.83 (3H, s, C18-$H_3$), 0.75 (1H, dt, *J* 12.2, 4.1 Hz); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 147.8 (C5), 125.0 (C4), 88.1 (C17), 68.5 (C3), 56.2, 51.6, 43.9, 38.5, 37.9, 37.2, 36.8, 33.9, 33.2, 29.9, 29.1, 24.4, 21.6, 19.4 (C18), 12.1 (C19); **LRMS (-ESI)**: *m/z* 369 (100%, $[C_{19}H_{29}O_5S]^-$); **HRMS (-ESI)**: calcd. for $[C_{19}H_{29}O_5S]^-$ 369.1736, found 369.1736.

Androst-4-ene-3β,17α-diol 17-sulfate, ammonium salt **66**

The reaction was conducted with epitestosterone (epiT) 17-sulfate, ammonium salt **67** [2] (derived from 95% conversion of epiT, assumed 16 µmol) as per the general procedure GP1 to yield the title compound **66** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C3-H protons showed 1:7 ratio of the 3α:3β diastereomers. Data is reported for the major diastereomer where relevant. **$R_f$** 0.59; **$^1$H NMR** (400 MHz, $CD_3OD$): δ

5.28 (1H, s, C4-H), 4.34 (1H, d, *J* 5.8 Hz, C17-H), 4.09 (1H, m, C3-H), 2.30-0.75 (19H, m), 1.10 (3H, s, C19-$H_3$), 0.79 (3H, s, C18-$H_3$); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 147.8 (C5), 124.9 (C4), 87.9 (C17), 68.5 (C3), 55.9, 50.7, 46.1, 38.5, 37.6, 36.9, 34.7, 33.3, 32.8, 31.2, 29.9, 25.6, 21.6, 19.4 (C18), 17.2 (C19); **LRMS (-ESI)**: *m/z* 369 (100%, $[C_{19}H_{29}O_5S]^-$); **HRMS (-ESI)**: calcd. for $[C_{19}H_{29}O_5S]^-$ 369.1736, found 369.1733.

Pregn-5-ene-3β,20*R*-diol 3-sulfate, ammonium salt (20*S*:20*R* = 1:6) **68**

The reaction was conducted with pregnenolone 3-sulfate, ammonium salt **69** (derived from pregnenolone, 5.0 mg, 16 μmol) as per the general procedure 2.4.4 to yield the title compound **68** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C18-$H_3$ protons showed 1:6 ratio of the 20*S*:20*R* diastereomers. **20*S*** = δ 0.70 (3H, s, C18-$H_3$), **20*R*** = δ 0.78 (3H, s, C18-$H_3$). Data is reported for the major diastereomer where relevant. **$R_f$** 0.53; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.39 (1H, m, C6-H), 4.13 (1H, tt, *J* 11.0, 4.8 Hz, C3-H), 3.64 (1H, m, C20-H), 2.53 (1H, dd, *J* 13.2, 3.0 Hz), 2.36 (1H, m), 2.19-0.94 (18H, m), 1.10 (3H, d, *J* 5.9 Hz, C21-$H_3$), 1.04 (3H, s, C19-$H_3$), 0.78 (3H, s, C18-$H_3$); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 141.6 (C5), 123.3 (C6), 79.9 (C3), 70.9 (C20), 59.3, 57.7, 51.8, 43.5, 40.8, 40.4, 38.5, 37.7, 33.1, 30.0, 26.8, 25.6, 23.8, 22.0, 19.8 (C18), 12.6 (C19), one carbon peak overlapping or obscured; **LRMS (-ESI)**: *m/z* 397 (100%, $[C_{21}H_{33}O_5S]^-$), 97 (35%, $[HSO_4]^-$); **HRMS (-ESI)**: calcd. for $[C_{21}H_{33}O_5S]^-$ 397.2049, found 397.2049.

Pregn-5-ene-3β,20*S*-diol 3-sulfate, ammonium salt (20*S*:20*R* = 1:1) **70**

The reaction was conducted according to literature method with minor modifications [16]. Pregnenolone tosylhydrazone 3-sulfate, ammonium salt **71** (derived from pregnenolone 3-sulfate **69**, 5.0 mg, 11 μmol) was dissolved in 10:1 2-propanol:water (312 μL). Sodium borohydride (3.0 mg, 72 μmol, 6.5 equiv.) was added to the solution, and the reaction mixture was stirred for 16 h at room temperature. The solution was then quenched with water (5 mL) and subjected to purification by SPE as per general procedure 2.4.2 to yield the title compound **70** as a colourless solid with > 98% conversion. The 400 MHz $^1$H NMR integration of the C18-$H_3$ protons showed a 1:1 ratio of the 20*S*:20*R* diastereomers. **20*S***: δ 1.21 (3H, d, *J* 6.2 Hz, C21-$H_3$), 0.70 (3H, s, C18-$H_3$), **20*R*:** δ 1.11 (3H, d, *J* 6.2 Hz, C21-$H_3$), 0.78 (3H, s, C18-$H_3$); **$R_f$** 0.50; **LRMS (-ESI)**: *m/z* 397 (100%, $[C_{21}H_{33}O_5S]^-$), 97 (25%, $[HSO_4]^-$).

## Glucuronylation reaction

### Glucuronylation of steroid

EA glucuronide, ammonium salt **17** [1]

The reaction was conducted with EA **22** (5.0 mg, 17 µmol) as per the general procedure 2.4.5. This gave the title compound **17** as a colourless solid with > 98% conversion as determined by 400 MHz $^{1}$H NMR integration of the C3-H protons. **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 4.41 (1H, d, *J* 7.9 Hz, C20-H), 3.77 (1H, m, C3-H), 3.56 (1H, d, *J* 9.1 Hz, C24-H), 3.46 (1H, t, *J* 9.3 Hz, C23-H), 3.38 (1H, t, *J* 9.0 Hz, C22-H), 3.18 (1H, t, *J* 8.2 Hz, C21-H), 2.42 (1H, dd, *J* 19.1, 8.8 Hz, C16-H), 2.06 (1H, dt, *J* 18.7, 8.9 Hz, C16-H), 1.99-0.98 (19H, m), 0.88 (3H, s, C18-$H_3$), 0.87 (3H, s, C19-$H_3$), 0.74 (1H, m). The $R_f$, $^{1}$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [1].

DHT glucuronide, ammonium salt **39** [1]

The reaction was conducted with DHT (5.0 mg, 17 µmol) as per the general procedure 2.4.5. This gave the title compound **39** as a colourless solid with 19% conversion as determined by 400 MHz $^{1}$H NMR integration of the C17-H protons. **$R_f$** 0.52; **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 4.35 (1H, d, *J* 7.8 Hz, C20-H), 3.82 (1H, t, *J* 8.6 Hz, C17-H), 3.51 (1H, d, *J* 9.7 Hz, C24-H), 3.48-3.33 (2H, m, C23-H and C22-H), 3.19 (1H, t, *J* 8.2 Hz, C21-H), 2.48 (1H, m), 2.36 (1H, t, *J* 14.6 Hz), 2.25-0.76 (20H, m), 1.07 (3H, s, C19-$H_3$), 0.87 (3H, s, C18-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 215.0 (C3), 176.8 (C25), 104.5 (C20), 89.3 (C17), 78.0 (C22), 76.4 (C24), 75.4 (C21), 73.8 (C23), 55.3, 52.1, 45.5, 44.4, 39.7, 39.7, 38.8, 36.9, 36.7, 32.5, 30.8, 30.0, 29.6, 24.3, 22.2, 12.1 (C18), 11.8 (C19); **LRMS (-ESI)**: *m/z* 465 (100%, $[C_{25}H_{37}O_8]^-$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{37}O_8]^-$ 465.2488, found 465.2482.

EpiDHT glucuronide **56**

The reaction was conducted with epiDHT **34** (5.0 mg, 17 µmol) as per the general procedure 2.4.5. This gave the title compound **56** as a colourless solid with 36% conversion as determined by 400 MHz $^{1}$H NMR integration of the C17-H protons. **$R_f$** 0.34; **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 4.25 (1H, d, *J* 7.8 Hz, C20-H), 3.93 (1H, d, *J* 5.5 Hz, C17-H), 3.52 (1H, d, *J* 9.5 Hz, C24-H), 3.45 (1H, t, *J* 9.1 Hz, C23-H), 3.39 (1H, t, *J* 8.9 Hz, C22-H), 3.19 (1H, t, *J* 8.4 Hz, C21-H), 2.48 (1H, m), 2.37 (1H, m), 2.22 (1H, m), 2.11-1.95 (2H, m), 1.84-0.77 (17H, m), 1.07 (3H, s, C19-H3), 0.74 (3H, s, C18-H3); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 215.2 (C3), 176.7 (C25), 101.8 (C20), 86.1 (C17), 78.0 (C22), 76.7 (C24), 74.9 (C21), 73.8 (C23), 55.1, 50.5, 48.3, 46.1, 39.9, 39.9, 37.1, 36.9, 33.4, 32.8, 30.1, 30.1, 29.9, 25.8, 22.1, 17.5 (C18),

11.8 (C19); **LRMS (-ESI)**: *m/z* 465 (100%, $[C_{25}H_{37}O_8]^-$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{37}O_8]^-$ 465.2488, found 465.2500.

Estrone glucuronide **43** [1]

The reaction was conducted with estrone (5.0 mg, 19 µmol) as per the general procedure 2.4.5. This gave the title compound **43** as a colourless solid with 37% conversion as determined by 400 MHz $^1$H NMR integration of the C1-H protons. **$R_f$** 0.38; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 7.19 (1H, d, *J* 8.5 Hz, C1-H), 6.89 (1H, d, *J* 8.5 Hz, C2-H), 6.84 (1H, s, C4-H), 3.72 (1H, d, *J* 9.0 Hz, C23-H), 3.55-3.47 (3H, m, C22-H, C21-H, and C20-H), 2.88 (2H, dd, *J* 9.2, 4.2 Hz, C6-$H_2$), 2.49 (1H, dd, *J* 18.3, 8.5 Hz), 2.44-1.29 (12H, m), 0.92 (3H, s, C18-$H_3$), C19-H not observed; **$^{13}$C NMR** (150 MHz, $CD_3OD$): δ 223.8 (C17), 176.5 (C24), 157.3 (C3), 138.8, 135.0, 127.2, 118.1, 115.7, 102.8 (C19), 77.9 (C21), 76.6 (C23), 74.8 (C20), 73.7 (C22), 51.7, 45.4, 39.8, 36.8, 32.8, 30.6, 27.7, 27.1, 22.5, 14.3 (C18), one carbon peak overlapping or obscured; **LRMS (-ESI)**: *m/z* 445 (30%, $[C_{24}H_{29}O_8]^-$); **HRMS (-ESI)**: *m/z* calcd. for $[C_{24}H_{29}O_8]^-$ 445.1862, found 445.1857.

Testosterone glucuronide **19** [1]

The reaction was conducted with testosterone **46** (5.0 mg, 17 µmol) as per the general procedure 2.4.5. This gave the title compound **19** as a colourless solid with 61% conversion as determined by 400 MHz $^1$H NMR integration of the C17-H protons. **$R_f$** 0.26; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.71 (1H, s, C4-H), 4.35 (1H, d, *J* 7.7 Hz, C20-H), 3.82 (1H, t, *J* 8.6 Hz, C17-H), 3.52 (1H, d, *J* 9.5 Hz, C24-H), 3.43 (1H, t, *J* 9.1 Hz, C23-H), 3.36 (1H, t, *J* 8.9 Hz, C22-H), 3.20 (1H, t, *J* 8.4 Hz, C21-H), 2.52-2.44 (2H, m), 2.32-2.27 (2H, m), 2.10-2.02 (3H, m), 1.89 (1H, m), 1.75-1.45 (6H, m), 1.36-1.26 (2H, m), 1.24 (3H, s, C19-$H_3$), 1.07-0.87 (3H, m), 0.90 (3H, s, C18-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 202.4 (C3), 175.3 (C25), 124.1, 104.5 (C20), 89.1 (C17), 78.0 (C22), 75.3 (C21), 73.8 (C23), 55.4, 51.7, 44.2, 40.1, 38.5, 36.8, 36.8, 34.7, 33.9, 32.9, 29.6, 24.2, 21.8, 17.7 (C18), 12.0 (C19), C24 not observed and one more carbon overlapping or obscured; **LRMS (-ESI)**: *m/z* 463 (100%, $[C_{25}H_{35}O_8]^-$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{35}O_8]^-$ 463.2332, found 463.2326.

Androst-4-ene-3β,17β-diol 3-glucuronide 17-propionate **48**

The reaction was conducted with androst-4-ene-3β,17β-diol 17-propionate **55** (derived from testosterone propionate **47**, 5.0 mg, 15 µmol, a 1:8 ratio of the 3α:3β diastereomers) as per the general procedure 2.4.5. This gave the title compound **48** as a colourless solid with a 42% conversion overall (50% conversion from 3β-diol to the 3-glucuronide, with the 3α-diol unreacted) as determined by 400 MHz $^1$H NMR integration of the C3-H protons. **$R_f$** 0.34; **$^1$H**

**NMR** (700 MHz, $CD_3OD$): δ 5.45 (1H, s, C4-H), 4.58 (1H, t, *J* 8.5 Hz, C17-H), 4.42 (1H, d, *J* 7.8 Hz, C20-H), 4.28 (1H, m, C3-H), 3.55 (1H, d, *J* 9.6 Hz, C24-H), 3.44 (1H, t, *J* 9.2 Hz, C23-H), 3.40 (1H, t, *J* 9.0 Hz, C22-H), 3.19 (1H, t, *J* 8.5 Hz, C21-H), 2.34-2.30 (2H, m), 2.23 (1H, m), 2.14 (1H, m), 2.06-2.04 (2H, m), 1.78-1.72 (3H, m), 1.66 (1H, m), 1.62-1.48 (4H, m), 1.40-1.28 (4H, m), 1.16 (1H, m), 1.11 (3H, t, *J* 7.6 Hz, C28-$H_3$), 1.07 (3H, s, C19-$H_3$), 0.89 (1H, m), 0.85 (3H, s, C18-$H_3$), 0.77 (1H, m); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.8 (C25), 176.3 (C26), 148.3 (C5), 122.2 (C4), 103.2 (C20), 84.1 (C17), 78.0 (C22), 76.5 (C3), 76.3 (C24), 75.0 (C21), 73.8 (C23), 56.0, 51.8, 43.8, 38.5, 38.1, 37.1, 36.8, 33.9, 33.2, 28.6, 28.5, 28.1, 24.5, 21.6, 19.3 (C18), 12.5 (C19), 9.6 (C28); **LRMS (-ESI)**: *m/z* 521 (100%, $[C_{28}H_{41}O_9]^-$); **HRMS (-ESI)**: calcd. for $[C_{28}H_{41}O_9]^-$ 521.2751, found 521.2749.

**Glucuronylation of steroid diol or diol mono-glucuronide**

5α-Androstane-3β,17α-diol bisglucuronide, ammonium salt **5**

The reaction was conducted with 5α-androstane-3β,17α-diol 17-glucuronide **35** (derived from 36% conversion of epiDHT **34**, assumed 6.1 μmol, a 1:8 ratio of the 3α:3β diastereomers) as per the general procedure 2.4.5. This gave the title compound **5** as a colourless solid with a 43% conversion overall (53% conversion from 3β-diol mono-glucuronide to the bis (glucuronide), with the 3α-diol mono-glucuronide unreacted) as determined by 400 MHz $^1$H NMR integration of the C3-H protons. Performing the C18 purification procedure eluting with methanol:water (25% v/v) as per Section 2.4.3 afforded the title compound **5** in pure form. **$R_f$** 0.21 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (700 MHz, $CD_3OD$): δ 4.41 (1H, d, *J* 7.8 Hz, C20-H), 4.24 (1H, d, *J* 7.8 Hz, C26-H), 3.93 (1H, d, *J* 5.5 Hz, C17-H), 3.77 (1H, m, C3-H), 3.57 (1H, d, *J* 9.7 Hz, C24-H), 3.52 (1H, d, *J* 9.8 Hz, C30-H), 3.45 (2H, dt, *J* 15.8, 9.3 Hz, C23-H and C29-H), 3.38 (2H, dt, *J* 11.1, 9.1 Hz, C22-H and C28-H), 3.21-3.16 (2H, m, C21-H and C27-H), 1.96 (1H, m), 1.89 (1H, m), 1.79-1.67 (5H, m), 1.60-1.49 (4H, m), 1.40-1.26 (6H, m), 1.18 (1H, m), 1.09 (1H, m), 1.03-0.98 (2H, m), 0.84 (3H, s, C18-$H_3$), 0.71 (3H, s, C19-$H_3$), 0.68 (1H, m); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.4 (C25 or C31), 176.4 (C25 or C31), 101.9 (C26), 101.7 (C20), 86.0 (C17), 78.7 (C3), 78.0 (C22 or C28), 77.9 (C22 or C28), 76.7 (C24 or C30), 76.4 (C24 or C30), 75.0 (C21 or C27), 74.9 (C21 or C27), 73.8 (C23 and C29), 55.7, 50.7, 46.1, 45.9, 38.5, 37.3, 36.8, 36.8, 33.7, 32.8, 30.3, 30.1, 29.8, 25.8, 21.9, 17.5 (C18), 12.8 (C19); **LRMS (-ESI)**: *m/z* 643 (50%, $[C_{31}H_{47}O_{14}]^-$), 467 (10%, $[C_{25}H_{39}O_8]^-$), 321 (100%, $[C_{31}H_{46}O_{14}]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{31}H_{47}O_{14}]^-$ 643.2966, found 643.2960.

Estradiol bisglucuronide, ammonium salt **9** [1]

**Method A**: The reaction was conducted with estradiol 3-glucuronide, ammonium salt **41** [1] (derived from 37% conversion of estrone, assumed 7.0 µmol) as per the general procedure 2.4.5. This gave the title compound **9** as a colourless solid with > 98% conversion as determined by 400 MHz $^{1}$H NMR integration of the C17-H protons. **$R_f$** 0.14 (5:2:1 ethyl acetate:methanol:water); **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 7.18 (1H, d, *J* 8.6 Hz, C1-H), 6.87 (1H, dd, *J* 8.6, 2.6 Hz, C2-H), 6.81 (1H, d, *J* 2.5 Hz, C4-H), 4.40 (1H, d, *J* 7.8 Hz, C25-H), 3.89 (1H, t, *J* 8.6 Hz, C17-H), 3.73 (1H, d, *J* 8.8 Hz, C23-H), 3.56 (1H, d, *J* 9.6 Hz, C29-H), 3.52-3.36 (5H, m, C22-H, C28-H, C21-H, C27-H, and C20-H), 3.21 (1H, t, *J* 8.4 Hz, C26-H), 2.84-2.81 (2H, m, C6-$H_2$), 2.31 (1H, m), 2.20-2.08 (3H, m), 1.88 (1H, m), 1.70 (1H, m), 1.48-1.20 (7H, m), 0.88 (3H, s, C18-$H_3$), C19-H not observed; **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 157.1 (C3), 139.0, 135.7, 127.2, 118.1, 115.5, 104.6 (C25), 102.8 (C19), 89.5 (C17), 78.0 (C27), 77.8 (C21), 75.3 (C26), 74.8 (C20), 73.8 (C28), 73.6 (C22), 51.2, 45.4, 44.6, 40.2, 38.8, 30.7, 29.7, 28.4, 27.6, 24.0, 12.11 (C18), C24, C30, C23, and C29 not observed; **LRMS (-ESI)**: *m/z* 623 (55%, $[C_{30}H_{39}O_{14}]^-$), 447 (60%, $[C_{24}H_{31}O_8]^-$), 113 (100%, $[C_5H_5O_3]^-$); **HRMS (-ESI)**: calcd. for $[C_{30}H_{39}O_{14}]^-$ 623.2340, found 623.2347.

**Method B**: The reaction was conducted with estradiol (5.0 mg, 18 µmol) as per the general procedure 2.4.5. The reaction was then purified by SPE as per Section 2.4.2 to yield the title compound **9** as a colourless solid containing a mixture of the title compound **9**, estradiol 17-glucuronide **42**, and estradiol 3-glucuronide **41** in a 2:2:1 ratio as determined by 400 MHz $^{1}$H NMR integration of the C1-H and C17-H protons (no starting steroid diol observed). Performing the C18 purification procedure eluting with methanol:water (10% v/v) as per Section 2.4.3 afforded the title compound **9** in pure form.

Androst-4-ene-3β,17β-diol bisglucuronide, ammonium salt **11**

**Method A**: The reaction was conducted with androst-4-ene-3β,17β-diol 17-glucuronide **45** (derived from 61% conversion of testosterone **46**, assumed 10 µmol, a 1:11 ratio of the 3α:3β diastereomers) as per the general procedure 2.4.5. This gave the title compound **11** as a colourless solid with a 60% conversion overall (66% conversion from 3β-diol mono-glucuronide to the bis(glucuronide), with the 3α-diol mono-glucuronide unreacted) as determined by 400 MHz $^{1}$H NMR integration of the C3-H protons. Performing the C18 purification procedure eluting with methanol:water (15% v/v) as per Section 2.4.3 afforded the title compound **11** in pure form. **$R_f$** 0.19 (5:2:1 ethyl acetate:methanol:water); **$^{1}$H NMR** (700 MHz, $CD_3OD$): δ 5.44 (1H, s, C4-H), 4.43 (1H, d, *J* 7.8 Hz, C20-H), 4.35 (1H, d, *J* 7.8

Hz, C26-H), 4.26 (1H, m, C3-H), 3.77 (1H, t, *J* 8.6 Hz, C17-H), 3.59 (1H, d, *J* 9.7 Hz, C24-H), 3.55 (1H, d, *J* 9.8 Hz, C30-H), 3.46-3.42 (2H, m, C23-H and C29-H), 3.39 (1H, t, *J* 9.0 Hz, C22-H), 3.36 (1H, t, *J* 9.0 Hz, C28-H), 3.20-3.18 (2H, m, C21-H and C27-H), 2.21 (1H, m), 2.09-2.02 (3H, m), 1.99 (1H, m), 1.77-1.71 (2H, m), 1.67-1.48 (5H, m), 1.39 (1H, m), 1.31-1.22 (2H, m), 1.19 (1H, m), 1.07 (3H, s, C19-$H_3$), 0.97 (1H, m), 0.88-0.82 (1H, m), 0.86 (3H, s, C18-$H_3$), 0.75 (1H, m); **$^{13}$C NMR** (150 MHz, $CD_3OD$): $\delta$ 176.3 (C25 or C31), 176.2 (C25 or C31), 148.6 (C5), 122.1 (C4), 104.7 (C26), 103.3 (C20), 89.7 (C17), 77.9 (C22 or C28), 77.9 (C22 or C28), 76.8 (C3), 76.4 (C24 or C30), 76.4 (C24 or C30), 75.3 (C27), 75.0 (C21), 73.8 (C23 or C29), 73.7 (C23 or C29), 56.1, 52.0, 44.3, 38.7, 38.6, 37.2, 36.8, 33.9, 33.3, 29.6, 28.1, 24.3, 21.8, 19.3 (C18), 12.0 (C19); **LRMS (-ESI)**: *m/z* 320 (100%, $[C_{31}H_{44}O_{14}]^{2-}$), 641 (25%, $[C_{31}H_{45}O_{14}]^{-}$); **HRMS (-ESI)**: calcd. for $[C_{31}H_{45}O_{14}]^{-}$ 641.2809, found 641.2809.

**Method B**: The reaction was conducted with androst-4-ene-3β,17β-diol 3-glucuronide **49** (derived from 42% conversion of testosterone propionate **47**, assumed 6.3 µmol) as per the general procedure 2.4.5. This gave the title compound **11** as a colourless solid with 42% conversion as determined by 400 MHz $^1$H NMR integration of the C17-H protons. Performing the C18 purification procedure eluting with methanol:water (15% v/v) as per Section 2.4.3 afforded the title compound **11** in pure form.

**Method C**: The reaction was conducted with androst-4-ene-3β,17β-diol **50** [12],[13] (derived from testosterone **46**, 5.0 mg, 17 µmol, a 1:9 ratio of the 3α:3β diastereomers) as per the general procedure 2.4.5. The reaction was then purified by SPE as per Section 2.4.2 to yield steroid glucuronides and starting steroid mixture as a colourless solid with a 90% conversion overall (> 98% conversion from 3β-diol to either 3- or 17-glucuronide or the bis(glucuronide), with the 3α-diol unreacted) as determined by 400 MHz $^1$H NMR integration of the C3-H protons. Performing SPE purification of this mixture as per Section 2.4.1 yielded a colourless solid containing a mixture of the title compound **11**, androst-4-ene-3β,17β-diol 17-glucuronide **45**, and androst-4-ene-3β,17β-diol 3-glucuronide **49** in a 1:2:4 ratio as determined by 400 MHz $^1$H NMR integration of the C20-H, C26-H, C3-H, and C18-$H_3$ protons. Performing the C18 purification procedure eluting with methanol:water (25% v/v) as per Section 2.4.3 afforded the title compound **11** in pure form.

