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Enzyme-assisted synthesis and structure characterization of glucuronide conjugates of eleven anabolic steroid metabolites

Laura Hintikka[a], *Tiia Kuuranne*[b,*], *Olli Aitio*[a], *Mario Thevis*[c], *Wilhelm Schänzer*[c], *Risto Kostiainen*[a]

[a] *Division of Pharmaceutical Chemistry, Faculty of Pharmacy, P.O. Box 56, Viikinkaari 5E, FIN-00014 University of Helsinki, Finland*
[b] *United Laboratories Ltd., P.O. Box 222, Höyläämötie 14, FIN-00380 Helsinki, Finland*
[c] *Center for Preventive Doping Research – Institute of Biochemistry, German Sport University, Carl-Diem Weg 6, 50933 Cologne, Germany*

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ABSTRACT

Enzyme-assisted *in vitro* synthesis of eleven glucuronide-conjugated anabolic androgenic steroid (AAS) metabolites was performed using biphenyl-induced rat liver microsomal enzymes. The substrates within the study were the main compounds and metabolites detected in human urine after dosing of, e.g. metandienone, metenolone, methyltestosterone, nandrolone, and testosterone. Yields of glucuronidation reactions were 13–28% for most compounds, but significantly higher (77–78%) for the substrates with 4-ene-3-one double bond system of the steroid A-ring. Characterization of glucuronide-conjugated AAS structures was based on nuclear magnetic resonance spectroscopy (^{1}H NMR) and on liquid chromatographic–mass spectrometric (LC–MS) and tandem mass spectrometric (LC–MS/MS) analyses in positive and negative ion mode electrospray ionization (ESI). Only minor differences were observed in optimal synthesis conditions between various substrates, which offer a potential to apply this *in vitro* assay as a default method for glucuronidation of new AAS substrates. The method allowed for a rapid production pathway of stereochemically pure AAS glucuronides in milligram amount, such as needed, e.g. in the development of analytical methods in forensic or pharmaceutical sciences, as well as in doping control.

1. Introduction

Anabolic-androgenic steroids (AAS) represent an important class of abused drugs in sport, and are classified as prohibited substances according to World Anti-Doping Agency (WADA). The main desired effects of these testosterone-derived compounds are their potential ability to improve physical performance of skeletal muscle and to balance catabolic condition in body after stress [1–2]. However, mediated mainly by their androgenic activity, the AAS have the potency also to cause serious health problems as side effects, e.g. cardiovascular or liver diseases [2].

Within the human body, the non-polar AAS are transformed extensively by phase-I and phase-II metabolic reactions. Enzymatically catalyzed phase-I reactions (oxidation, reduction or hydroxylation) introduce more functionalities into steroid structure, which increase the polarity and serve as sites for phase-II reactions. Phase-II reactions (glucuronidation, sulfation, methylation, acetylation and conjugation with amino acids or glutathione) are conjugation reactions, which

* *Corresponding author*. Tel.: +358 9 5060 5441; fax: +358 9 5060 5420.
E-mail address: tiia.kuuranne@yhtyneetlaboratoriot.fi (T. Kuuranne).
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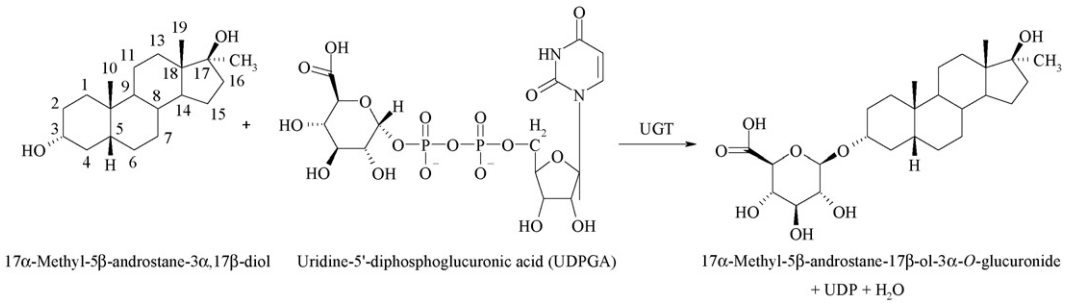
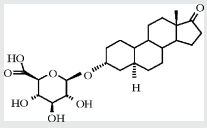
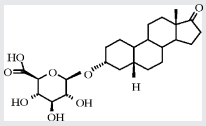
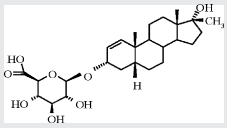
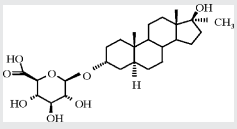
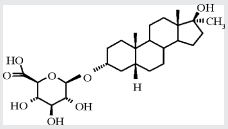
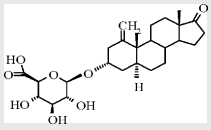


Fig. 1 – Formation of 5β-MTG in glucuronidation reaction.

terminate the activity of the compound and enhance elimination. The most common pathways of AAS conjugation in human body are glucuronidation and sulfation. Steroids possessing a 3β-hydroxyl structure are mostly excreted as sulfates, whereas steroids with 3α- or 17β-hydroxyl group favor glucuronide-conjugation [3].

