A simple method for the small scale synthesis and solid-phase extraction purification of steroid sulfates

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Abstract

Steroid sulfates are a major class of steroid metabolite that are of growing importance in fields such as anti-doping analysis, the detection of residues in agricultural produce or medicine. Despite this, many steroid sulfate reference materials may have limited or no availability hampering the development of analytical methods. We report simple protocols for the rapid synthesis and purification of steroid sulfates that are suitable for adoption by analytical laboratories. Central to this approach is the use of solid-phase extraction (SPE) for purification, a technique routinely used for sample preparation in analytical laboratories around the world. The sulfate conjugates of sixteen steroid compounds encompassing a wide range of steroid substitution patterns and configurations are prepared, including the previously unreported sulfate conjugates of the designer steroids furazadrol (17β-hydroxyandrostan-[2,3-d]isoxazole), isofurazadrol (17β-hydroxyandrostan[3,2-c]isoxazole) and trenazone (17β-hydroxyestra-4,9-dien-3-one). Structural characterisation data, together with NMR and mass spectra are reported for all steroid sulfates, often for the first time. The scope of this approach for small scale synthesis is highlighted by the sulfation of one microgram of testosterone (17β-hydroxyandrost-4-en-3-one) as monitored by liquid chromatography-mass spectrometry (LCMS).

Keywords

Steroid, Sulfate ester, Steroid sulfate, Sulfation, Solid-phase extraction, Anti-doping

1. Introduction

Steroid sulfates are a major class of phase II steroid metabolite that are of growing importance in fields such as anti-doping analysis [1], the detection of residues in agricultural produce [2] and medicine [3]. This is in part driven by improvements in liquid chromatography mass spectrometry (LCMS) technology that empower the direct detection of phase II conjugates [4,5] but also arises due to the important information unveiled by a thorough analysis of phase II metabolism [6,7]. In the field of anti-doping science the analysis of human sulfate metabolites can afford greater retrospectivity for the detection of steroidal agents [8–10] and also serve as markers to distinguish between steroids of exogenous and endogenous origin [11–13]. Although there are a range of reliable approaches to analyse for phase II metabolites in both humans and animals, the range of available steroidal sulfate reference materials is incomplete and the ability to rapidly make and manipulate steroid sulfates as standards or reference materials has limitations [1]. This may mean that significant steroid sulfate markers cannot be quantified or even identified [11,13].

A range of methods have been developed to access steroid sulfates including the reaction of the parent steroid with sulfate salts and acetic anhydride [14], chlorosulfonic acid [15], amine complexes of sulfur trioxide [16–19], sulfuric acid and carbodiimides [20], sulfamic acid [21], or more recently by novel sulfuryl imidazolium salts [22,23]. These reactions however, whilst effective in affording the desired sulfate compounds, generally require significant chemical expertise and may also require harsh or hazardous conditions [15,19,20], specialised reagents [22,23], or complicated purification methods. These factors make small scale synthesis of steroid sulfates for analytical purposes a somewhat challenging undertaking. Simple synthetic access to steroid sulfates would facilitate the identification of metabolites and assist in the development of methods targeting these analytes. In this paper we report a general method for the small scale synthesis and purification of steroid sulfate compounds for anti-doping research and other analytical applications that is suitable for adoption by analytical laboratories. The method takes advantage of a rapid purification by solid-phase extraction (SPE), a technique familiar to analytical laboratories but with untapped potential in chemical synthesis.

2. Experimental

2.1. Materials

Chemicals and solvents including sulfur trioxide pyridine complex (SO_3.py), sulfur trioxide triethylamine complex (SO_3.NEt_3) and 1,4-dioxane, were purchased from Sigma–Aldrich (Castle Hill, Australia) and were used as supplied unless otherwise stated. Androsterone (3α-hydroxy-5α-androst-17-one), epiandrosterone (3β-hydroxy-5α-androst-17-one), etiocholanolone (3α-hydroxy-5β-androst-17-one), methandriol (17α-methylandrost-5-ene-3β,17β-diol) and testosterone (17β-hydroxyandrost-4-en-3-one) were obtained from Steraloids (Newport RI, USA). Estra-4,9-dien-3,17-one was obtained from AK Scientific (Union City CA, USA). Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) was obtained from BDH (Poole, UK). Lithocholic acid (3α-Hydroxy-5β-cholan-24-oic acid) was obtained from L. Light & Co. (Colnbrook, UK). Epitestosterone (17α-hydroxyandrost-4-en-3-one) was synthesised from testosterone using literature methods [24]. MilliQ water was used in all aqueous solutions and in the liquid chromatography mobile phase. Liquid chromatography (gradient) grade methanol was obtained from Merck (Kilsyth, Australia) and was used for preparing the liquid chromatography mobile phase and steroid standard solutions. *N*,*N*-Dimethylformamide (DMF) and aqueous ammonia solution were obtained from Chem-Supply (Gillman, Australia). Formic acid was obtained from Ajax Chemicals (Auburn, Australia). Ethyl formate and methanol were distilled separately from calcium hydride under a nitrogen atmosphere before use. Chlorosulfonic acid [CAUTION!] was distilled under a nitrogen atmosphere before use. Tetrahydrofuran was distilled from sodium wire before use. Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis weak anion exchange (WAX) 6cc cartridges (186004647).