<u>Androst-5-ene-3β,17β-diol bisglucuronide, ammonium salt **10**</u>

The reaction was conducted with androst-5-ene-3β,17β-diol (5.0 mg, 17 µmol) as per the general procedure 2.4.5. The reaction was then purified by SPE as per Section 2.4.2 to yield

the title compound **10** as a colourless solid containing a mixture of the title compound **10** and androst-5-ene-3β,17β-diol 3-glucuronide in a 1:1 ratio as determined by 400 MHz $^{1}$H NMR integration of the C20-H and C26-H protons (no starting steroid diol observed). Performing the C18 purification procedure eluting with methanol:water (20% v/v) as per Section 2.4.3 afforded the title compound **10** in pure form. **$R_f$** 0.19 (5:2:1 ethyl acetate:methanol:water); **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 5.38 (1H, s, C6-H), 4.40 (1H, d, *J* 7.8 Hz, C20-H), 4.36 (1H, d, *J* 7.8 Hz, C26-H), 3.81 (1H, t, *J* 8.5 Hz, C17-H), 3.65 (1H, m, C3-H), 3.58-3.37 (6H, m, C24-H, C30-H, C23-H, C29-H, C22-H, C28-H), 3.21-3.16 (2H, m, C21-H and C27-H), 2.43 (1H, m), 2.25 (1H, m), 2.11-1.85 (5H, m), 1.71-1.46 (7H, m), 1.30-1.20 (2H, m), 1.14-0.93 (3H, m), 1.04 (3H, s, C19-$H_3$), 0.86 (3H, s, C18-$H_3$); **$^{13}$C NMR** (150 MHz, $CD_3OD$): δ 175.7 (C25 or C31), 175.6 (C25 or C31), 142.0 (C5), 122.5 (C6), 104.8 (C26), 102.4 (C20), 89.8 (C17), 79.8 (C3), 77.9 (C22 or C28), 77.8 (C22 or C28), 76.6 (C24 or C30), 76.5 (C24 or C30), 75.3 (C27), 75.0 (C21), 73.7 (C23 or C29), 73.6 (C23 or C29), 52.6, 51.8, 44.1, 39.7, 38.7, 38.6, 38.0, 33.2, 32.6, 30.6, 29.7, 24.3, 21.8, 19.9 (C18), 12.0 (C19); **LRMS (-ESI)**: *m/z* 320 (100%, $[C_{31}H_{44}O_{14}]^{2-}$), 641 (50%, $[C_{31}H_{45}O_{14}]^{-}$); **HRMS (-ESI)**: calcd. for $[C_{31}H_{45}O_{14}]^{-}$ 641.2809, found 641.2809.

<u>19-Norandrost-4-ene-3β,17β-diol bisglucuronide, ammonium salt **12**</u>

The reaction was conducted with 19-norandrost-4-ene-3β,17β-diol **51** [14] (derived from nandrolone, 5.0 mg, 18 μmol, a 1:6 ratio of the 3α:3β diastereomers) as per the general procedure 2.4.5. The reaction was then purified by SPE as per Section 2.4.2 to yield steroid glucuronides and starting steroid mixture as a colourless solid with a 85% conversion overall (> 98% conversion from 3β-diol to either 3-glucuronide or the bis(glucuronide), with the 3α-diol unreacted) as determined by 400 MHz $^{1}$H NMR integration of the C4-H protons. Performing SPE purification of this mixture as per Section 2.4.1 yielded a colourless solid containing a mixture of the title compound **12** and 19-norandrost-4-ene-3β,17β-diol 3-glucuronide in a 5:1 ratio as determined by 400 MHz $^{1}$H NMR integration of the C19-H and C25-H protons. Performing the C18 purification procedure eluting with methanol:water (20% v/v) as per Section 2.4.3 afforded the title compound **12** in pure form. **$R_f$** 0.18 (5:2:1 ethyl acetate:methanol:water); **$^{1}$H NMR** (700 MHz, $CD_3OD$): δ 5.53 (1H, s, C4-H), 4.43 (1H, d, *J* 7.8 Hz, C19-H), 4.35 (1H, d, *J* 7.9 Hz, C25-H), 4.28 (1H, m, C3-H), 3.80 (1H, t, *J* 8.6 Hz, C17-H), 3.57 (1H, d, *J* 9.6 Hz, C23-H), 3.52 (1H, d, *J* 9.7 Hz, C29-H), 3.45-3.35 (4H, m, C22-H, C28-H, C21-H, C27-H), 3.20-3.17 (2H, m, C20-H and C26-H), 2.26 (1H, m), 2.13 (1H, m), 2.10-1.97 (4H, m), 1.78-1.71 (3H, m), 1.64 (1H, m), 1.57 (1H, m), 1.41 (1H, m), 1.28-1.20 (4H, m), 1.10 (1H, m), 1.02 (1H, m), 0.91-0.85 (1H, m), 0.88 (3H, s, C18-$H_3$), 0.60

(1H, m); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.6 (C24 and C30), 144.1 (C5), 122.9 (C4), 104.6 (C25), 103.2 (C19), 89.6 (C17), 78.0 (C21 or C27), 77.9 (C21 or C27), 76.5 (C23 or C29), 76.4 (C23 or C29), 76.2 (C3), 75.3 (C26), 75.0 (C20), 73.8 (C22 or C28), 73.8 (C22 or C28), 51.9, 51.1, 44.5, 43.3, 42.2, 38.7, 36.2, 32.7, 31.0, 29.6, 27.1, 26.9, 24.1, 12.1 (C18); **LRMS (-ESI)**: *m/z* 313 (5%, $[C_{30}H_{42}O_{14}]^{2-}$), 627 (100%, $[C_{30}H_{43}O_{14}]^-$); **HRMS (-ESI)**: calcd. for $[C_{30}H_{43}O_{14}]^-$ 627.2653, found 627.2655.

5β-Cholane-3α,24-diol bisglucuronide, ammonium salt **13**

The reaction was conducted with 5β-cholane-3α,24-diol (5.0 mg, 14 µmol) as per the general procedure 2.4.5. The reaction was then purified by SPE as per Section 2.4.2 to yield a colourless solid containing a mixture of the title compound **13** and 5β-cholane-3α,24-diol 24-glucuronide in a 2:1 ratio as determined by 400 MHz $^1$H NMR integration of the C25-H and C31-H protons (no starting steroid diol observed). Performing the C18 purification procedure eluting with methanol:water (50% v/v) as per Section 2.4.3 afforded the title compound **13** in pure form. **$R_f$** 0.23 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.41 (1H, d, *J* 7.7 Hz, C25-H), 4.25 (1H, d, *J* 7.7 Hz, C31-H), 3.96 (1H, m, C24-$H_A$), 3.82 (1H, m, C3-H), 3.55 (2H, d, *J* 9.3 Hz, C29-H and C35-H), 3.49-3.36 (5H, m, C28-H, C34-H, C27-H, C33-H, C24-$H_B$), 3.23-3.17 (2H, m, C26-H and C32-H), 2.02 (1H, m), 1.95-1.79 (5H, m), 1.72 (1H, m), 1.65-1.59 (2H, m), 1.55-0.88 (19H, m), 0.96-0.94 (6H, m, C21-$H_3$ and C19-$H_3$), 0.69 (3H, s, C18-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.8 (C30 and C36), 104.3 (C31), 101.8 (C25), 79.2, 78.0 (C27 or C33), 77.9 (C27 or C33), 76.3 (C29 or C35), 76.2 (C29 or C35), 75.0 (C26 or C32), 75.0 (C26 or C32), 73.8 (C28 or C34), 73.8 (C28 or C34), 71.4, 58.0, 57.8, 43.9, 43.6, 41.9, 41.6, 37.3, 37.0, 36.3, 35.8, 35.2, 33.3, 29.4, 28.3, 27.7, 27.5, 27.4, 25.3, 23.9, 22.0, 19.1 (C18), 12.5 (C19); **LRMS (-ESI)**: *m/z* 356 (100%, $[C_{36}H_{56}O_{14}]^{2-}$), 713 (30%, $[C_{36}H_{57}O_{14}]^-$); **HRMS (-ESI)**: calcd. for $[C_{36}H_{57}O_{14}]^-$ 713.3748, found 713.3749.

5α-Pregnane-3β,20*S*-diol bisglucuronide, ammonium salt **14**

The reaction was conducted with 5α-pregnane-3β,20*S*-diol (5.0 mg, 16 µmol) as per the general procedure 2.4.5. The reaction was then purified by SPE as per the general procedure 2.4.2 to yield a colourless solid containing a mixture of the title compound **14** and 5α-pregnane-3β,20*S*-diol 3-glucuronide in a 1:1 ratio as determined by 400 MHz $^1$H NMR integration of the C22-H and C28-H protons (no starting steroid diol observed). Performing the C18 purification procedure eluting with methanol:water (23% v/v) as per Section 2.4.3 afforded the title compound **14** in pure form. **$R_f$** 0.26 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (700 MHz, $CD_3OD$): δ 4.40 (1H, d, *J* 7.8 Hz, C22-H), 4.35 (1H, d, *J* 7.8 Hz, C28-H),

3.78 (1H, m, C3-H), 3.64 (1H, m, C20-H), 3.55-3.53 (2H, m, C26-H and C32-H), 3.45-3.42 (2H, m, C25-H and C31-H), 3.40-3.36 (2H, m, C24-H and C30-H), 3.19-3.16 (2H, m, C23-H and C29-H), 2.05 (1H, m), 1.94-1.89 (2H, m), 1.74-1.69 (3H, m), 1.63-1.26 (10H, m), 1.33 (3H, d, *J* 6.0 Hz, C21-$H_3$), 1.16-1.09 (3H, m), 1.06-0.99 (2H, m), 0.93 (1H, m), 0.84 (3H, s, C18-$H_3$), 0.69 (3H, s, C18-$H_3$), 0.67 (1H, m); **$^{13}$C NMR** (150 MHz, $CD_3OD$): δ 105.8 (C22), 101.9 (C28), 82.8 (C20), 78.8 (C3), 78.0 (C24 or C30), 77.9 (C24 or C30), 76.6 (C26 or C32), 76.3 (C26 or C32), 75.5 (C29), 75.0 (C23), 73.8 (C25 or C31), 73.7 (C25 or C31), 59.2, 57.7, 55.9, 46.0, 42.9, 40.5, 38.3, 36.8, 36.7, 35.3, 33.4, 30.3, 30.1, 27.8, 25.2, 23.2, 22.1, 12.9 (C18), 12.7 (C19), C27 and C33 not observed; **LRMS (-ESI)**: *m/z* 335 (100%, $[C_{33}H_{50}O_{14}]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{33}H_{51}O_{14}]^-$ 671.3279, found 671.3254.

Pregn-5-ene-3β,20*R*-diol bisglucuronide, ammonium salt (20*S*:20*R* = 1:5) **16**

The reaction was conducted with pregn-5-ene-3β,20*R*-diol **52** [15] (5.0 mg, 16 µmol, a 1:6 ratio of the 20*S*:20*R* diastereomers) as per the general procedure 2.4.5. The reaction was then purified by SPE as per the general procedure 2.4.2 to yield a colourless solid containing a mixture of the title compound **16** and pregn-5-ene-3β,20-diol 20-glucuronide in a 9:1 ratio as determined by 400 MHz $^1$H NMR integration of the C22-H and C28-H protons (no starting steroid diol observed). Performing the C18 purification procedure eluting with methanol:water (40% v/v) as per Section 2.4.3 afforded the title compound **16** in pure form. The 400 MHz $^1$H NMR integration of the C18-$H_3$ protons showed a 1:5 ratio of the 20*S*:20*R* diastereomers. **20*S***: δ 0.72 (3H, s, C18-$H_3$), **20*R***: δ 0.83 (3H, s, C18-$H_3$). Data is reported for the major diastereomer where relevant. **$R_f$** 0.21 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.36 (1H, d, *J* 5.3 Hz, C6-H), 4.40 (1H, d, *J* 7.8 Hz, C22-H), 4.36 (1H, d, *J* 7.7 Hz, C28-H), 3.98 (1H, m, C20-H), 3.63 (1H, m, C3-H), 3.59-3.52 (2H, m, C26-H and C32-H), 3.45-3.35 (4H, m, C25-H, C31-H, C24-H, C30-H), 3.19 (2H, m, C23-H and C29-H), 2.42 (1H, m), 2.31 (1H, m), 2.25 (1H, m), 1.99-1.94 (2H, m), 1.87 (1H, m), 1.69-1.46 (8H, m), 1.30-0.91 (6H, m), 1.11 (3H, d, *J* 5.9 Hz, C21-$H_3$), 1.02 (3H, s, C19-$H_3$), 0.83 (3H, s, C18-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.3 (C27 or C33), 175.7 (C27 or C33), 142.1 (C5), 122.7 (C6), 102.3 (C22), 100.8 (C28), 79.6 (C3), 77.9 (C24 or C30), 77.8 (C24 or C30), 77.1 (C26 or C32), 76.4 (C26 or C32), 75.7, 75.2, 75.0, 73.7 (C25 or C31), 73.6 (C25 or C31), 57.9, 57.6, 52.0, 43.6, 40.5, 39.7, 38.6, 38.0, 33.2, 33.2, 30.6, 26.8, 25.5, 22.2, 19.9 (C18), 18.7, 12.1 (C19); **LRMS (-ESI)**: *m/z* 334 (100%, $[C_{33}H_{48}O_{14}]^{2-}$), 669 (5%, $[C_{33}H_{49}O_{14}]^-$); **HRMS (-ESI)**: calcd. for $[C_{33}H_{49}O_{14}]^-$ 669.3122, found 669.3137.

Pregn-5-ene-3β,20*S*-diol bisglucuronide, ammonium salt (20*S*:20*R* = 1:1) **15**

The reaction was conducted with pregn-5-ene-3β,20*S*-diol **53** (5.0 mg, 16 µmol, a 2:1 ratio of the 20*S*:20*R* diastereomers) as per the general procedure 2.4.5. The reaction was then purified by SPE as per the general procedure 2.4.2 to yield a colourless solid containing a mixture of the title compound **15** and pregn-5-ene-3β,20-diol 3-glucuronide in a 4:1 ratio as determined by 400 MHz $^{1}$H NMR integration of the C22-H and C28-H protons (no starting steroid diol observed). Performing the C18 purification procedure eluting with methanol:water (40% v/v) as per Section 2.4.3 afforded the title compound **15** in pure form. The 400 MHz $^{1}$H NMR integration of the C18-$H_3$ protons showed a 1:1 ratio of the 20*S*:20*R* diastereomers. **20*S***: δ 1.34 (3H, d, *J* 6.1 Hz, C21-$H_3$), 0.72 (3H, s, C18-$H_3$), **20*R***: δ 1.10 (3H, d, *J* 6.2 Hz, C21-$H_3$), 0.84 (3H, s, C18-$H_3$); **LRMS (-ESI)**: *m/z* 334 (100%, $[C_{33}H_{48}O_{14}]^{2-}$), 669 (25%, $[C_{33}H_{49}O_{14}]^{-}$).

## Glucuronylation of steroid diol mono-sulfate

Androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide, ammonium salt **24**

The reaction was conducted with androst-5-ene-3β,17β-diol 3-sulfate, ammonium salt **57** (derived from DHEA **1**, 5.5 mg, 19 µmol) as per the general procedure 2.4.5. This gave the title compound **24** as a colourless solid with 15% conversion as determined by 400 MHz $^{1}$H NMR integration of the C17-H protons. Performing the C18 purification procedure eluting with methanol:water (23% v/v) as per Section 2.4.3 afforded the title compound **24** in pure form. **$R_f$** 0.25 (5:2:1 ethyl acetate:methanol:water); **$^{1}$H NMR** (700 MHz, $CD_3OD$): δ 5.39 (1H, d, *J* 5.1 Hz, C6-H), 4.37 (1H, d, *J* 7.8 Hz, C20-H), 4.14 (1H, tt, *J* 11.0, 4.5 Hz, C3-H), 3.78 (1H, t, *J* 8.5 Hz, C17-H), 3.59 (1H, d, *J* 9.4 Hz, C24-H), 3.46 (1H, t, *J* 9.4 Hz, C23-H), 3.36 (1H, t, *J* 9.1 Hz, C22-H), 3.20 (1H, t, *J* 8.0 Hz, C21-H), 2.54 (1H, ddd, *J* 13.4, 5.0, 2.3, C16-H), 2.35 (1H, m, C16-H), 2.08-0.89 (17H, m), 1.04 (3H, s, C19-$H_3$), 0.86 (3H, s, C18-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 141.7 (C5), 123.1 (C6), 104.9 (C20), 90.0 (C17), 79.8 (C3), 77.8 (C22), 75.3 (C21), 73.6 (C23), 52.5, 51.7, 44.1, 40.4, 38.6, 38.5, 37.8, 33.1, 32.6, 30.0, 29.7, 24.3, 21.8, 19.8 (C18), 11.9 (C19), C25 and C24 not observed; **LRMS (-ESI)**: *m/z* 567 (15%, $[C_{25}H_{36}O_{11}SNa]^{-}$), 545 (20%, $[C_{25}H_{37}O_{11}S]^{-}$), 469 (10%, $[C_{23}H_{33}O_8S]^{-}$), 369 (40%, $[C_{19}H_{29}O_5S]^{-}$), 272 (100%, $[C_{25}H_{36}O_{11}S]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{37}O_{11}S]^{-}$ 545.2057, found 545.2055.

Estradiol 3-sulfate 17-glucuronide, ammonium salt **25**

The reaction was conducted with estradiol 3-sulfate, ammonium salt **44** [2] (derived from 90% conversion of estrone, assumed 20 µmol) as per the general procedure 2.4.5. This

gave the title compound **25** as a colourless solid with 97% conversion as determined by 400 MHz $^1$H NMR integration of the C18-$H_3$ protons. **$R_f$** 0.25 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (400 MHz, $CD_3OD$): $\delta$ 7.23 (1H, d, *J* 8.5 Hz, C1-H), 7.03 (1H, dd, *J* 8.5, 2.6 Hz, C2-H), 7.00 (1H, d, *J* 2.5 Hz, C4-H), 4.41 (1H, d, *J* 7.8 Hz, C19-H), 3.88 (1H, t, *J* 8.6 Hz, C17-H), 3.60 (1H, d, *J* 9.5 Hz, C23-H), 3.48-3.35 (2H, m, C22-H and C21-H), 3.22 (1H, t, *J* 8.4 Hz, C20-H), 2.86-2.83 (2H, m, C6-$H_2$), 2.34-1.21 (13H, m), 0.89 (3H, s, C18-$H_3$); **$^{13}$C NMR** (100 MHz, $CD_3OD$): $\delta$ 151.6 (C3), 138.8, 138.1, 127.0, 122.5, 119.7, 104.7 (C19), 89.6 (C17), 77.9 (C21), 75.3 (C20), 73.8 (C22), 51.2, 45.5, 44.6, 40.1, 38.8, 30.6, 29.7, 28.3, 27.6, 24.0, 12.1 (C18), C24 and C23 not observed; **LRMS (-ESI)**: *m/z* 549 (15%, $[C_{24}H_{30}O_{11}SNa]^-$), 451 (30%, $[C_{22}H_{27}O_8S]^-$), 351 (90%, $[C_{18}H_{23}O_5S]^-$), 263 (100%, $[C_{24}H_{30}O_{11}S]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{24}H_{31}O_{11}S]^-$ 527.1587, found 527.1588.

<u>Estradiol 3-glucuronide 17-sulfate, ammonium salt **28**</u>

The reaction was conducted with estradiol 17-sulfate, ammonium salt **72** [2] (derived from 70% conversion of estradiol, assumed 13 µmol) as per the general procedure 2.4.5. This gave the title compound **28** as a colourless solid with 86% conversion as determined by 400 MHz $^1$H NMR integration of the C1-H protons. Performing the C18 purification procedure eluting with methanol:water (10% v/v) as per Section 2.4.3 afforded the title compound **28** in pure form. **$R_f$** 0.21 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (400 MHz, $CD_3OD$): $\delta$ 7.19 (1H, d, *J* 8.6 Hz, C1-H), 6.87 (1H, dd, *J* 8.5, 2.7 Hz, C2-H), 6.81 (1H, d, *J* 2.6 Hz, C4-H), 4.85 (1H, C19-H assigned by COSY cross peak analysis), 4.31 (1H, t, *J* 8.6 Hz, C17-H), 3.74 (1H, d, *J* 9.3 Hz), 3.55-3.46 (3H, m), 2.85-2.82 (2H, m, C6-$H_2$), 2.35-1.22 (13H, m), 0.85 (3H, s, C18-$H_3$); **$^{13}$C NMR** (100 MHz, $CD_3OD$): $\delta$ 157.1 (C3), 138.9, 135.7, 127.2, 118.0, 115.5, 102.8 (C19), 88.1 (C17), 77.8 (C21), 74.8 (C20), 73.6 (C22), 50.8, 45.4, 44.2, 40.2, 38.0, 30.7, 29.2, 28.4, 27.4, 24.1, 12.2 (C18), C24 and C23 not observed; **LRMS (-ESI)**: *m/z* 549 (20%, $[C_{24}H_{30}O_{11}SNa]^-$), 527 (95%, $[C_{24}H_{31}O_{11}S]^-$), 351 (100%, $[C_{18}H_{23}O_5S]^-$), 263 (20%, $[C_{24}H_{30}O_{11}S]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{24}H_{31}O_{11}S]^-$ 527.1587, found 527.1591.

<u>5α-Androstane-3β,17β-diol 3-glucuronide 17-sulfate, ammonium salt **26**</u>

The reaction was conducted with 5α-androstane-3β,17β-diol 17-sulfate, ammonium salt **60** (derived from DHT, 5.5 mg, 19 µmol, a 1:9 ratio of the 3α:3β diastereomers) as per the general procedure 2.4.5. This gave the title compound **26** as a colourless solid with a 83% conversion overall (92% conversion from 3β-diol to the 3-glucuronide, with the 3α-diol unreacted) as determined by 400 MHz $^1$H NMR integration of the C3-H protons. Performing the C18 purification procedure eluting with methanol:water (25% v/v) as per Section 2.4.3

afforded the title compound **26** in pure form. **$R_f$** 0.25 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.41 (1H, d, *J* 7.7 Hz, C20-H), 4.21 (1H, t, *J* 8.0 Hz, C17-H), 3.77 (1H, tt, *J* 10.8, 5.1 Hz, C3-H), 3.56 (1H, d, *J* 9.2 Hz, C24-H), 3.46-3.35 (2H, m, C23-H and C22-H), 3.17 (1H, t, *J* 8.3 Hz, C21-H), 2.15 (1H, ddt, *J* 15.0, 9.3, 6.2 Hz), 1.96-0.89 (20H, m), 0.85 (3H, s, C18-$H_3$), 0.79 (3H, s, C19-$H_3$), 0.68 (1H, dt, *J* 12.2, 4.3 Hz); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.1 (C25), 102.1 (C20), 88.2 (C17), 78.9 (C3), 77.9 (C22), 76.4 (C24), 75.0 (C21), 73.7 (C23), 55.9, 51.9, 46.0, 44.0, 38.3, 38.1, 36.9, 36.8, 35.3, 32.8, 30.3, 30.0, 29.2, 24.4, 21.8, 12.7 (C18), 12.2 (C19); **LRMS (-ESI)**: *m/z* 569 (15%, $[C_{25}H_{38}O_{11}SNa]^-$), 471 (15%, $[C_{23}H_{35}O_8S]^-$), 371 (40%, $[C_{19}H_{31}O_5S]^-$), 273 (100%, $[C_{25}H_{38}O_{11}S]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{39}O_{11}S]^-$ 547.2213, found 547.2216.

5α-Androstane-3β,17α-diol 3-glucuronide 17-sulfate, ammonium salt **27**

The reaction was conducted with 5α-androstane-3β,17α-diol 17-sulfate, ammonium salt **62** (derived from epiDHT **34**, 5.0 mg, 17 μmol, a 1:10 ratio of the 3α:3β diastereomers) as per the general procedure 2.4.5. This gave the title compound **27** as a colourless solid with a 62% conversion overall (79% conversion from 3β-diol to the 3-glucuronide, with the 3α-diol unreacted) as determined by 400 MHz $^1$H NMR integration of the C3-H protons. Performing the C18 purification procedure eluting with methanol:water (25% v/v) as per Section 2.4.3 afforded the title compound **27** in pure form. **$R_f$** 0.23 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (600 MHz, $CD_3OD$): δ 4.42 (1H, d, *J* 7.8 Hz, C20-H), 4.31 (1H, d, *J* 5.7 Hz, C17-H), 3.76 (1H, tt, *J* 9.3, 5.2 Hz, C3-H), 3.59 (1H, d, *J* 9.3 Hz, C24-H), 3.46-3.36 (2H, m, C23-H and C22-H), 3.17 (1H, t, *J* 8.3 Hz, C21-H), 2.14 (1H, m), 1.94 (1H, m), 1.75-0.66 (20H, m), 0.85 (3H, s, C18-$H_3$), 0.74 (3H, s, C19-$H_3$); **$^{13}$C NMR** (150 MHz, $CD_3OD$): δ 102.0 (C20), 88.0 (C17), 78.9 (C3), 77.9 (C22), 75.0 (C21), 73.8 (C23), 55.6, 50.9, 46.2, 46.0, 38.4, 37.2, 36.8, 35.3, 33.7, 32.9, 31.2, 30.3, 30.1, 25.6, 21.8, 17.3 (C18), 12.7 (C19), C25 and C24 not observed; **LRMS (-ESI)**: *m/z* 569 (15%, $[C_{25}H_{38}O_{11}SNa]^-$), 547 (60%, $[C_{25}H_{39}O_{11}S]^-$), 471 (10%, $[C_{23}H_{35}O_8S]^-$), 371 (30%, $[C_{19}H_{31}O_5S]^-$), 273 (100%, $[C_{25}H_{38}O_{11}S]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{39}O_{11}S]^-$ 547.2213, found 547.2210.

Androst-4-ene-3β,17β-diol 3-glucuronide 17-sulfate, ammonium salt **29**

The reaction was conducted with androst-4-ene-3β,17β-diol 17-sulfate, ammonium salt **64** (derived from testosterone **46**, 5.5 mg, 19 μmol, a 1:13 ratio of the 3α:3β diastereomers) as per the general procedure 2.4.5. This gave the title compound **29** as a colourless solid with a 80% conversion overall (86% conversion from 3β-diol to the 3-glucuronide, with the 3α-diol unreacted) as determined by 400 MHz $^1$H NMR integration of the C3-H protons.

Performing the C18 purification procedure eluting with methanol:water (15% v/v) as per Section 2.4.3 afforded the title compound **29** in pure form. **$R_f$** 0.25 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.44 (1H, s, C4-H), 4.43 (1H, d, *J* 7.8 Hz, C20-H), 4.27 (1H, m, C3-H), 4.21 (1H, t, *J* 8.5 Hz, C17-H), 3.58 (1H, d, *J* 9.3 Hz, C24-H), 3.46-3.35 (2H, m, C23-H and C22-H), 3.19 (1H, t, *J* 8.0 Hz, C21-H), 2.26-0.85 (18H, m), 1.07 (3H, s, C19-$H_3$), 0.83 (3H, s, C18-$H_3$), 0.76 (1H, dt, *J* 12.1, 4.1 Hz); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 148.5 (C5), 122.1 (C4), 103.2 (C20), 88.1 (C17), 77.9 (C22), 76.7, 75.0 (C21), 73.7 (C23), 56.1, 51.6, 43.9, 38.6, 37.9, 37.2, 36.8, 33.9, 33.2, 29.1, 28.1, 24.4, 21.6, 19.3 (C18), 12.1 (C19), C25 and C24 not observed; **LRMS (-ESI)**: *m/z* 567 (20%, $[C_{25}H_{36}O_{11}SNa]^-$), 545 (90%, $[C_{25}H_{37}O_{11}S]^-$), 369 (45%, $[C_{19}H_{29}O_5S]^-$), 272 (85%, $[C_{25}H_{36}O_{11}S]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{37}O_{11}S]^-$ 545.2057, found 545.2059.

<u>Androst-4-ene-3β,17α-diol 3-glucuronide 17-sulfate, ammonium salt **30**</u>

The reaction was conducted with androst-4-ene-3β,17α-diol 17-sulfate, ammonium salt **66** (derived from 95% conversion of epiT, assumed 16 μmol, a 1:7 ratio of the 3α:3β diastereomers) as per the general procedure 2.4.5. This gave the title compound **30** as a colourless solid with a 42% conversion overall (47% conversion from 3β-diol to the 3-glucuronide, with the 3α-diol unreacted) as determined by 400 MHz $^1$H NMR integration of the C3-H protons. Performing the C18 purification procedure eluting with methanol:water (15% v/v) as per Section 2.4.3 afforded the title compound **30** in pure form. **$R_f$** 0.25 (5:2:1 ethyl acetate:methanol:water); **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.44 (1H, s, C4-H), 4.44 (1H, d, *J* 7.8 Hz, C20-H), 4.32 (1H, d, *J* 5.7 Hz, C17-H), 4.27 (1H, m, C3-H), 3.59 (1H, d, *J* 9.3 Hz, C24-H), 3.47-3.37 (2H, m), 3.19 (1H, t, *J* 8.2 Hz, C21-H), 2.27-0.74 (19H, m), 1.07 (3H, s, C19-$H_3$), 0.77 (3H, s, C18-$H_3$); **$^{13}$C NMR** (150 MHz, $CD_3OD$): δ 148.6 (C5), 122.1 (C4), 103.4 (C20), 87.9 (C17), 77.9 (C22), 76.9 (C3), 76. 4 (C24), 75.0 (C21), 73.7 (C23), 55.8, 50.7, 46.1, 38.6, 37.6, 36.8, 34.7, 33.4, 32.8, 31.2, 28.1, 25.6, 21.6, 19.3 (C18), 17.2 (C19), C25 not observed; **LRMS (-ESI)**: *m/z* 567 (30%, $[C_{25}H_{36}O_{11}SNa]^-$), 545 (40%, $[C_{25}H_{37}O_{11}S]^-$), 369 (50%, $[C_{19}H_{29}O_5S]^-$), 272 (95%, $[C_{25}H_{36}O_{11}S]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{37}O_{11}S]^-$ 545.2057, found 545.2055.

<u>Pregn-5-ene-3β,20*R*-diol 3-sulfate 20-glucuronide, ammonium salt (20*S*:20*R* = 1:11) **31**</u>

The reaction was conducted with pregn-5-ene-3β,20*R*-diol 3-sulfate, ammonium salt **68** (derived from pregnenolone, 5.0 mg, 16 μmol, a 1:6 ratio of the 20*S*:20*R* diastereomers) as per the general procedure 2.4.5. This gave the title compound **31** as a colourless solid with a 96% conversion as determined by 400 MHz $^1$H NMR integration of the C18-$H_3$ protons.

The 400 MHz $^{1}$H NMR integration of the C18-$H_3$ protons showed a 1:11 ratio of the 20*S*:20*R* diastereomers. **20*S*** = δ 0.72 (3H, s, C18-$H_3$), **20*R*** = δ 0.84 (3H, s, C18-$H_3$). Data is reported for the major diastereomer where relevant. **$R_f$** 0.37 (5:2:1 ethyl acetate:methanol:water); **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 5.38 (1H, m, C6-H), 4.36 (1H, d, *J* 7.7 Hz, C22-H), 4.13 (1H, tt, *J* 11.0, 4.8 Hz, C3-H), 3.99 (1H, m, C20-H), 3.52-3.35 (3H, m, C24-H, C25-H, and C26-H), 3.19 (1H, dd, *J* 9.0, 7.8 Hz, C23-H), 2.52 (1H, ddd, *J* 13.3, 5.1, 2.2 Hz), 2.33 (1H, m), 2.07-0.88 (18H, m), 1.11 (3H, d, *J* 6.0 Hz, C21-$H_3$), 1.03 (3H, s, C19-$H_3$), 0.84 (3H, s, C18-$H_3$); **$^{13}$C NMR** (200 MHz, $CD_3OD$): δ 141.7 (C5), 123.3 (C6), 100.6 (C22), 79.9 (C3), 78.0 (C20), 75.5, 75.2, 73.8, 57.9, 57.6, 51.9, 43.6, 40.5, 40.4, 38.5, 37.8, 33.2, 33.2, 30.0, 26.8, 25.5, 22.1, 19.8 (C18), 18.7, 12.1 (C19), C27 and C26 not observed; **LRMS (-ESI)**: *m/z* 573 (25%, $[C_{27}H_{41}O_{11}S]^-$), 497 (10%, $[C_{25}H_{37}O_8S]^-$), 397 (30%, $[C_{21}H_{33}O_5S]^-$), 286 (100%, $[C_{27}H_{40}O_{11}S]^{2-}$); **HRMS (-ESI)**: calcd. for $[C_{27}H_{41}O_{11}S]^-$ 573.2370, found 573.2371.

Pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide, ammonium salt (20*S*:20*R* = 2:1) **32**

The reaction was conducted with pregn-5-ene-3β,20*S*-diol 3-sulfate, ammonium salt **70** (derived from pregnenolone, 5.0 mg, 16 µmol, a 1:1 ratio of the 20*S*:20*R* diastereomers) as per the general procedure 2.4.5. This gave the title compound **32** as a colourless solid with a 83% conversion as determined by 400 MHz $^{1}$H NMR integration of the C18-$H_3$ protons. Performing the C18 purification procedure eluting with methanol:water (40% v/v) as per the general procedure 2.4.3 afforded the title compound **32** in pure form. The 400 MHz $^{1}$H NMR integration of the C18-$H_3$ protons showed a 2:1 ratio of the 20*S*:20*R* diastereomers. **20*S***: δ 1.34 (3H, d, *J* 6.1 Hz, C21-$H_3$), 0.72 (3H, s, C18-$H_3$), **20*R***: δ 1.11 (3H, d, *J* 5.8 Hz, C21-$H_3$), 0.84 (3H, s, C18-$H_3$); **$R_f$** 0.37 (5:2:1 ethyl acetate:methanol:water); **LRMS (-ESI)**: *m/z* 573 (25%, $[C_{27}H_{41}O_{11}S]^-$), 497 (10%, $[C_{25}H_{37}O_8S]^-$), 397 (20%, $[C_{21}H_{33}O_5S]^-$), 286 (75%, $[C_{27}H_{40}O_{11}S]^{2-}$), 97 (100%, $[HSO_4]^-$).

## Sulfation reaction

### Sulfation of steroid

EA sulfate, ammonium salt **73** [2]

The reaction was conducted with EA **22** (5.5 mg, 19 µmol) as per the general procedure GP2 to yield the title compound **73** as a colourless solid with > 98% conversion. **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 4.26 (1H, tt, *J* 11.3, 5.6 Hz, C3-H), 2.43 (1H, dd, *J* 19.1, 8.8 Hz, C16-H), 2.11-0.99 (20H, m), 0.88 (3H, s, C18-$H_3$), 0.87 (3H, s, C19-$H_3$), 0.75 (1H, ddd, *J* 12.2, 10.4, 4.1 Hz). The $R_f$, $^{1}$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [2].

DHEA sulfate, ammonium salt **58** [2]

The reaction was conducted with DHEA **1** (5.5 mg, 19 µmol) as per the general procedure GP2 to yield the title compound **58** as a colourless solid with > 98% conversion. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.44 (1H, d, *J* 5.2 Hz, C6-H), 4.14 (1H, tt, *J* 11.5, 4.8 Hz, C3-H), 2.56 (1H, ddd, *J* 13.2, 5.0, 2.3 Hz, C16-H), 2.44 (1H, dd, *J* 19.2, 8.4 Hz), 2.37 (1H, m, C16-H), 2.16-1.02 (16H, m), 1.07 (3H, s, C19-$H_3$), 0.90 (3H, s, C18-$H_3$). The $R_f$, $^1$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [2].

Estrone sulfate, ammonium salt **59** [2]

The reaction was conducted with estrone (5.9 mg, 22 µmol) as per the general procedure GP2 to yield the title compound **59** as a colourless solid with 90% conversion. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 7.25 (1H, d, *J* 8.3 Hz, C1-H), 7.06-7.02 (2H, m, C2-H and C4-H), 2.92-2.89 (2H, m, C6-$H_2$), 2.50 (1H, dd, *J* 18.4, 8.5 Hz), 2.45-1.40 (12H, m), 0.93 (3H, s, C18-$H_3$). The $R_f$, $^1$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [2].

Estradiol 17-sulfate, ammonium salt **72** [2]

The reaction was conducted according to the literature with minor modification [2]. A solution of estradiol (5.0 mg, 18 µmol) in DMF (200 µL) was treated with a solution of sulfur trioxide-pyridine complex (15 mg, 94 µmol, 5.2 equiv.), stirred for 21 h, and purified by SPE as per the general procedure GP2 to yield the title compound **72** as a colourless solid with 70% conversion. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 7.08 (1H, d, *J* 8.4 Hz, C1-H), 6.53 (1H, dd, *J* 8.4, 2.7 Hz, C2-H), 6.47 (1H, d, *J* 2.5 Hz, C4-H), 4.31 (1H, t, *J* 8.6 Hz, C17-H), 2.78-2.74 (2H, m, C6-$H_2$), 2.29-1.17 (13H, m), 0.83 (3H, s, C18-$H_3$). The $R_f$, $^1$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [2].

DHT sulfate, ammonium salt **61** [2]

The reaction was conducted with DHT (5.5 mg, 19 µmol) as per the general procedure GP2 to yield the title compound **61** as a colourless solid with > 98% conversion. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.22 (1H, t, *J* 8.7 Hz, C17-H), 2.49 (1H, dt, *J* 14.8, 6.7 Hz), 2.37 (1H, t, *J* 14.4 Hz), 2.25-0.76 (20H, m), 1.07 (3H, s, C19-$H_3$), 0.83 (3H, s, C18-$H_3$). The $R_f$, $^1$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [2].

EpiDHT sulfate, ammonium salt **63**

The reaction was conducted with epiDHT (5.0 mg, 17 µmol) as per the general procedure GP2 to yield the title compound **63** as a colourless solid with > 98% conversion. **$R_f$** 0.47; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.33 (1H, d, *J* 5.8 Hz, C17-H), 2.49 (1H, dt, *J* 14.7, 6.7 Hz), 2.37

(1H, t, *J* 14.4 Hz), 2.25-0.74 (20H, m), 1.07 (3H, s, C19-$H_3$), 0.77 (3H, s, C18-$H_3$); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 214.8 (C3), 87.9 (C17), 55.0, 50.8, 48.1, 46.2, 45.5, 39.8, 38.9, 37.0, 36.9, 33.3, 32.9, 31.2, 30.1, 25.6, 22.0, 17.3 (C18), 11.7 (C19); **LRMS (-ESI)**: *m/z* 369 (100%, $[C_{19}H_{29}O_5S]^-$), 97 (20%, $[HSO_4]^-$); **HRMS (-ESI)**: calcd. for $[C_{19}H_{29}O_5S]^-$ 369.1736, found 369.1741.

Testosterone sulfate, ammonium salt **65** [2]

The reaction was conducted with testosterone **46** (5.5 mg, 19 µmol) as per the general procedure GP2 to yield the title compound **65** as a colourless solid with > 98% conversion. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.71 (1H, s, C4-H), 4.23 (1H, t, *J* 8.5 Hz, C17-H), 2.54-0.95 (19H, m), 1.24 (3H, s, C19-$H_3$), 0.87 (3H, s, C18-$H_3$). The $R_f$, $^1$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [2].

EpiT sulfate, ammonium salt **67** [2]

The reaction was conducted with epiT (5.0 mg, 17 µmol) as per the general procedure GP2 to yield the title compound **67** as a colourless solid with 95% conversion. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.71 (1H, s, C4-H), 4.34 (1H, d, *J* 5.8 Hz, C17-H), 2.53-0.96 (19H, m), 1.24 (3H, s, C19-$H_3$), 0.81 (3H, s, C18-$H_3$). The $R_f$, $^1$H NMR, $^{13}$C NMR, LRMS, and HRMS matched the literature [2].

Pregnenolone sulfate, ammonium salt **69**

The reaction was conducted with pregnenolone (5.0 mg, 16 µmol) as per the general procedure GP2 to yield the title compound **69** as a colourless solid with > 98% conversion. **$R_f$** 0.55; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.40 (1H, m, C6-H), 4.14 (1H, tt, *J* 11.4, 4.7 Hz, C3-H), 2.65 (1H, t, *J* 8.9 Hz), 2.54 (1H, ddd, *J* 13.2, 5.1, 2.3 Hz), 2.35 (1H, m), 2.18-1.01 (17H, m), 2.13 (3H, s, C21-$H_3$), 1.04 (3H, s, C19-$H_3$), 0.63 (3H, s, C18-$H_3$); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 212.4 (C20), 141.6 (C5), 123.1 (C6), 79.7 (C3), 64.7, 58.1, 51.5, 45.1, 40.4, 39.9, 38.5, 37.7, 33.2, 32.9, 31.7, 30.0, 25.5, 23.8, 22.2, 19.7 (C18), 13.6 (C19); **LRMS (-ESI)**: *m/z* 395 (100%, $[C_{21}H_{31}O_5S]^-$), 97 (35%, $[HSO_4]^-$); **HRMS (-ESI)**: calcd. for $[C_{21}H_{31}O_5S]^-$ 395.1892, found 395.1893.

## Hydrolysis reaction

### PaS enzyme hydrolysis reaction

Estradiol 17-glucuronide **42** [1]

A solution of estradiol 3-sulfate 17-glucuronide, ammonium salt **25** (1.0 mg, 1.8 µmol) in MilliQ water (450 µL) was added to a falcon tube containing MilliQ water (9 mL), Tris-HCl

buffer (500 µL, 1 M, pH 8.2), and PaS wild type enzyme (50 µL, 60 mg $mL^{-1}$) [4] and left to stand at room temperature for 3 h. The reaction was then adjusted to pH 7 (universal indicator strips) by the addition of aqueous acetic acid (500 µL, 1 M) and purified by SPE as per Section 2.4.2 to yield the title compound **42** as a colourless solid with a > 98% conversion. **$R_f$** 0.33; **$^1$H NMR** (400 MHz, $CD_3OD$): δ 7.07 (1H, d, *J* 8.5 Hz, C1-H), 6.53 (1H, dd, *J* 8.8, 2.3 Hz, C2-H), 6.47 (1H, s, C4-H), 4.40 (1H, d, *J* 7.8 Hz, C19-H), 3.90 (1H, t, *J* 8.7 Hz, C17-H), 3.55 (1H, d, *J* 9.3 Hz, C23-H), 3.47-3.36 (2H, m, C22-H and C21-H), 3.21 (1H, t, *J* 8.0 Hz, C20-H), 2.79-2.75 (2H, m, C6-$H_2$), 2.28 (1H, m), 2.19-2.10 (3H, m), 1.87 (1H, m), 1.68 (1H, m), 1.47-1.21 (7H, m), 0.89 (3H, s, C18-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.2 (C24), 155.9 (C3), 138.8, 132.7, 127.2, 116.0, 113.7, 104.6 (C19), 89.5 (C17), 77.9 (C21), 75.3 (C20), 73.8 (C22), 51.2, 45.3, 44.6, 40.4, 38.9, 30.7, 29.7, 28.5, 27.7, 24.0, 12.1 (C18), C23 not observed; **LRMS (-ESI)**: *m/z* 447 (45%, $[C_{24}H_{31}O_8]^-$); **HRMS (-ESI)**: *m/z* calcd. for $[C_{24}H_{31}O_8]^-$ 447.2019, found 447.2020.

**Base hydrolysis reaction**

Androst-4-ene-3β,17β-diol 3-glucuronide **49**

A solution of androst-4-ene-3β,17β-diol 3-glucuronide 17-propionate **48** (derived from 42% conversion of testosterone propionate **47**, assumed 6.3 µmol) in methanol (200 µL) was treated with 5 M aqueous sodium hydroxide (20 µL, 0.10 mmol, 16 equiv.), and stirred for 3 h at room temperature. The reaction mixture was then quenched with water (3 mL) and purified by SPE as per Section 2.4.2 to yield the title compound **49** as a colourless solid with > 98% conversion. **$R_f$** 0.32; **$^1$H NMR** (700 MHz, $CD_3OD$): δ 5.44 (1H, s, C4-H), 4.42 (1H, d, *J* 7.7 Hz, C20-H), 4.27 (1H, m, C3-H), 3.60-3.54 (2H, m, C17-H and C24-H), 3.47-3.37 (2H, m, C23-H and C22-H), 3.19 (1H, t, *J* 8.3 Hz, C21-H), 2.22 (1H, m), 2.06-2.00 (2H, m), 1.96 (1H, m), 1.84 (1H, m), 1.75 (1H, m), 1.63-1.22 (9H, m), 1.07 (3H, s, C19-$H_3$), 1.04-0.71 (4H, m), 0.75 (3H, s, C18-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 148.5 (C5), 122.1 (C4), 103.3 (C20), 82.4 (C17), 78.0 (C22), 76.6 (C3), 75.0 (C21), 73.7 (C23), 56.2, 52.1, 44.0, 38.6, 38.0, 37.4, 36.8, 34.0, 33.3, 30.6, 28.1, 24.3, 21.7, 19.3 (C18), 11.6 (C19), C25 and C24 not observed; **LRMS (-ESI)**: *m/z* 465 (100%, $[C_{25}H_{37}O_8]^-$); **HRMS (-ESI)**: calcd. for $[C_{25}H_{37}O_8]^-$ 465.2488, found 465.2487.

## Tosylhydrazone reaction

### Tosylhydrazone of steroid

Pregnenolone tosylhydrazone **54**

The reaction was conducted according to literature method with minor modifications [18]. Pregnenolone (5.0 mg, 16 µmol) was dissolved in DMF (150 µL). *para*-Toluenesulfonyl hydrazide (6.0 mg, 32 µmol, 2 equiv.) was then added to the solution. The reaction mixture was stirred for 16 h at room temperature. The solution was then quenched with water (5 mL) and subjected to purification by WAX SPE as per Section 2.4.1 but with different eluting solutions: 0.1 M aqueous sodium hydroxide (15 mL), water (15 mL), and methanol (15 mL). The methanol fraction was concentrated *in vacuo* to yield the title compound **54** as a colourless solid with > 98% conversion. **$R_f$** 0.23 (1:1 ethyl acetate:n-hexane); **$^1$H NMR** (400 MHz, $CD_3OD$): $\delta$ 7.80 (2H, d, *J* 8.4 Hz), 7.35 (2H, d, *J* 7.8 Hz), 5.33 (1H, d, *J* 5.5 Hz, C6-H), 3.39 (1H, m, C3-H), 2.42 (3H, s), 2.25-2.14 (4H, m), 1.97 (1H, m), 1.87-0.91 (15H, m), 1.78 (3H, s), 0.99 (3H, s), 0.30 (3H, s); **$^{13}$C NMR** (100 MHz, $CD_3OD$): $\delta$ 159.9, 145.0, 142.2 (C5), 137.5, 130.2 (2C), 129.3 (2C), 122.3 (C6), 72.4 (C3), 60.0, 57.7, 51.7, 44.9, 43.0, 39.9, 38.5, 37.7, 33.4, 32.9, 32.3, 25.3, 24.2, 22.1, 21.5, 21.5, 19.8 (C18), 13.2 (C19); **LRMS (+ESI)**: *m/z* 485 (100%, $[C_{28}H_{41}N_2O_3S]^+$); **HRMS (+ESI)**: calcd. for $[C_{28}H_{41}N_2O_3S]^+$ 485.2838, found 485.2834.

### Tosylhydrazone of steroid mono-sulfate

Pregnenolone tosylhydrazone 3-sulfate, ammonium salt **71**

The reaction was conducted according to literature method with minor modifications [18]. Pregnenolone 3-sulfate, pyridinium salt (5.0 mg, 11 µmol) was dissolved in DMF (105 µL). *para*-Toluenesulfonyl hydrazide (3.9 mg, 21 µmol, 2 equiv.) was then added to the solution. The reaction mixture was stirred for 16 h at room temperature. The solution was then quenched with water (5 mL) and subjected to purification by SPE as per general procedure 2.4.1 but with different eluting solutions: 0.1 M aqueous sodium hydroxide (15 mL), water (15 mL), methanol (15 mL), and saturated aqueous ammonia solution in methanol (5% v/v, 15 mL). The methanolic ammonia fraction was concentrated *in vacuo* to yield the title compound **71** as a colourless solid with > 98% conversion. **$R_f$** 0.50; **$^1$H NMR** (400 MHz, $CD_3OD$): $\delta$ 7.80 (2H, d, *J* 8.3 Hz), 7.35 (2H, d, *J* 8.0 Hz), 5.38 (1H, m, C6-H), 4.12 (1H, tt, *J* 11.5, 4.7 Hz, C3-H), 2.53 (1H, ddd, *J* 13.3, 5.0, 2.3 Hz), 2.42 (3H, s), 2.37-0.93 (19H, m), 1.78 (3H, s), 1.00 (3H, s), 0.29 (3H, s); **$^{13}$C NMR** (100 MHz, $CD_3OD$): $\delta$ 159.9, 145.0, 141.6 (C5), 137.5, 130.2 (2C), 129.3 (2C), 123.1 (C6), 79.8 (C3), 60.0, 57.6, 51.6, 44.9, 40.4, 39.9, 38.5, 37.7, 33.3, 32.9, 30.0, 25.2, 24.2, 22.1, 21.5, 19.7 (C18), 18.2, 13.2 (C19); **LRMS (-**

**ESI)**: *m/z* 563 (100%, $[C_{28}H_{39}N_2O_6S_2]^-$), 395 (15%, $[C_{21}H_{31}O_5S]^-$), 97 (10%, $[HSO_4]^-$); **HRMS (-ESI)**: calcd. for $[C_{28}H_{39}N_2O_6S_2]^-$ 563.2250, found 563.2248.