At present, the analytical methods for the detection of glucuronide-conjugated AAS are based on gas chromatographic (GC) separation and mass spectrometric (MS) detection of hydrolyzed and derivatized compounds. Although the methods are robust and sensitive, sample preparation of the indirect method is time-consuming and the GC–MS sample throughput is relatively low [4]. Thus, the development of more straightforward methods based on the direct analysis of steroid conjugates is of great interest. Application of liquid chromatographic (LC) separation interfaced by soft ionization techniques, such as electrospray ionization (ESI), with tandem mass spectrometry (MS/MS) offers an effective analytical tool for the direct monitoring of AAS glucuronides. One significant drawback of the conjugate analysis, however, is still the lack of reference material, which is a necessity in the development and application of analytical methods. In addition to doping

Table 1A – Nomenclature, precursors, and structures of the AAS glucuronides, as well as sources of the steroid aglycones used as starting material

Abbreviation	Compound	Structure	Precursor	Source
5α-NG	5α-Estran-17-one-3α-O-glucuronide		Nandrolone	S
5β-NG	5β-Estran-17-one-3α-O-glucuronide		Nandrolone	S
5β-EPIMG	17β-Methyl-5β-androst-1-ene-17α-ol-3α-O-glucuronide		Metandienone	DSHS
5α-MTG	17α-Methyl-5α-androstane-17β-ol-3α-O-glucuronide		Mestanolone Methyltestosterone Oxymetholone	S
5β-MTG	17α-Methyl-5β-androstane-17β-ol-3α-O-glucuronide		Metandienone Metandriol Methyltestosterone	S
5α-MEG	1-Methylen-5α-androstan-17-one-3α-O-glucuronide		Metenolone	DSHS

DSHS = Institute of Biochemistry, Cologne, Germany; S = Steraloids, Wilmington, USA.

control analytical purposes, the conjugated reference standards are also needed within steroid metabolism research in pharmaceutical industry and research institutes.

In vivo production of reference substances in human runs often into ethical as well as practical problems associated with the isolation of pure metabolites from urine. Chemical synthesis methods for several metabolites of AAS have been developed, both phase-I [5,6] and recently also phase-II metabolites [7–9]. Some issues related to chemical synthesis, e.g. formation of racemic reaction mixtures has been reported earlier [10]. An alternative approach to chemical *in vitro* glucuronide synthesis is enzyme-assisted synthesis catalyzed with microsomal uridine diphosphoglucuronosyl-transferase (UGT) enzymes (Fig. 1). One of the main advantages of the enzyme-assisted synthesis over the chemical synthesis is the stereospecificity of the enzymes, which allows synthesis of stereospecifically pure conjugates. Secondly, enzyme-assisted synthesis offers a practical pathway for the rapid production of small amounts of glucuronides, such as needed in the build-up of an analytical method. Rat liver preparations as a source of conjugating enzymes have been used for several groups of substrates, e.g. *p*-nitrophenol [11], nitrogen containing antidepressants [12], catechols [13,14] nitrocatechols [15,16], androsterone, androstanediol, dihydrotestosterone [17], epitestosterone [18], and testosterone [19].

The aim of this study was to synthesize glucuronide conjugate standards of eleven AAS. The selection of the compounds in this work represent the parent compounds and metabolites which can be detected in human urine after dosing of exogenous anabolic steroids, e.g. metandienone, metenolone, methyltestosterone and nandrolone, as well as endogenous testosterone (Tables 1A and 1B), which all are reported to be misused by sportsmen [20]. The group of compounds represents also steroids with small differences in the substitution of carbons 1, 3, 5, 10 and 17. The glucuronides were synthesized by using enzyme-assisted synthesis method, which was optimized in respective to substrate, UDPGA and protein concentration. The synthesized products were characterized by nuclear magnetic resonance (NMR) spectroscopy and ESI tandem mass spectrometry, and used as reference material for the development of an LC–MS-based method suitable for UGT enzyme kinetic studies.

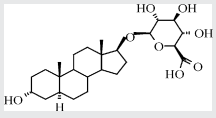
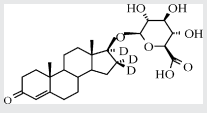
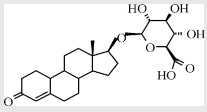
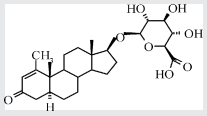
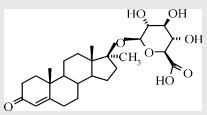
2. Methods

2.1. Substrates and reagents

Nomenclature, precursors, and structures of the AAS glucuronides, as well as sources of the steroid aglycones of this study are presented in Tables 1A and 1B. Uridine-5′-diphosphoglucuronic acid (UDPGA) and D-saccharic acid 1,4-lactone were purchased from Sigma (MO, USA) and Aroclor 1254 (RCS-088/Analabs, Lot No K040) was from the Foxboro Company (CT, USA). Acetonitrile (Rathburn, Scotland), dichloromethane (Baker, The Netherlands), and methanol (Baker) were of HPLC-grade. For the NMR studies methanol-d_4 (99.50%, Acros Organics, Belgium) and acetone-d_6 (99.9%, Aldrich, Germany) were used.

Liver microsomes were prepared from Aroclor 1254 induced (a single dose of 500 mg/4.5 mL olive oil/body weight male Wistar rats ($n = 5$) at the department of Industrial Hygiene and Toxicology (Finnish Institute of Occupational Health, Helsinki, Finland) according to ear-

Table 1B – Nomenclature, precursors, and structures of the AAS glucuronides, as well as sources of the steroid aglycones used as starting material

Abbreviation	Compound	Structure	Precursor	Source
AG	5α-Androstane-3α-ol-17β-O-glucuronide		Testosterone	DSHS
d_3-TG	[16,16,17-$^2H_3$]4-androsten-3-one-17β-O-glucuronide		–	DSHS
NG	Estr-4-en-3-one-17β-O-glucuronide		Nandrolone	Diosynth
5α-1-MEG	1-Methyl-5α-androst-1-en-3-one-17β-O-glucuronide		Metenolone	DSHS
MTG	17α-Methyl-5β-androstane-3α-ol-17β-O-glucuronide		Methyltestosterone	DSHS

DSHS = Institute of Biochemistry, Cologne, Germany; S = Steraloids, Wilmington, USA.

Table 2 – Optimized conditions of the synthesis of AAS glucuronides

Abbreviation	Substrate (μM)	UDPGA (mM)	Protein (mg/mL)
5α-NG	250	5	1
5β-NG	500	5	0.75
5β-EPIMG	500	5	1
5α-MTG	1000	2.5	0.5
5β-MTG	1000	2.5	0.5
5α-MEG	500–1000	2.5	0.75
AG	500	2.5	1
d_3-TG	500	5	1
NG	500	5	1
5α-1-MEG	500	5	1
MTG	1000	7.5	0.5

See Tables 1A and 1B for nomenclature.

lier described procedure [21]. The treatment of the animals was approved by the local ethical committee for animal studies. BCA protein assay kit (Pierce, IL, USA) was used for the determination of protein concentration (18.5 mg/mL), which was used to standardize the amount of microsomal enzymes.

2.2. *Enzyme-assisted synthesis and purification*

Synthesis conditions were optimized in small-scale studies in total volume of 100 μL with respect to concentration of the substrate 1–1000 μM (AAS or its phase-I metabolite), UDPGA 0.5–10 mM, and enzymatic protein 0.1–1.85 mg/mL. Optimization of each parameter was carried out in turn while keeping the other parameters constant in an overnight incubation. The constant concentrations of each component were 50 μM (substrate), 5 mM (co-substrate), 0.5 mg/mL (protein) and incubation time of 2 h. D-Saccharic acid 1,4-lactone (5 mM) was added to the reaction mixture as β-glucuronidase inhibitor to prevent the effect of bond-breaking enzymes of the tissue preparation. Incubation reaction was carried out in 50 mM phosphate buffer (pH 7.4) with 5 mM $MgCl_2$. After centrifugation the internal standard (d_3-5β-MTG) was added to the supernatant of the small-scale samples and purified in SPE prior to LC–MS/MS analysis.

For the synthesis of AAS glucuronide steroid aglycone was diluted in 2 mL of methanol and filled up to the total volume of 20 mL of earlier described phosphate buffer. The reaction was initiated with the addition of UDPGA, carried out in water bath of 37 °C for 12–15 h with continuous magnet stirring, and terminated with transferring the incubation mixture

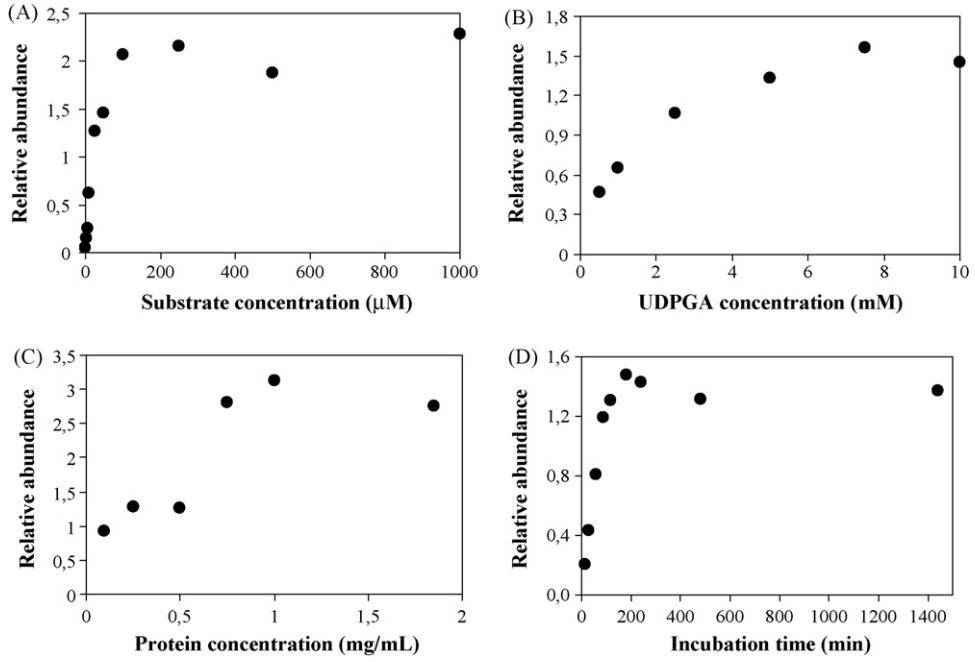


Fig. 2 – Optimization of synthesis conditions for 5α-NG. Formation of the conjugate as function of (A) substrate concentration, (B) UDPGA concentration, (C) microsomal protein concentration, and (D) incubation time. Constant conditions: 50 μM substrate, 5 mM UDPGA, 0.5 mg/mL microsomal protein, and 2 h incubation time.

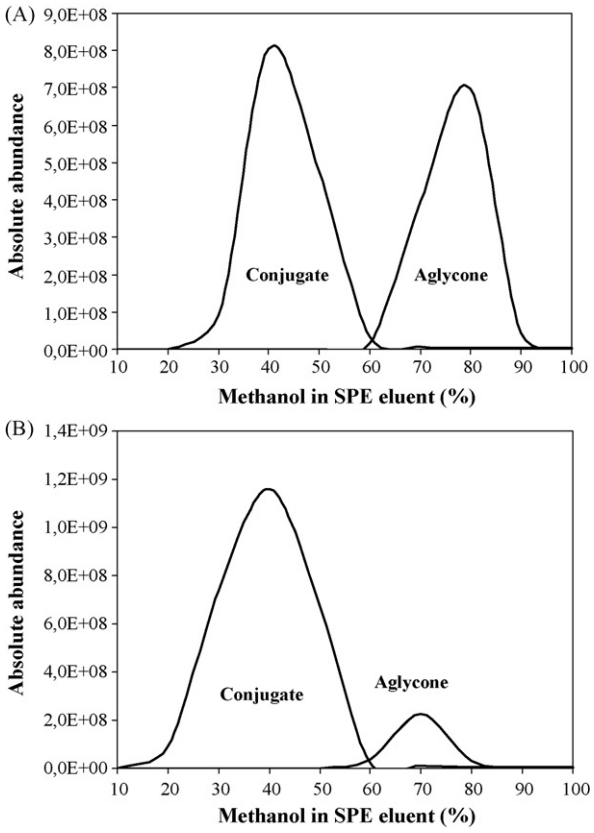


Fig. 3 – Elution of AAS glucuronide and steroid aglycone from the reaction mixture of 5α-1-MEG (A) and NG (B) as function of methanol concentration in the eluent of solid-phase extraction.

to ice bath, and precipitating the enzymatic protein with dichloromethane, which also extracted the excess of starting material. The aqueous phase of the synthesis, containing the AAS glucuronide, was removed and further purified by solid-phase extraction (SPE) using C_{18} cartridge (Isolute, 100 mg, International Sorbent Technology, UK) according to the method described earlier by Borts and Bowers [22], except to an additional SPE step, which was added to the procedure to ensure the purity of steroid glucuronide fraction with the final elution of 60% methanol in water. AAS glucuronide fraction was then evaporated to dryness in 60 °C with nitrogen and reconstituted in LC–MS/MS solvents A:B (75:25).

3. Analytical methods

3.1. *Liquid chromatography*

The LC separation was done by a Hewlett-Packard model 1100 binary pump and autosampler (Hewlett-Packard, Palo Alto, CA, USA), using an endcapped Purospher RP-18 column (125 mm × 3 mm i.d., particle size 5 μm) (Merck). Eluent A was 15 mM ammonium acetate–formic acid buffer (pH 4.2) in water–acetonitrile (9:1, v/v) and eluent B contained the same buffer, with the water–acetonitrile ratio 1:9 (v/v). The injection volume was 5 μL. In gradient run eluent B was increased linearly from 25 to 40% in 11 min, followed by a rapid raise to 90% for 2 min and equilibration of 3 min. The LC flow rate of 0.5 mL/min was post-column split (1:10) (Acurate; LC-Packings, The Netherlands) prior to introduction to ESI source.

3.2. *Mass spectrometry*

The instrument used in MS and MS/MS measurements was Applied Biosystems API3000 (Perkin-Elmer SCIEX, Concord, ON, Canada) triple quadrupole instrument with a turbo ionspray source. The ionspray voltages were optimized to 5500 and 4500 V, and the orifice (declustering) voltages to 20 V, in positive and negative ion mode ESI, respectively. Purified air (custom-made air purifier; Atlas Copco, Belgium) was used as the nebulizing gas (0.82 L/min) and curtain gas. Nitrogen (Generator Systems 75–72; Whatman, USA) was used as the collision gas and turbo gas (6 L/min) and the ion source temperature was adjusted to 350 °C. The MS and MS/MS spectra were measured by using direct infusion of the purified compounds with flow rate of 10 μL/min. The samples were dissolved in the LC eluent consisting of 7.5 mM ammonium acetate in water–methanol (50–50, v/v). In negative ion mode the eluent was neutral, whereas in positive ion mode the pH was adjusted to 4.2 with formic acid. The recorded mass ranges were *m/z* 100–600 and *m/z* 50–550 in MS and MS/MS experiments, respectively, with the scan speed of 5 s. Collision offset voltages 20–30 and 40 V were applied in positive and negative ion mode, respectively, adjusted to produce the spectrum of representative fragmentation for each glucuronide.

Table 3 – Synthesized steroid glucuronides and their yields

Compound	Yield (mg)	Yield (%)	Compound	Yield (mg)	Yield (%)
5α-NG	1.3	16	AG	1.9	16
5β-NG	1.1	13	d_3-TG	6.5	77
5β-EPIMG	1.4	25	NG	3.5	77
5α-MTG	2.1	22	5α-1-MEG	1.7	24
5β-MTG	2.6	26	MTG	1.2	14
5α-MEG	2.5	28			

See Tables 1A and 1B for nomenclature.

3.3. NMR spectroscopy

The ^{1}H NMR experiments were carried out on a Varian Unity 500 spectrometer at 23 °C using a gHX nano-NMR probe. Acetone-d_6 was used as solvent for MTG, 5β-EPIM and 5β-EPIMG. The rest of the compounds were dissolved in methanol-d_4. The ^{1}H chemical shifts were referenced to residual acetone or methanol, 2.05 and 3.31 ppm, respectively.

4. Results and discussion

4.1. Enzyme-assisted synthesis

Glucuronidation synthesis was optimized using sample volumes of 100 μL with respect to substrate (steroid aglycone), co-substrate (UDPGA) and UGT-enzymes (microsomal protein) concentration, in order to rationalize the consumption of expensive starting materials, especially that of UDPGA. The optimization experiments were carried out with 10% methanol, which was a compromise between dissolution of non-polar steroids and easily denaturizing enzymal protein. All the tested steroid substrates were possible to conjugate via enzyme-assisted pathway using rat liver microsomes, and only slight differences were detected in the optimal conditions for the various substrates (Table 2). Substrate inhibition was not observed for any of the steroids within the concentration range of 1–1000 μM, but when examining the effect of substrate concentration, a plateau of glucuronidation was observed at 500–1000 μM, except for 5α-NG, for which it was 250 μM (Fig. 2A). The first point of the plateau was selected as the concentration level for the bulk synthesis. The increase of UDPGA concentration increased the yield of the glucuronides until the plateau was achieved (Fig. 2B). Optimal concentration of UDPGA was 2.5–5 mM for all the other compounds except for methyltestosterone, which required 7.5 mM concentration of co-substrate for maximal yield of MTG. The formation of the steroid glucuronides was linearly proportional to the concentration of protein up to 0.5–1 mg/mL (Fig. 2C). The effect of incubation time was tested up to 24 h, and observed to reach the plateau after 6 h of incubation at the latest (Fig. 2D). However, there was no evidence of breakdown of the glucuronide conjugate during that time, which allowed an overnight incubation (12–15 h).

The larger scale enzymatic synthesis of the steroid glucuronides were carried out under optimized conditions (Table 2) using 20 mL reaction volume. The excess of starting material was removed in liquid–liquid extraction (LLE) with dichloromethane, and recycled in prospective (non-quantitative) synthesis. The more polar steroid glucuronide remained in aqueous layer, but also some residue of starting material was found to remain in that fraction. To increase purity the sample was further purified with SPE. Retention of steroid substrate and glucuronide conjugate in SPE was examined as a function of methanol percentage (1–100%) (Fig. 3). The difference between the polarities of the two compounds was sufficient for the complete isolation of the AAS glucuronide when methanol–water (60:40, v/v) was applied in the final elution.

The synthesis method was suitable for producing steroid glucuronides in milligram amounts (1.1–6.5 mg), with yields of 13–78% (Table 3). Steroid substrates with 4-ene-3-one structure and 17β-OH position solely available for *O*-glucuronidation (NG, d_3-NG and d_3-TG) showed superior yields of 77–78% in comparison to the substrates with completely saturated or 1-ene A-ring structure (13–28%). Glucuronidation of methyltestosterone was exceptional (14%), as the only possible site for *O*-glucuronidation in methyltestosterone is the 17β-OH-group, sterically hindered by the neighboring 17α-CH_3, which is likely to inhibit the catalysis of the bulky UGT enzymes. Based on the detected formation of MTG, although in low yield, it may be proposed that the structure still allows conjugation to some extent in the absence of a more favorable functionality. Within the group of selected compounds 5α-androstane-3α,17β-diol and its d_5-labeled analog were the only ones with sterically non-hindered 3α- and 17β-hydroxyl groups.

5. Structure characterization

5.1. Mass spectrometry

The abundant ammonium adduct ion $[M+NH_4]^+$ was detected in positive ion ESI-MS for the glucuronides with saturated A-ring system, whereas for d_3-TG, NG and d_3-NG with 4-ene-3-one structure protonated molecule $[M+H]^+$ was detected as base peak. The ratio of protonated molecule and ammonium adduct ion was found to strongly depend on the proton affinity (PA) of the compound, higher proton affinity resulting into formation of stronger protonated molecule. Proton affinity of the 4-ene-3-one structure in the steroid ring results higher proton affinity than hydroxyl group or carbonyl group without conjugated double bond [23]. For 5α-1-MEG and MTG, the relative abundance of $[M+NH_4]^+$ and $[M+H]^+$ was approximately equal. Formation of sodium adduct $[M+Na]^+$ was also detected for most of the compounds, although with variable relative abundances. In negative ion ESI an intensive deprotonated molecule $[M-H]^-$ was observed as the base peak for all AAS glucuronides (Table 4). In negative ion ESI the most potential

Table 4 – Positive and negative ion ESI-MS of AAS glucuronides

Compound	POS ESI			NEG ESI
	$[M+H]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[M-H]^-$
5α-NG	n.d.	470 (100)	n.d.	451 (100)
5β-NG	n.d.	470 (100)	475 (43)	451 (100)
5β-EPIMG	n.d.	498 (100)	n.d.	479 (100)
5α-MTG	n.d.	500 (100)	505 (36)	481 (100)
5β-MTG	n.d.	500 (100)	505 (38)	481 (100)
5α-MEG	479 (2)	496 (57)	501 (100)	477 (100)
AG	n.d.	486 (100)	491 (6)	467 (100)
d_3-TG	468 (100)	485 (41)	490 (66)	466 (100)
NG	451 (100)	n.d.	473 (50)	449 (100)
5α-1-MEG	479 (89)	496 (100)	501 (11)	477 (100)
MTG	479 (94)	496 (100)	501 (15)	477 (100)

See Tables 1A and 1B for nomenclature.

site for deprotonation $[M-H]^-$ is the carboxylic acid moiety of the glucuronic acid, as the steroid aglycones are not ionized in negative ion ESI at all. These results are in good agreement with earlier studies by Bowers and Sanaullah [24], Williams et al. [25] and Kuuranne et al. [26,27], and gave evidence on the corrected molecule weights of the synthesized steroid glucuronides.

The positive ion MS/MS spectra (Table 3) showed abundant product ions formed by loss of glucuronide moiety without or with loss of one or two water molecules. The positive charge stayed predominantly on the aglycone side, and only weak fragments were detected from glucuronic acid side, e.g. $[Glu+H]^+ = 177$, $[Glu+H-H_2O]^+ = 159$, $[Glu+H-2H_2O]^+ = 141$ (Table 5). The negative ion MS/MS spectra of $[M-H]^-$ showed intense product ions derived from the deprotonated glucuronic acid $[Glu-H]^-$ (Table 6). The spectra showed also weak product ions formed by the cleavage of glucuronic acid moiety $[M-H-Glu]^-$, followed by the abstraction of two hydrogens $[M-H-Glu-2H]^-$, and losses of water $[M-H-Glu-nH_2O]^-$. The positive and negative ion MS and MS/MS spectra indicate clearly the presence of glucuronide moiety. However, the MS data does not provide unambiguous information on the site of glucuronidation.

5.2. *NMR spectroscopy*

One-dimensional ^1H NMR was used to determine the position of the glycosidic linkage and the anomericity of the glucuronic acid moiety in the steroid glucuronides. Each glucuronide spectrum contained the five methine signals arising from the glucuronic acid. The measured vicinal $^3J_{GH1,GH2}$ coupling constants of approximately 7.8 Hz confirmed the β-configuration for the glucuronic acid for each glucuronide. Integration of the signals gave a 1:1 glucuronic acid/aglycone ratio indicating the correct stoichiometry.

As the steroid substrates resulting d_4-5α-NG, 5α-MEG, d_3-TG, NG, d_3-NG, 5α1-MEG and MTG contain only one possible site for glucuronidation there was no ambiguity about the position of the glycosidic linkage in the corresponding glucuronides. In the structures of AG and 5β-EPIMG there are two possible glucuronidation sites, namely hydroxyl groups in positions 3 and 17. For these compounds ^1H spectra of both 5α-androstane-3α,17β-diol (A, steroid aglycone) and the steroid glucuronide (AG) were measured. In 5α-androstane-3α,17β-diol H3 and H17 resonate downfield to the steroid bulk region and can be distinguished from each other due to their different coupling patterns. H17 of steroid aglycone is a triplet [28] with a coupling constant of 8.5 Hz while H3 is typically a multiplet resulting from several small couplings [29]. Comparison of the steroid aglycone spectrum with its glucuronide-conjugated product (AG) spectrum showed that the H17 signal shifted by 0.25 ppm downfield upon glucuronidation and on the other hand that H3 signal did not shift, which suggests that the glucuronide indeed is a 17-glucuronide.

For 5β-EPIMG none of the methyl signals, including 17β-methyl, shifted upon glucuronidation while H1, H2 and H3 shifted downfield by 0.09, 0.12 and 0.16 ppm, respectively. This suggests that 5β-EPIMG is a 3-glucuronide. 5α-NG, 5β-NG, 5α-MTG and 5β-MTG have been fully characterized by NMR previously [30].

Table 5 – Positive ion ESI-MS/MS of AAS glucuronides

Ions	5α-NG, 20 V	5β-NG, 20 V	5β-EPIMG, 20 V	5α-MTG, 20 V	5β-MTG, 20 V	5α-MEG, 20 V	AG, 20 V	d_3-TG, 25 V	NG, 30 V	5α-1-MEG, 30 V	MTG, 30 V
Precursor											
$[M+NH_4]^+$	470 (11)	470 (100)	498 (5)	500 (13)	500 (9)	496 (7)	486 (4)	468 (100)	451 (100)	496 (1)	496 (2)
$[M+H]^+$											
Product ions											
$[M+NH_4\text{-}NH_3]^+$	453 (6)	453 (23)	481 (4)	n.d.	n.d.	479 (1)	n.d.			479 (100)	479 (100)
$[M+NH_4-H_2O]^+$	n.d.	n.d.		482 (4)	482 (4)	n.d.	n.d.			n.d.	n.d.
$[M+H-H_2O]^+$	435 (28)	435 (21)	n.d.	465 (15)	465 (13)	461 (6)	451 (7)	n.d.	n.d.	n.d.	461 (2)
$[M+H-2H_2O]^+$	417 (74)	417 (92)	n.d.	447 (5)	447 (7)	443 (2)	433 (1)	n.d.	n.d.	n.d.	n.d.
$[M+H-Glu]^+$	277 (60)	277 (67)	305 (3)	n.d.	n.d.	303 (8)	n.d.	292 (45)	275 (71)	303 (18)	303 (32)
$[M+H-Glu-H_2O]^+$	259 (100)	259 (42)	287 (4)	289 (44)	289 (58)	285 (100)	275 (34)	274 (13)	257 (24)	285 (11)	285 (71)
$[M+H-Glu-2H_2O]^+$	241 (14)	241 (13)	269 (100)	271 (100)	271 (100)	267 (10)	257 (100)	256 (8)	239 (14)	267 (4)	267 (34)
$[Glu+H]^+$	177 (22)	177 (4)	177 (2)	177 (19)	177 (6)	n.d.	177 (13)	177 (5)	n.d.	177 (1)	177 (9)
$[Glu+H-H_2O]^+$	159 (16)	159 (4)	159 (1)	159 (7)	159 (3)	159 (1)	159 (7)	159 (8)	159 (12)	159 (4)	159 (15)
$[Glu+H-2H_2O]^+$	141 (16)	141 (5)	n.d.	141 (5)	141 (4)	141 (1)	141 (6)	141 (5)	141 (6)	141 (5)	141 (4)

Collision offset voltages indicated separately for each steroid glucuronide.

Table 6 – Negative ion ESI-MS/MS of AAS glucuronides at applied collision offset voltage of 40 V

Ions	5α-NG	5β-NG	5β-EPIMG	5α-MTG	5β-MTG	5α-MEG	AG	d_3-TG	NG	5α-1-MEG	MTG
Precursor											
$[M-H]^-$	451 (52)	451 (57)	479 (100)	481 (89)	481 (100)	477 (56)	467 (26)	466 (10)	449 (11)	477 (14)	477 (15)
Product ions											
$[M-H-H_2O]^-$	433 (5)	433 (3)	461 (8)	463 (6)	463 (6)	459 (7)	449 (1)	n.d.	431 (1)	459 (1)	459 (2)
$[M-H-Glu]^-$	n.d.	n.d.	303 (9)	305 (1)	n.d.	301 (5)	291 (6)	290 (6)	273 (32)	301 (26)	301 (15)
$[M-H-Glu-2H]^-$	273 (12)	273 (3)	301 (1)	303 (1)	303 (4)	n.d.	289 (11)	n.d.	271 (23)	299 (22)	n.d.
$[M-H-Glu-H-D]^-$								n.d.			
$[M-H-Glu-2D]^-$								286 (3)			
$[Glu-H]^-$	175 (2)	n.d.	175 (6)	175 (28)	175 (15)	175 (4)	175 (2)	175 (3)	175 (4)	175 (2)	175 (2)
$[Glu-H-H_2O]^-$	157 (11)	157 (9)	157 (9)	157 (36)	157 (18)	157 (11)	157 (6)	157 (6)	157 (9)	157 (5)	157 (4)
$[Glu-H-H_2O-CO]^-$	n.d.	129 (6)	129 (9)	129 (16)	129 (9)	129 (5)	129 (6)	129 (5)	129 (6)	129 (6)	129 (5)
m/z 113	(75)	(55)	(57)	(90)	(59)	(100)	(47)	(82)	(75)	(55)	(48)
m/z 99	(6)	(6)	(9)	(4)	(4)	(5)	(5)	(3)	(8)	(9)	(9)
m/z 97	(1)	n.d.	(4)	(1)	(1)	n.d.	(4)	n.d.	(4)	(4)	n.d.
m/z 95	(12)	(7)	(8)	(10)	(6)	(8)	(18)	(11)	(11)	(15)	(12)
m/z 85	(100)	(100)	(61)	(100)	(41)	(55)	(100)	(100)	(100)	(100)	(100)
m/z 75	(82)	(82)	(40)	(89)	(49)	(85)	(60)	(72)	(77)	(70)	(63)
m/z 59	(6)	(5)	(4)	(12)	(3)	(18)	(6)	(5)	(6)	(7)	(6)

6. Conclusions

Glucuronide-conjugated metabolites of anabolic androgenic steroids were produced by *in vitro* enzyme-assisted synthesis using induced rat liver microsomes as the source of the conjugating uridine diphosphoglucuronosyl-transferase enzymes. The method offers a practical pathway for the rapid production of stereochemically pure AAS glucuronides in milligram amount, such as needed in the development of analytical methods. The yields of syntheses were mostly 13–28%, though for substrates with conjugated 4-ene-3-one double bond system significantly higher (77–78%). Due to relatively simple reaction mixture and only minor differences between the optimal conditions for the various AAS substrates, the addition of a new structural analogue to the test set should be straightforward and thus also easily applied.

The intact AAS glucuronides can be ionized in both positive and negative ion ESI. The positive ion ESI mass the base peak was either $[M+H]^+$ or $[M+NH_4]^+$, depending on the A-ring structure of the steroid. In negative ion mode an intense deprotonated molecule $[M-H]^-$ was observed similarly with each analyte, completing the molecular weight data of the synthesized conjugates. The most representative mass spectrometric information on the AAS glucuronide structure was obtained from MS/MS fragmentation studies in positive ion ESI, which is also the method of choice for the routine LC–MS/MS analysis of AAS glucuronides.

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