2.2. Instruments

Melting points were determined using a SRS Optimelt MPA 100 melting point apparatus and are uncorrected. Optical rotational were determined using a Perkin–Elmer 241MC polarimeter (sodium D line, 298 K) in the indicated solvents. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded using either Varian 400 MHz, Bruker Ascend 400 MHz, Bruker Avance 400 MHz or Bruker Avance 600 MHz spectrometers at 298 K using deuterated methanol solvent unless otherwise specified. Data is reported in parts per

million (ppm), referenced to residual protons or ^{13}C in deuterated methanol solvent (CD_3OD: ^{1}H 3.31 ppm, ^{13}C 49.00 ppm) unless otherwise specified, with multiplicity assigned as follows: br = broad, s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet. Coupling constants *J* are reported in Hertz. Low-resolution mass spectrometry (LRMS) and high-resolution mass spectrometry (HRMS) were performed using positive electron ionisation (+EI) on a Micromass VG Autospec mass spectrometer or negative electrospray ionization (–ESI) on a Micromass ZMD ESI-Quad, or a Waters LCT Premier XE mass spectrometer. Reactions were monitored by analytical thin layer chromatography (TLC) using Merck Silica gel 60 TLC plates (7:2:1 ethyl acetate: methanol: water, unless otherwise specified) and were visualised by staining with a solution of potassium permanganate [$KMnO_4$ (3 g), K_2CO_3 (20 g), NaOH (0.25 g), H_2O (305 mL)], with heating as required. Liquid chromatography mass spectrometry (LCMS) was performed using an Agilent Technologies Infinity 1260 LC system equipped with an Agilent 1290 HTS LC Injector and an Agilent 6120 Quadrupole detector. Injections (10 μL) were resolved with an Agilent Zorbax SP-C18 UPLC column (2.1 mm x 50 mm, 1.8 μm, 600 bar) with an isocratic mobile phase consisting of 38% aqueous ammonium acetate (26.3 mM): 62% methanol. The column and sample modules were set to 30 °C and 4 °C respectively.

2.3. Chemical synthesis

2.3.1. General method for the small scale steroid sulfation reaction with purification by SPE

A solution of SO_3.py (10.0 mg, 62.8 mmol) in DMF (100 μL) was added to a solution of steroid (1.0 mg) in 1,4-dioxane (100 μL) and the resulting solution was then stirred in a capped vial at room temperature for 4 h. The reaction was then quenched with water (1.5 mL) and subjected to purification by SPE. An Oasis WAX SPE cartridge (6 cc) was pre-conditioned with methanol (5 mL) followed by water (15 mL). The reaction mixture (1.7 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 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 sulfate as the corresponding ammonium salt.

2.3.2. General method for the small scale steroid sulfation reaction with conversion determined by ^{1}H NMR analysis

A steroid sulfation reaction was performed as per 2.3.1 above. A modified SPE protocol 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 yielded a mixture containing both the starting steroid and the corresponding steroid sulfate as the ammonium salt. A ^{1}H NMR spectrum was obtained and integration of a suitable signal (typically C3-H or C17-H) of both steroid and steroid sulfate provided a ratio of the two compounds which was used to determine the percent conversion of the sulfation reaction. The mixture was then subjected to a second SPE purification using the conditions outlined in 2.3.1 to yield pure steroid sulfate as the corresponding ammonium salt.

2.3.3. General method for the steroid sulfation reaction with purification by recrystallisation

Sulfation was performed by minor modification of known literature procedures [17]. A solution of steroid (100 mg) in pyridine (1 mL) was added drop-wise to solid SO_3.NEt_3 or SO_3.py (1.1–1.6 equiv). The resulting solution was stirred at room temperature for 20 h unless otherwise specified. The reaction was then added to diethyl ether (20 mL) and the resulting precipitate was collected by filtration. The crude steroid sulfate salt was recrystallised from refluxing dichloromethane/diethyl ether, washed with small portions of cold diethyl ether and finally dried *in vacuo* to yield the desired steroid sulfate as its corresponding triethylammonium or pyridinium salt.