## Labelled fluoro sugar synthesis

### Synthesised $^{18}O$ labelled fluoro sugar

{$^{18}O$}-α-D-Glucuronyl fluoride, ammonium salt {$^{18}O$}-**2**

Acetonitrile (110 µL) and sodium bicarbonate buffer ($c_i$ = 1.0 M, 110 µL, $c_f$ = 0.5 M, pH 9; prepared in $D_2{}^{18}O$) was added to vacuum-dried α-D-glucopyranosyl fluoride {$^{18}O$}-**36** (10 mg, 0.055 mmol), BAIB (39 mg, 0.12 mmol) and TEMPO (1.7 mg, 0.011 mmol). The reaction mixture was stirred, initially on ice which was allowed to warm to room temperature. After 24 h, the reaction was diluted with water (500 µL) and then washed with chloroform (3 x 500 µL). The aqueous layer was then collected and concentrated under reduced pressure giving a crude colourless solid with a > 98% conversion as determined by 400 MHz $^1$H NMR integration of the C1-H protons. The crude was then subjected to anion exchange column chromatography (Dowex®, 1x8, 200-400 mesh, $HCO_3^-$ form). The column was eluted with milliQ water (4 column volumes) then 50 mM ammonium bicarbonate (2 column volumes), followed by 0.1 M ammonium bicarbonate (6 column volumes), then by 0.2 M ammonium bicarbonate until complete elution of the target compound as indicated by TLC. Appropriate fractions were combined and concentrated under reduced pressure to afford the title compound {$^{18}O$}-**2** as a colourless solid (7.6 mg, 0.035 mmol, 64%). $\mathbf{R_f}$ 0.40 (5:2:1 EtOAc:MeOH:$H_2O$); **$^1$H NMR** (400 MHz, $D_2O$): δ 5.70 (1H, dd, $J_{H1\text{-}F}$ 53.4, $J_{H1\text{-}H2}$ 2.8 Hz, C1-H), 4.08 (1H, d, $J_{H4\text{-}H5}$ 10.1 Hz, C5-H), 3.76 (1H, t, $J_{H3\text{-}H4} \approx J_{H2\text{-}H3}$ 9.2 Hz, C3-H), 3.66 (1H, ddd, $J_{H2\text{-}F}$ 26.0, $J_{H2\text{-}H3}$ 9.8, $J_{H1\text{-}H2}$ 2.8 Hz, C2-H), 3.57 (1H, t, $J_{H4\text{-}H5} \approx J_{H3\text{-}H4}$ 9.4 Hz, C4-H); **LRMS (-ESI)**: *m/z* 199 (25%, $[C_6H_8O_4[^{18}O_2]F]^-$), 197 (13%, $[C_6H_8O_5[^{18}O_1]F]^-$), 179 (15%, $[C_6H_7O_4[^{18}O_2]]^-$), 177 (10%, $[C_6H_7O_5[^{18}O_1]]^-$); **HRMS (-ESI)**: *m/z* calcd. for $[C_6H_8O_4[^{18}O_2]F]^-$ 199.0390, found 199.0390. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [9].

### Synthesised $^{13}C$ labelled fluoro sugar

1,2,3,4,6-Penta-*O*-acetyl-{$^{13}C_6$}-β-D-glucopyranoside {$^{13}C_6$}-**74**

Acetic anhydride (10 mL, 0.11 mol) was added to sodium acetate (0.16 g, 20 mmol), and an anomeric mixture of {$^{13}C_6$}-D-glucose (1.00 g, 5.38 mmol) and stirred at 100 °C. The reaction mixture was then stirred for 2 h, at which time a further aliquot of acetic anhydride (5.0 mL, 53 mmol) was added. The reaction was then left for a further 2 h, where full consumption of the starting material was observed by TLC. Upon cooling the reaction was diluted with

dichloromethane (20 mL), and poured into a stirring solution of aqueous sodium thiosulfate (30 mL, 10% w/v) cooled on ice. The organic layer was then extracted and washed with saturated sodium bicarbonate solution (3 x 20 mL). The organic layer was then dried with anhydrous $MgSO_4$ and concentrated under reduced pressure, giving the crude product as a mixture of anomers. Recrystallisation from methanol (10 mL per gram of crude solid) gave the title compound {$^{13}C_6$}-**74** as a colourless solid (1.04 g, 2.48 mmol, 46%). The 400 MHz $^1$H NMR integration of the C1-H protons showed 1:33 ratio of the α:β anomers. Data is reported for the major anomer where relevant. **$R_f$** 0.30 (9:1 $CHCl_3$:EtOH); **mp**: 128-130 ºC (lit.[19],[20] 130-131 ºC); **[α]**$_{23}^{D}$ +5.5 (c 0.8, $CHCl_3$) [lit.[19] **[α]**$_{23}^{D}$ +4.5 (c 4.6, $CHCl_3$)]; **IR** (ATR): 2945 (C-H), 1752 (C=O), 1434, 1374, 1215, 1031, 975, 906 cm$^{-1}$; **$^1$H NMR** (400 MHz, $CDCl_3$): δ 5.71 (1H, dd, $^1J_{H1-C1}$ 167.0 Hz, $^3J_{H1-H2}$ 8.3 Hz, C1-H), 5.39-4.97 (3H, m, C2-H, C3-H, C4-H), 4.43-3.97 (2H, m, C6-$H_a$ and C6-$H_b$), 3.82 (1H, m, C5-H), 2.11 (3H, s), 2.08 (3H, s), 2.03-2.02 (6H, m), 2.01 (3H, s); **$^{13}$C NMR** (100 MHz, $CDCl_3$): δ 170.6 (C=O), 170.1 (C=O), 169.4 (C=O), 169.3 (C=O), 169.0 (C=O), 91.7 (dt, $^1J_{C1-C2}$ 48.2 Hz, $^2J_{C1-C3}$ ≈ $^3J_{C1-C4}$ 5.0 Hz, C1), 73.8-72.0 (2C, m, C3 and C4), 70.3 (ddd, $^1J_{C1-C2}$ 48.2 Hz, $^1J_{C2-C3}$ 40.8 Hz, $^2J_{C2-C4}$ 3.5 Hz, C2), 67.8 (td, $^1J_{C5-C6}$ ≈ $^1J_{C4-C5}$ 41.6 Hz, $^2J_{C3-C5}$ 3.5 Hz, C5), 61.5 (dt, $^1J_{C5-C6}$ 44.8 Hz, $^2J_{C4-C6}$ ≈ $^3J_{C3-C6}$ 4.2 Hz, C6), 20.9 ($CH_3$), 20.7 ($CH_3$), 20.6 (3x$CH_3$); **LRMS (+ESI):** *m/z* 419 (100%, [{$^{13}C_6$}$C_{10}H_{22}O_{11}Na]^+$); **HRMS (+ESI)** *m/z* calcd. for [{$^{13}C_6$}$C_{10}H_{22}O_{11}Na]^+$ 419.1261, found 419.1260. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [19].

2,3,4,6-Tetra-*O*-acetyl-{$^{13}C_6$}-α-D-glucopyranosyl fluoride {$^{13}C_6$}-**75**

Hydrogen fluoride-pyridine (70%, 3 mL) was added to 1,2,3,4,6-penta-*O*-acetyl-{$^{13}C_6$}-β-D-glucopyranoside {$^{13}C_6$}-**74** (1.00 g, 2.39 mmol) dissolved in dry dichloromethane, under nitrogen at room temperature. The reaction was stirred for 6 h, then poured into a solution of diethyl ether (5 mL) and quenched with aqueous potassium fluoride (17 mL, 10% w/v). The organic layer was collected, and aqueous layer was washed with ether:hexane 3:1 (3 x 20 mL). The organic layers were combined and washed with aqueous potassium fluoride (3 x 20 mL, 10% w/v), saturated aqueous sodium bicarbonate (20 mL) and then brine (20 mL). The organic layer was dried over anhydrous $MgSO_4$, and then concentrated under reduced pressure at 30 ºC. The resulting crude oil was purified by column chromatography (silica, 9:1 $CH_3Cl$:EtOAc), which afforded the title compound {$^{13}C_6$}-**75** as a colourless solid (752 mg, 1.98 mmol, 83%). **$R_f$** 0.50 (9:1 $CHCl_3$:EtOH); **mp**: 104-106 ºC (lit.[20] 108 ºC); **[α]**$_{26}^{D}$ +65.0 (c 0.26, $CHCl_3$) [lit.[20] **[α]**$_{20}^{D}$ +90.1 (c 3, $CHCl_3$)]; **IR** (ATR): 2951 (C-H), 1748 (C=O), 1343, 1373, 1215, 1139, 1031, 914, 760 cm$^{-1}$; **$^1$H NMR** (400 MHz,$CDCl_3$): δ 5.95-5.32 (2H,

m, C1-H and C4-H), 5.28-4.77 (2H, m, C2-H and C3-H), 4.42-3.97 (3H, m, C5-H and C6-$H_2$), 2.10 (3H, s), 2.10 (3H, s), 2.04 (3H, s), 2.02 (3H, s); **$^{13}$C NMR** (100 MHz, $CDCl_3$): δ 170.7 (C=O), 170.1 (2xC=O), 169.6 (C=O), 103.7 (m, C1), 71.2-68.9 (3C, m, C2, C3, C4), 67.5 (m, C5), 61.3 (dt, $^1J_{C5\text{-}C6}$ 44.5 Hz, $^2J_{C4\text{-}C6}$ ≈ $^3J_{C3\text{-}C6}$ 3.6 Hz, C6), 20.8 ($CH_3$), 20.8 ($CH_3$), 20.7 (2x$CH_3$); **LRMS (+ESI)**: *m/z* 379 (100%, [{$^{13}C_6$}$C_8H_{19}O_9FNa]^+$); **HRMS (+ESI)**: *m/z* calcd. for [{$^{13}C_6$}$C_8H_{19}O_9FNa]^+$ 379.1112, found 379.1116. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [21].

<u>{$^{13}C_6$}-α-D-Glucopyranosyl fluoride {$^{13}C_6$}-**36**</u>

Sodium methoxide (1 mL, 0.1 mM, in MeOH) was added dropwise to a solution of 2,3,4,6-tetra-*O*-acetyl-{$^{13}C_6$}-α-D-glucopyranosyl fluoride {$^{13}C_6$}-**75** (714 mg, 1.88 mmol) dissolved in dry methanol (7.5 mL), on ice, under a nitrogen atmosphere. The reaction was stored in a 4 $^oC$ fridge for 18 h, then quenched with silica (1 g) and concentrated under reduced pressure. The crude was subjected to column chromatography (silica, 5:2 EtOAc:EtOH), which afforded the title compound {$^{13}C_6$}-**36** as a colourless solid (318 mg, 1.51 mmol, 80%). **$R_f$** 0.45 (7:2:1 EtOAc:MeOH:$H_2O$); **mp**: 110-115 $^oC$ (lit.[22] 112-119 $^oC$); **[α]**$_{27}^D$ +79.8 (c 0.15, $H_2O$) [lit.[22] **[α]**$_{24}^D$ +97.6 (c 1.5, $H_2O$)]; **IR** (ATR): 3376 (br, O-H), 2975 (C-H), 1455, 1357, 1145, 1066, 996, 877, 758 $cm^{-1}$; **$^1$H NMR** (400 MHz, $D_2O$): δ 5.61 (1H, m, C1-H), 4.11-3.28 (6H, m);**$^{13}$C NMR** (100 MHz, $D_2O$): δ 107.3 (ddt, $^1J_{C1\text{-}F}$ 222.5 Hz, $^1J_{C1\text{-}C2}$ 45.0 Hz, $^2J_{C1\text{-}C3}$ ≈ $^3J_{C1\text{-}C4}$ 2.9 Hz, C1), 75.4-70.3 (3C, m, C2, C3, C4), 68.5 (m, C5), 60.1 ( dt, $^1J_{C6\text{-}C5}$ 43.2 Hz, $^2J_{C6\text{-}C4}$ ≈ $^3J_{C6\text{-}C3}$ 3.5 Hz, C6); **LRMS (+ESI)**: *m/z* 211 (100%, [{$^{13}C_6$}$H_{11}O_5FNa]^+$); **HRMS (+ESI)**: *m/z* calcd. for [{$^{13}C_6$}$H_{11}O_5FNa]^+$ 211.0690, found 211.0692. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [22].

<u>{$^{13}C_6$}-α-D-Glucuronyl fluoride, ammonium salt {$^{13}C_6$}-**2**</u>

Acetonitrile (0.6 mL) and sodium bicarbonate buffer ($c_i$ = 1.0 M, 0.6 mL, $c_f$ = 0.5 M, pH 9) was added to {$^{13}C_6$}-α-D-glucopyranosyl fluoride {$^{13}C_6$}-**36** (50 mg, 0.24 mmol), BAIB (200 mg, 0.62 mmol) and TEMPO (7.5 mg, 48 µmol). The reaction mixture was stirred, initially on ice which was allowed to warm to room temperature. After 24 h, the reaction was diluted with water (5 mL) and then washed with chloroform (3 x 5 mL). The aqueous layer was then collected and then concentrated under reduced pressure giving a crude colourless solid. The crude was then subjected to anion exchange column chromatography (Dowex®, 1x8, 200-400 mesh, $HCO_3^-$ form). The column was eluted with milliQ water (4 column volumes) then 50 mM ammonium bicarbonate (2 column volumes), followed by 0.1 M ammonium bicarbonate (6 column volumes), then by 0.2 M ammonium bicarbonate until complete

elution of the target compound as indicated by TLC. Appropriate fractions were combined and concentrated under reduced pressure to afford the title compound {$^{13}C_6$}-**2** as a translucent colourless solid (38.3 mg, 0.19 mmol, 79%). **$R_f$** 0.44 (5:2:1 EtOAc:MeOH:$H_2O$); **mp**: 115-120 ºC (lit.[6] 112-119 ºC); **[α]**$_{27}^{D}$ +7.0 (c 0.2, $H_2O$) [lit.[9] **[α]**$_{24}^{D}$ +46.6 (c 1.1, $H_2O$)]; **IR** (ATR): 3200 (O-H), 2922 (C-H), 1538 ($CO_2^-$), 1402 ($CO_2^-$), 1276, 1139, 1011, 653 $cm^{-1}$; **$^1$H NMR** (400 MHz, $D_2O$): δ 6.72 (1H, m, C1-H), 4.27 (1H, m, C4-H), 3.99-3.29 (3H, m); **$^{13}$C NMR** (100 MHz, $D_2O$): δ 175.5 (dt, $^1J_{C5-C6}$ 59.3 Hz, $^2J_{C4-C6} \approx {}^3J_{C3-C6}$ 4.1 Hz, C6), 106.9 (m, C1), 106.9 (4C, m, C2, C3, C4, C5); **LRMS (-ESI)**: *m/z* 201 (100%, [{$^{13}C_6$}$H_8O_6F$]$^-$); **HRMS (-ESI)**: *m/z* calcd. for [{$^{13}C_6$}$H_8O_6F$]$^-$ 201.0509, found 201.0506. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [9].

## Labelled steroid mono-glucuronides synthesis

### Synthesised $^{18}$O labelled steroid mono-glucuronides

EA {$^{18}$O}-glucuronide, ammonium salt {$^{18}$O}-**17**

The reaction was conducted with EA **22** (1.0 mg, 3.4 µmol) and {$^{18}$O}-α-D-glucuronyl fluoride {$^{18}$O}-**2** as per the general procedure 2.4.5. This gave the title compound {$^{18}$O}-**17** as a colourless solid with > 98% conversion as determined by 400 MHz $^1$H NMR integration of the C3-H protons. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.41 (1H, d, *J* 7.9 Hz, C20-H), 3.77 (1H, m, C3-H), 3.56 (1H, d, *J* 9.1 Hz, C24-H), 3.46 (1H, t, *J* 9.3 Hz, C23-H), 3.38 (1H, t, *J* 9.0 Hz, C22-H), 3.18 (1H, t, *J* 8.2 Hz, C21-H), 2.42 (1H, dd, *J* 19.1, 8.8 Hz, C16-H), 2.06 (1H, dt, *J* 18.7, 8.9 Hz, C16-H), 1.99-0.98 (19H, m), 0.88 (3H, s, C18-$H_3$), 0.87 (3H, s, C19-$H_3$), 0.74 (1H, m); **LRMS (-ESI)**: *m/z* 469 (100%, [$C_{25}H_{37}O_6${$^{18}O_2$}]$^-$), 467 (60%, [$C_{25}H_{37}O_7${$^{18}O_1$}]$^-$); **HRMS (-ESI)**: *m/z* calcd. for [$C_{25}H_{37}O_6${$^{18}O_2$}]$^-$ 469.2573, found 469.2573. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [1].

### Synthesised $^{13}$C labelled steroid mono-glucuronides

Testosterone {$^{13}C_6$}-glucuronide, ammonium salt {$^{13}C_6$}-**19**

The reaction was conducted with testosterone **46** (5.0 mg, 17 µmol) and {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2** by general procedure 2.4.5. This gave the title compound {$^{13}C_6$}-**19** as a colourless solid with 41% conversion as determined by 400 MHz $^1$H NMR integration of the C17-H protons. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.71 (1H, s, C4-H), 4.35 (1H, dd, $^1J_{H20-C20}$ 158.3 Hz, $^3J_{H20-H21}$ 7.7 Hz, C20-H), 3.83 (1H, m), 3.58 (1H, m), 3.35 (1H, m), 3.27-2.97 (2H, m), 2.55-2.41 (2H, m), 2.34-2.24 (2H, m), 2.17-1.99 (3H, m), 1.89 (1H, m), 1.78-1.44 (6H, m), 1.37-1.25 (2H, m), 1.24 (3H, s, C18-$H_3$), 1.09-0.92 (3H, m), 0.90 (3H, s, C19-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 202.4 (C=O), 176.6 (dt, $^1J_{C24-C25}$ 58.8 Hz, $^2J_{C23-C25} \approx {}^3J_{C22-}$

$_{C25}$ 4.9 Hz, C25), 104.5 (dt, $^{1}J_{C20\text{-}C21}$ 47.1 Hz, $^{2}J_{C20\text{-}C22} \approx {}^{3}J_{C20\text{-}C23}$ 4.9 Hz, C20), 89.2 (C17), 79.0-71.9 (4C, m, C21, C22, C23, C24), 69.0, 55.4, 51.7, 44.2, 40.0, 38.5, 36.8, 34.7, 33.9, 32.8, 31.4, 29.6, 24.2, 21.8,17.7 (C18), 12.0 (C19), one peak overlapping or obscured; **LRMS (-ESI)**: *m/z* 469 (100%, $[C_{19}\{^{13}C_6\}H_{35}O_8]^-$); **HRMS (-ESI)**: *m/z* calcd. for $[C_{19}\{^{13}C_6\}H_{35}O_8]^-$ 469.2533, found 469.2530. Spectroscopic data was found to be consistent with the unlabelled compound [1].

<u>EpiT $\{^{13}C_6\}$-glucuronide, ammonium salt $\{^{13}C_6\}$-**20**</u>

The reaction was conducted with epiT (5.0 mg, 17 µmol) and $\{^{13}C_6\}$-α-D-glucuronyl fluoride $\{^{13}C_6\}$-**2** as per the general procedure 2.4.5. This gave the title compound $\{^{13}C_6\}$-**20** as a colourless solid with 6% conversion as determined by 400 MHz $^{1}$H NMR integration of the C17-H protons. **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 5.71 (1H, s, C4-H), 4.17 (1H, dd, $^{1}J_{H20\text{-}C20}$ 157.0 Hz, $^{3}J_{H20\text{-}H21}$ 7.6 Hz, C20-H), 3.98 (1H, m, C17-H), 3.75-3.39 (3H, m), 2.99 (1H, m), 2.56-2.42 (2H, m), 2.30 (1H, m), 2.15-1.89 (3H, m), 1.88-1.36 (8H, m), 1.29 (2H, s), 1.24 (3H, s, C18-$H_3$), 1.15-0.84 (3H, m), 0.78 (3H, s, C19-$H_3$); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 202.5 (C=O), 176.8 (d, $J_{C24\text{-}C25}$ 58.8 Hz, C25), 169.9, 124.0, 101.7 (m, C20), 85.7, 79.8-71.5 (4C, m, C21, C22, C23, C24), 57.5, 55.3, 45.9, 40.1, 37.3, 36.9, 34.8, 34.1, 33.7, 32.6, 29.8, 25.7, 21.7, 17.8 (C18), 17.3 (C19); **LRMS (-ESI)**: *m/z* 469 (100%, $[C_{19}\{^{13}C_6\}H_{35}O_8]^-$); **HRMS (-ESI)**: *m/z* calcd. for $[C_{19}\{^{13}C_6\}H_{35}O_8]^-$ 469.2533, found 469.2531. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [1].

<u>DHEA $\{^{13}C_6\}$-glucuronide, ammonium salt $\{^{13}C_6\}$-**4**</u>

The reaction was conducted with DHEA **1** (5.0 mg, 17 µmol) and $\{^{13}C_6\}$-α-D-glucuronyl fluoride $\{^{13}C_6\}$-**2** as per the general procedure 2.4.5. This gave the title compound $\{^{13}C_6\}$-**4** as a colourless solid with 71% conversion as determined by 400 MHz $^{1}$H NMR integration of the C3-H protons. **$^{1}$H NMR** (400 MHz, $CD_3OD$): δ 5.42 (1H, m, C6-H), 4.40 (1H, dd, $^{1}J_{H20\text{-}C20}$ 157.9 Hz, $^{3}J_{H20\text{-}H21}$ 7.6 Hz, C20-H), 3.77-3.53 (2H, m), 3.42-3.32 (2H, m), 3.00 (1H, m), 2.52-2.40 (2H, m), 2.27 (1H, m), 2.17-1.93 (4H, m), 1.90 (1H, m), 1.79 (1H, m), 1.78-1.45 (7H, m), 1.43-1.21 (2H, m), 1.14 (1H, m), 1.07 (3H, s, C18-$H_3$), 0.90 (3H, s, C19-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.8 (d, $J_{C24\text{-}C25}$ 58.3 Hz, C25), 142.2, 122.1, 102.2 ( dt, $^{1}J_{C20\text{-}21}$ 47.0 Hz, $^{2}J_{C20\text{-}C22} \approx {}^{3}J_{C20\text{-}C23}$ 4.8 Hz, C20), 79.3, 78.9-67.1 (4C, m, C21, C22, C23, C24), 53.0, 51.8, 39.6, 38.5, 38.0, 36.7, 32.8, 32.7, 31.9, 30.5, 30.5, 22.8, 21.4, 19.9 (C18), 13.9 (C19), one peak overlapping or obscured; **LRMS (-ESI)**: *m/z* 469 (100%, $[C_{19}\{^{13}C_6\}H_{35}O_8]^-$); **HRMS (-ESI)**: *m/z* calcd. for $[C_{19}\{^{13}C_6\}H_{35}O_8]^-$ 469.2533, found 469.2533. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [1].

EA {$^{13}C_6$}-glucuronide, ammonium salt {$^{13}C_6$}-**17**

The reaction was conducted with EA **22** (5.0 mg, 17 µmol) and {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2** as per the general procedure 2.4.5. This gave the title compound {$^{13}C_6$}-**17** as a colourless solid with > 98% conversion as determined by 400 MHz $^1$H NMR integration of the C3-H protons. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.42 (1H, dd, $^1J_{H20\text{-}C20}$ 158.2 Hz, $^3J_{H20\text{-}H21}$ 7.7 Hz, C20-H), 3.76 (1H, m), 3.59 (1H, m), 3.23 (1H, m), 3.00 (1H, m), 2.43 (1H, dd, $J_{H15'\text{-}H16}$ 19.1 Hz, $J_{H15''\text{-}H16}$ 8.7 Hz, C16-H), 2.07 (1H, m), 1.95-1.45 (9H, m), 1.44-0.95 (11H, m), 0.88 (3H, s, C18-$H_3$), 0.87 (3H, s, C19-$H_3$), 0.74 (1H, m); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 177.0 (dt, $^1J_{C24\text{-}C25}$ 58.4 Hz, $^2J_{C23\text{-}C25} \approx {}^3J_{C22\text{-}C25}$ 4.7 Hz, C25), 101.9 (dt, $^1J_{C20\text{-}C21}$ 47.0 Hz, $^2J_{C20\text{-}C22} \approx {}^3J_{C20\text{-}C23}$ 4.8 Hz, C20), 79.0, 79.0-71.0 (4C, m, C21, C22, C23, C24), 55.9, 52.8, 46.0, 38.3, 36.9, 36.9, 36.4, 35.3, 32.8, 32.1, 30.3, 29.8, 22.8, 21.6, 21.6, 14.2 (C18), 12.7 (C19), one peak overlapping or obscured; **LRMS (-ESI)**: *m/z* 471 (100%, [$C_{19}${$^{13}C_6$}$H_{37}O_8$]$^-$); **HRMS (-ESI)**: *m/z* calcd. for [$C_{19}${$^{13}C_6$}$H_{37}O_8$]$^-$ 471.2690, found 471.2690. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [1].

Etiocholanolone {$^{13}C_6$}-glucuronide, ammonium salt {$^{13}C_6$}-**18**

The reaction was conducted with etiocholanolone (5.0 mg, 17 µmol) and {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2** as per the general procedure 2.4.5. This gave the title compound {$^{13}C_6$}-**18** as a colourless solid with < 5% conversion as determined by 400 MHz $^1$H NMR integration of the C3-H protons. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.41 (1H, dd, $^1J_{H20\text{-}C20}$ 158.0 Hz, $^3J_{H20\text{-}H21}$ 7.6 Hz, C20-H), 3.82 (1H, m), 3.69 (1H, m), 3.66-3.49 (2H, m), 3.01 (1H, m), 2.43 (1H, dd, $J_{H15'\text{-}H16}$19.1 Hz, $J_{H15''\text{-}H16}$ 8.6 Hz, C16-H), 2.08 (1H, m), 2.02-1.72 (5H, m), 1.71-1.51 (5H, m), 1.50-1.18 (8H, m), 1.03 (1H, m), 0.98 (3H, s, C18-$H_3$), 0.92 (1H, m), 0.87 (3H, s, C19-$H_3$); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.9 (d, $J_{C24\text{-}C25}$ 58.3, C25), 101.8 (dt, $^1J_{C20\text{-}C21}$ 47.0 Hz, $^2J_{C20\text{-}C22} \approx {}^3J_{C20\text{-}C23}$ 4.8 Hz, C20), 79.1-68.9 (4C, m, C21, C22, C23, C24), steroidal carbons not observed; **LRMS (-ESI)**: *m/z* 471 (100%, [$C_{19}${$^{13}C_6$}$H_{37}O_8$]$^-$); **HRMS (-ESI)**: *m/z* calcd. for [$C_{19}${$^{13}C_6$}$H_{37}O_8$]$^-$ 471.2690, found 471.2690. Spectroscopic data was found to be consistent with the literature for the unlabelled compound [1].

## Synthesised $^{13}$C labelled steroid bisglucuronides

Estradiol 3,17{$^{13}C_6$}-bisglucuronide, ammonium salt {$^{13}C_6$}-**9**

The reaction was conducted with estradiol 3-glucuronide, ammonium salt **41** [1] (1.0 mg, 2.1 µmol, see SI section) and {$^{13}C_6$}-α-D-glucuronyl fluoride {$^{13}C_6$}-**2** by general procedure 2.4.5. This gave the title compound {$^{13}C_6$}-**9** as a colourless solid with > 98% conversion as determined by 400 MHz $^1$H NMR integration of the C17-H protons. **$^1$H NMR** (700 MHz,

$CD_3OD$): δ 7.18 (1H, d, *J* 8.6 Hz, C1-H), 6.87 (1H, dd, *J* 8.6, 2.5 Hz, C2-H), 6.81 (1H, d, *J* 2.5 Hz, C4-H), 4.40 (1H, dd, *J* 158.5, 7.6 Hz, C25-H), 3.89 (1H, m, C17-H), 3.74 (1H, d, *J* 9.5 Hz, C23-H), 3.67-3.09 (7H, m, C29-H, C22-H, C28-H, C21-H, C27-H, C20-H, and C26-H), 2.83-2.81 (2H, m, C6-$H_2$), 2.31(1H, m), 2.22-2.06 (3H, m), 1.88 (1H, m), 1.69 (1H, m), 1.49-1.22 (7H, m), 0.88 (s, 3H, C18-$H_3$), C19-H not observed; **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 176.0 (m, C30), 157.1, 139.0, 135.7, 127.2, 118.1, 115.5, 104.6 (m, C25), 102.8 (C19), 89.6, 78.6-72.7 (4C, m, C26, C27, C28, C29), 51.2, 45.4, 44.6, 40.2, 38.8, 30.7, 29.7, 28.4, 27.6, 24.0, 12.1 (C18), C20-24 obscured by carbons C26-30 signals; **LRMS (-ESI)**: *m/z* 314 ($[C_{24}\{^{13}C_6\}H_{38}O_{14}]^{2-}$); **HRMS (-ESI)**: *m/z* calcd. for $[C_{24}\{^{13}C_6\}H_{39}O_{14}]^-$ 629.2533, found 629.2536.

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# Chapter 3 – Steroid Bis(sulfates)

## 3.1. Foreword

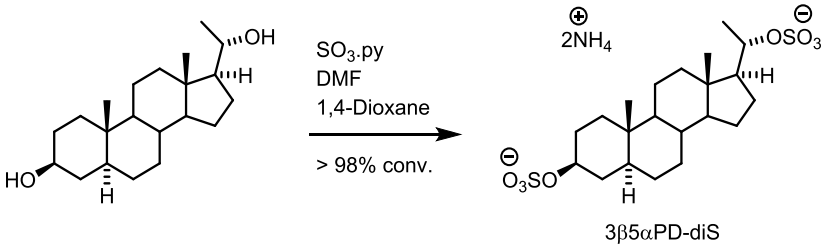
At the time this thesis was written, the following manuscript below had been accepted for publication in “Journal of Molecular Endocrinology”. This publication describes the use of steroid bis(sulfate) metabolites as markers in the prenatal diagnosis of disease. Permission has been granted by BioScientifica Ltd. via RightsLink for the reproduction of this publication within this thesis (License number: 4306480240229). This publication was authored by Dr. Oscar J. Pozo, Mr. Josep Marcos, Mr. Olha Khymenets, Mr. Andy Pranata, Mr. Christopher C. Fitzgerald, Associate Professor Malcolm D. McLeod and Dr. Cedric Shackleton. This publication was produced through the contributions of all authors, and was coordinated by Dr Oscar J. Pozo. Specific contributions of Mr. Andy Pranata were listed below:

- The synthesis, purification, and characterisation of 5α-pregnane-3β,20*S*-diol bis(sulfate), ammonium salt reference material.
- The synthesis and characterisation of 21-hydroxypregnenolone bis(sulfate), ammonium salt reference material.

In general, the steroid bis(sulfate) metabolites in the article were only identified through a process of small scale synthesis and LC-MS analysis of reference materials with the absence of detailed spectroscopic data. The synthesis and spectroscopic characterisation of bis(sulfate) reference materials reported below provided additional certainty regarding the identity of the reported bis(sulfate) metabolites. The two bis(sulfate) reference materials were prepared and sent overseas to the collaborators to allow direct comparison with urinary metabolites as part of the prenatal diagnosis study.

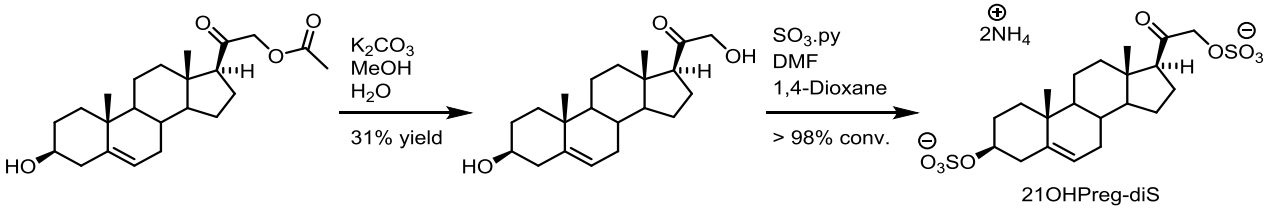
The synthesis of 5α-pregnane-3β,20*S*-diol bis(sulfate), ammonium salt was performed by a one-step sulfation reaction of 5α-pregnane-3β,20*S*-diol. The procedure was adapted from previous literature methods [32]. 5α-Pregnane-3β,20*S*-diol was reacted with sulfur trioxide pyridine complex and purified by solid phase extraction (SPE) using C18 cartridge (Scheme 4, Section 3.3.3). This purification method was different from the literature that used a WAX cartridge but was adopted as it was more cost effective and could be conducted on larger scale (~10 mg). It is important to note that no starting steroid diol remained after the sulfation reaction and no monosulfates products were observed, thus C18 cartridge could be used for the purification. The first wash used during the purification method was saturated aqueous ammonia solution in water (5% v/v). This eluent exchanged the counter-ions of the

bis(sulfate) reference material from the pyridinium to the ammonium salt.




Scheme 4. Synthesis of 5α-pregnane-3β,20*S*-diol bis(sulfate), ammonium salt

The synthesis of 21-hydroxypregnenolone bis(sulfate), ammonium salt was achieved by the hydrolysis of 21-acetoxypregnenolone to give 21-hydroxypregnenolone followed by the one-step sulfation reaction (Scheme 5). A mild basic hydrolysis reagent, potassium carbonate in methanol, was used and successfully gave the desired 21-hydroxypregnenolone (Section 3.3.4) [33]. The subsequent sulfation reaction was straightforward. Sulfur trioxide pyridine complex was used as reported in a literature procedure [32] and C18 cartridge was again employed for purification.




Scheme 5. Synthesis of 21-hydroxypregnenolone bis(sulfate), ammonium salt

## 3.2. Steroid sulfation pathways targeted by disulfates determination. Application to prenatal diagnosis.

**Steroid sulfation pathways targeted by disulfates determination. Application to prenatal diagnosis**

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**Short title**

Steroid disulfates in prenatal diagnosis

**Keywords**

Steroid bis-sulfates, steroid disulfates, steroid sulfation, prenatal diagnosis, LC-MS/MS, steroid metabolomics

**Corresponding author: Oscar J. Pozo**

**Word count of the full article** 4200

**ABSTRACT**

The steroid disulfates (*aka* bis-sulfates or bis(sulfates)) are a significant but minor fraction of the urinary steroid metabolome that have not been widely studied because major components are not hydrolyzed by the commercial sulfatases commonly used in steroid metabolomics. In early studies, conjugate fractionation followed by hydrolysis using acidified solvent (solvolysis) was used for the indirect detection of this fraction by GC-MS. This paper describes the application of a specific LC-MS/MS method for the direct identification of disulfates in urine, and their use as markers for the prenatal diagnosis of disorders causing reduced estriol production: STSD (Steroid Sulfatase Deficiency), SLOS (Smith-Lemli-Opitz Syndrome) and PORD (P450 Oxido-Reductase Deficiency). Disulfates were detected by monitoring a constant-ion-loss (CIL) from the molecular di-anion. While focused on disulfates, our methodology included an analysis of intact steroid glucuronides and monosulfates because steroidogenic disorder diagnosis usually requires an examination of the complete steroid profile. In the disorders studied, a few individual steroids (as disulfates) were found particularly informative: pregn-5-ene-3β,20*S*-diol, pregn-5-ene-3β,21-diol (STSD, neonatal PORD) and 5α-pregnane-3β,20*S*-diol (pregnancy PORD). Authentic steroid disulfates were synthesized for use in this study as aid to characterization. Tentative identification of 5ξ-pregn-7-ene-3ξ,20*S*-diol and 5ξ-pregn-7-ene-3ξ,17,20*S*-triol disulfates was also obtained in samples from SLOS affected pregnancies. Seven ratios between the detected metabolites were applied to distinguish the three selected disorders from control samples. Our results show the potential of the direct detection of steroid conjugates in the diagnosis of pathologies related with steroid biosynthesis.

## INTRODUCTION

From the earliest days of steroid metabolomics, the principal conjugated forms of steroids (sulfates and glucuronides) have been hydrolyzed prior to analysis, and for decades the instrument of choice for steroid separation and measurement has been GC-MS (Shackleton and Marcos 2006). While this technique remains the gold-standard for steroid profiling, LC-MS/MS has been increasingly adopted because of the simplified sample preparation and speed of analysis, mainly provided by absence of a derivatization step. This is in spite of the poor ionization for fully reduced steroids by electrospray (ESI) (Pozo, et al. 2007). While an advance, this methodology still retains the most time-consuming step of sample preparation, the enzymatic or chemical hydrolysis of conjugates (Gomes, et al. 2009). Hydrolysis itself can take several hours and requires a further solid phase extraction (SPE). Necessary chemical derivatization for GC-MS can also take hours.

Intact steroid conjugates have been analyzed by mass spectrometry since the introduction of particle beam ionization (e.g. Fast Atom Bombardment, FAB) in the 1980s (Shackleton and Straub 1982; Shackleton 1983). Their spectra have dominant deprotonated molecules $[M-H]^-$ in negative ion mode allowing ease of mass determination. Conjugate analysis was simplified with the introduction of electrospray ionization (ESI) and incorporation of HPLC and MS/MS. Glucuronides can be analyzed in both positive and negative ionization modes by monitoring $[M+NH_4]^+$ and $[M-H]^-$ respectively (Fabregat, et al. 2013). In the case of monosulfates, collision-induced-dissociation (CID) of the strong $[M-H]^-$ ions shows a distinctive hydrogen sulfate ($HSO_4^-$) fragment at *m/z* 97 (Shackleton 1983; Galuska, et al. 2013). Direct detection of steroid conjugates also circumvents the ionization problems of reduced steroids (Pozo, et al. 2007) as phase II metabolites have readily ionized functionality (i.e. a carboxylic acid in glucuronides and an acidic sulfate ester in sulfates).

While mono-conjugates dominate the sulfate fraction of urinary steroids, it has been known since the 1960s that disulfates (diS, also referred to as bis(sulfates) or bis-sulfates to distinguish them from compounds containing the disulfate ($S_2O_7^{2-}$) unit) are significant components of the metabolome (Pasqualini and Jayle 1962; Arcos and Lieberman 1967; Shackleton, et al. 1968a; Shackleton, et al. 1968b; Jänne, et al. 1969). Early studies by GC-MS of separated conjugate fractions showed that, in addition to the classic 3β-sulfated steroids, hydroxyls at positions 16β-, 17-(α and β) and 18- in androgens and 20- and 21- in pregnanes were prone to sulfation (Jänne, et al. 1969; Jänne and Vihko 1970; Laatikainen, et al, 1972; Meng and Sjövall 1997).

Since these original studies, disulfates have been a largely ignored component of the metabolome that nevertheless had significant potential to expand the understanding of steroid biosynthetic and metabolic pathways. Given this, we sought to develop LC-MS/MS methodology to target this group. It was found that constant-ion-loss (CIL) of hydrogen sulfate ($HSO_4^-$) fragment at *m/z* 97 from the molecular di-anion $[M-2H]^{2-}$ was the most useful reaction to monitor (McLeod, et al. 2017).

The ease of steroid disulfate analysis led us to investigate their use in diagnosis of steroid biosynthetic disorders. One particular area of interest to the authors has been the pre-natal diagnosis of single-gene disorders of estriol (E3) synthesis by urine analysis, of which we have studied three conditions by GC-MS, viz., Steroid Sulfatase Deficiency (STSD), Smith-Lemli-Opitz Syndrome (SLOS, 7-dehydrosterol reductase deficiency) and cytochrome P450 Oxido-Reductase Deficiency (PORD) (Marcos et al. 2009; Shackleton, et al. 2004a; Shackleton et al. 2004b; Arlt et al. 2004; Reisch, et al. 2013; Shackleton et al. 2007). This communication offers our preliminary observations of the disulfated steroids excreted in these disorders at around mid-pregnancy. While focusing on disulfates, selected monosulfates and glucuronides were also included; evaluating the complete steroid profile is crucial to diagnosing aberrant steroid biosynthesis (Shackleton and Marcos 2006).

## MATERIALS AND METHODS

### *Reagents and chemicals*

Steroid starting materials were obtained from Steraloids (Newport, RI, USA). Chemicals and solvents including sulfur trioxide pyridine complex ($SO_3$·py), *N*,*N*-dimethylformamide (DMF) and ammonium formate (HPLC grade) were purchased from Sigma-Aldrich (St Louis, MO, USA). Aqueous ammonia solution (25%), and acetonitrile and formic acid (LC-MS grade) were from Merck (Darmstadt, Germany). MilliQ water was obtained using a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

### *Synthesis of reference steroid disulfates*

The qualitative synthesis of steroid disulfates as the ammonium salts was performed as previously described (McLeod, et al. 2017) with small modifications. Briefly, 1 mg of each steroid standard was directly dissolved in a freshly prepared solution of $SO_3$·py complex (20 mg, 124 µmol, ~38 eq/steroid or ~19eq/hydroxyl group) in DMF (100 µL) and incubated at room temperature for 72 hours. The success of synthesis was confirmed by analysis of reaction using both LC-MS in scan mode and LC-MS/MS for collision induced dissociation studies. The purification of synthesised disulfates was performed using SPE as previously described (McLeod et al, 2017).

Steroid disulfate reference materials isolated as the corresponding ammonium salts and used in this study included: 5α-pregnane-3β,20*S*-diol bis(sulfate), (3β5αPD-diS); 3β,21-dihydroxypregn-5-en-20-one bis(sulfate), (21-hydroxypregnenolone bis(sulfate), 21OHPreg-diS); androst-5-ene-3β,17α-diol bis(sulfate), (5AD(17α)-diS); androst-5-ene-3β,17β-diol bis(sulfate), (5AD(17β)-diS); 3β,16α-dihydroxyandrost-5-en-17-one bis(sulfate), (16α-hydroxydehydroepiandrosterone bis(sulfate), 16αOHDHEA-diS); 3β,16β-dihydroxyandrost-5-en-17-one bis(sulfate) (16β-

hydroxydehydroepiandrosterone bis(sulfate), 16βOHDHEA-diS); pregn-5-ene-3β,17α,20*S*-triol 3,20 bis(sulfate), (5PT-diS); pregn-5-ene-3α,20*S*-diol bis(sulfate), (5PD-diS); 5β-pregnane-3β,20*S*-diol bis(sulfate); 5β-pregnane-3α,20*S*-diol bis(sulfate); 5α-pregnane-3α,20*S*-diol bis(sulfate); 5β-pregnane-3β,20*R*-diol bis(sulfate), 5α-pregnane-3β,20*R*-diol bis(sulfate); 5β-pregnane-3α,20*R*-diol bis(sulfate). In this manuscript the IUPAC terms for the 20-hydroxypregnane diastereomers are used, S and R, in some publications often trivialized to α and β, respectively.

Two reference materials (3β5αPD-diS and 21OHPreg-diS), were prepared on larger scale and subjected to characterisation by spectroscopic methods. Experimental details and characterization data for these new compounds, together with copies of the $^{1}$H NMR, $^{13}$C NMR, and ESI LRMS spectra are available from the authors (MM).

***Urine Samples***

One of our laboratories (Children's Hospital Oakland, Dr. Cedric Shackleton) has been the recipient for urine samples from patients with suspected abnormal steroidogenesis in an attempt to characterize the defects. The studies were approved by the Children's Hospital Institutional Review Board (IBR#2010-038)). Many of the samples used in this study were remnants of those sent to the laboratory for investigation of low pregnancy estriol (generally defined as individuals with serum unconjugated estriol < 0.3 MoM, multiples of median). Other samples were from women who had had a previously affected SLOS child or other symptomatic reasons for concern regarding steroidogenesis. The samples have generally been collected between week 16 and 30 of gestation. They have been stored frozen at -20 °C. Eleven STSD samples were analyzed, and six samples from SLOS affected pregnancies. The neonatal PORD samples were collected at 7, 18 and 23 days. Urine samples from unaffected pregnancies were from a collection held by IMIM (Institut Hospital del Mar

d'Investigacions Mèdiques, Barcelona). Normal neonatal urine specimens were from a control urine collection at the Institute of Metabolism and Systems Research (IMSR), University of Birmingham UK.

***Sample treatment***

Urine extraction was by C18 SPE. Generally, a 2 mL aliquot of urine was passed through a pre-conditioned cartridge. After a washing step with 3 mL water, steroid conjugate analytes were eluted using 2 mL of methanol. After evaporation of a 200 µL aliquot of the elution solvent, the extract was reconstituted in 100 µL of water and 5 µL was injected into the UHPLC-MS/MS system. Stably labelled 17-S$\{^{18}O\}_3$-5α-androstane-3α,17β-diol 3,17-bis(sulfate) and 17-S$\{^{18}O\}_3$-5α-androstane-3β,17β-diol bis(sulfate) were used as internal standards. The labelled sulfate residue was introduced to the steroidal diol mono-sulfate using labelled S$\{^{18}O\}_3$.py generated in situ from labelled sulfuric acid (95% atom) and acetic anhydride in pyridine. Experimental details and characterization data for these internal standards, together with copies of the $^{1}H$ NMR, $^{13}C$ NMR, and ESI LRMS spectra are available from the authors (MM)

***UHPLC-MS/MS analysis***

*Disulfates*

The study was carried out using a triple quadrupole (XEVO TQ-S micro) mass spectrometer equipped with an ESI source and interfaced to an Acquity UPLC system for the chromatographic separation (all from Waters Associates, Milford, MA, USA). Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set to approximately 1200 L/h, and the cone gas flow was 50 L/h. A cone voltage of 30 V and

a capillary voltage of 0.4 kV were used in negative ionization mode. The nitrogen desolvation temperature was set to 600 °C, and the source temperature was 150 °C.

The UHPLC separation was performed using an Acquity UPLC CSH Phenyl-Hexyl column (2.1 × 100 mm i.d., 1.7 µm) (Waters Associates), at a flow rate of 300 µL/min. Water and acetonitrile:water (9:1) both with formic acid (0.01% v/v) and ammonium formate (25 mM) were selected as mobile phase solvents. A gradient program was used; the percentage of organic solvent was linearly changed as follows: 0 min, 15%; 0.5 min, 15%; 25 min, 30%; 26 min, 100%; 27 min, 100%; 28 min, 15%; 30 min, 15%. The total analysis time was 30 min.

For the constant ion loss (CIL) scan, dwell times of 6 ms and collision energies of 15 eV were selected for each ion transition. Due to the molecular masses of steroid hormones and metabolites (250-400 Da), the precursor ions of disulfates ($[M-2H]^{2-}$) were restricted to the range from *m/z* 199 to *m/z* 274. A Selected Reaction Monitoring (SRM) approach containing 75 preselected transitions was used for the simultaneous detection of steroid disulfates. Among them, the transition 228→359 corresponded to the internal standards used in the analysis.

*Monoconjugates*

While the focus has been on steroid disulfates we have acquired data on steroid monosulfates and glucuronides previously reported as relevant for the studied disorders. Based on previous studies (Gomez, et al. 2014) the product ions at *m/z* 97 and *m/z* 75 for sulfates and glucuronides respectively were chosen (Table 1). Exceptions were estriol conjugates due to the influence of the aromatic ring. The neutral loss of the conjugate (80 Da and 176 Da for sulfates and glucuronides respectively were detected).

*Quantification*

For this study, accurate quantitative measurements have not been conducted for two reasons: 1) lack of some authentic compounds prevented the determination of relative responses of analyte transitions to internal standard transitions; 2) the urine samples were random “spot” collections and not accurate 24 h collections. Instead, we have determined “diagnostic-ratios” from raw mass spectrometric transition responses. These ratios are of an analyte known to be *overproduced* to one known to be *underproduced* in a particular disorder. Such ratios have long been used in GC-MS analysis (Shackleton and Marcos,2006).

## RESULTS AND DISCUSSION

### *Method development*

This communication applies recent LC-MS/MS studies on steroid disulfate analysis using the constant-ion-loss (CIL) from the di-anionic precursor $[M\text{-}2H]^{2-}$ (McLeod, et al. 2017). The method was developed for untargeted detection, and designed for the analysis of a maximum number of natural disulfates. The use of this precursor ion and the fact that the product ion has a higher *m/z* value is unusual for small molecules. Determination of disulfates under these conditions gives clean chromatograms and the main interferences observed in the chromatograms are due to the relatively high natural abundance of the $^{34}S$ isotope (4.25%). The transition coming from the *m/z* 97 loss from an unsaturated ($\Delta^4$, $\Delta^5$, etc.) $\{^{34}S\}_1$-disulfate isotope is completely indistinguishable from the one coming from an A-ring reduced steroid disulfate.

To maximize isobaric steroid metabolite separation (e.g. pregnenediol-diS, the pregnanediol-disulfates and the androstenediol-disulfates) in this study, a phenyl-hexyl column with a relatively high amount of ammonium formate (25 mM) was required to obtain sharp and well resolved chromatographic peaks. Column temperature was critical for this purpose with 30 °C determined as optimum. Under these conditions, a 25 min gradient from 15% to 30% of organic solvent provided desired separation (Figure 1A).

Under optimized conditions the elution order of disulfates was dihydroxyandrostanones < dihydroxypregnanones < androstenediols < pregnanediols. In a specific group, 17β hydroxysteroid disulfates eluted earlier than their 17α-counterparts and 20*S*-hydroxysteroid disulfates eluted earlier than their 20*R* counterparts. Regarding A ring derivatives, $\Delta^5$ steroid disulfates eluted before the fully reduced metabolites, the elution order of the reduced steroids being 3β,5β < 3β,5α < 3α,5β < 3α,5α. The chromatographic conditions were also able to separate

the two estriol glucuronide isomers i.e. the 16-glucuronide and 3-glucuronide. Unfortunately, sulfate and glucuronide conjugates of two useful steroids in PORD diagnosis, androsterone and etiocholanolone, could not be separated under the selected conditions even after increasing the gradient to 1 h (Figure 1B).

***Application to prenatal detection of disorders affecting estriol synthesis***

We report preliminary studies to determine whether steroid disulfates in urine can be useful markers in the prenatal detection of disorders affecting estriol synthesis; until now only monoconjugates had been used. The background to this study being that unconjugated serum E3 is frequently measured at mid-pregnancy as a marker for Down's syndrome as part of a test called triple- or quad- marker screening (Haddow, et al. 1994). If results are low the question remains as to the reason, and our original research was directed to diagnosis of Smith-Lemli-Opitz Syndrome (SLOS), the clinically most severe cause of low E3 (Shackleton, *et al*. 2007). These studies led to investigation of other causes such as STSD and PORD.

Diagnostic ratios are frequently employed in steroid metabolomics and E3 frequently used as denominator. Dominant E3 conjugates are 3- and 16-glucuronides (30% and 60%, respectively) with about 2.5% as monosulfate and estriol-3-glucuronide-16-sulfate (6.5%) (Tikkanen, *et al*. 1973). We assessed E3 excretion from the measurement of glucuronide and monosulfate conjugates (Table 1).

*Steroid sulfatase deficiency (STSD) (OMIM , 308100, location, Xp22.31)*

This X-linked disorder prevents the release of steroid from steroid sulfates. A summary of the biosynthetic pathway leading to estriol is shown in Figure 2, illustrating that inactivity of the enzyme in placenta prevents 16$\alpha$OHDHEA-S conversion to E3. This fetal 16$\alpha$OHDHEA-S, androst-5-ene-3$\beta$,16$\alpha$,17$\beta$-triol sulfate

(5AT-S) and other steroid sulfates pass through the placenta and mother to be excreted in urine largely unchanged (Taylor and Shackleton, 1979).

STSD urine samples (N=11) and 11 controls were analyzed using the CIL scan method for disulfates complemented with the acquisition of 16αOHDHEA-S and E3 monoconjugates (Table 1). Among the disulfates measured by the CIL method, we found that the response ratio between six of them, namely 16αOHDHEA-diS, 5AD(17α)-diS, 5AD(17β)-diS, 5PT-diS, 21OHPreg-diS and 5PD-diS against E3 glucuronide (measured as sum of 3- and 16-glucuronides) was markedly increased in STSD.

Representative chromatograms of a normal pregnancy urine and one with an STSD affected fetus are shown in Figure 3. The ratio values for our normal and STSD data sets are shown in Figure 4A and show all analytes clearly distinguish STSD from normal. Additionally, we used the ratios to evaluate the relative efficacy of each analyte in diagnosis. The best steroid discriminatory ratio would show greatest difference between the lowest steroid sulfate/E3-G ratio value in STSD, and the highest ratio found in controls (Figure 4B). Interestingly, the ratios that gave the greatest differential were $\Delta^5$ pregnenes; 5PD-diS, 5PT-diS and 21OHPreg-diS, not the $C_{19}$ steroid sulfates on the direct biosynthetic pathway to E3. Combining ratio data can give an even greater separation for normal and affected; note the combined data for 21OHPreg-diS and 5PD-diS in Figure 4C. Such pregnene metabolites should be incorporated in MS based methodologies for detection of the disorder.

*P450 oxido-reductase deficiency (PORD) (POR OMIM 124015 location: 7q11.23)*

Several pregnant women carrying PORD fetuses have been studied by GC-MS (Shackleton, et al. 2004; Reisch, et al. 2013), but for only two were samples available for this study. Shackleton and co-workers (2004a) deduced that the dominant “feto-

placental" maternal urinary steroid in PORD pregnancies was 3β5αPD-diS. While this steroid is also present in the disulfate fraction of normal pregnancy urine it is in much greater amount in PORD affected pregnancies. From its dominance together with reduced E3, it was concluded that this metabolite is a maternal excretory product of fetal pregnenolone. An intermediate precursor would be fetal steroid 5PD-diS. Excess pregnenolone and its sulfate are the result of an apparent "block" in 17-hydroxylase/C17-20 lyase secondary to attenuated POR activity (Figure 5). This block, together with suppressed 16$\alpha$-hydroxylase (also due to PORD) causes reduction of fetal 16αOHDHEA-S production leading to low maternal E3 production and excretion. The precise sequence of reactions from fetal pregnenolone to 3β5$\alpha$PD-diS, and localities of the conversions (fetal adrenal, liver, placenta and mother) is yet to be determined. The process is multi-step, probably including placental 3β-desulfation and likely 3β-hydroxysteroid dehydrogenase/isomerase. It has long been known that both 3β5$\alpha$PD-diS and 5PD-diS are prominent disulfates in umbilical cord blood (Laatikainen, et al. 1972) so are freely synthesized and transported in the feto-placental unit. The synthetic sequence for pregnenolone conversion to urinary metabolites in normal and PORD affected pregnancies and neonate are shown in Figure 5. Evidence suggests the corresponding conversion of pregnenolone sulfate to DHEA-S is not an available pathway (Neunzig, et al. 2014; Sanchez-Guijo, et al. 2016; Rege, et al. 2017).

Besides the increased excretion of 3β5$\alpha$PD-diS, we also observed an increase in the transitions corresponding to 5PD-diS and 3β,21-dihydroxy-5α-pregnan-20-one disulfate (21OHPreg3$\beta$5$\alpha$-diS), the latter in spite of a likely POR requirement by fetal 21-hydroxylase. However, it should be noted that this fetal enzyme differs from that coded by CYP21A2 required in cortisol synthesis (Guerami et al., 1988, Corsan, Macdonald and Casey., 1997).

In Figure 6 we illustrate the chromatographic profiles of the 3β5αPD-diS, 5PD-diS and 21OHPreg3β5α-diS and the E3 conjugates in a control (Figure 6A) and affected pregnancy (Figure 6B). The dominance of the 3β5αPD-diS in the affected pregnancies is striking. In GC-MS diagnosis of PORD prenatally the ratio of 3β5αPD/E3 was used, i.e. the ratio of principal PORD fetal metabolite to E3, the conventional feto/maternal metabolite. In Figure 6C are shown ratios for intact conjugates in PORD and controls. For the denominator (E3) we summed the total of both glucuronylated forms.

One of the GC-MS prenatal diagnostic ratios for PORD remains a challenge for LC-MS/MS under conditions developed for this study. With fetal PORD there is increased androsterone production as a result of the "alternative pathway" activity (Arlt, et al. 2004) resulting in markedly increased androsterone/etiocholanolone ratio (Shackleton et al 2004a). That ratio should theoretically be determined by direct analysis of glucuronides and this separation has been already reported by C18 columns both in glucuronides (Pozo et al. 2008) and unconjugated (Marcos and Pozo, 2016). Unfortunately, under current chromatographic conditions developed for the disulfates such isobaric monoconjugates (sulfates or glucuronides) could not be resolved.

*Postnatal detection of PORD:*

While this paper has focused on prenatal diagnosis of PORD by 3β5αPD-diS measurement, Shackleton and co-workers (Shackleton et al. 2004b) have shown that its precursor 5PD-diS is a key analyte in diagnosing the condition in the first months of life suggested its inclusion here. In PORD neonatal samples this steroid is dominant, excretory values exceeding the classical major metabolites such as 16αOHDHEA-S and 16α-hydroxypregnenolone sulfate whose biosynthesis by 16α-hydroxylation is also

POR dependent. In the first weeks of life the fetal zone of the adrenal is still dominant, but diminishing, and is responsible for producing a large amount of 3β-OH-$\Delta^5$ steroids.

Figure 7 illustrates the separation of steroid disulfates in an affected PORD infant and normal infant. We have included 16αOHDHEA-S as analyte to act as denominator for a potential diagnostic ratio 5PD-diS/16αOHDHEA-S. This ratio is shown for three affected infants and normal controls in Figure 7C, clearly defining the condition. Interestingly, one of the first steroid disulfates to be identified in the neonatal period were 5AD(17$\alpha$ and 17$\beta$)-diS (Shackleton, et al. 1968a, Laatikainen, et al. 1972), and 16βOHDHEA-diS (Shackleton, et al. 1968b, Laatikainen, et al. 1972) and these are clearly separated with this methodology (Figure 7).

*Smith-Lemli-Opitz Syndrome. "7-dehydrosterol reductase" deficiency. (SLOS) (OMIM 602858* location: 11q13,4)

This condition is caused by deficiency in 7-dehydrosterol reductase and the notable feature is a build-up of 7- and 8-dehydrocholesterol, which can be used to diagnose the condition when measured in amniotic fluid (Kelley,1994). The affected fetus can use these sterols as steroid precursors, resulting in the appearance in maternal urine of dehydro (DH) versions of common natural steroids. For instance, 5β-pregn-7(and 8)-ene-3α,17α,*20S*-triol, (7(8)-DHPT) and an estriol equivalent, principally 8-dehydroestriol (8-DHE3) (Guo, et al. 2001, Shackleton, et al. 1999). The biosynthesis of steroids in SLOS pregnancy is illustrated in Figure 8.

SLOS steroids are mainly excreted as glucuronides. Thus, distinct peaks corresponding to different isomers of 8-DHE3-G were found in all SLOS samples (Figure 9). On the other hand, the detection of 7(8)-DHPT-G provided more difficulties due to endogenous interferences probably coming from other pregnenetriols and DH-hydroxypregnenolones which would share the same transition (Figure 9).

A systematic study has not been made of steroid sulfates in this condition and lack of appropriate authentic compounds has meant that only *candidate* chromatographic peaks were provisionally identified. Such peaks were chosen by having the expected CIL transitions for steroids with additional unsaturation and to be accepted as SLOS-specific "candidate" analytes these peaks had to be present in all six confirmed SLOS pregnancies, and be absent from controls. Two main metabolites were found. Peaks with the expected transitions for DHPT-diS and DHPD-diS were observed in all SLOS samples. Additionally, other minor metabolites such as DH-androstenediol-diS were also found. In Figure 9 the chromatograms of the proposed steroid disulfate analytes with DH-pregnanetriol glucuronide and DHE3-glucuronide are shown. Little information can be stated on stereochemistry of candidate analytes; not only that of 3- and 5-positions but both $\Delta^7$ and $\Delta^8$ isomers are likely present. The chromatograms illustrated were from one affected pregnancy and one control. Similar chromatograms were produced for the other five affected pregnancies and controls. Clearly this is the most challenging of the three conditions for conjugate LC-MS/MS analysis although aberrant steroid conjugate peaks definitive for SLOS were detected.

*Distinguishing the disorders: summary*

This study has focused on the mass spectrometric analysis of steroid disulfates, but steroid monosulfates and glucuronides have been included where required to determine ratios used for diagnosis. In order to evaluate the potential of the approach based on the combined screening of glucuronides, monosulfates and disulfates, we propose a panel of markers able to differentiate between the selected disorders and control samples. We found that using the ratios 16αOHDHEA-S/E3-G, 3β5αPD-diS/E3-G, 5PD-diS/16αOHDHEA-S, 21OHPreg-diS/E3-G, 21OHPreg-diS/16αOHDHEA-S, 8DHE3-G/E3-G and DHPT-diS/E3-G allowed for the successful differentiation between the controls and the different disorders

Hopefully the study emphasizes the potential of LC-MS analysis of all conjugate types in future development of steroid metabolomics.

***General discussion***

The steroid disulfates are a minor fraction of the urinary steroid metabolome, but may provide significant markers of aberrant steroid biosynthesis. As a family, intact steroid disulfates have not been recently subject to detailed study due a lack of suitable analytical methodology. In the past, studyingthis family always involved time-consuming fractionation of free and conjugate families followed by solvolysis and GC-MS analysis. Most of the available literature stems from the 60's and 70's and it was shown early which secondary positions (assuming the primary sulfated position is the 3-hydroxyl) could be sulfated. These were 17 ($\alpha$-and $\beta$), 16$\beta$- and 18 in $C_{19}$ steroids and 20*S*- and 21- in $C_{21}$ steroids. During that early research period the dominant biological materials chosen to study were associated with pregnancy. In that respect our current studies have followed this lead and the major disulfate components reported here were also noted in the early publications (Shackleton, et al. 1968a; Shackleton, et al. 1968b; Jänne, et al. 1969; Jänne and Vihko 1970; Laatikainen et al 1972; Meng and Sjövall 1997).

There is little definitive evidence as to which sulfotransferases are responsible for the secondary sulfation (Mueller et al 2015), and how disulfates are transported (Grosser et al 2017). Available sulfation evidence points solely to SULT2A1 which appears to have an active site capable of encompassing a wide variety of steroid substrates (both free and monosulfated) and conduct sulfation at either end of the steroid molecule. Thus, it can sulfate free steroids or steroid monosulfates (Cook, et al. 2009).

A question remains as to whether disulfation is purely a catabolic reaction or if such steroids could be transportable reservoirs of active hormone precursors, as is likely the case for DHEA and estrone sulfates. Guerami and co-workers (1988) have

proposed that 21OHPreg-diS is an 11-deoxycorticosterone (DOC) precursor during pregnancy, particularly since circulating levels of this mineralocorticoid and its sulfate are increased during gestation (Corsan, Macdonald and Casey 1997). It is known that the placenta is capable of hydrolyzing 21-sulfates and the enzyme responsible is the usual STS as 21-desulfation does not occur in STSD (Guerami, et al. 1988). Another possible reservoir for disulfates is 5AD(17β)-diS, potentially a testosterone or estradiol precursor. This steroid is also subject to STS action in mammals. In contrast, it is believed that human sulfatases are inactive on 17α- ($C_{19}$ steroids) or 20*S*--sulfates, a situation shared with the commercial snail and mollusk enzymes used for hydrolysis in steroid analysis (Stevenson, et al. 2014).

In summary, we have provided analytical data on the steroid disulfates through their measurement as intact molecules by LC-MS/MS, employing CIL scan monitoring. We have attempted to use these additional members of the steroid metabolome to distinguish fetal disorders of steroid synthesis. To the best of our knowledge, this is the first time that direct analysis of steroid disulfates has proved its value for clinical diagnosis.

The ultimate goal of these studies is the ability to quantify the whole urinary steroid metabolome as unhydrolyzed conjugates, the monosulfates, disulfates, glucuronides and mixed sulfate-glucuronide conjugates. Studies of the plasma steroid metabolome should also be included. To achieve this goal will require the synthesis of a multitude of authentic steroids including appropriate internal standards and an improvement in chromatographic resolution.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported

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## FIGURE LEGENDS

**Figure 1**

Chromatographic separation obtained for (A) seven synthesized isomers of pregnanediol disulfates and (B) androsterone and etiocholanolone glucuronide. Note that the method optimized for the separation of isomeric disulfate metabolites was not able to separate epimeric glucuronides.

**Figure 2**

Steroid synthesis in STSD pregnancy starting from fetal adrenal pregnenolone. Inactivity of STS prevents conversion of 16αOHDHEA-S to estriol in placenta so the former (and its metabolites) is excreted as sulfates by mother. $C_{21}$ steroid sulfates upstream from 16αOHDHEA-S also pass the placenta without de-sulfation and are directly excreted in maternal urine.

**Figure 3**

Selected urinary $\Delta^5$ steroid sulfate and estriol conjugate analysis in a control and STSD affected pregnancy. Note the markedly increased $\Delta^5$ steroid mono and disulfates in STSD. Regarding the estriol conjugates it must be noted that glucuronides give lower MS transition responses than sulfates under the reported conditions. In reality, E3-S is a minor metabolite of estriol compared to the 3- and 16-glucuronides, although it appears contrary in the chromatograms.

**Figure 4**

Panel A: Ratios of steroid sulfates to E3 glucuronide (3+16) in STSD. The scale represents the ratios of raw peak areas of transitions, not the actual amount quantified. All ratios show separation of controls from affected pregnancies. Panel B: a measure of the difference between the lowest STSD ratio and highest control ratio. The higher this value, the greater the separation between affected and normal. Two $\Delta^5$ pregnene di-sulfates are the most efficacious diagnostic analytes. Panel C: combining data from 5PD-diS and 21OHPreg-diS increases discrimination between normal and STSD.

**Figure 5**

Steroid biosynthesis and metabolism in PORD and normal pregnancies and neonates. *Normal pregnancy:* fetal adrenal pregnenolone is converted to maternally excreted estriol conjugates (POR essential). *PORD pregnancy*: excess adrenal pregnenolone (due to PORD) is metabolized primarily to maternally excreted 3β5αPD-diS. *Normal neonate*: Excretion product 16αOHDHEA-S and other sulfates. *PORD neonate*: major pregnenolone excretory product 5PD-diS.

**Figure 6**

Selected Reaction Monitoring (SRM) transition chromatograms of estriol conjugates and steroid disulfates in control (A) and PORD affected pregnancies (B). Note high excretion of 3β5αPD-diS and 21OHPreg3β5α-diS. (C) Graph shows peak area ratios (analyte/E3-G) for 2 affected pregnancies and 11 controls.

**Figure 7.**

SRM chromatograms of control (A) and affected PORD babies (B). The key analyte is the pregnenolone metabolite 5PD-diS and its relative excess is determined by peak area ratio to 16αOHDHEA-S, normally a dominant metabolite in neonates. Discrimination obtained by the use of 5PD-diS/16αOHDHEA-S (C) and 21OHPreg-diS/16αOHDHEA-S (D) between healthy and PORD babies.

**Figure 8.**

Deficiency of 7-dehydrosterol reductase (DHCR7, SLOS] causes 7- or 8- dehydro-cholesterol to be used as fetal precursor for downstream steroids which retain B- ring unsaturation. Dehydro-pregnanetriol (DHPT) and dehydroestriol (DHE3) glucuronides have been used classically for diagnosis but here are candidate disulfates tentatively identified, compounds not seen in controls.

**Figure 9.**

SRM chromatograms of candidate analytes in SLOS pregnancy. (A) control pregnancy and (B) affected pregnancy. Transition chromatograms for known diagnostic steroid glucuronides and candidate disulfates useful for diagnosis. While authentic steroids are not available, these steroids, with appropriate transitions were only present in affected pregnancies. Steroid A/B ring stereochemistry including Δ7/Δ8 unsaturation is as yet unknown. In the control chromatograms E3 conjugates are shown but all SLOS candidate disulfates and glucuronides are absent.

**Table 1**. SRM parameters of selected steroids

| Analyte | Disorder | MW | Retention time (min) | Precursor ion (*m/z*) | Product ion (*m/z*) | Collision energy (eV) |
|---|---|---|---|---|---|---|
| *Monoconjugates* | | | | | | |
| E3-3G | all | 464 | 2.3 | 463 | 287 | 30 |
| E3-16G | all | 464 | 9.3 | 463 | 287 | 30 |
| E3-3S | all | 368 | 7.3 | 367 | 287 | 35 |
| 16OHDHEA-S | STSD/PORD* | 384 | 16.0 | 383 | 97 | 40 |
| DHE3-G | SLOS | 462 | 8.6/9.1 | 461 | 285 | 30 |
| DHPT-G | SLOS | 510 | 21.3 | 509 | 75 | 30 |
| *Disulfates* | | | | | | |
| 5AD(17α)-diS | STSD | 450 | 15.5 | 224 | 351 | 15 |
| 5AD(17β)-diS | STSD | 450 | 13.4 | 224 | 351 | 15 |
| 16αOHDHEA-diS | STSD | 464 | 10.0 | 231 | 365 | 15 |
| 16βOHDHEA-diS | STSD | 464 | 8.2 | 231 | 365 | 15 |
| 5PT-diS | STSD | 494 | 10.5 | 246 | 395 | 15 |
| 21OHPreg-diS | STSD/PORD | 492 | 18.2 | 245 | 393 | 15 |
| 5PD-diS | STSD/PORD* | 478 | 16.7 | 238 | 379 | 15 |
| 3β5αPD-diS | PORD | 480 | 17.6 | 239 | 381 | 15 |
| DH5AD-diS | SLOS | 448 | 11.4 | 223 | 349 | 15 |
| DHPT-diS | SLOS | 494 | 12.6 | 246 | 395 | 15 |
| DHPD-diS | SLOS | 478 | 15.6 | 238 | 379 | 15 |

* PORD neonatal

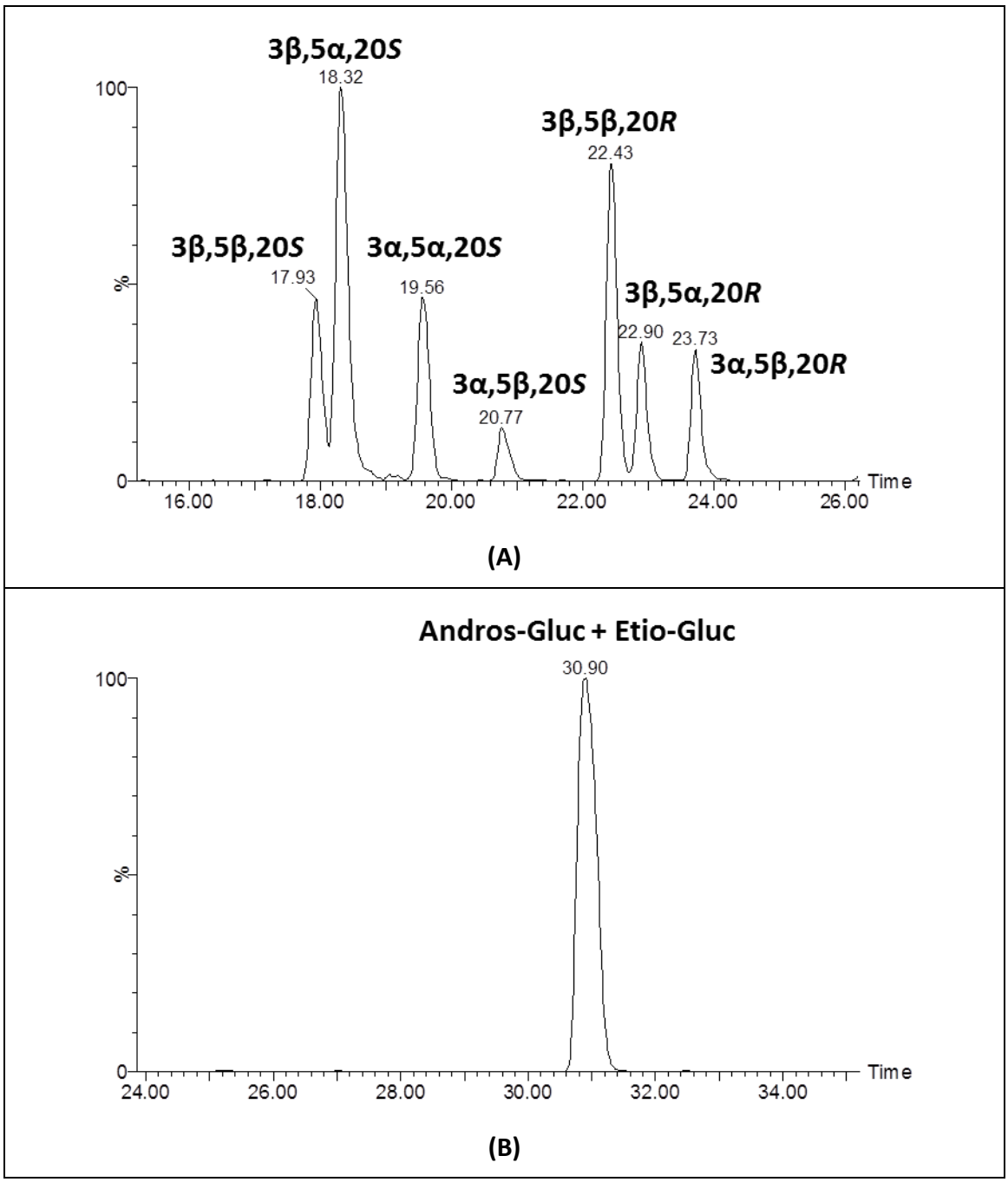
3β,5α,20S
18.32
3β,5β,20R
22.43
3β,5β,20S
17.93
3α,5α,20S
19.56
3β,5α,20R
22.90
23.73
3α,5β,20R
3α,5β,20S
20.77
100
%
0
16.00
18.00
20.00
22.00
24.00
26.00
Time
(A)
Andros-Gluc + Etio-Gluc
30.90
100
%
0
24.00
26.00
28.00
30.00
32.00
34.00
Time
(B)

**Figure 1**

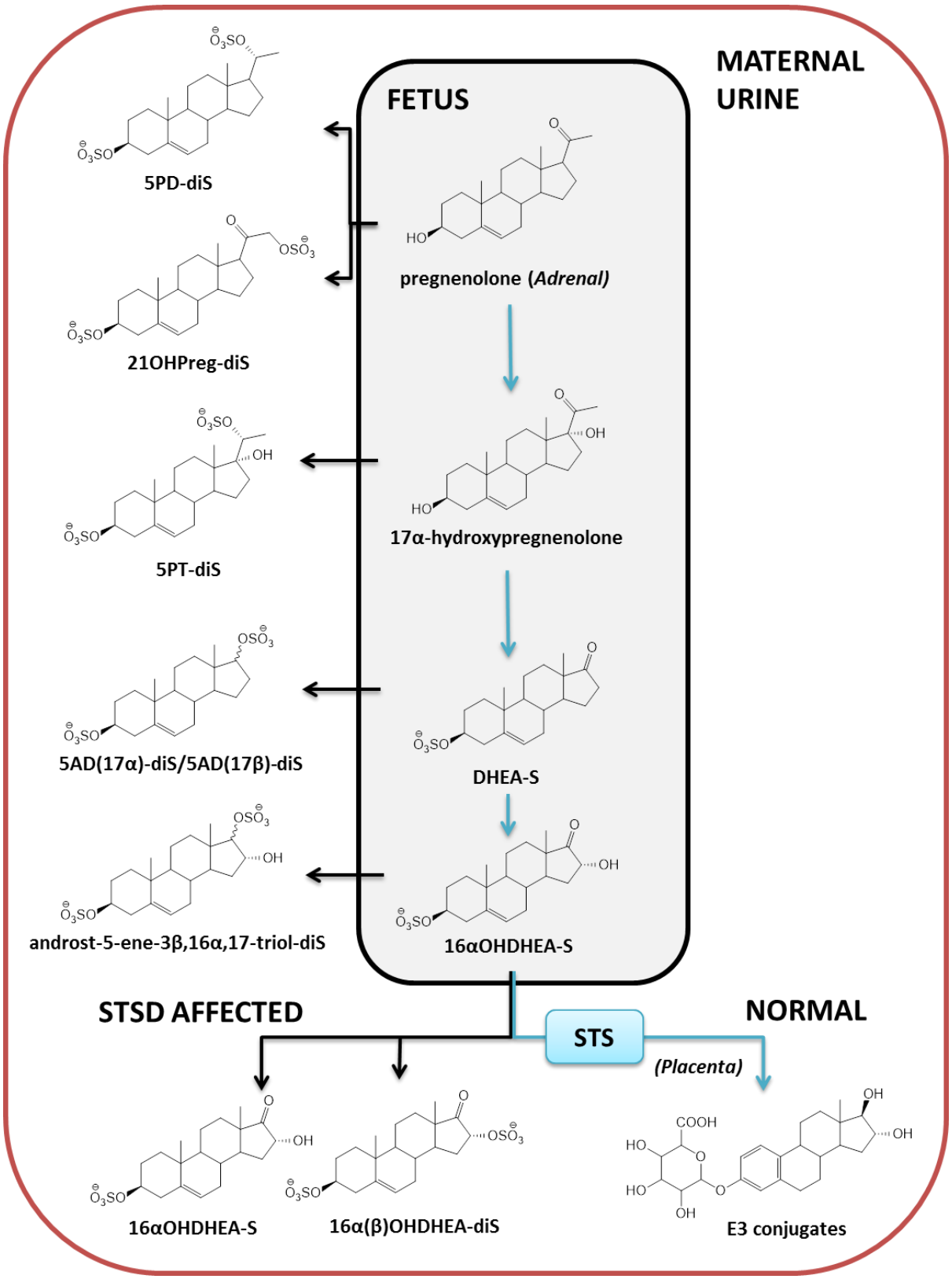
MATERNAL URINE
FETUS
5PD-diS
pregnenolone (Adrenal)
21OHPreg-diS
17α-hydroxypregnenolone
5PT-diS
DHEA-S
5AD(17α)-diS/5AD(17β)-diS
16αOHDHEA-S
androst-5-ene-3β,16α,17-triol-diS
STSD AFFECTED
NORMAL
STS
(Placenta)
16αOHDHEA-S
16α(β)OHDHEA-diS
E3 conjugates

**Figure 2**

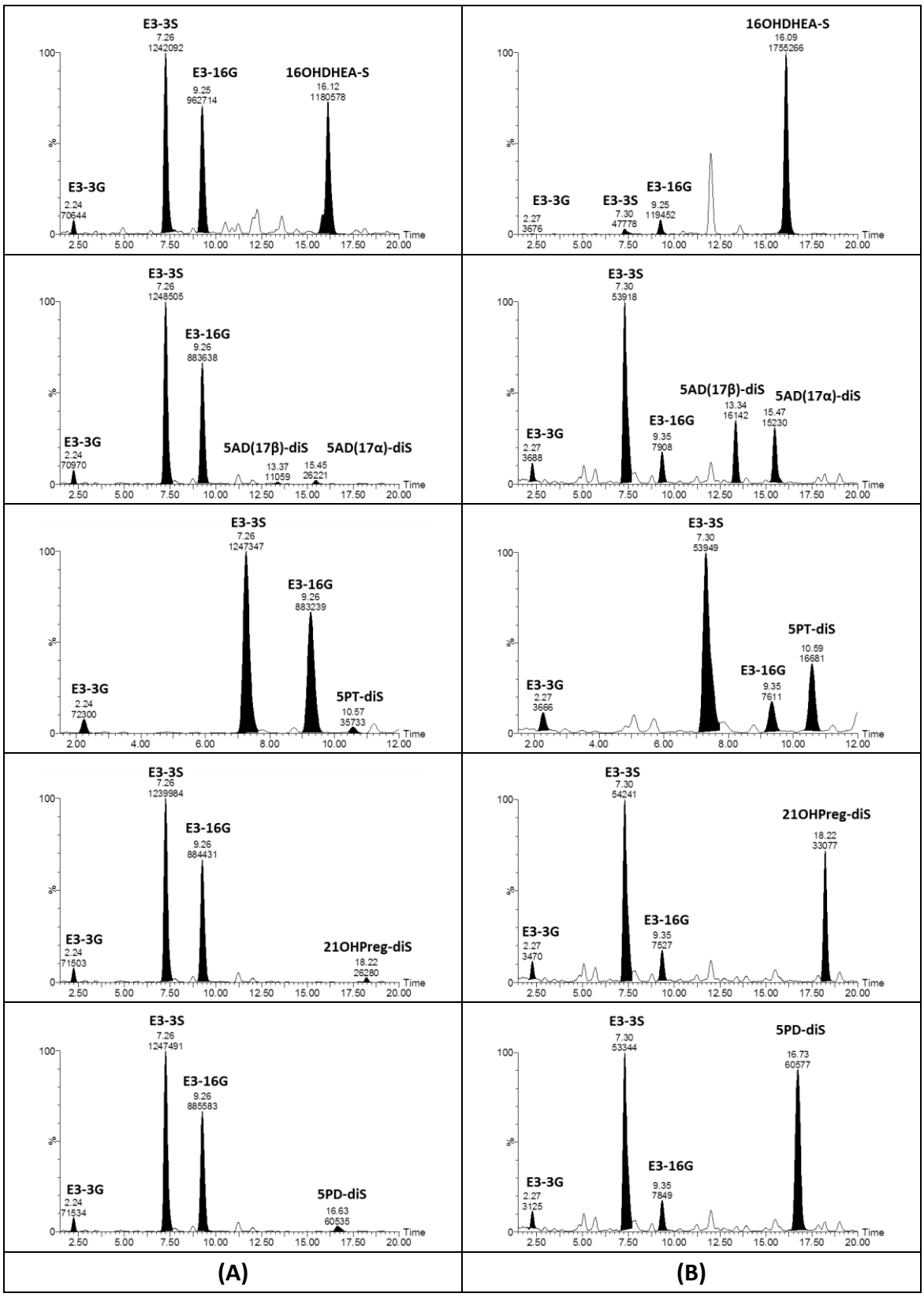
E3-3S
7.26
1242092
E3-16G
9.25
962714
16OHDHEA-S
16.12
1180578
E3-3G
2.24
70644
16OHDHEA-S
16.09
1755266
E3-3G
2.27
3676
E3-3S
7.30
47778
E3-16G
9.25
119452
E3-3S
7.26
1248505
E3-16G
9.26
883638
E3-3G
2.24
70970
5AD(17β)-diS
13.37
11059
5AD(17α)-diS
15.45
26221
E3-3S
7.30
53918
5AD(17β)-diS
13.34
16142
5AD(17α)-diS
15.47
15230
E3-16G
9.35
7908
E3-3G
2.27
3688
E3-3S
7.26
1247347
E3-16G
9.26
883239
E3-3G
2.24
72300
5PT-diS
10.57
35733
E3-3S
7.30
53949
5PT-diS
10.59
16681
E3-16G
9.35
7611
E3-3G
2.27
3666
E3-3S
7.26
1239984
E3-16G
9.26
884431
E3-3G
2.24
71503
21OHPreg-diS
18.22
26280
E3-3S
7.30
54241
21OHPreg-diS
18.22
33077
E3-16G
9.35
7527
E3-3G
2.27
3470
E3-3S
7.26
1247491
E3-16G
9.26
885583
E3-3G
2.24
71534
5PD-diS
16.63
60535
E3-3S
7.30
53344
5PD-diS
16.73
60577
E3-16G
9.35
7849
E3-3G
2.27
3125
%
Time
(A)
(B)

**Figure 3**

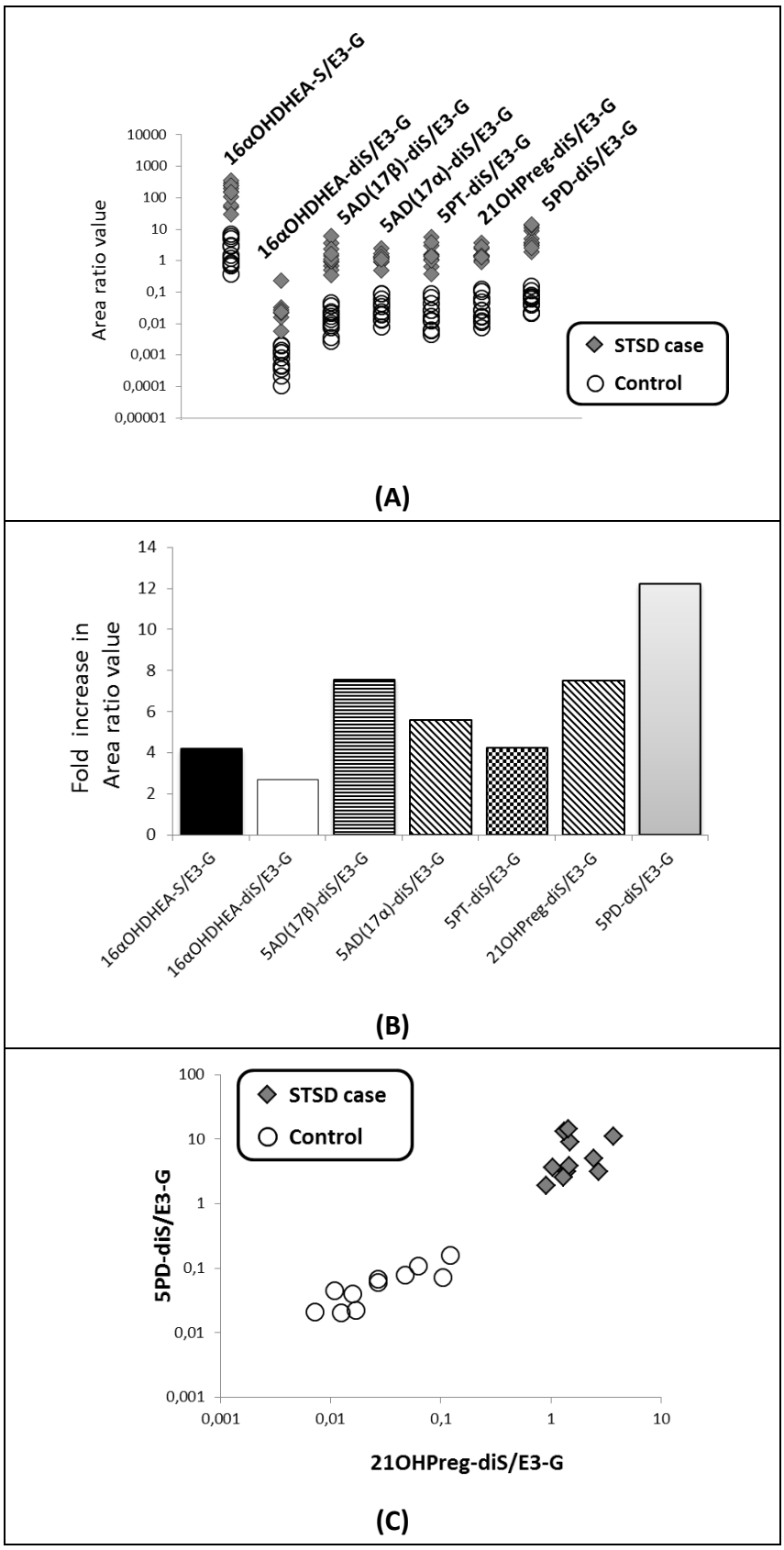
16αOHDHEA-S/E3-G
16αOHDHEA-diS/E3-G
5AD(17β)-diS/E3-G
5AD(17α)-diS/E3-G
5PT-diS/E3-G
21OHPreg-diS/E3-G
5PD-diS/E3-G
Area ratio value
10000
1000
100
10
1
0,1
0,01
0,001
0,0001
0,00001
STSD case
Control
(A)
Fold increase in Area ratio value
14
12
10
8
6
4
2
0
16αOHDHEA-S/E3-G
16αOHDHEA-diS/E3-G
5AD(17β)-diS/E3-G
5AD(17α)-diS/E3-G
5PT-diS/E3-G
21OHPreg-diS/E3-G
5PD-diS/E3-G
(B)
5PD-diS/E3-G
21OHPreg-diS/E3-G
100
10
1
0,1
0,01
0,001
0,001
0,01
0,1
1
10
STSD case
Control
(C)

**Figure 4**

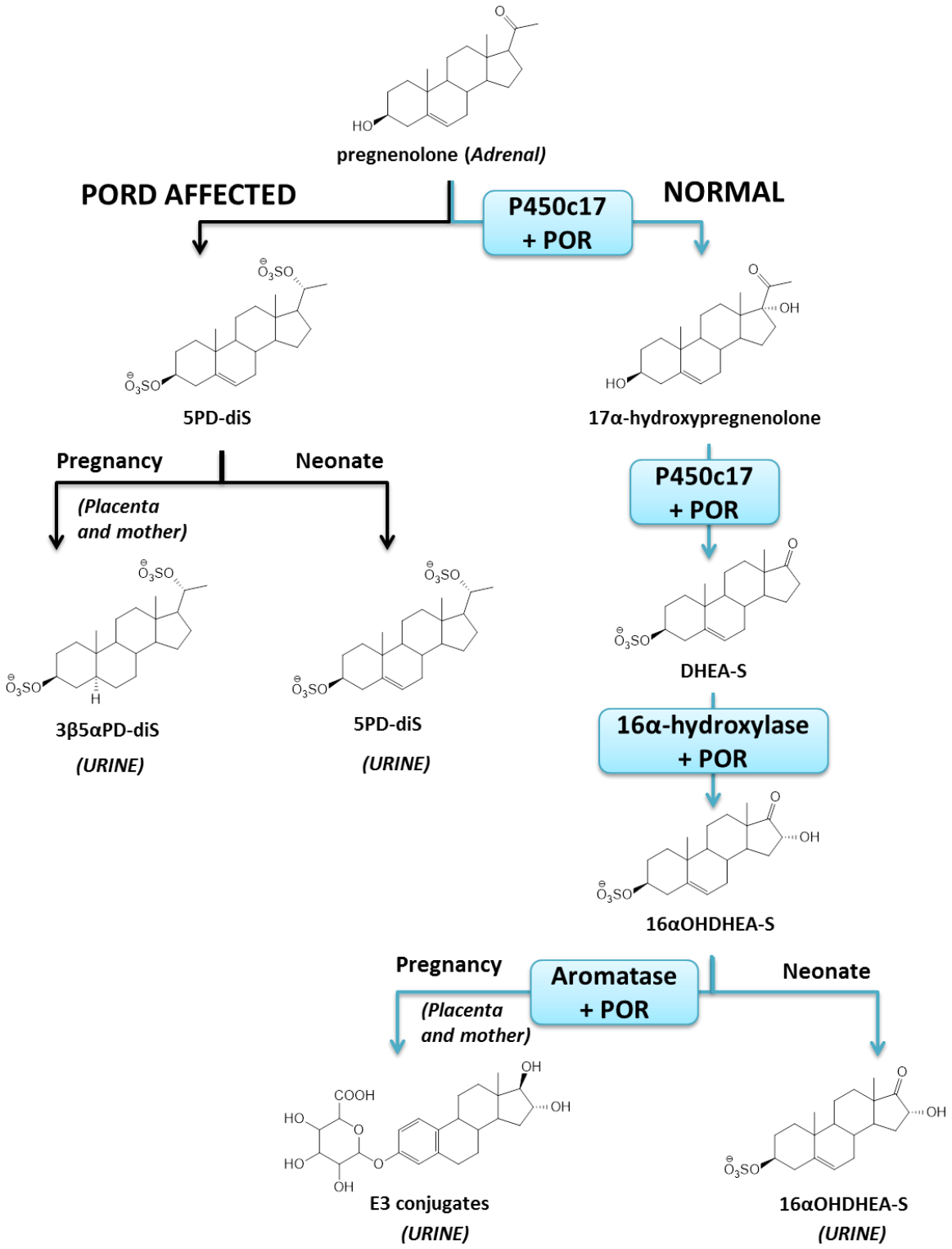
pregnenolone (*Adrenal*)
PORD AFFECTED
P450c17
+ POR
NORMAL
5PD-diS
17α-hydroxypregnenolone
Pregnancy
Neonate
(Placenta
and mother)
P450c17
+ POR
DHEA-S
16α-hydroxylase
+ POR
3β5αPD-diS
(URINE)
5PD-diS
(URINE)
16αOHDHEA-S
Pregnancy
Aromatase
+ POR
Neonate
(Placenta
and mother)
E3 conjugates
(URINE)
16αOHDHEA-S
(URINE)

**Figure 5**

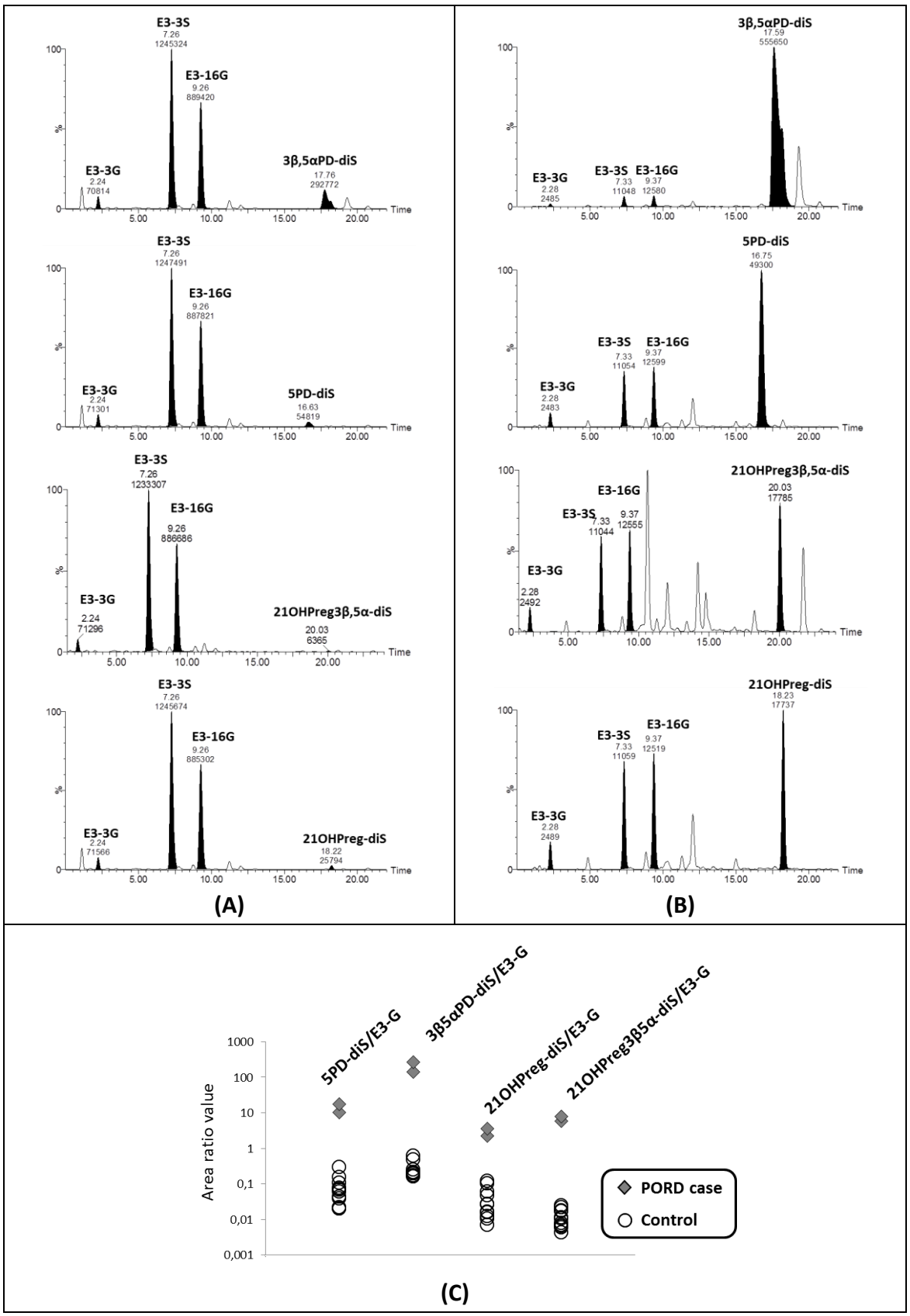
E3-3S
7.26
1245324
E3-16G
9.26
889420
E3-3G
2.24
70814
3β,5αPD-diS
17.76
292772
E3-3S
7.26
1247491
E3-16G
9.26
887821
E3-3G
2.24
71301
5PD-diS
16.63
54819
E3-3S
7.26
1233307
E3-16G
9.26
886686
E3-3G
2.24
71296
21OHPreg3β,5α-diS
20.03
6365
E3-3S
7.26
1245674
E3-16G
9.26
885302
E3-3G
2.24
71566
21OHPreg-diS
18.22
25794
Time
(A)
3β,5αPD-diS
17.59
555650
E3-3G
2.28
2485
E3-3S
7.33
11048
E3-16G
9.37
12580
5PD-diS
16.75
49300
E3-3S
7.33
11054
E3-16G
9.37
12599
E3-3G
2.28
2483
21OHPreg3β,5α-diS
20.03
17785
E3-16G
E3-3S
7.33
11044
9.37
12555
E3-3G
2.28
2492
21OHPreg-diS
18.23
17737
E3-3S
7.33
11059
E3-16G
9.37
12519
E3-3G
2.28
2489
(B)
5PD-diS/E3-G
3β5αPD-diS/E3-G
21OHPreg-diS/E3-G
21OHPreg3β5α-diS/E3-G
Area ratio value
1000
100
10
1
0,1
0,01
0,001
PORD case
Control
(C)

**Figure 6**

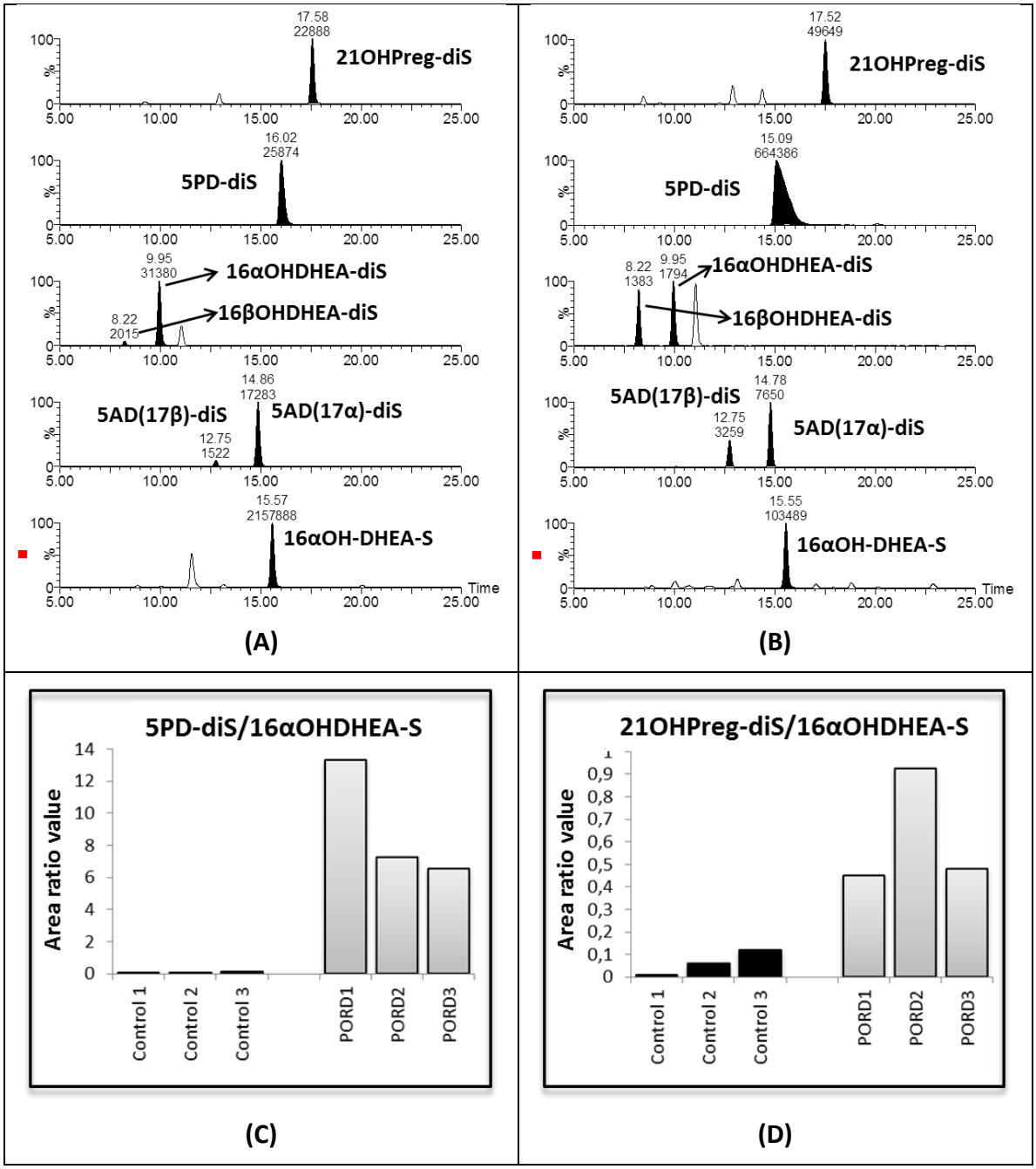
17.58
22888
21OHPreg-diS
16.02
25874
5PD-diS
9.95
31380
16αOHDHEA-diS
8.22
2015
16βOHDHEA-diS
14.86
17283
5AD(17β)-diS
5AD(17α)-diS
12.75
1522
15.57
2157888
16αOH-DHEA-S
Time
(A)
17.52
49649
21OHPreg-diS
15.09
664386
5PD-diS
8.22
1383
9.95
1794
16αOHDHEA-diS
16βOHDHEA-diS
5AD(17β)-diS
14.78
7650
12.75
3259
5AD(17α)-diS
15.55
103489
16αOH-DHEA-S
Time
(B)
5PD-diS/16αOHDHEA-S
Area ratio value
Control 1
Control 2
Control 3
PORD1
PORD2
PORD3
(C)
21OHPreg-diS/16αOHDHEA-S
Area ratio value
Control 1
Control 2
Control 3
PORD1
PORD2
PORD3
(D)

**Figure 7**

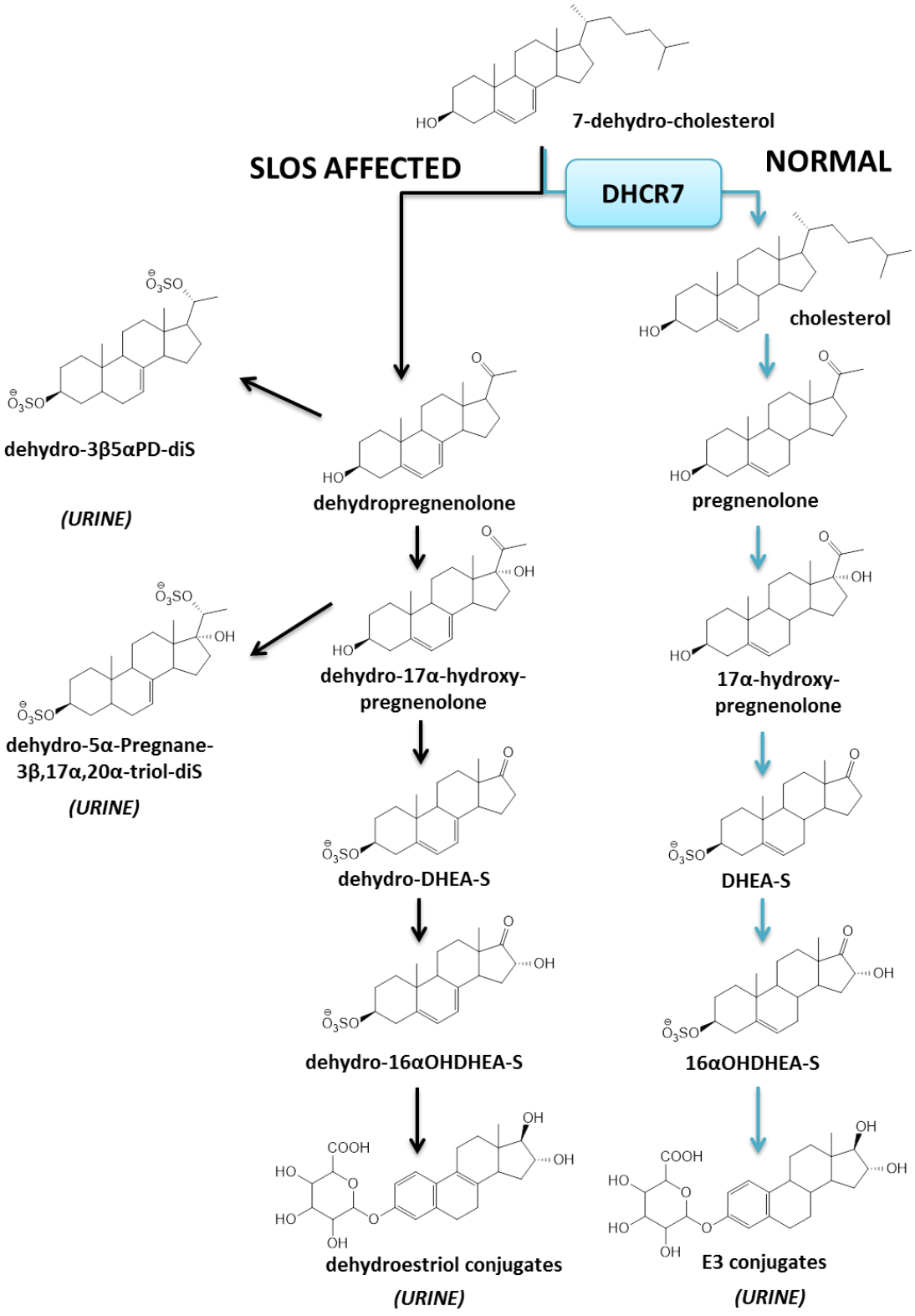
7-dehydro-cholesterol
SLOS AFFECTED
DHCR7
NORMAL
cholesterol
dehydro-3β5αPD-diS
(URINE)
dehydropregnenolone
pregnenolone
dehydro-17α-hydroxy-pregnenolone
17α-hydroxy-pregnenolone
dehydro-5α-Pregnane-3β,17α,20α-triol-diS
(URINE)
dehydro-DHEA-S
DHEA-S
dehydro-16αOHDHEA-S
16αOHDHEA-S
dehydroestriol conjugates
(URINE)
E3 conjugates
(URINE)

**Figure 8**

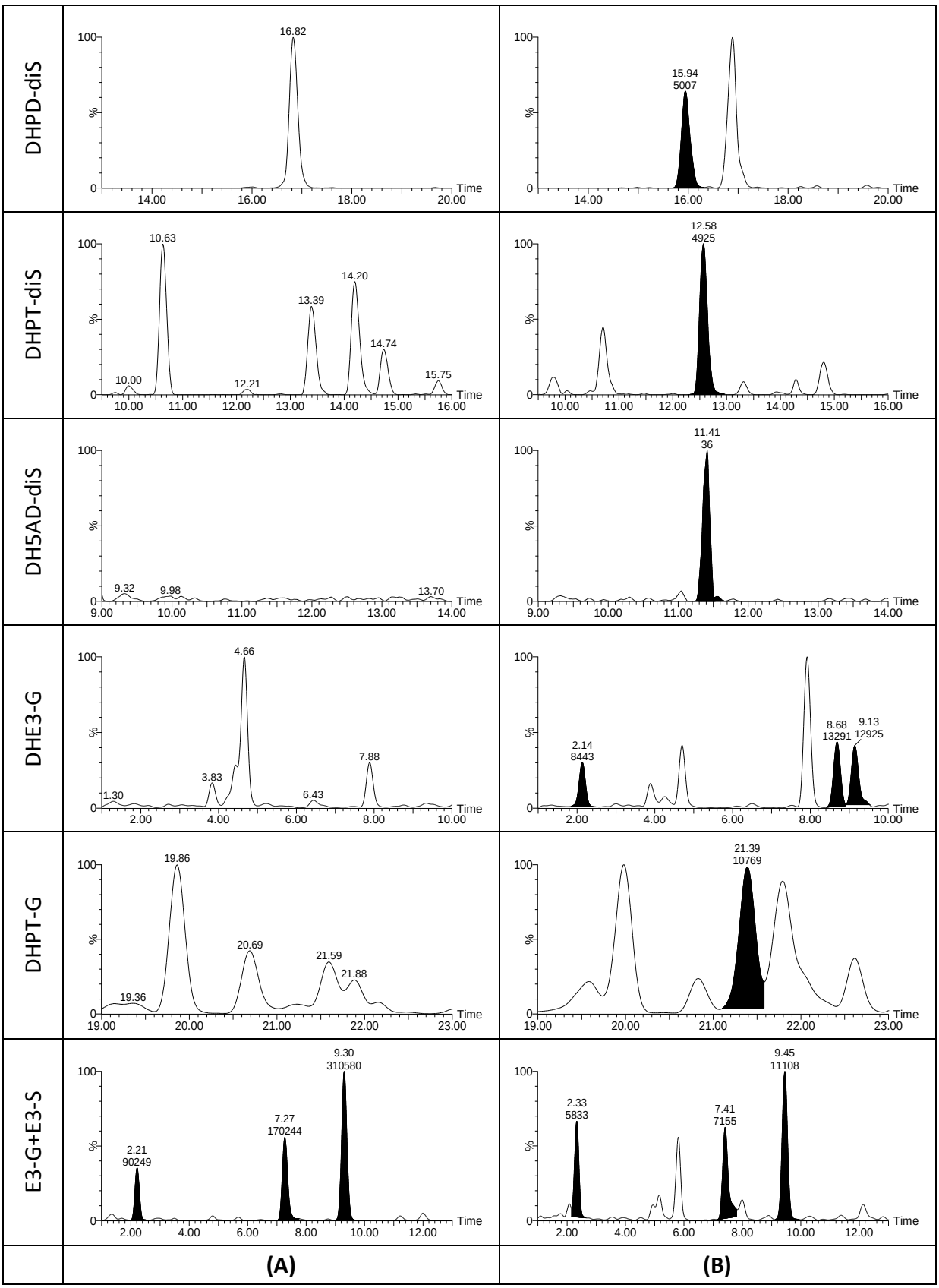
DHPD-diS
DHPT-diS
DH5AD-diS
DHE3-G
DHPT-G
E3-G+E3-S
(A)
(B)
Time
%
16.82
15.94
5007
10.63
10.00
12.21
13.39
14.20
14.74
15.75
12.58
4925
11.41
36
9.32
9.98
13.70
4.66
1.30
3.83
6.43
7.88
2.14
8443
8.68
13291
9.13
12925
19.86
19.36
20.69
21.59
21.88
21.39
10769
9.30
310580
7.27
170244
2.21
90249
2.33
5833
7.41
7155
9.45
11108

**Figure 9.**

### 3.3. Experimental section

Described below are the materials, instruments, and methods to synthesise the reference materials that were made by Mr. Andy Pranata and were discussed in the relevant publication. Copies of the $^{1}$H NMR, $^{13}$C NMR and LRMS spectra for these compounds are available electronically in the supplementary information for this thesis or on request from Associate Professor Malcolm D. McLeod.

#### 3.3.1. Materials

Chemicals and solvents including sulfur trioxide-pyridine complex ($SO_3$·py) were purchased from Sigma–Aldrich (Castle Hill, Australia) unless specified otherwise. 5α-Pregnane-3β,20*S*-diol and 21-acetoxypregnenolone (21-acetoxy-3β-hydroxypregn-5-en-20-one) were purchased from Steraloids (Newport RI, USA). MilliQ water was used in all aqueous solutions. *N*,*N*-Dimethylformamide (DMF), aqueous ammonia solution, and potassium carbonate were obtained from Chem-Supply (Gillman, Australia). Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Sep-Pak Vac C18 3cc cartridges (PN 186004619).

#### 3.3.2. Instruments

$^{1}$H and $^{13}$C nuclear magnetic resonance (NMR) spectra were recorded using either a Bruker Avance 400 MHz, 700 MHz, or 800 MHz spectrometer at 298 K using deuterated methanol solvent. Data is reported in parts per million (ppm), referenced to residual protons or $^{13}$C in deuterated methanol solvent ($CD_3OD$: $^{1}$H 3.31 ppm, $^{13}$C 49.00 ppm), with multiplicity assigned as follows: s = singlet, d = doublet, t = triplet, m = multiplet. Coupling constants *J* are reported in Hertz (Hz). Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) for reference material characterisation were performed using negative or positive electrospray ionisation (-ESI or +ESI) on a Micromass ZMD ESI-Quad, or a Waters LCT Premier XE mass spectrometer. Infrared spectra were recorded on a Perkin-Elmer 1800 Series FTIR spectrometer. Melting points were measured on an SRS Opti-melt MPA 100 automated melting point system and are uncorrected. Optical rotations were recorded in $CHCl_3$ using a Rudolph Research Analytical Autopol I Automatic Polarimeter (sodium D line, 298 K). Reactions were monitored by analytical thin layer chromatography (TLC) using Merck (Bayswater, Australia) silica gel 60 TLC plates and were visualised by staining with a solution of 5% (v/v) sulfuric acid in methanol, with heating as required.

### 3.3.3. 5α-Pregnane-3β,20*S*-diol bis(sulfate), ammonium salt

The reaction was conducted according to the literature [32]. A solution of $SO_3$·py (50.0 mg, 314 µmol) in DMF (500 µL) was added to a solution of 5α-pregnane-3β,20*S*-diol (5.0 mg, 16 µmol) in 1,4-dioxane (500 µL) and the resulting solution was then stirred in a capped vial at room temperature for 16 h. The reaction was then quenched with water (7.5 mL) and subjected to purification by SPE. A C18 SPE cartridge (3 cc) was pre-conditioned with methanol (2 mL) followed by water (4 mL). The reaction mixture was then loaded onto the cartridge and eluted under a positive pressure of nitrogen at a flow rate of approximately 2 mL $min^{-1}$ with the following solutions: saturated aqueous ammonia solution in water (5% v/v, 6 mL), water (6 mL), and methanol (6 mL). The methanol fraction was concentrated *in vacuo* to yield the title compound as colourless solid with > 98% conversion as determined by 400 MHz $^1$H NMR integration of the C3-H and C20-H protons. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 4.38 (1H, m, C20-H), 4.25 (1H, m, C3-H), 2.02 (1H, m), 1.94-1.88 (2H, m), 1.82-1.29 (13H, m), 1.38 (3H, d, *J* 6.2 Hz, C21-$H_3$), 1.19-0.91 (6H, m), 0.85 (3H, s), 0.73 (3H, s), 0.69 (1H, m); **$^{13}$C NMR** (175 MHz, $CD_3OD$): δ 80.1 (C20), 79.7 (C3), 58.3, 57.8, 55.7, 46.3, 42.9, 40.4, 38.2, 36.6, 36.5, 36.4, 33.3, 29.9, 29.8, 27.1, 25.0, 22.1, 21.7, 13.0 (C18), 12.6 (C19); **LRMS (-ESI)**: *m/z* 479 (5%, $[C_{21}H_{35}O_8S_2]^-$), 381 (10%), 239 (100%, $[C_{21}H_{34}O_8S_2]^{2-}$), 111 (10%), 97 (20%, $[HSO_4]^-$); **HRMS (-ESI)**: calcd. for $[C_{21}H_{35}O_8S_2]^-$ 479.1773, found 479.1770.

### 3.3.4. 21-Hydroxypregnenolone [33]

The reaction was conducted according to the literature [33]. A solution of 21-acetoxypregnenolone (500 mg, 1.34 mmol) in methanol (10 mL) and water (1 mL) was treated with potassium carbonate (200 mg, 1.45 mmol) and the mixture was stirred at reflux for 30 mins. The reaction mixture was concentrated *in vacuo*, diluted with water (50 mL), and extracted with ethyl acetate (3 x 50 mL). The combined organic extract was washed with water, dried over sodium sulfate, filtered, and then concentrated *in vacuo*. The solid residue was recrystallised from ethyl acetate to afford the title compound, 139 mg (0.42 mmol, 31% yield) as colourless solid. **$R_f$** 0.23 (50:50 ethyl acetate:hexane); **m.p.** 158-165 °C (lit. [34] m.p. 160-165 °C); **$[\alpha]_D^{25}$** +5.0 (*c* 0.90, $CHCl_3$) (lit. [34] $[\alpha]_D$ +7.3 (*c* 0.907, $CHCl_3$)); **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.35 (1H, d, *J* 5.4 Hz, C6-H), 4.23-4.12 (2H, m, C21-$H_2$), 3.39 (1H, m, C3-H), 2.58 (1H, t, *J* 8.9 Hz, C17-H), 2.28-2.14 (3H, m), 2.05-0.98 (16H, m), 1.03 (3H, s), 0.66 (3H, s); **$^{13}$C NMR** (100 MHz, $CD_3OD$): δ 211.9 (C20), 142.3 (C5), 122.2 (C6), 72.4, 70.2, 59.9, 58.2, 51.5, 45.5, 43.0, 39.7, 38.6, 37.7, 33.3, 32.9, 32.3, 25.7, 23.9, 22.2, 19.9 (C18), 13.8 (C19); **IR** (ATR): 3306 (br, O-H), 2930 (=C-H), 2847 (C-H), 2453 (br, weak),

1698 (C=O), 1048 (C-O) $cm^{-1}$; **LRMS (+ESI)**: *m/z* 687 (20%, $[C_{42}H_{64}O_6Na]^+$), 355 (100%, $[C_{21}H_{32}O_3Na]^+$); **HRMS (+ESI)**: calcd. for $[C_{21}H_{32}O_3Na]^+$ 355.2249, found 355.2245.

### 3.3.5. 21-Hydroxypregnenolone bis(sulfate), ammonium salt

The reaction was conducted according to the literature [32]. A solution of $SO_3$·py (50.0 mg, 314 µmol) in DMF (500 µL) was added to a solution of 21-hydroxypregnenolone (5.0 mg, 15 µmol) in 1,4-dioxane (500 µL) and the resulting solution was then stirred in a capped vial at room temperature for 16 h. The reaction was then quenched with water (7.5 mL) and subjected to purification by SPE. A C18 SPE cartridge (3 cc) was pre-conditioned with methanol (2 mL) followed by water (4 mL). The reaction mixture was then loaded onto the cartridge and eluted under a positive pressure of nitrogen at a flow rate of approximately 2 mL $min^{-1}$ with the following solutions: saturated aqueous ammonia solution in water (5% v/v, 6 mL), water (6 mL), and methanol (6 mL). The methanol fraction was concentrated *in vacuo* to yield the title compound as colourless solid with > 98% conversion as determined by 400 MHz $^1$H NMR integration of the C3-H and C20-H protons. **$^1$H NMR** (400 MHz, $CD_3OD$): δ 5.40 (1H, d, *J* 5.4 Hz, C6-H), 4.59 (1H, d, *J* 16 Hz, C21-H), 4.47 (1H, d, *J* 16 Hz, C21-H), 4.14 (1H, m, C3-H), 2.84 (1H, t, *J* 8.9 Hz, C17-H), 2.55 (1H, m), 2.35 (1H, m), 2.22-1.10 (17H, m), 1.04 (3H, s), 0.66 (3H, s); **$^{13}$C NMR** (200 MHz, $CD_3OD$): δ 208.0 (C20), 141.6 (C5), 123.1 (C6), 79.8 (C3), 73.5 (C21), 59.8, 58.2, 51.4, 45.8, 40.4, 39.6, 38.4, 37.7, 33.3, 32.9, 30.0, 25.6, 23.8, 22.2, 19.7 (C18), 13.8 (C19); **LRMS (-ESI)**: *m/z* 245 (100%, $[C_{21}H_{30}O_9S_2]^{2-}$), 111 (30%), 97 (30%, $[HSO_4]^-$), 80 (5%, $[^{\bullet}SO_3]^-$); **HRMS (-ESI)**: calcd. for $[C_{21}H_{31}O_9S_2]^-$ 491.1410, found 491.1419.

# Chapter 4 – Conclusions and Future Work

In conclusion, the development of the glucuronylsynthase enzyme has been very useful for the synthesis of steroid bisglucuronides and sulfate glucuronides. This enzymatic glucuronylation technique, combined with sulfation and reduction reaction yielded a library of ten steroid bisglucuronides and ten steroid sulfate glucuronides. These compounds dramatically expanded the number of reference materials prepared and characterised that can be used for analytical method development. Comparing with older synthesis routes that needed harsher conditions and some protection and deprotection steps, the syntheses described in this thesis were milder and practically easier to perform. A method of selective labelling of steroid bisglucuronides and sulfate glucuronides was also described using the glucuronylsynthase enzyme and $^{13}C$ labelled α-D-glucuronyl fluoride. This method could easily produce stable isotope labelled internal standards or mass spectrometry probes.

Detection of bis(sulfate) metabolites using GC-MS was performed in the past through extensive fractionation, hydrolysis, and derivatisation prior to analysis. Two steroid bis(sulfates) were synthesised in this thesis, and these were used as reference materials to directly detect markers of disease in the unborn child using the LC-MS methods. Similarly, studies of steroid bisglucuronides and sulfate glucuronides were also performed in the past using GC-MS that needed a long preparation process. The newly synthesised and characterised compounds described in this thesis can be used as reference materials to develop new and direct LC-MS methods to study these neglected metabolite families.

In the future, analysis of steroidal bisconjugates using LC-MS can be performed in areas including anti-doping analysis or medical diagnosis. If these compounds were found to be useful, more steroid bisconjugates can be synthesised as reference materials. Although the glucuronylsynthase enzyme has a broad substrate scope, it was observed to fail in some cases. Given this, the development of this enzyme through protein engineering to enhance substrate scope or activity could also be a topic for future work.

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