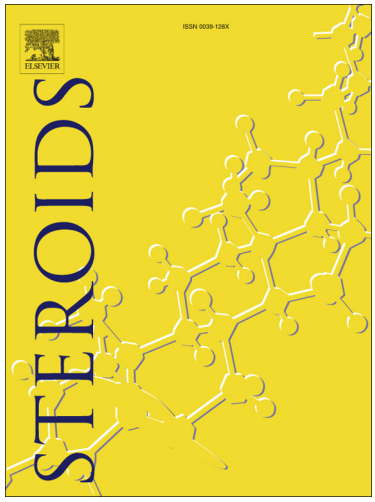

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Synthesis of Steroid Bisglucuronide and Sulfate Glucuronide Reference Materials: Unearthing Neglected Treasures of Steroid Metabolism

Andy Pranata[a], Christopher C. Fitzgerald[a], Olha Khymenets[b], Erin Westley[a], Natasha J. Anderson[a], Paul Ma[a], Oscar J. Pozo[b], Malcolm D. McLeod[a,*]

[a] Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia.

[b] Integrative Pharmacology and Systems Neuroscience Group, IMIM, Hospital del Mar, Doctor Aiguader 88, Barcelona, Spain.

* Corresponding author.

E-mail address: malcolm.mcleod@anu.edu.au (M. D. McLeod)

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. 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 the bisconjugated steroid reference materials has been studied. Preliminary targeted ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis of the reference materials prepared revealed the presence of three steroid sulfate glucuronides as endogenous human urinary metabolites.

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
- Three steroid sulfate glucuronide metabolites confirmed in human urine

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, LC-MS/MS = liquid chromatography-tandem 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, UHPLC-MS/MS = ultra-high-performance liquid chromatography–tandem mass spectrometry, WAX = weak anion exchange.

1. 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 [1]. Many current therapeutic interventions target steroid biosynthesis or signalling pathways [2],[3],[4] and this knowledge is also exploited in steroid abuse that remains a major problem for world sport and wider society [5]. In mammalian systems, steroids undergo two phases of metabolism [6]. 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 [7],[8] and glucuronic acid [9],[10]. 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 [11]. However, phase II conjugates also serve other important roles in steroid transport and regulation, with steroids such as dehydroepiandrosterone (DHEA) sulfate and estrone sulfate serving as an endogenous depot in steroid hormone metabolism [12],[13].

Traditionally, steroid analysis has been conducted using gas chromatography-mass spectrometry (GC-MS) [14]. 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 [11]. 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],[15]. Milder enzymatic hydrolysis with *Escherichia coli* (*E. coli*) β-glucuronidase neglects the contribution of steroid sulfate metabolites and can result in incomplete hydrolysis [16], while the use of crude enzyme preparations containing glucuronidase and sulfatase enzymes can also lead to undesired steroid conversions [11] and is unable to hydrolyse sulfates at several positions such as steroid 20-sulfates [17]. More recently, purified bacterial arylsulfatase enzymes have been developed [18] 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 [19]. 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, as they ionise well by electrospray ionisation (ESI), and time-consuming hydrolysis and derivatisation steps are not required [20]. Typically, mono-conjugated steroid sulfate and glucuronide conjugates have been studied using this approach [16],[21]. 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 [22],[23], typically coupled to GC-MS analysis [24],[25]. 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-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) constant ion loss (CIL) scan method for the direct and untargeted detection of steroid bis(sulfate) metabolites was reported [26]. 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) [27].

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 [26]. 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 [28],[29],[30], sulfate glucuronides [31],[32],[33] or diglucuronides [34] have been reported. Examples of small scale, qualitative chemical and biochemical synthesis of steroid

bisglucuronides [35],[36],[34],[37],[38] and sulfate glucuronides [39] 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 three of these steroid sulfate glucuronides have been confirmed as endogenous human urinary metabolites by UHPLC-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.

2. Experimental

Materials and instruments associated with the chemical synthesis and characterisation of bisconjugates are reported in the supplementary material.

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

Negative mode LC-MS/MS studies for the ionisation and fragmentation of reference materials was undertaken using a Waters Acquity triple quadrupole mass spectrometer equipped with an ESI source and interfaced to a Waters 2695 Alliance Separations Module. Nitrogen was used as desolvation and cone gas. The desolvation gas flow was 602 L/h, and the cone gas flow was 1 L/h. The nitrogen desolvation temperature was 349 °C, and the source temperature was 148 °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) or targeted MS/MS mode (*m/z* 50-700, collision energy 10-50 eV) and with 4 kV capillary voltage. The LC separation of reference materials was performed using a Waters Symmetry C18 column (150 x 2.1 mm i.d., 5 µm) and a column temperature of 30 °C, eluting with a gradient consisting of the following mobile phases: methanol and water, both containing 0.01% formic acid and 10 mM ammonium formate. A gradient elution program was applied, where the percentage of organic solvent was linearly changed at a flow rate

of 0.3 mL min^{-1}: 0 min, 30%; 9 min, 90%; 10 min, 30%; 15 min, 30%. The total analysis time was 15 min.

The UHPLC-MS/MS analysis of urine samples was carried out using a Waters XEVO TQ-S micro triple quadrupole mass spectrometer equipped with an ESI source and interfaced to a Waters Acquity UPLC system for the chromatographic separation. Nitrogen was used as desolvation and cone gas. The desolvation gas flow was 1200 L/h, and the cone gas flow was 50 L/h. The nitrogen desolvation temperature was 600 °C, and the source temperature was 150 °C. A cone voltage of 26 V and a capillary voltage of 0.4 kV were used in negative ionization mode. The UHPLC separation for urine samples was performed using a Waters Acquity UPLC CSH Phenyl-Hexyl column (100 x 2.1 i.d., 1.7 µm) and a column temperature of 30 °C, eluting with a gradient consisting of the following mobile phases: acetonitrile:water (9:1, v/v), and water, both containing 0.01% formic acid and 25 mM ammonium formate (VWR Prolabs Chemicals, Leuven, Belgium). A gradient elution program was applied, where the percentage of organic solvent was linearly changed at specified flow rates: 0 min (0.4 mL/min), 15%; 0.5 min (0.4 mL/min), 15%; 26 min (0.4 mL/min), 32%; 27 min (0.5 mL/min), 100%; 28 min (0.5 mL/min), 100%; 29 min (0.5 mL/min), 15%; 29.5 min (0.4 mL/min), 15%, 30 min (0.4 mL/min) 15%. The total analysis time was 30 min.

2.2. Urine sample preparation

The collection of human urine samples was conducted with approval of the Comité Ètic d'Investigació Clínica of our institute (CEIC-IMAS no. 94/467) and the Spanish Health Ministry (DGFPS no. 95/75). Three sub-sets of urine samples were selected for confirmation of the presence of steroid sulfate glucuronide metabolites: urine samples from healthy female volunteers (n=11, age 23-40 years), urine samples from healthy male volunteers (n=11, age 26-46 years), and spot urine samples from healthy pregnant women (n=8, age 30-39 years) at mid-term (gestation week 18-28). The collection time was not specified for these samples. All urine samples were stored frozen at -20 °C prior to analysis.

The procedure was adapted from the literature with minor modifications [40]. An aliquot of urine (2 mL) was fortified with stably labelled 5α-androstan-3α,17β-diol 3,17{$^{18}O_3$}-bis(sulfate) and 5α-androstan-3β,17β-diol 3,17{$^{18}O_3$}-bis(sulfate), (25 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 an Oasis weak

anion exchange (WAX) solid phase extraction (SPE) cartridge (Waters, 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 (150 µL) and transferred to a sealed vial. The extract was injected (5 µL) into the instrument for analysis by UHPLC-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 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 **3** 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 UHPLC-MS using an Agilent 6120 quadrupole mass spectrometer equipped with and API-ES source and coupled to an Agilent 1290 Infinity LC injector, HTS sampler and 1260 Infinity UHPLC system. Nitrogen was used as drying and nebulizing gas. The drying gas flow was 3.0 L/min, and the nebulizing pressure was 20 psig. The drying gas temperature was 300 °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 3 kV capillary voltage. The UHPLC separation was performed with an Agilent Poroshell 120

C18 column (30 mm x 2.1 i.d. mm, 2.7 μm) and column temperature at 30 °C, eluting with a gradient consisting of the following mobile phases: 10% v/v methanol:water, and 90% v/v methanol:water, both containing 10 mM ammonium acetate. A gradient elution program was applied, where the percentage of organic solvent was linearly changed at a flow rate of 0.2 mL min^{-1}: 0 min, 42%; 1 min, 42%; 6 min, 80%; 7 min, 100%; 8 min, 100%; 9 min, 42%; 14 min, 42%. The total analysis time was 14 min.

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 [37],[41]. 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 min^{-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 [41]. 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 ^1H NMR spectrum was obtained and integration of a suitable signal (typically C3-H, C16-H or C17-H) 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 [37]. 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 equivalents) 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** [26] (5.0 mg, 17 μmol, see the supplementary material) 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 (> 95% by ^{1}H NMR). Characterisation data is reported in the supplementary material.

The steroid bisglucuronides **5** and **9**-**16** (Figure 2), namely 5α-androstane-3β,17α-diol bisglucuronide **5**, estradiol bisglucuronide **9**, androst-5-ene-3β,17β-diol bisglucuronide **10**, androst-4-ene-3β,17β-diol bisglucuronide **11**, estr-4-ene-3β,17β-diol bisglucuronide **12**, 5β-cholane-3α,24-diol bisglucuronide **13**, 5α-pregnane-3β,20*S*-diol bisglucuronide **14**, pregn-5-ene-3β,20*S*-diol bisglucuronide **15**, and pregn-5-ene-3β,20*R*-diol bisglucuronide **16**, were prepared using similar methods (see the supplementary material).

Figure 2.

2.4.6.2 Method B. The reaction was conducted with 5α-androstane-3β,17β-diol 17-glucuronide, ammonium salt [18] (derived in 19% conversion from dihydrotestosterone, assumed 3.2 μmol, a 1:9 ratio of the 3α:3β diastereomers, see the supplementary material) 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 (> 95% by ^{1}H NMR).

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 [18] (1.0 mg, 2.1 μmol, a 1:7 ratio of the 3α:3β diastereomers, see the supplementary material) 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 (> 95% by ^{1}H NMR). Characterisation data is reported in the supplementary material.

The steroid {$^{13}C_6$}-bisglucuronide estradiol 3,17{$^{13}C_6$}-bisglucuronide {$^{13}C_6$}-**9**, and {$^{13}C_6$}-monoglucuronides DHEA {$^{13}C_6$}-glucuronide {$^{13}C_6$}-**4**, epiandrosterone (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** (Figure 3), were prepared using similar methods (see the supplementary material).

Figure 3.

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

The reaction was conducted with EA sulfate, ammonium salt [41] (derived from EA **22**, 5.5 mg, 19 μmol, see the supplementary material) by general procedure 2.4.4 to yield the title compound **21** as a colourless solid with > 98% conversion. Characterisation data is reported in the supplementary material.

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 (> 95% by ^{1}H NMR). Characterisation data is reported in the supplementary material.

The steroid sulfate glucuronides **24**-**32** (Figure 4), namely androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24**, estradiol 3-sulfate 17-glucuronide **25**, 5α-androstane-3β,17β-diol 3-glucuronide 17-sulfate **26**, 5α-androstane-3β,17α-diol 3-glucuronide 17-sulfate **27**, estradiol 3-glucuronide 17-sulfate **28**, androst-4-ene-3β,17β-diol 3-glucuronide 17-sulfate **29**, androst-4-ene-3β,17α-diol 3-glucuronide 17-sulfate **30**, pregn-5-ene-3β,20*R*-diol 3-sulfate 20-glucuronide **31**, pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32**, were prepared using similar methods (see the supplementary material)

Figure 4.

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 (> 95% by ^{1}H NMR). Characterisation data is reported in the supplementary material.

3. 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 [37]. 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 [37]. 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 equivalents) and the glucuronylsynthase enzyme, as shown for 5α-androstane-3β,17β-diol **7** (Scheme 1) [37].

Scheme 1.

After the reaction, SPE using an Oasis 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** (Figure 2). 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 [37], 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 3β,20*R*- or 3α,24-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*. [37], with 3β(5α), 3β(5-ene), 3(phenolic), 20*R* and 24 hydroxy groups showing the highest reactivity, while 3α(5β), 17β, 17α and 20*S* hydroxy groups showed lower reactivity, and 3α(5α) hydroxy 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 (Scheme 2). This was despite earlier work [37], 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 (Figure 2).

Scheme 2.

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.

Figure 5.

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 the supplementary material) 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 clearer conversions 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 increase synthetic efficiency. 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 (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) in acetonitrile and sodium bicarbonate buffer. These modified conditions avoided the use of aqueous bleach as stoichiometric oxidant [42] 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). For example, elimination of unlabelled water from aldehyde hydrate {^{18}O}-**38**, followed by addition of labelled water and oxidation would afford the doubly labelled {$^{18}O_2$}-α-D-glucuronyl fluoride. 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.

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 the ^{13}C isotope. A four-step synthesis of α-D-glucuronyl fluoride {$^{13}C_6$}-**2** was employed, starting from {$^{13}C_6$}-D-glucose [42]. 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 hydroxy groups on the sugar ring, and subsequent sulfation could then occur unselectively on any of the available hydroxy groups.

Scheme 5.

The sulfation reaction was performed using sulfur trioxide-pyridine complex according to a literature method [41]. Ketone reduction was performed using sodium borohydride [41], or with additional cerium (III) chloride heptahydrate (Luche conditions) for α,β-unsaturated ketones like testosterone and epitestosterone sulfate [43]. 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 [14]. 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 [44],[45]. Reduction of the toluenesulfonylhydrazone derivative of pregnenolone sulfate under conditions similar to those reported by Tada *et al*. [46], 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 [37] 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* hydroxy 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 [37], 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 [47]. 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 [37]. 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 the sulfation reaction. After sulfation, C3-H or C17-H shifted downfield by 0.65-0.76 ppm as expected from the previously reported data [41]. 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 the glucuronylation reaction, the oxymethine proton shifts were smaller than after the sulfation reaction as mentioned above and as previously reported for monoglucuronides [37]. 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 scan MS with 70 V cone voltage, the mono-anion $[M-H]^-$ was the major ion observed with some minor $[M-H-gluc]^-$ 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 $[M-H-gluc]^-$ 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 $[M-H-2gluc]^-$ *m/z* 271 (15%) in the scan MS. In contrast, scan MS with 26 V cone voltage formed the di-anion $[M-2H]^{2-}$ as the major ion, while still forming mono-anion $[M-H]^-$ with 50-100% relative abundance. In-source fragmentation was only observed for estradiol bisglucuronide **9**, giving 5% $[M-H-gluc]^-$. A recent MS study on a library of crude chromatographically resolved steroid bisglucuronides reported similar findings [38]. 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 $[M-H]^-$, the major fragments at 50 eV collision energy that retained the steroid backbone were $[M-H-gluc]^-$ and $[M-H-C_2H_4O_3]^-$, 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 $[M-H-2gluc]^-$ (*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 for steroid glucuronides [16],[38]. Fragmentation data for all steroid bisglucuronide mono-anion precursors is tabulated in the supplementary material (Table S2).

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 $[M\text{-}2H\text{-}(gluc\text{-}H)]^-$ (equivalent to $[M\text{-}H\text{-}gluc]^-$ above) and $[M\text{-}2H\text{-}(C_2H_3O_3)]^-$ (equivalent to $[M\text{-}H\text{-}C_2H_4O_3]^-$ 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 CIL scan method [26]. Estradiol bisglucuronide **9** showed a fragment ion at *m/z* 271 ($[M\text{-}2H\text{-}(gluc\text{-}H)\text{-}gluc]^-$) 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 ($[M\text{-}2H\text{-}(gluc\text{-}H)\text{-}H_2O]^-$) 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. Fragmentation data for all steroid bisglucuronide di-anion precursors is tabulated in the supplementary material (Table S3).

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 ($[M\text{-}H]^-$) of 5α-androstane-3β,17β-diol 3{$^{13}C_6$},17-bisglucuronide {$^{13}C_6$}-**6** was fragmented, two ions *m/z* 467 ($[M\text{-}H\text{-}(\{^{13}C_6\}\text{-}gluc)]^-$) and 473 ($[M\text{-}H\text{-}gluc]^-$) were observed with similar intensity (Table 1). 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 only one fragment *m/z* 453 ($[M\text{-}H\text{-}gluc]^-$) where the unlabelled glucuronide at 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, 157/163, 113/118, 85/89, 75/77) appeared for both selectively labelled bisglucuronides.

Fragmentation of the di-anions ($[M\text{-}2H]^{2-}$) showed similar behaviour (Figure 6, Table 2). For the estradiol 3,17{$^{13}C_6$}-bisglucuronide, the preferential loss of the unlabelled glucuronide was also observed. In this case, neutral loss of the unlabelled glucuronide unit to give *m/z* 226 ($[M\text{-}2H\text{-}gluc]^{2-}$) was accompanied by reductions in the relative intensity of the unlabelled glucuronide fragment ions *m/z* 85 and 75 relative to their labelled counterparts *m/z* 89 and 77. Ion loss of the unlabelled glucuronide was also observed to give m/z 453 ($[M\text{-}2H\text{-}(gluc\text{-}H)]^-$). Both labelled and unlabelled glucuronide fragments (*m/z* 113/118, 85/89, 75/77) appeared for both selectively labelled bisglucuronides. In conclusion, selectively mono-labelled bisglucuronide reference materials provide a means

to study the selectivity of fragmentation associated with both glucuronide units of a bisglucuronide compound. Collision induced dissociation was also performed on EA glucuronide and the stable isotope labelled EA glucuronides {$^{18}O_2$}-**17** and {$^{13}C_6$}-**17**, and these results are reported in the supplementary material (Table S4).

Figure 6.

Table 1.

Table 2.

3.6. MS analysis of steroid sulfate glucuronide reference materials

3.6.1. Ionisation

During the scan MS analysis, 26 V and 70 V cone voltages were used to maximise the response of the di-anion ($[M-2H]^{2-}$) and mono-anion ($[M-H]^-$) respectively. When the 26 V cone voltage was used, the formation of the di-anion ($[M-2H]^{2-}$) was favoured, with the mono-anion ($[M-H]^-$) observed at lower relative abundance (15-55%). The in-source fragment $[M-2H-(gluc-H)]^-$ was also observed at relatively low 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 ($[gluc-H]^-$).

When using 70 V cone voltage, mono-anion ($[M-H]^-$) but no di-anion was observed. Similar to above, an in-source fragment was typically observed corresponding to loss of the dehydrated glucuronic acid ($[M-H-gluc]^-$, 25-100%, corresponding to $[M-2H-(gluc-H)]^-$ above). Other in-source fragments such as $[M-H-C_4H_6O_5]^-$, $[M-H-C_7H_{12}O_7]^-$ and $[M-H-gluc-H_2O]^-$ were also commonly observed. The estradiol 3-sulfate 17-glucuronide **25** formed an in-source fragment *m/z* 271, corresponding to ($[M-H-gluc-SO_3]^-$).

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 ion was found at *m/z* 97 corresponding to hydrogen sulfate ion ($[HSO_4]^-$). The *m/z* 97 ion was typically the

most 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 SO_3 rather than 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 $[M\text{-}H\text{-}SO_3]^-$ and $[M\text{-}H\text{-}gluc\text{-}SO_3]^-$, respectively, were only seen for estradiol 3-sulfate 17-glucuronide **25**. The ion at *m/z* 80 corresponding to sulfur trioxide radical anion ($[^{\bullet}SO_3]^-$) was also formed from this compound. Fragmentation data for all steroid sulfate glucuronide mono-anion precursors is tabulated in the supplementary material (Table S5).

Fragmentation of the di-anion precursor $[M\text{-}2H]^{2-}$ at 20 eV collision energy gave a greater number of fragments than the mono-anion $[M\text{-}H]^-$. Similar to the mono-anion, loss of the glucuronide derived anion ($[M\text{-}2H\text{-}(gluc\text{-}H)]^-$, corresponding to $[M\text{-}H\text{-}gluc]^-$ 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, $[M\text{-}2H\text{-}(gluc\text{-}H)\text{-}H_2O]^-$ (corresponding to $[M\text{-}H\text{-}gluc\text{-}H_2O]^-$ 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 $[M\text{-}2H\text{-}(C_2H_3O_3)]^-$ (corresponding to $[M\text{-}H\text{-}C_2H_4O_3]^-$ above) similar to the steroid bisglucuronide di-anion fragmentation. Once again, the ion loss fragmentation from $[M\text{-}2H]^{2-}$ to $[M\text{-}2H\text{-}(C_2H_3O_3)]^-$, $[M\text{-}2H\text{-}(gluc\text{-}H)]^-$, and $[M\text{-}2H\text{-}(gluc\text{-}H)\text{-}H_2O]^-$ lead to an increase in *m/z* ratio. Other fragments were also observed including $[M\text{-}2H\text{-}(C_3H_3O_5)]^-$ and $[M\text{-}2H\text{-}(C_3H_5O_5)]^-$. 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 $[M\text{-}2H\text{-}(gluc\text{-}H)\text{-}SO_3]^-$ and $[M\text{-}2H\text{-}(C_7H_{11}O_7)\text{-}SO_3]^-$. On the other hand, the aromatic glucuronide estradiol 3-glucuronide 17-sulfate **28** fragmented to give $[gluc\text{-}H]^-$ (*m/z* 175), while only glucuronide fragments (*m/z* 113, 85, 75) were usually observed in the other library members. Fragmentation data for all steroid sulfate glucuronide di-anion precursors is tabulated in the supplementary material (Table S6).

Figure 7.

3.6.3. UHPLC-MS/MS analysis of real urine samples

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. The selected transitions for three of the analytes (**23**, **24** and **32**) are summarized in Table 3.

This SRM method was applied to a set of 30 real urine samples collected in our laboratory. Confirmation of the analyte identity was performed using the World Anti-Doping Agency (WADA) MS criteria for retention time and the relative abundance of three diagnostic transitions [48]. Three of the synthesized analytes, namely pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32**, 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** and androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24** could be detected in most of the samples. The occurrence of all of them could be confirmed in several samples by both the ion ratios of several transitions and the retention time.

The acquisition of the most specific transition (286 → 397) allowed for the detection of pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32** in all the samples analysed (Table 4) with retention times commonly within 1% of deviation. The identity of that conjugate in human urine could be confirmed by the relative abundance of the second transition (286 → 497) in all but a few diluted samples. Additionally, the third acquired transition could be observed within criteria in more than half of the samples. An example of the confirmation of a male urine is illustrated in Figure 8a. The 20*R* isomer **31** was also detected and confirmed in one of the male samples analysed using the same transitions as those of the 20*S* isomer **32**.

5α-Androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** could be detected in all male and female samples by using the transition 273 → 371 (Table 4) with retention time deviations normally below 1%. In contrast, that analyte could only be detected in one of the urines collected during pregnancy. The identity of the compound could be confirmed in six out of the 30 samples analysed by using a second transition. The three selected transitions could be observed within criteria in three male samples. An example is provided in Figure 8b.

Finally, androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24** could be detected in all samples except for three urines collected during pregnancy (Table 4). Similar to the other two analytes, conjugate **24** did not substantially differ its retention time from that of the reference material (typical deviation below 1%) and could be confirmed in several of the

analysed samples from both male and female (14 out of 22 with two transitions, and 11 out of 22 with three transitions). However, its identity could not be confirmed in any of the urines collected during pregnancy. An example is provided in Figure 8c.

In summary, our results confirmed the existence of at least three previously unreported steroid sulfate glucuronide conjugates as endogenous urinary 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 UHPLC-MS methods for the direct and untargeted detection of this metabolite family.

Table 3.

Table 4.

Figure 8.

4. 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 [22],[23], followed by GC-MS detection [24],[25]. 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 [26]. 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 [26], and for the analysis of maternal urine to provide discriminating prenatal diagnosis of inborn errors of steroid biosynthesis associated with SLOS, STSD, and PORD [27]. 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 [26].

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 [37],[42],[49]. The glucuronylsynthase is an engineered

glycosynthase variant [50],[51] of the *E. coli* β-glucuronidase enzyme that is widely employed in chemical analysis for steroid glucuronide hydrolysis in sample preparation [11]. 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 [37]. This study extends the glucuronylsynthase approach to the synthesis of ten steroid bisglucuronide and ten steroid sulfate glucuronide, reference materials. The synthesis was performed 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 [52],[53] 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 [42]. The glucuronylsynthase method is also distinct from chemical methods of glucuronylation that employ protected and activated glucuronide donors and may require multiple protection and deprotection steps [54],[55]. 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 [31]. 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 hydroxy 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 an established route [42] 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 {$^{13}C_6$}-**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*S*-diol 3-sulfate 20-glucuronide **32**, 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** and androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24** as endogenous human urinary metabolites by UHPLC-MS/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 steroidal bisconjugates in the steroid profile, unearthing these neglected treasures of steroidal metabolism.

5. 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 material

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

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Tables

Table 1. 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	$[M-H]^-$	$[M-H-C_2H_4O_3]^-$	$[M-H-gluc]^-$	$[M-H-2gluc]^-$	$[gluc-H]^-$	$[gluc-H-H_2O]^-$	$[gluc-H-H_2O-CO_2]^-$	$[gluc-H-H_2O-CO_2-CO]^-$	$[C_2H_3O_3]^-$
5α-androstane-3β,17β-diol bisglucuronide	643 (20)	567 (5)	467 (10)	-	-	157 (5)	113 (50)	85 (100)	75 (60)
5α-androstane-3β,17β-diol 3{$^{13}C_6$},17-bisglucuronide	649 (20)[a]	573 (3)[a]	467 (6) 473 (10)[a]	-	-	157 (5) 163 (5)[a]	113 (50) 118 (40)[a]	85 (100) 89 (80)[a]	75 (60) 77 (60)[a]
estradiol 3,17-bisglucuronide	623 (5)	-	447 (35)	271 (40)	175 (10)	157 (5)	113 (85)	85 (100)	75 (50)
estradiol 3,17{$^{13}C_6$}-bisglucuronide	629 (5)[a]	-	453 (50)[a]	271 (50)	175 (10)	157 (5) 163 (3)[a]	113 (100) 118 (30)[a]	85 (75) 89 (75)[a]	75 (30) 77 (35)[a]

Table 2. 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	$[M-2H]^{2-}$	$[M-2H-(gluc-H)]^{-}$	$[M-2H-(gluc-H)-gluc]^{-}$	$[gluc-H-H_2O-CO_2]^{-}$	$[gluc-H-H_2O-CO_2-CO]^{-}$	$[C_2H_3O_3]^{-}$
5α-androstane-3β,17β-diol 3,17-bisglucuronide	321 (5)	467 (5)	-	113 (25)	85 (90)	75 (100)
5α-androstane-3β,17β-diol 3{$^{13}C_6$},17-bisglucuronide	324 (10)[a]	467 (5) 473 (5)[a]	-	113 (30) 118 (25)[a]	85 (100) 89 (85)[a]	75 (100) 77 (100)[a]
estradiol 3,17-bisglucuronide	311 (5)	447 (5)	271 (40)	113 (40)	85 (100)	75 (95)
estradiol 3,17{$^{13}C_6$}-bisglucuronide	314 (3)[a]	453 (5)[a]	271 (50)	113 (25) 118 (25)[a]	85 (45) 89 (100)[a]	75 (40) 77 (90)[a]

Table 3. Diagnostic transitions for pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32** (20*S*:20*R* = 2:1), 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** and androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24**. * Indicates the most sensitive transition.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone Voltage (V)	Collision Energy (eV)	Proposed product ion
32	286	497	26	15	$[M\text{-}2H\text{-}(C_2H_3O_3)]^-$
	286	397*	26	15	$[M\text{-}2H\text{-}(gluc\text{-}H)]^-$
	286	97	26	25	$[HSO_4]^-$
23	273	471	26	15	$[M\text{-}2H\text{-}(C_2H_3O_3)]^-$
	273	371*	26	15	$[M\text{-}2H\text{-}(gluc\text{-}H)]^-$
	273	353	26	20	$[M\text{-}2H\text{-}(gluc\text{-}H)\text{-}H_2O]^-$
24	272	469	26	15	$[M\text{-}2H\text{-}(C_2H_3O_3)]^-$
	272	369*	26	15	$[M\text{-}2H\text{-}(gluc\text{-}H)]^-$
	272	97	26	25	$[HSO_4]^-$

Table 4. Confirmation of the presence of pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32**, 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** and androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24** in human urine. Comparison of the relative abundances obtained for the selected transitions in the real urine samples with the ones obtained for the reference material. In brackets, the number of samples in which each transition was observed within criteria. * Indicates the most sensitive transition. ^ Not detected.

		Relative abundance (%)			
Analyte	Transition	Reference material	Male samples (n=11)	Female samples (n=11)	Pregnancy samples (n=8)
32	286→497	25%	20%-30% (n=9)	21%-29% (n=9)	22%-30% (n=8)
	286→397*	100%	100% (n=11)	100% (n=11)	100% (n=8)
	286→97	2%	1%-3% (n=5)	1%-3% (n=5)	1%-3% (n=6)
23	273→471	34%	34%-40% (n=4)	28%-37% (n=2)	n.d.^
	273→371*	100%	100% (n=11)	100% (n=11)	100% (n=1)
	273→353	6%	5%-6% (n=3)	n.d.^	n.d.^
24	272→469	14%	10%-16% (n=6)	10%-16% (n=5)	n.d.^
	272→369*	54%	50%-58% (n=11)	50%-59% (n=11)	(n=5)
	272→97	100%	100% (n=8)	100% (n=6)	n.d.^

Figure legends

Figure 1. Examples of singly and doubly conjugated steroid metabolites

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.

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.

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**.

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).

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.

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**.

Figure 8. Chromatogram of the three selected SRM transitions for a male urine sample containing (a) pregn-5-ene-3β,20*S*-diol 3-sulfate 20-glucuronide **32** (b) 5α-androstane-3β,17β-diol 3-sulfate 17-glucuronide **23** and (c) androst-5-ene-3β,17β-diol 3-sulfate 17-glucuronide **24**. Relative abundances were in agreement with those obtained for the reference material (see Table 4).

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.

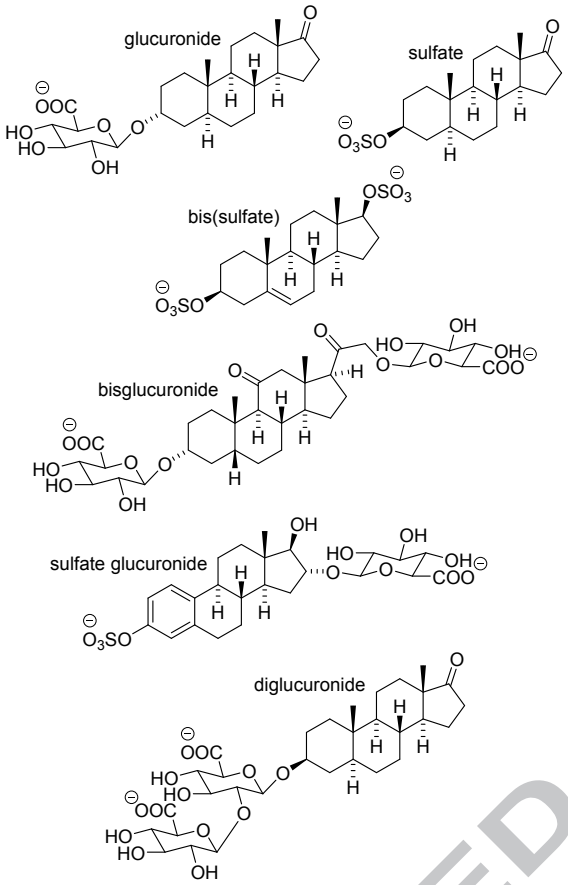
Scheme 2. Step-wise synthesis of 5α-androstane-3β,17α-diol 3,17-bisglucuronide **5** from epidihydrotestosterone **34**.

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

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.

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

Figures



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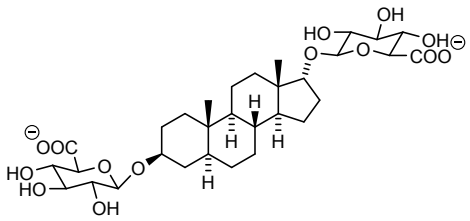

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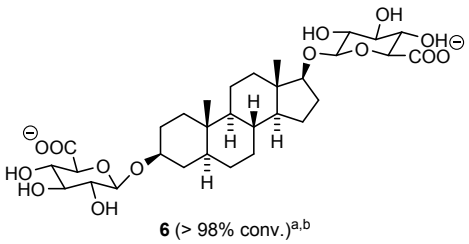
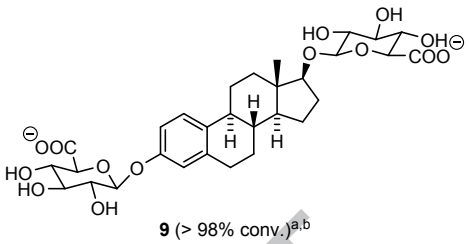
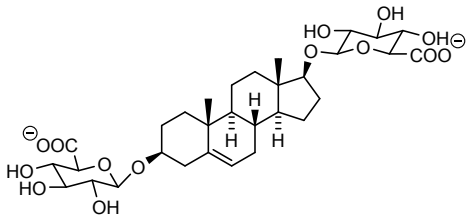


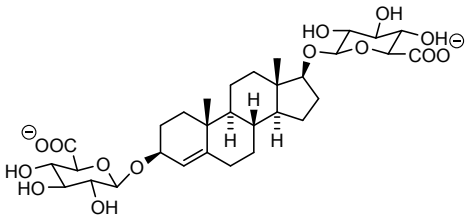
6 (> 98% conv.)[a,b]
2:1 bisglucuronide:3-glucuronide



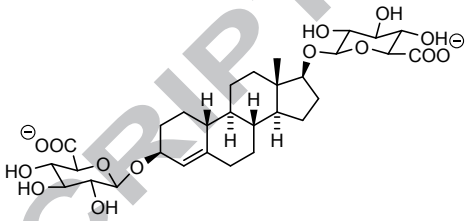
9 (> 98% conv.)[a,b]
2:2:1 bisglucuronide:17-glucuronide:3-glucuronide



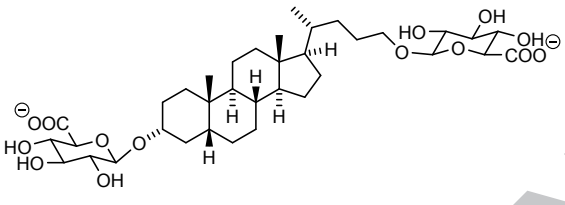
10 (> 98% conv.)[b]
1:1 bisglucuronide:3-glucuronide



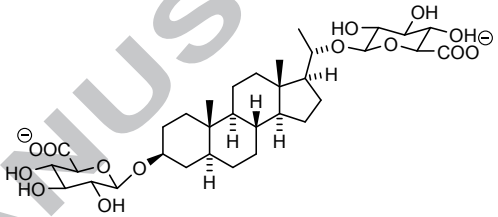
11 (90% conv.)[a,b,c]
1:2:4 bisglucuronide:17-glucuronide:3-glucuronide



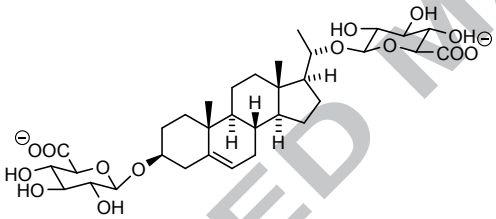
12 (85% conv.)[b,d]
5:1 bisglucuronide:3-glucuronide



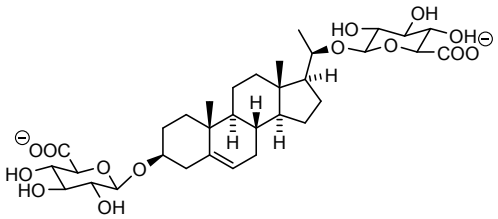
13 (> 98% conv.)[b]
2:1 bisglucuronide:24-glucuronide



14 (> 98% conv.)[b]
1:1 bisglucuronide:3-glucuronide



15 (20*S*:20*R* = 1:1, > 98% conv.)[b,e]
4:1 bisglucuronide:3-glucuronide



16 (20*S*:20*R* = 1:5, > 98% conv.)[b,f]
9:1 bisglucuronide:20-glucuronide

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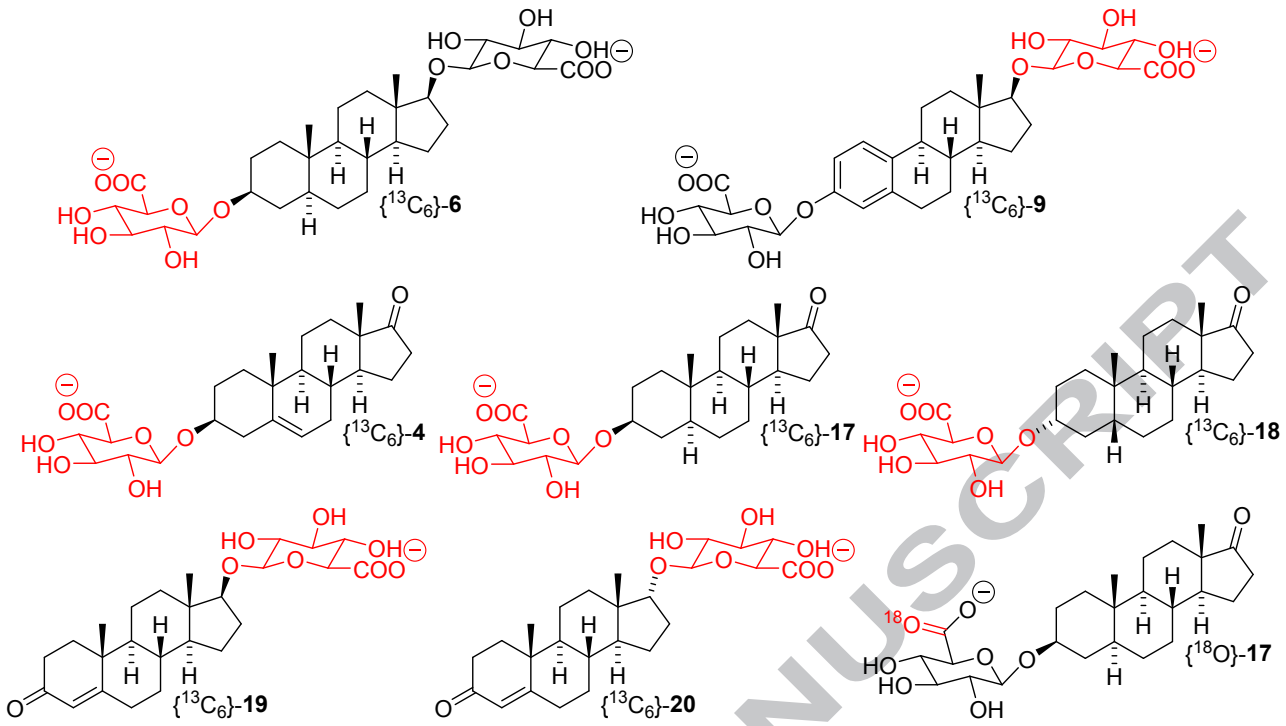

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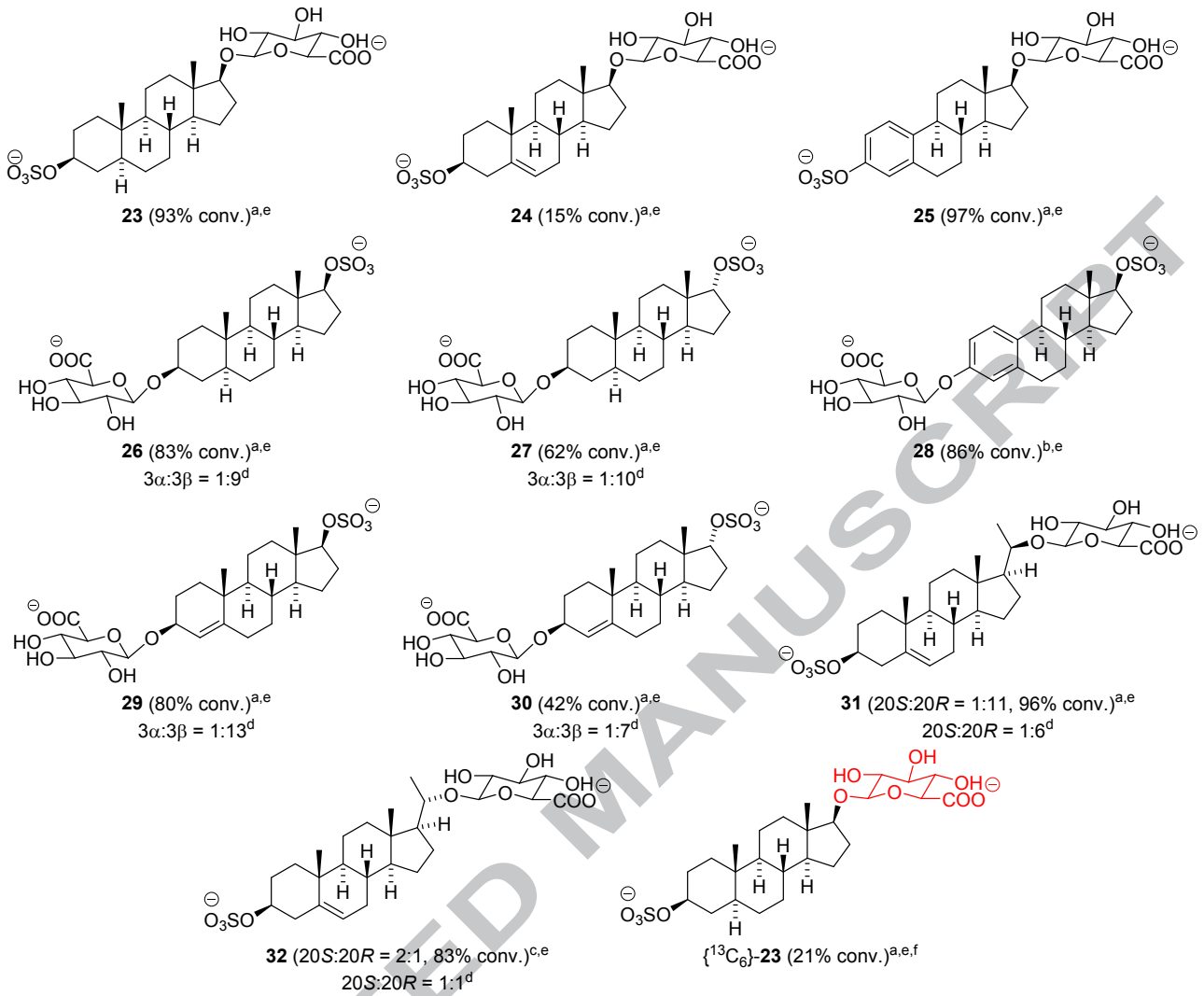

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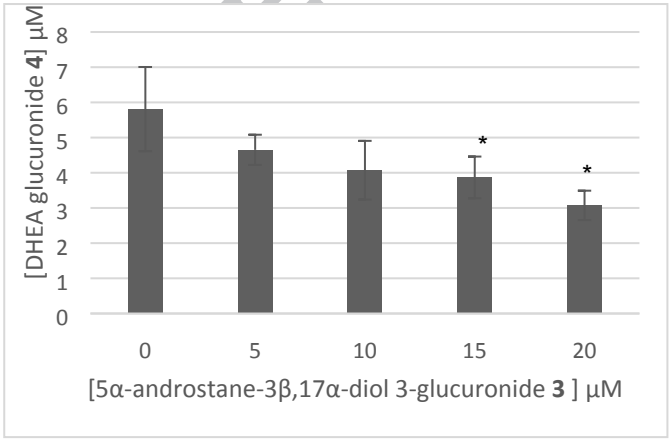

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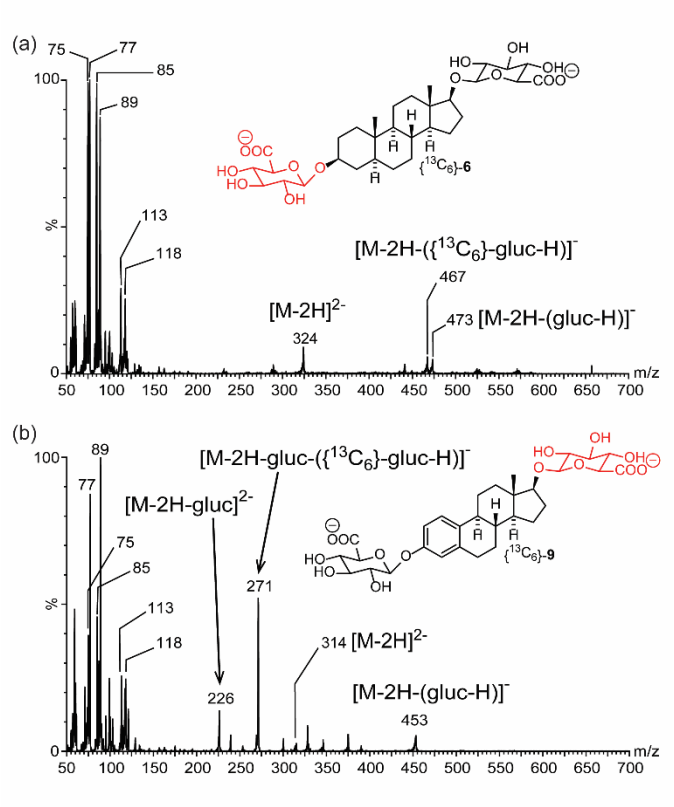

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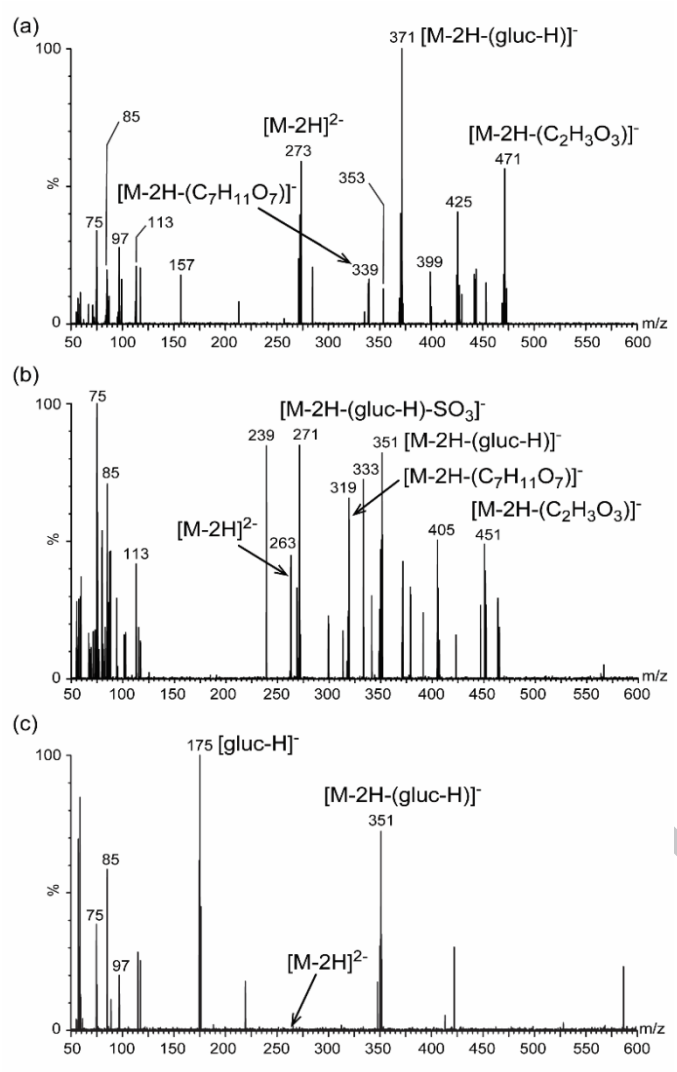

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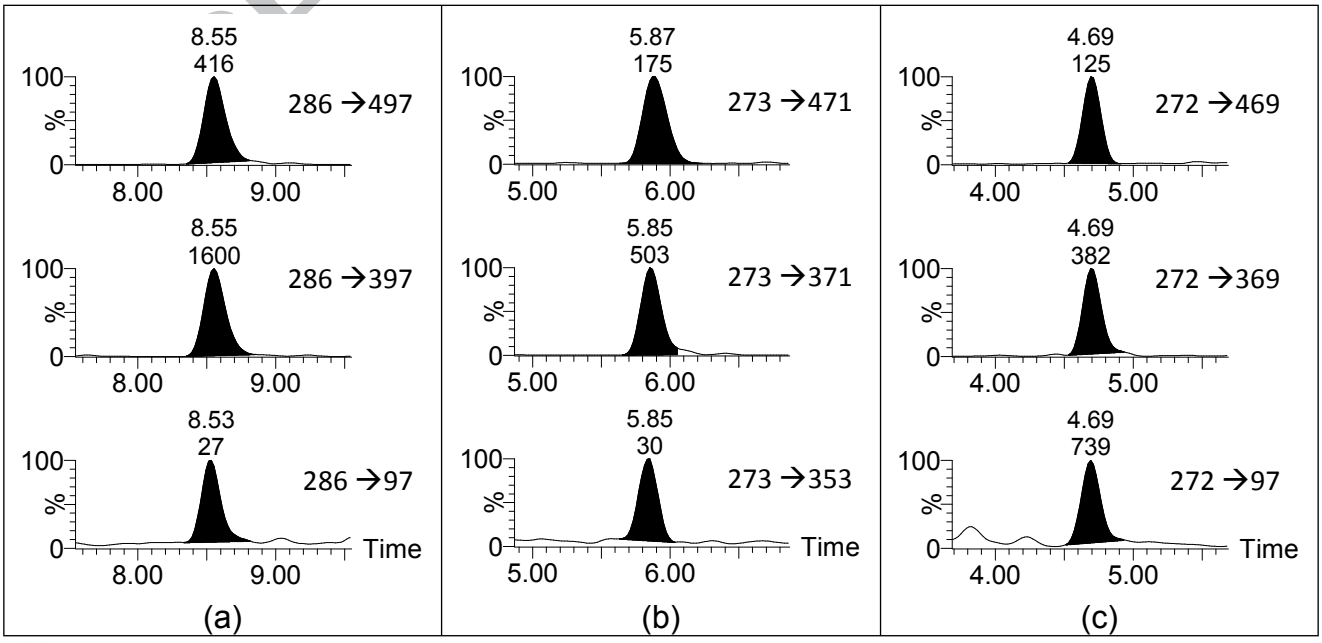

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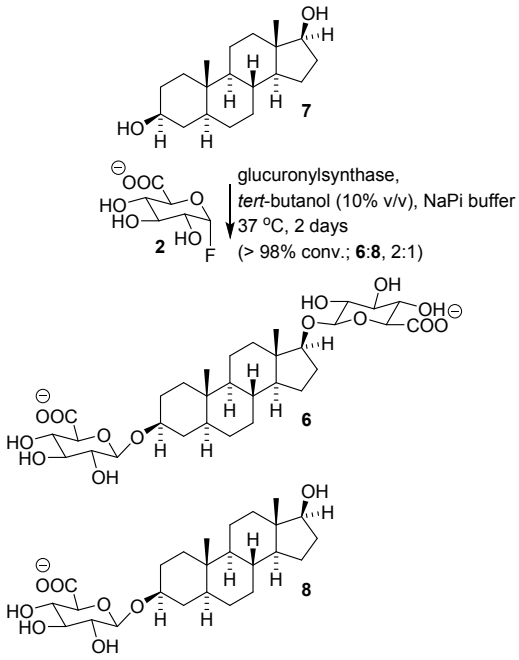

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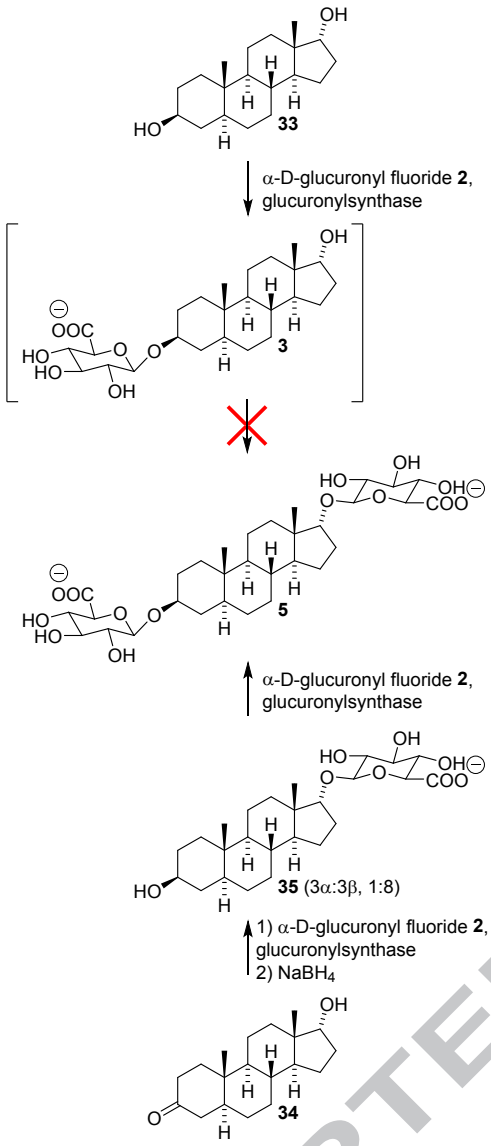

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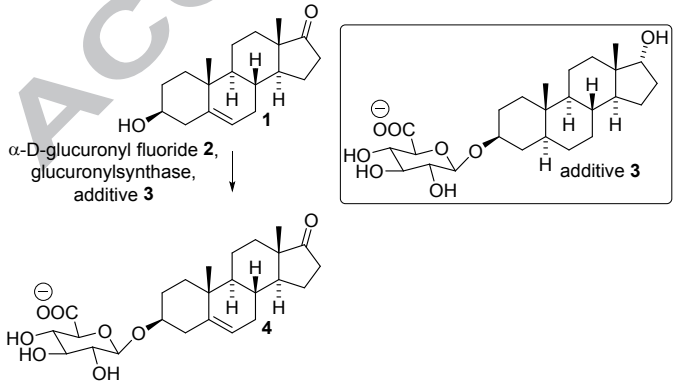

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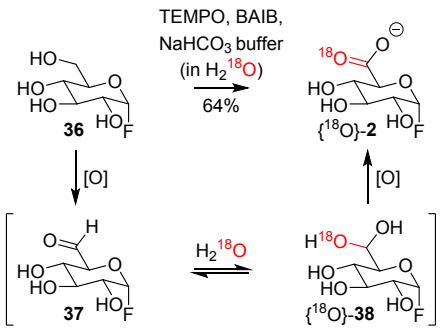

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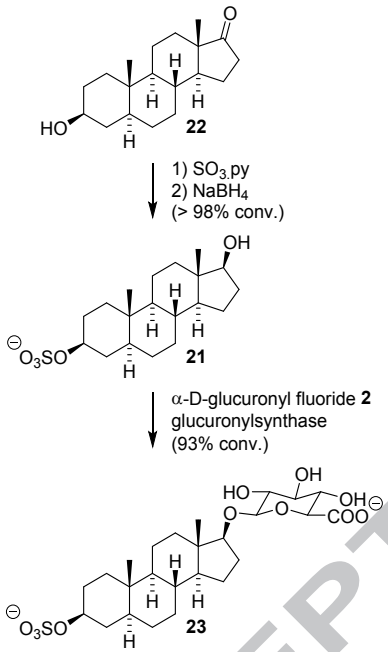

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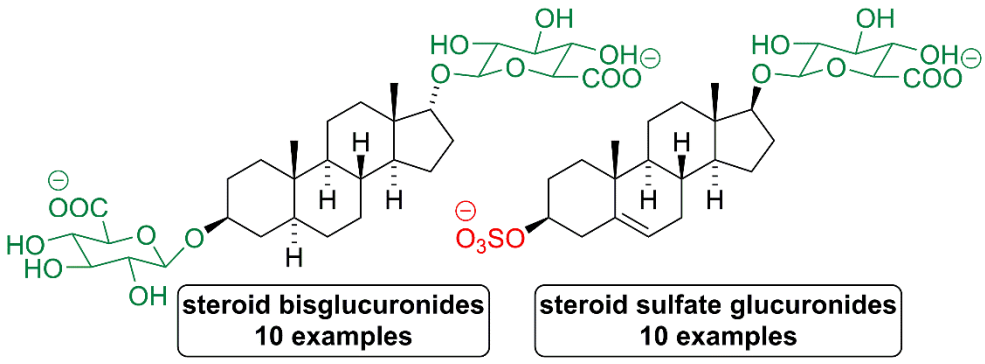

Highlights

- 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
- Three steroid sulfate glucuronide metabolites confirmed in human urine