*2.3.4. Testosterone 17-sulfate, ammonium salt **1a*** [17]

A solution of testosterone (1.0 mg, 3.47 μmol) in 1,4-dioxane (100 μL) was treated with a solution of SO_3.py (10.0 mg, 62.8 μmol, 18.1 equiv) in DMF (100 μL) and purified by SPE as per 2.3.1 to yield the *title compound* **1a** as a white solid. Performing the sulfation reaction as per 2.3.2 showed full conversion. R_f 0.34; δ_H (400 MHz): 5.71 (s, 1H, C4-H), 4.34 (t, *J* 8.6 Hz, 1H, C17-H), 2.53-0.95 (m, 19H), 1.24 (s, 3H, C18-H_3), 0.87 (s, 3H, C19-H_3); δ_C (100 MHz): 202.4 (C3), 175.2 (C5), 124.1 (C4), 87.9 (C17), 55.4, 55.1, 51.3, 43.8, 40.0, 37.7, 36.8, 34.7, 33.9, 32.8, 29.1, 24.3, 21.6, 17.7 (C18), 12.0 (C19); LRMS (–ESI): *m/z* 367 (100%,

$[C_{19}H_{27}O_5S]^-$); HRMS (–ESI): found 367.1579, $[C_{19}H_{27}O_5S]^-$ requires 367.1579. Copies of the 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR and –ESI LRMS are reproduced in the supporting information. Experimental details, data and spectra for the steroid sulfate ammonium salts **2**–**13a** presented in Table 1 and Scheme 2 are reported in the supporting information.

*2.3.5. Testosterone 17-sulfate, triethylammonium salt **1b*** [17]

Testosterone (239 mg, 0.83 mmol) in pyridine (2 mL) was treated with solid $SO_3.NEt_3$ (236 mg, 1.30 mmol, 1.6 equiv) and stirred for 2 h as per 2.3.3 to yield the *title compound* **1b** (155 mg, 40%) as an off white solid. R_f 0.34; mp 164–166 °C (lit [17] 158–163 °C); $[\alpha]^{25}_D$ +58 (c 10, $CHCl_3$) (lit [17] $[\alpha]^{25}_D$ +64 [$CHCl_3$]); δ_H (400 MHz): 5.71 (s, 1H, C4-H), 4.23 (t, *J* 8.4 Hz, 1H, C17-H), 3.10 (q, *J* 7.3 Hz, 6H, NC*H*$_2$CH$_3$), 2.55–0.90 (m, 19H), 1.32 (t, *J* 7.4 Hz, 9H, NCH$_2$C*H*$_3$), 1.24 (s, 3H, C18-H$_3$), 0.87 (s, 3H, C19-H$_3$); δ_C (100 MHz): 202.4 (C3), 175.2 (C5), 124.1 (C4), 87.8 (C17), 55.4, 51.3, 47.9 (N*C*H$_2$CH$_3$), 43.9, 40.0, 37.7, 36.8, 34.7, 33.9, 32.8, 29.2, 24.3, 21.7, 17.7 (C18), 12.1 (C19), 9.2 (NCH$_2$*C*H$_3$), one carbon overlapping or obscured; LRMS (–ESI): *m/z* 367 (100%, $[C_{19}H_{27}O_5S]^-$); HRMS (–ESI): found 367.1579, $[C_{19}H_{27}O_5S]^-$ requires 367.1579. Copies of the 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR and –ESI LRMS are reproduced in the supporting information. Experimental details, data and spectra for the steroid sulfate triethylammonium salts **2**–**5**, **7** and **9b** presented in Table 1 are reported in the supporting information.

*2.3.6. Testosterone 17-sulfate, pyridinium salt **1c*** [25]

Testosterone (216 mg, 0.75 mmol) in pyridine (2 mL) was treated with solid SO_3.py (134 mg, 0.84 mmol, 1.1 equiv) as per 2.3.3 to yield the *title compound* **1c** (145 mg, 43%) as an off white solid. R_f 0.34; mp 140–145 °C (lit [25] 138–140 °C); $[\alpha]^{25}_D$ +52 (c 10, $CHCl_3$) (lit [26] $[\alpha]^{25}_D$ +200 [c 10, MeOH]); δ_H (400 MHz): 8.88 (m, 2H, *o*-pyridinium), 8.68 (m, 1H, *p*-pyridinium), 8.14 (m, 2H, *m*-pyridinium), 5.71 (s, 1H, C4-H), 4.30 (t, *J* 8.6 Hz, 1H, C17-H), 2.55–0.86 (m, 19H), 1.24 (s, 3H, C18-H$_3$), 0.86 (s, 3H, C19-H$_3$); δ_C (100 MHz): 202.4 (C3), 175.1 (C5), 148.5 (*o*-pyridinium), 143.0 (*p*-pyridinium), 128.9 (*m*-pyridinium), 124.2 (C4), 87.9 (C17), 55.4, 51.3, 43.8, 40.1, 37.7, 36.7 (2 C), 32.7, 32.6, 29.2, 24.3, 21.7, 17.7 (C18), 12.1 (C19), one carbon overlapping or obscured; LRMS (–ESI): *m/z* 367 (100%, $[C_{19}H_{27}O_5S]^-$), 97 (20%, $[HSO_4]^-$); HRMS (–ESI) found 367.1579, $[C_{19}H_{27}O_5S]^-$ requires 367.1579. Copies of the 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR and –ESI LRMS are reproduced in the supporting

information. Experimental details, data and spectra for etiocholanolone 3-sulfate pyridinium salt **6c** presented in Table 1 is reported in the supporting information.

*2.3.7. Estrone (3-hydroxyestra-1,3,5(10)-triene-17-one) 3-sulfate, ammonium salt **14a*** [23]

Chlorosulfonic acid [CAUTION! Corrosive. Use in an efficient fume hood.] (10 μL, 150.0 μmol, 8.1 equiv) was slowly added to a solution of estrone (5.0 mg, 18.5 μmol) in pyridine (50 μL) with cooling on ice. After the vigorous reaction had subsided, the reaction was capped, allowed to warm and then stirred at room temperature for 20 h. The reaction was quenched by the slow addition of water (1.5 mL) and then purified by SPE as per 2.3.1 to yield the *title compound* **14a** as a yellow-brown solid. Performing the sulfation reaction as per 2.3.2 showed 65% conversion. R_f 0.49; mp 150–155 °C; δ_H (400 MHz): 7.25 (d, *J* 8.4 Hz, 1H, C1-H), 7.08–7.01 (m, 2H, C2-H and C4-H), 2.95–2.87 (m, 2H, C6-H_2), 2.55–1.40 (m, 13H), 0.92 (s, 3H, C18-H_3); δ_C (100 MHz): 223.7 (C17), 151.8 (C3), 138.7, 137.6, 127.0, 119.8, 112.5, 51.7, 45.5, 39.7, 36.7, 32.8, 30.5, 27.6, 27.0, 23.3, 22.5, 14.2 (C18); LRMS (–ESI): *m/z* 349 (100%, $[C_{18}H_{21}O_5S]^-$), 269 (40%), 97 (20%, $[HSO_4]^-$), 80 (25%, $[SO_3]^-$); HRMS (–ESI): found 349.1110, ($[C_{18}H_{21}O_5S]^-$) requires 349.1110. Copies of the 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR and –ESI LRMS are reproduced in the supporting information.

*2.3.8. Estrone 3-sulfate, pyridinium salt **14c*** [25,26]

Chlorosulfonic acid [CAUTION! Corrosive. Use in an efficient fume hood.] (620 μL, 9.33 mmol, 5.0 equiv) was added drop-wise to a rapidly stirring solution of estrone (505 mg, 1.87 mmol) in pyridine with cooling on ice. After the vigorous reaction subsided the reaction was allowed to warm to room temperature and then stirred for 20 h. The reaction was then added to aqueous potassium hydroxide solution (0.1 M, 30 mL) and extracted into ethyl acetate (4 x 50 mL) and 3:1 chloroform-isopropanol solution (4 x 40 mL). The combined organic extracts were dried with magnesium sulfate and evaporated to dryness. The crude steroid sulfate salt was recrystallised as per 2.3.3 to yield the *title compound* **14c** (199 mg, 25%) as a white solid. R_f 0.45; mp 165–169 °C (lit [26] 170–175 °C); $[\alpha]^{25}_D$ +79 (c 10, $CHCl_3$) (lit [26] $[\alpha]^{25}_D$ +84 [c 0.96, $CHCl_3$]); δ_H (400 MHz): 8.88 (m, 2H, *o*-pyridinium), 8.68 (m, 1H, *p*-pyridinium), 8.12 (m, 2H, *m*-pyridinium), 7.24 (d, *J* 8.4 Hz, 1H, C1-H), 7.07–7.01 (m, 2H, C2-H and C4-H), 2.95–2.90 (m, 2H, C6-H_2), 2.55–1.40 (m, 13H), 0.92 (s, 3H, C18-H_3); δ_C (100 MHz): 224.1 (C17), 151.5 (C3), 148.2 (*o*-pyridinium), 142.8 (*p*-pyridinium), 138.2,

137.5, 128.9 (*m*-pyridinium), 127.2, 120.0, 112.7, 51.7, 45.4, 39.8, 36.8, 32.9, 30.3, 27.5, 27.0, 23.2, 22.6, 14.3 (C18); LRMS (–ESI): *m/z* 349 (100%, $[C_{18}H_{21}O_5S]^-$), 269 (40%), 97 (20%, $[HSO_4]^-$), 80 (25%, $[SO_3]^-$); HRMS (–ESI): found 349.1110, $[C_{18}H_{21}O_5S]^-$ requires 349.1110. Copies of the 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR and –ESI LRMS are reproduced in the supporting information.

2.3.9. Estradiol (estra-1,3,5(10)-triene-3,17β-diol) 3-sulfate, ammonium salt ***15a***

A solution of estrone 3-sulfate, ammonium salt **14a** (derived from estrone, 5.0 mg, 18.5 μmol) in methanol (100 μL) was slowly added to solid sodium borohydride (7 mg, 185 μmol 10.0 equiv) with cooling on ice. After the vigorous reaction had subsided the reaction was capped, allowed to warm to room temperature and stirred for 4 h. The reaction was quenched by the slow addition of water (3 mL), adjusted to pH 7 (universal indicator strips) by addition of aqueous hydrochloric acid (0.1 M, ~2 mL) and then purified by SPE as per 2.3.1 to yield the *title compound* **15a** as a white solid. Performing the reduction reaction above with SPE purifications as per 2.3.2 showed full conversion. R_f 0.54; δ_H (400 MHz): 7.23 (d, *J* 8.4 Hz, 1H, C1-H), 7.08–6.98 (m, 2H, C2-H and C4-H), 3.67 (t, *J* 8.6 Hz, 1H, C17-H), 2.89–2.83 (m, 2H, C6-H_2), 2.40–1.15 (m, 13H), 0.78 (s, 3H, C18-H_3); δ_C (100 MHz): 151.7 (C3), 138.8, 138.1, 127.0, 122.5, 119.7, 82.5 (C17), 51.4, 45.5, 44.4, 40.3, 38.0, 30.7, 30.6, 28.4, 27.5, 24.0, 11.7 (C18); LRMS (–ESI): *m/z* 351 (100%, $[C_{18}H_{23}O_5S]^-$); HRMS (–ESI): found 351.1266, $[C_{18}H_{23}O_5S]^-$ requires 351.1266. Copies of the 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR and –ESI LRMS are reproduced in the supporting information.

2.3.10. Estrone 2-sulfonate, ammonium salt ***16a***

Employing conditions developed by Dusza, Joseph and Bernstein [19], solid estrone (5.0 mg, 18.5 μmol) was mixed with solid SO_3.py (15 mg, 94.2 μmol, 5.1 equiv) and heated at 180 °C for 15 min in an oil bath. The resulting brown melt was allowed to cool, quenched with water (1.5 mL) and then purified by SPE as per 2.3.1 to yield the *title compound* **16a** as a yellow-brown solid. Performing the sulfation reaction as per 2.3.2 showed full conversion. R_f 0.36; mp 180–183 °C; δ_H (400 MHz): 7.57 (s, 1H, C1-H), 6.59 (s, 1H, C4-H), 2.90–2.83 (m, 2H, C6-H_2), 2.55–1.40 (m, 13H), 0.92 (s, 3H, C18-H_3); δ_C (100 MHz): 223. 7 (C17), 152.8, 142.7, 132.3, 127.3, 125.3, 117.7, 51.6, 45.1, 39.7, 36.7, 32.7, 30.3, 27.5, 26.9, 22.5, 14.3 (C18), one carbon overlapping or obscured; LRMS (–ESI): *m/z* 349 (100%, $[C_{18}H_{21}O_5S]^-$), 97 (30%,

$[HSO_4]^-$), 80 (20%, $[SO_3]^-$); HRMS (–ESI): found 349.1097, $[C_{18}H_{21}O_5S]^-$ requires 349.1110. Copies of the 400 MHz ^{1}H NMR, 100 MHz ^{13}C NMR and –ESI LRMS are reproduced in the supporting information.

3. Results and discussion

3.1. Sulfation reaction conditions

Of the variety of conditions available in the literature, the application of sulfur trioxide amine complexes appeared to offer the greatest utility due to their commercial availability, ease of handling, reasonable stability to residual moisture and mild reaction conditions as opposed to competing methods [14–23]. In our hands sulfur trioxide pyridine complex could be briefly weighed in the laboratory without special precautions. A solution of sulfur trioxide pyridine complex in DMF (100 mg mL^{-1}) was used for the sulfation reactions and when stored in a sealed vial at 4 °C maintained activity for 2 weeks. In contrast to typical steroid sulfation reactions which use pyridine as the reaction solvent [16–18], DMF and 1,4-dioxane were used instead to maintain compatibility with the SPE protocol (2.3.1) and to reduce toxicity and odour concerns. Our reaction protocol involved treatment of a solution of steroid in 1,4-dioxane with a solution of excess sulfur trioxide pyridine complex in DMF, followed by quenching with water and SPE as shown in Scheme 1, below. Under these conditions testosterone (1 mg) could be reliably converted to testosterone 17-sulfate **1a** with >98% conversion. On a larger scale (10 mg) synthesis of testosterone 17-sulfate **1a** the isolated yield (94%) showed reasonable concordance with this high conversion. These conditions proved quite general for the synthesis of a wide range of secondary alcohol-derived steroid sulfates **1**–**13a** (Table 1, Scheme 2). Modified conditions were required for the synthesis of estrone 3-sulfate derivatives (3.5 below).

To further confirm the identity of these steroid sulfate ammonium salts prepared by small scale synthesis and SPE, a range of known reference compounds were also prepared using larger scale sulfation and recrystallisation [17] as either the triethylammonium salts **1**–**5**, **7** and **9b** or pyridinium salts **1** and **6c** (Table 1). These materials allowed for comparison of the different salts by NMR and MS as detailed below (3.3)

<Scheme 1 here>

3.2. Solid-phase extraction purification

Traditionally one or more recrystallizations or chromatographic separations are required to purify steroid sulfate compounds to an acceptable standard for analytical use [16–18]. This is not practical however when utilising small quantities of material, as often might be the case with rare or expensive steroids or isotope-labelled samples. To circumvent this problem we adopted SPE which has been routinely used in anti-doping laboratories for the extraction of steroids and other compounds from biological matrices such as blood and urine [27–29]. Rather surprisingly, despite the extensive application of SPE in chemical analysis, it has not been widely used for the preparation of steroid sulfates, and where employed, typically forms only part of the purification process [30–32].

Our SPE purification protocol adopted Oasis WAX cartridges which contain a mixed-mode polymeric/weak anion exchange resin allowing fractionation of the anionic steroid sulfate from any residual neutral steroidal alcohol and other non-volatile reaction components. Cartridges were pre-conditioned with methanol followed by water then loaded directly with the quenched reaction mixture. Washing the cartridge sequentially with aqueous formic acid solution (2% v/v), water and finally methanol eluted any residual steroidal alcohol and other reaction components. A final elution with saturated aqueous ammonia in methanol (5% v/v) afforded the desired steroid sulfate as the ammonium salt after removal of eluant under reduced pressure. Alternatively, the methanolic ammonia fraction could be dried at 60 °C for 1 h under stream of nitrogen to give the steroid sulfate, albeit with trace amounts of ammonium formate observable in the ^{1}H NMR spectrum. During development, this SPE protocol was shown to cleanly and efficiently separate an equimolar mixture of testosterone and testosterone 17-sulfate **1a**. Purification of reactions conducted on 5 mg of steroidal alcohol or less were readily conducted using a single 500 mg resin (6 cc) SPE cartridge, with larger scale synthesis requiring purification in parallel.

The SPE protocol outlined above resulted in the efficient purification of all steroid sulfates investigated with the exception of the lithocholic acid 3-sulfate **9a**. In this case the lithocholic acid starting material contains a carboxylate sidechain that can also interact with the anion exchange resin leading to co-elution with lithocholic acid sulfate **9a** in the

methanolic ammonia wash. In this instance, washing the cartridge with formic acid in methanol (2% v/v, 15 mL) was employed to elute lithocholic acid, with lithocholic acid sulfate **9a** then eluted cleanly by methanolic ammonia in the final step.

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 (2.3.1) resulted in the elution by methanolic ammonia of a combined fraction containing both free steroid and steroid sulfate. This was then subjected to 400 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, sulfation occurred with 97% conversion or greater. This contrasted with the larger scale sulfation reactions that employed purification by recrystallisation, which afforded moderate 21–76% isolated yields (Table 1, entries **1**–**7** and **9b/c**).

3.3. NMR and MS analysis of steroid sulfates

For each steroid investigated, (Table 1, Scheme 2), conducting the reaction on a 1–2 mg scale afforded sufficient pure steroid sulfate to conduct 400 or 600 MHz ^{1}H NMR analysis. The compounds obtained by this sulfation protocol were of high purity (>95%) as assessed by this technique (copies of NMR spectra for each steroid sulfate are reproduced in the supporting information). For the secondary steroidal alcohols studied, sulfation resulted in a 0.58–0.74 ppm downfield shift of the oxymethine proton at the reaction site, as expected based on electronic considerations. For the phenolic substrates estrone and estradiol, sulfation similarly resulted in a 0.50 ppm downfield shift of the protons *ortho* to the sulfate ester and a smaller downfield shift of 0.18 ppm for H1 relative to the parent steroid. Overlay of the ^{1}H NMR spectra derived from the steroid sulfate ammonium salts **1**–**7**, **9a** and **14a** prepared by small scale synthesis and SPE with the triethylammonium salts **1**–**5**, **7**, and **9b**, or pyridinium salts **1**, **6** and **14c**, prepared by large scale synthesis and recrystallisation showed identical signals for the protons of the steroidal nucleus, further supporting the identity of these compounds. Further, SPE purification of testosterone 17-sulfate, pyridinium salt **1c** by the general method (2.3.1) afforded testosterone 17-sulfate,

ammonium salt **1a** which was identical in all respects with that obtained directly from testosterone using the direct small scale synthesis (2.3.4).

During this study we found that the literature contained little or no characterisation data for several of the steroid sulfate ammonium salt products that were prepared in the screen. To address this, we scaled up our syntheses of these steroid sulfates **3**–**6**, **8**, **13** and **15a** specifically those derived from androstanolone (17β-hydroxy-5α-androstan-3-one), androsterone, epiandrosterone, estradiol, etiocholanolone and methandriol, obtaining full ^{1}H, ^{13}C NMR, and MS data for each. Further, both the small and large scale synthesis provided significant additional ^{1}H, ^{13}C NMR, and MS data, as well as spectra for the majority of steroid sulfates shown in table 1. The designer steroids furazadrol (17β-hydroxyandrostan-[2,3-d]isoxazole), isofurazadrol (17β-hydroxyandrostan[3,2-c]isoxazole) and trenazone (17β-hydroxyestra-4,9-dien-3-one) gave rise to previously un-reported sulfate conjugates **10**–**12a** that were also prepared on a scale suitable for full characterisation.

Mass spectrometry was also used to characterise the steroid sulfates prepared. In each case the steroid sulfate, ammonium salts exhibited satisfactory purity and identity by –ESI LRMS and HRMS respectively. Further the steroid sulfate ammonium salts **1**–**7**, **9** and **14a** prepared by small scale synthesis and SPE showed identical, albeit simple, –ESI LRMS behaviour with the triethylammonium salts **1**–**5**, **7** and **9b**, or pyridinium salts **1, 6** and **14c**, prepared by large scale synthesis and recrystallisation.

<Table 1 here>

3.4. Sulfation of secondary steroidal alcohols

The small scale synthesis and purification of steroid sulfates proved highly effective for a wide variety of secondary steroidal alcohol substitution patterns and configurations (Table 1, Scheme 2). For methandriol (Table 1, entry **8a**), which possesses both secondary 3β- and tertiary 17β-hydroxyl groups, mono-sulfation was observed to take place regioselectively at the secondary hydroxyl group to cleanly give methandriol 3-sulfate **8a**. A 0.74 ppm downfield shift the H3 proton was observed in the ^{1}H NMR spectrum consistent with sulfation at C3 as expected based on steric considerations. The sulfation of tertiary hydroxyl

groups has been reported on a larger scale with sulfur trioxide pyridine complex [33] or the more reactive chlorosulfonic acid reagent [34]. The tertiary alkyl sulfate esters so derived are typically unstable and undergo elimination, rearrangement or hydrolysis under ambient conditions [33,35,36]. The sulfation of lithocholic acid which possesses both a secondary 3β-hydroxyl group and a carboxylic acid also proceeded without incident using a modified SPE method.

3.5. Sulfation of estrogenic steroidal alcohols

Although estrogenic steroids are not typically considered candidates for use as performance enhancing substances in sport, they may offer indirect advantages [37]. Additionally, these compounds and their *in vivo* metabolites play host a wide range of biological functions that have important applications in medical science [3]. Sulfate conjugates of the estrogens were therefore attractive targets for synthesis. Attempted sulfation of estrone under standard conditions (2.3.1) failed to afford the desired estrone 3-sulfate, reflecting significant differences in reactivity for aliphatic versus phenolic hydroxyl groups. This is despite literature reports describing the formation of phenolic sulfates under similar conditions [17,18]. This observation proved useful for the selective sulfation under standard conditions (2.3.1) of estradiol to afford estradiol 17-sulfate **13a** in which reaction occurs at the secondary alkyl C17 hydroxyl group (Scheme 2) [18].

<Scheme 2 here>

A range of conditions were investigated in efforts to effect sulfation of estrone on a small scale. Employing an excess of chlorosulfonic acid [CAUTION] in pyridine gave estrone 3-sulfate **14a** with 65% conversion [15]. Fortunately, these conditions were compatible with our existing SPE method (2.3.1) providing the sulfate in pure form. A 0.50 ppm downfield shift the H2 and H4 protons were observed in the ^{1}H NMR spectrum consistent with sulfation at H3. To extend this chemistry, sodium borohydride reduction of the resulting estrone 3-sulfate **14a** was used to selectively afford estradiol 3-sulfate **15a** after SPE purification.

During efforts to obtain estrone 3-sulfate we also investigated the so-called “fusion” method whereby a neat mixture of the steroid and sulfur trioxide pyridine complex are

heated together at 180 °C, thereby conducting the sulfation reaction in the melt [19]. This method led to the unexpected formation of the isomeric steroid derivative estrone 2-sulfonate **16a**, a product of electrophilic aromatic substitution presumably promoted by the high reaction temperature. The 400 MHz ^{1}H NMR spectrum showed two singlets at δ 7.57 and 6.59 consistent with the proposed structure. This result suggests some caution is warranted in applying the fusion method for the synthesis of aromatic sulfates.

3.6. Sulfation on the microgram scale

Finally, we wished to explore the suitability of this approach to the synthesis of analytical quantities of material by applying this protocol to a microgram scale synthesis of testosterone 17-sulfate **1a**. Success with such small quantities would have important applications for the sulfation of rare and expensive steroid compounds such as novel designer steroids or isotope labelled compounds [38] where significant time, resource or financial costs are involved in obtaining sufficient quantities of material for analysis. In addition, it raised the prospect of generating phase II steroid sulfates from phase I steroid metabolites isolated from biological samples. When the protocol was applied to a series of reactions with testosterone (100 μg to 1 μg), analysis by LCMS allowed detection of the product steroid sulfate by single ion monitoring (SIM, *m/z* –367) even at the lowest concentration trialled (1 μg), and near-quantitative conversion (1.28±0.04 μg testosterone sulfate, 95±3% HPLC yield, mean±sem, n=3) to the desired sulfate was still observed at these levels. These results highlight the power of this approach for the small scale synthesis of steroid sulfate compounds for analytical purposes.

Conclusion

Employing a small scale sulfation protocol with purification by SPE, sixteen steroid sulfates have been synthesised on a milligram scale with near-complete conversion, including the previously unreported sulfate conjugates of the designer steroids furazadrol, isofurazadrol and trenazone. The scope of this approach for small scale synthesis is highlighted by the sulfation of one microgram of testosterone as monitored by LCMS. This quick and simple protocol employs commercially available consumables and reagents and allows the rapid

and efficient synthesis and purification of steroid sulfate compounds. The method is suitable for use in analytical laboratories and should serve to expand the availability of steroid sulfate reference materials for a range of analytical applications.

Acknowledgements

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Supplementary material

Supporting information containing experimental procedures, characterisation data, NMR and MS spectra for all compounds can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids……

References

[1] Gomes RL, Meredith W, Snape CE, Sephton MA. Analysis of conjugated steroid androgens: Deconjugation, derivatisation and associated issues. J Pharm Biomed Anal 2009;49:1133–40.

[2] Anizan S, Di Nardo D, Bichon E, Monteau F, Cesbron N, Antignac J-P, et al. Targeted phase II metabolites profiling as new screening strategy to investigate natural steroid abuse in animal breeding. Anal Chim Acta 2011;700:105–13.

[3] Nussbaumer P, Billich A. Steroid sulfatase inhibitors. Med Res Rev 2004;24:529–76.

[4] Tudela E, Deventer K, Geldof L, Van Eenoo P. Urinary detection of conjugated and unconjugated anabolic steroids by dilute-and-shoot liquid chromatography-high resolution mass spectrometry. Drug Test Anal 2014: in press, DOI: 10.1002/dta.1650

[5] Deventer K, Pozo OJ, Verstraete AG, Van Eenoo P. Dilute-and-shoot-liquid chromatography-mass spectrometry for urine analysis in doping control and analytical toxicology. TrAC Trends Anal Chem 2014;55:1–13.

[6] Thevis M, Kuuranne T, Geyer H, Schänzer W. Annual banned-substance review: analytical approaches in human sports drug testing. Drug Test Anal 2013;5:1–19.

[7] Kuuranne T. Phase-II metabolism of androgens and its relevance for doping control analysis. In: Thieme D, Hemmersbach P, editors. Doping in Sports, Springer–Verlag: Berlin; 2010, p. 65–75.

[8] Gómez C, Pozo OJ, Marcos J, Segura J, Ventura R. Alternative long-term markers for the detection of methyltestosterone misuse. Steroids 2013;78:44–52.

[9] Strahm E, Baume N, Mangin P, Saugy M, Ayotte C, Saudan C. Profiling of 19-norandrosterone sulfate and glucuronide in human urine: Implications in athlete's drug testing. Steroids 2009;74:359–64.

[10] Torrado S, