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ARTICLE

The Escherichia coli glucuronylsynthase promoted synthesis of steroid glucuronides: improved practicality and broader scope†

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A library of steroid glucuronides was prepared using the glucuronylsynthase derived from *Escherichia coli* β-glucuronidase, followed by purification using solid-phase extraction. A representative range of steroid substrates were screened for synthesis on the milligram scale under optimised conditions with conversions dependent on steroid substitution and stereochemistry. Epiandrosterone (3β-hydroxy-5α-androstane-17-one) provided the highest conversion of 90% (84% isolated yield). The previously unreported glucuronide conjugates of methandriol (17α-methylandrost-5-ene-3β,17β-diol), cholest-5-ene-3β,25-diol and the designer steroid trenazone (17β-hydroxyestr-4,9-dien-3-one) were prepared on a multi-milligram scale suitable for characterisation by ^{1}H and ^{13}C NMR spectroscopy. The glucuronide conjugate of d_5-etiocholanolone (2,2,3,4,4-d_5-3α-hydroxy-5β-androstane-17-one), a target developed by the World Anti-Doping Agency as a certified reference material, was also prepared on a milligram scale. The improved *E. coli* glucuronylsynthase method provides for the rapid synthesis and purification of steroid glucuronides on a scale suitable for a range of analytical applications.

INTRODUCTION

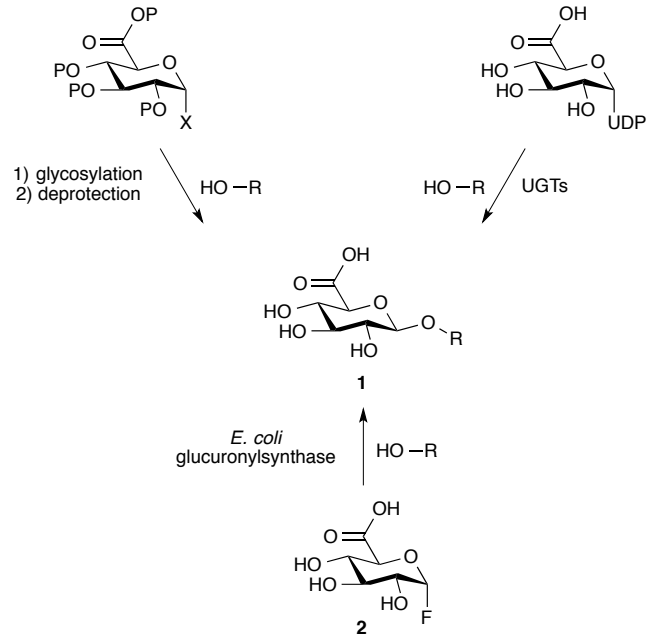
The conjugation of glucuronic acid to an acceptor in phase II metabolism is a major pathway for the excretion of endogenous and exogenous substances in mammalian systems. Glucuronide conjugates **1** (Scheme 1) thus play an important role in various fields such as pharmacology,[1,2] sports drug testing,[3,4] and detection of agricultural residues in the environment.[5] In addition, glucuronides have been used in linkers for targeted delivery of cytotoxic agents coupled to monoclonal antibodies,[6,7] as well as in pro-drugs, which use the body's glucuronidase enzymes to unmask bioactive species.[1,8,9]

There have been many protocols developed for the synthesis of glucuronides and these fall under two broad approaches: chemical or enzymatic (Scheme 1). The chemical approach, that includes the Koenigs–Knorr glycosylation,[10] and more modern variants, is still the most widely-used method for glucuronide synthesis.[1,11] However, it can result in the production of ortho ester or acyl transfer side-products that serve to reduce the yield and complicate purification of the desired glucuronide.[1,11] In addition, the need for water-sensitive activators and glycosyl donors, and the requirement for protecting groups makes small-scale synthesis for analytical purposes challenging.

The enzymatic approach consists of two major methods, the first of which utilises mammalian uridine 5′-diphospho-glucuronosyltransferase (UGT) enzyme family to perform the glucuronylation in a single step.[12–15] This method has the advantage of avoiding protecting groups, but employs a sensitive and expensive uridine 5′-diphosphoglucuronic acid donor and requires expression of the membrane-bound UGT enzymes.[16] Difficulties in the expression and purification of UGTs mean that animal sacrifice is often used to obtain sufficient quantities of enzyme for synthesis, which gives rise to additional ethical considerations.[12–14]

The second enzymatic method developed by the McLeod group employs the glucuronylsynthase derived from wild-type *E. coli* β-glucuronidase.[17,18] The glucuronylsynthase is derived by single-point mutation of the wild-type enzyme, which disables glycosyl hydrolase activity but allows for glycoside synthesis when used in combination with a synthetically-derived α-D-glucuronyl fluoride **2** donor.[19,20] This method also performs glucuronylation in a single step but has some added advantages over the UGT-based approach in that the *E. coli* glucuronylsynthase is a soluble bacterial enzyme that is readily expressed and purified and the α-D-glucuronyl fluoride **2** substrate is readily accessible in four synthetic steps from D-glucose.

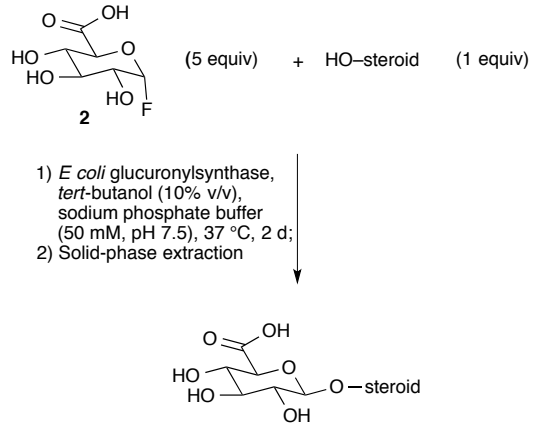
Steroid glucuronides are an important class of compounds in the field of sports drug testing, with many endogenous and exogenous steroids excreted primarily as glucuronide conjugates.[21] As a result, significant work has been undertaken on both the synthesis and analysis of steroid glucuronides.[13,14,22,23] In previous work we outlined a three-step approach based on the temporary introduction of solubilising substituents for the glucuronylsynthase-mediated synthesis of steroid glucuronides.[17] Although effective, the addition and deletion of solubilising substituents proved somewhat unwieldy and lacking in generality. In this paper we report on the development of an improved method for the single-step glucuronylsynthase-mediated synthesis and purification of steroid glucuronides on a scale suitable for a range of analytical applications.



Scheme 1 Preparation of glucuronide conjugates.

Results and discussion

The glucuronylation protocol developed has three main components: the glucuronylsynthase enzyme, the synthetically-derived α-D-glucuronyl fluoride **2** donor and a suitable acceptor alcohol (Scheme 2).[17] In this paper the substrates were steroidal alcohols of different constitutions and stereochemistries. The reactions were carried out in a mixed 10% v/v *tert*-butanol/sodium phosphate buffer at pH 7.5 and 37 °C for 2 d. We observed that conducting the reactions in the presence of *tert*-butanol led to a significant increase in steroid solubility without the co-solvent serving as a substrate or having a deleterious effect on enzyme activity, obviating the need for solubilising substituents to obtain reasonable solubility and hence useful enzyme-catalysed reaction rates.[17] This contrasts with the influence of *tert*-butanol co-solvent (5 or 10% v/v) on the glucuronylation of 2-phenylethanol that shows moderately reduced enzyme activity.[17] The wild-type β-glucuronidase-mediated hydrolysis of *para*-nitrophenyl β-D-glucuronide also shows reduced activity in the presence of *tert*-butanol co-solvent (5 or 10% v/v), albeit with similar long-term enzyme stability relative to buffer alone.[17]

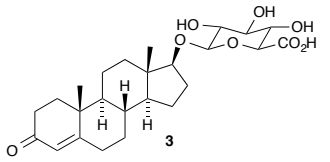
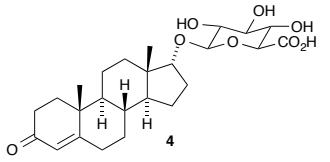
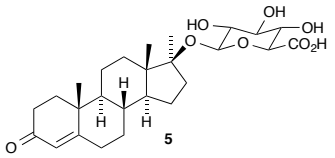
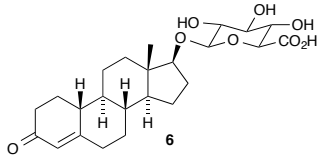
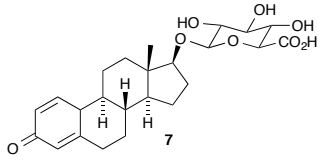
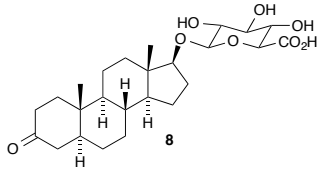
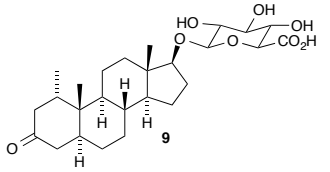
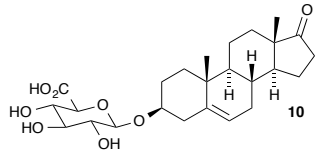
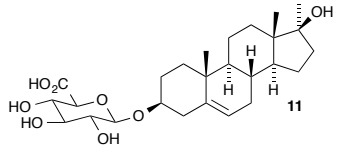
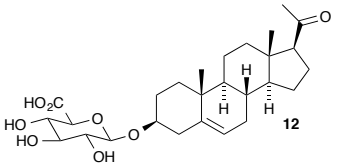
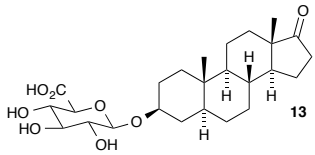
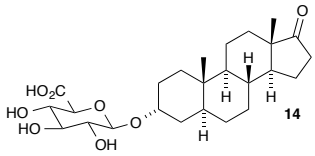
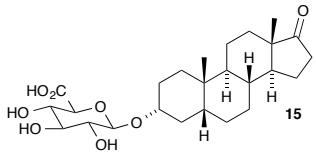
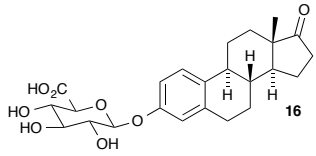
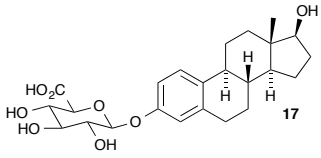
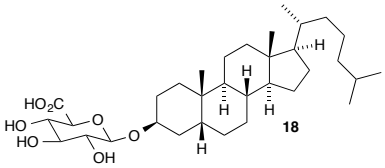
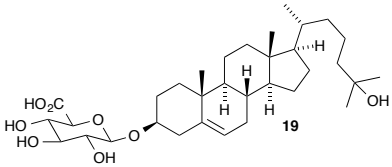
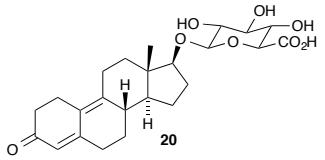


Scheme 2 The glucuronylsynthase protocol.

To facilitate the rapid purification of the glucuronide product from residual steroidal alcohol, non-volatile salts and other reaction components we developed a method based on commercially available solid-phase extraction (SPE) cartridges. Our SPE purification employed Oasis WAX cartridges containing a mixed-mode polymeric/weak anion exchange resin. Cartridges were conditioned with methanol and water and then loaded with the crude reaction mixture. The cartridge was then washed with 2% aqueous formic acid, water and then methanol to elute any residual steroidal alcohol substrate. The steroidal glucuronide was eluted with saturated aqueous ammonium hydroxide in methanol (5% v/v). The target glucuronides were afforded in high purity after evaporation of the eluant under reduced pressure. The ^{1}H NMR spectra of the SPE purified materials so obtained were fully consistent with the proposed structures and are reproduced in the Electronic Supplementary Information (ESI). Our investigations revealed that reactions using 1 mg of steroidal alcohol were readily purified using a single 60 mg resin (3 cc) SPE cartridge. Larger scale synthesis required purification in parallel or the use of larger 500 mg resin (6 cc) cartridges.

Given the typically small scale of the synthesis, determining the mass of product and hence chemical yield with precision was not feasible. To address this we elected to monitor the conversion of starting material to product by ^{1}H NMR integration. Omitting the methanol wash step in the SPE method outlined above resulted in the elution by methanolic ammonia of a combined fraction containing both free steroid and steroid glucuronide. This was then subjected to 400 or 600 MHz ^{1}H NMR integration of selected steroidal protons in both the starting material and product allowing for the determination of reaction conversion as reported in Table 1. For the secondary steroidal alcohols studied, glucuronylation typically resulted in

Table 1 Synthesis of steroid glucuronides

Entry[a]	Glucuronide product	Conv. (%)[b] (Yield %)[c]
1	 **3**	50[d]
2	 **4**	28[d]
3	 **5**	<5[e]
4	 **6**	64[d]
5	 **7**	8[d]
6	 **8**	13[d]
7	 **9**	12[d]
8	 **10**	87[d,f] (94)
9	 **11**	32[d,f] (15)
10	 **12**	36[d,f] (26)
11	 **13**	90[d,f] (84)
12	 **14**	<5[e]
13	 **15**	25[d] (20)
14	 **16**	33[d]
15	 **17**	88[d,g]
16	 **18**	<5[e]
17	 **19**	20[d,f] (8)
18	 **20**	21[d,f] (34)

[a]Reactions conditions: steroid (1.0 equiv), α-D-glucuronyl fluoride (5.0 equiv), *E. coli* glucuronylsynthase (0.2 mg mL^{-1}), *tert*-butanol (10% v/v), sodium phosphate buffer (50 mM, pH 7.5), 37 °C, 2 d. [b]Determined by 400 or 600 MHz ^{1}H NMR integration. [c]Isolated yield of pure glucuronide for reactions on 5–10 mg scale. [d]The 400 or 600 MHz ^{1}H NMR spectrum is provided in the ESI. [e]Conversion <5% as determined by 400 MHz ^{1}H NMR integration. [f]The 150 MHz ^{13}C NMR spectrum is provided in the ESI. [g]Eluted as a 1.0:1.6:1.1 mixture of 3-mono-, 17-mono- and 3,17-bis-glucuronides as determined by 600 MHz ^{1}H NMR integration.

a 0.24–0.31 ppm downfield shift of the oxymethine proton at the reaction site, with the anomeric proton showing a coupling constant ($^3J_{1,2}$ = 7.7-8.0 Hz) consistent with β-glycoside formation. For the phenolic substrates estrone and estradiol, glucuronylation resulted in a 0.32–0.34 ppm downfield shift of the protons ortho to the reaction site, with the anomeric proton obscured by water from the deuterated methanol solvent. The conversion showed good reproducibility for the glucuronylation of dehydroepiandrosterone (87, 90 and 94% conversion, n = 3), testosterone (48 and 50%), methandriol (32 and 33%) and estrone (29, 30 and 33%), such that we now routinely assess enzyme activity by monitoring the small scale reaction conversion of dehydroepiandrosterone. In examples where larger scale synthesis was conducted (entries 8–11, 13, 17, 18) the isolated yields showed reasonable concordance with the reported conversions.

The glucuronide products **3**–**20** resulting from a screen of eighteen different steroidal alcohols possessing a range of different constitutions and stereochemistries are shown in Table 1. Several trends were evident in the observed conversions with steroids possessing 3β- or 17β-hydroxy groups giving higher conversions under the glucuronylation conditions than those with 3α- or 17α-hydroxy groups (*cf.* entries 1 and 2, 11 and 12). In addition, 3β-hydroxyandrostane derivatives (entries 8–11) generally gave higher conversions than their 17β-hydroxy congeners (entries 1, 4–7 and 18), which may be attributed to the slightly higher steric bulk resulting from the neighbouring C18 methyl group or other unfavourable enzyme-substrate interactions. The two steroids with highest conversion were dehydroepiandrosterone (entry 8) and epiandrosterone (entry 11), while stereochemically distinct substrates such as etiocholanolone (entry 13) and androsterone (entry 12) were less productive. Despite the changes in reactivity, the glucuronylation was effective for a wide range of common 3, 5 and 17 steroidal stereochemistries and substitution patterns. In the majority of cases (Table 1), conducting the reaction on a 1–2 mg scale afforded pure glucuronide on a sufficient scale to conduct 400 or 600 MHz ^{1}H NMR analysis. The broad substrate scope of this enzyme catalysed synthesis is noteworthy but has parallels in the use of wild-type *E. coli* β-glucuronidase in analytical applications. During routine screening for the presence of steroidal compounds in athlete samples, β-glucuronidase is employed to hydrolyse the combined glucuronide fraction to give free steroids prior to derivatisation and analysis by GCMS.[24] This protocol relies on the promiscuity of the enzyme to catalyse the hydrolysis of the wide range of steroid glucuronides present with few exceptions.[25,26] The glucuronylsynthase enzyme used in this study appears to retain analogous promiscuity, albeit with an efficacy that depends on steroid substitution patterns.

Methyltestosterone (entry 3) was one of three tertiary alcohols that were examined, with the corresponding glucuronide **5** was obtained in only trace amounts (<5% conversion), detectable by mass spectrometry but not ^{1}H NMR. This is likely due to the high steric hindrance afforded by both the C18 and C20 methyl groups that impede access of the 17β-hydroxy group to the glycosyl donor. This parallels the low reactivity observed for methyltestosterone in the Koenigs–Knorr glycosylation and deprotection sequence, affording a low 3.5% yield of the methyltestosterone 17-glucuronide.[27] Little or no reactivity for methyltestosterone glucuronylation is observed for recombinant human UGTs and human liver microsomes.[15] Low yields are also reported for glucuronylation using rat liver microsomes.[13] For methandriol (entry 9), which possesses both a secondary 3β- and tertiary 17β-hydroxy groups, and cholest-5-ene-3β,25-diol (entry 17) which possesses both a secondary 3β- and tertiary 25-hydroxy groups, glucuronylation was observed to take place regioselectively at the secondary hydroxy group. In both examples a 0.26 ppm downfield shift the H3 proton was observed consistent with glucuronylation at H3. Estradiol which possesses both phenolic 3- and secondary 17β-hydroxy groups afforded a mixture of 3-mono-, 17-mono- and 3,17-bis-glucuronides in an 1.0:1.6:1.1 ratio as determined by 600 MHz ^{1}H NMR integration.

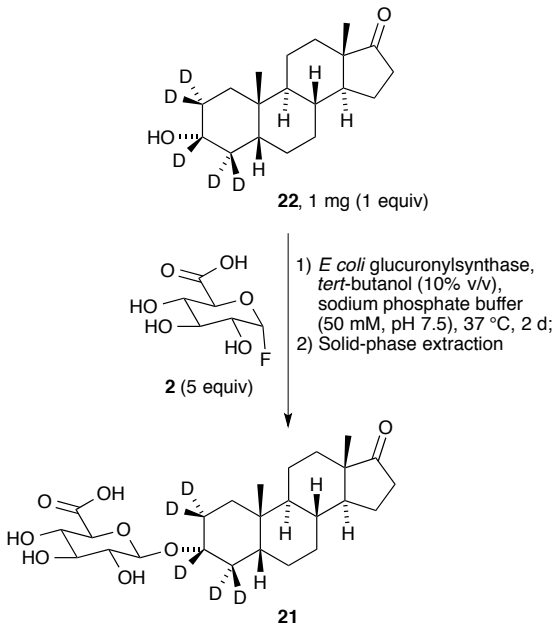
For some substrates such as androstanolone (entry 6) and the cholesterol derivatives (entries 16, 17), solubility posed a particular problem that likely contributed to the low conversion – for example, only trace amounts of coprostanol 3-glucuronide (entry 16) was observed. The co-solvent *tert*-butanol was used to enhance steroid solubility in the aqueous reaction media and was able to dissolve the majority of steroid substrates used in this study at a final concentration of ~0.69 mM, but for the substrates mentioned above only partial dissolution was achieved and so the reactions were conducted on the resulting saturated solutions.

During this study we found that the literature contained little NMR characterisation data for several known glucuronide products that were prepared in the screen. To redress this, we scaled up our syntheses for these substrates, specifically for pregnenolone and epiandrosterone (entries 10 and 11), obtaining full ^{1}H and ^{13}C NMR data for each. Three of the substrates investigated, methandriol, cholest-5-ene-3β,25-diol and the designer steroid trenazone (entries 9, 17 and 18),[28] gave rise to previously un-reported glucuronide conjugates **11**, **19** and **20** that were also prepared on a scale suitable for characterisation by ^{1}H and ^{13}C NMR spectroscopy.

One of the aims of our work was to provide a simple and effective alternative protocol for the synthesis of steroid glucuronides, in particular for small scale research applications where traditional methods of glucuronide synthesis fail or encounter significant practical difficulties. One such application is the synthesis of glucuronide conjugates from rare and expensive deuterated steroids. The deuterated steroid conjugate d_5-etiocholanolone 3-glucuronide **21** is a compound developed by the World Anti-Doping Agency (WADA) as a reference material to facilitate the longitudinal monitoring of testosterone abuse.[29] In our hands a commercially-derived one milligram sample of d_5-etiocholanolone **22** was converted to the corresponding glucuronide **21** conjugate on a sufficient scale to conduct 600 MHz NMR analysis (Scheme 3). The resulting conjugate showed an absence of the H3 proton signal of the non-deuterated counterpart at δ_H 3.81 together with

simplification of the aliphatic region from δ_H 1.98–0.98 consistent with per-deuteration of the A-ring. High-resolution mass spectrometry under negative ESI also revealed an ion ($[M - H]^-$, *m/z* 470.2800) consistent with the d_5-etiocholanolone 3-glucuronide **21**.

The glucuronylation of two additional[17,18] non-steroidal acceptors was investigated that hints at a wider substrate scope for the glucuronylsynthase protocol (Table 2). This resulted in the formation 4-methylumbeliferone 7-glucuronide **23**[30] and chloramphenicol 3-glucuronide **24**.[31] Chloramphenicol glucuronide has previously been prepared by UGT-mediated synthesis.[31] During comparison with the literature data we noted that the published ^{1}H NMR data and spectra were incorrectly referenced but accounting for this, the data and spectra corresponded well with the material produced in this work. The regioselectivity of glucuronylation was supported by the downfield shift (0.19 and 0.10 ppm) of the H3 protons and a HMBC three bond correlation between one H3 proton (4.00 ppm) and the anomeric carbon (104.2 ppm). Finally, although the results of this study have focussed on small scale synthesis compatible with rapid SPE purification, we also note that the glucuronylsynthase method reported here is readily scalable. In previous work the glucuronylsynthase has been used for the larger scale synthesis of a testosterone 17-glucuronide derivative (72% yield, 54 mg) and 2-phenylethanol glucuronide (96% yield, 718 mg).[17,18]

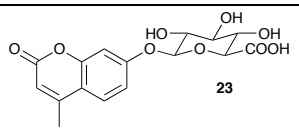
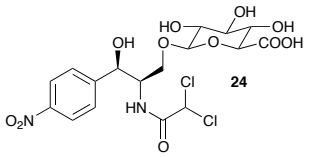


Scheme 3 Synthesis of d_5-etiocholanolone 3-glucuronide **21**.

Conclusion

A library of steroid glucuronides was prepared using the glucuronylsynthase derived from *Escherichia coli* β-glucuronidase, followed by purification using solid-phase extraction. The improved protocol demonstrated broad substrate scope, with fifteen out of eighteen substrates providing glucuronide conjugate in high purity and on a scale suitable for ^{1}H NMR analysis. The method allows for the rapid synthesis and purification of glucuronide conjugates on a small scale and avoids many of the complications associated with traditional chemical or enzymatic approaches. The reliability and efficiency of this method is highlighted by the preparation of a d_5-etiocholanolone 3-glucuronide **21** from a commercially-derived one milligram sample of the deuterated steroid d_5-etiocholanolone **22**. It is envisaged that the *E. coli* glucuronylsynthase system will help meet the future demand for glucuronide standards in analytical applications. Further engineering, directed to increasing the substrate scope and improving the catalytic efficiency of the glucuronylsynthase enzyme is in progress and will be reported in due course.

Table 2 Synthesis of non-steroidal glucuronides

Entry[a]	Glucuronide product	Conv. (%)[b]
1	 **23**	53[c]
2	 **24**	11[c,d]

[a]Reactions conditions: acceptor (1.0 equiv), α-D-glucuronyl fluoride (5.0 equiv), *E. coli* glucuronylsynthase (0.2 mg mL^{-1}), *tert*-butanol (10% v/v), sodium phosphate buffer (50 mM, pH 7.5), 37 °C, 2 d. [b]Determined by 400 or 800 MHz ^{1}H NMR integration. [c]The 400 or 800 MHz ^{1}H NMR spectrum is provided in the ESI. [d]The 800 MHz 2D HSQC and HMBC spectra are provided in the ESI.

Experimental

Epiandrosterone 3-glucuronide 13.[32] *Method A (conversion by ^{1}H NMR analysis):* Epiandrosterone (1.00 mg, 3.44 μmol) was dissolved in *tert*-butanol (500 μL) and sodium phosphate buffer (3.22 mL, 50 mM, pH 7.5). Glucuronylsynthase (0.92 mL, 1.09 mg mL^{-1}, final concentration 0.2 mg mL^{-1}) and α-D-glucuronyl fluoride **2** (3.89 mg, 18.2 μmol, 5 equiv) dissolved in sodium phosphate buffer (365 μL, 50 mM) were added and the reaction incubated without agitation at 37 °C for 2 days. The reaction was then subjected to solid-phase extraction. An Oasis WAX SPE cartridge (60 mg, 3 mL) was pre-conditioned with methanol (1 mL) and milliQ water (3 mL). The crude reaction was loaded onto the cartridge and washed with aqueous formic acid (3 mL, 2% v/v), milliQ water (3 mL), and finally with saturated aqueous ammonium hydroxide in methanol (9 mL, 5% v/v). The appropriate fractions were combined and the solvent removed under reduced pressure at 40 °C to afford a mixture of epiandrosterone and epiandrosterone 3-glucuronide with a 90% conversion as

determined by 600 MHz ^{1}H NMR integration of the H3 protons. A copy of the ^{1}H NMR conversion spectrum is reproduced in the ESI.

Method B (synthesis): The reaction was carried out as for method A on a larger scale with epiandrosterone (5.03 mg, 1.73 μmol). The reaction was then subjected to solid-phase extraction. An Oasis WAX SPE cartridge (500 mg, 6 mL) was pre-conditioned with methanol (5 mL) and milliQ water (15 mL). The crude reaction was loaded onto the cartridge and washed with aqueous formic acid (15 mL, 2% v/v), milliQ water (15 mL), methanol (15 mL) to elute free steroid, and finally with saturated aqueous ammonium hydroxide in methanol (30 mL, 5% v/v) to elute the steroid glucuronide. The appropriate fractions were combined and the solvent removed under reduced pressure at 40 °C to afford epiandrosterone 3-glucuronide **13** as a white solid (6.8 mg, 84%). Copies of the ^{1}H and ^{13}C NMR spectra are reproduced in the ESI. R_f 0.44 (7:2:1 EtOAc : MeOH : H_2O); δ_H (600 MHz, CD_3OD) 4.41 (1 H, d, $J_{H20–H21}$ 8.0, H20), 3.78 (1 H, m, H3), 3.55 (1 H, d, $J_{H24–H23}$ 9.5, H24), 3.43 (1 H, t, $J_{H23–H24} \approx J_{H23–H22}$ 9.2, H23), 3.39 (1 H, t, $J_{H22–H23} \approx J_{H22–H21}$ 8.9, H22), 3.18 (1 H, t, $J_{H21–H22} \approx J_{H21–H20}$ 8.4, H21), 2.43 (1 H, dd, J 19.3, 8.7), 2.06 (1 H, dt, J 19.3, 9.4) 1.97–1.91 (2 H, m), 1.83 (1 H, m), 1.75–1.49 (7 H, m), 1.40–1.28 (5 H, m), 1.23 (1 H, m), 1.15 (1 H, m), 1.06–1.01 (2 H, m), 0.88 (3 H, s, CH_3), 0.87 (3 H, s, CH_3), 0.75 (1 H, m); δ_C (150 MHz, CD_3OD) 224.0 (C17), 176.7 (C25), 101.8 (C20), 78.5 (C3), 77.8 (C22), 75.4 (C24), 74.9 (C21), 73.7 (C23), 55.8, 52.6, 45.9, 38.1, 36.8, 36.6, 36.3, 35.1, 32.7, 32.0, 30.2, 29.7, 22.6, 21.5, 14.1 (CH_3), 12.6 (CH_3), one carbon overlapping or obscured; LRMS (–ESI) *m/z*: 465 ($[M - H]^-$); HRMS (–ESI) *m/z*: Calcd. for $C_{25}H_{37}O_8$ ($[M - H]^-$) 465.2488, found 465.2488.

Abbreviations

Androstanolone	17β-hydroxy-5α-androstan-3-one
Androsterone	3α-hydroxy-5α-androstan-17-one
Boldenone	17β-hydroxyandrost-1,4-dien-3-one
Coprostanol	5β-cholestan-3β-ol
Dehydroepiandrosterone	3β-hydroxyandrost-5-en-17-one
Epiandrosterone	3β-hydroxy-5α-androstan-17-one
Epitestosterone	17α-hydroxyandrost-4-en-3-one
Estradiol	estra-1,3,5(10)-triene-3,17β-diol
Estrone	3-hydroxyestra-1,3,5(10)-trien-17-one
Etiocholanolone	3α-hydroxy-5β-androstan-17-one
d_5-Etiocholanolone	2,2,3,4,4-d_5-3α-hydroxy-5β-androstan-17-one
Mesterolone	17β-hydroxy-1α-methyl-5α-androstan-3-one
Methandriol	17α-methylandrost-5-ene-3β,17β-diol
Methyltestosterone	17β-hydroxy-17α-methylandrost-4-en-3-one
Nandrolone	17β-hydroxyestr-4-en-3-one
Pregnenolone	3β-hydroxypregn-5-en-20-one
SPE	solid-phase extraction
Testosterone	17β-hydroxyandrost-4-en-3-one
Trenazone	17β-hydroxyestra-4,9-dien-3-one
UGT	uridine 5′-diphosphoglucuronosyl-transferase

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Notes and references

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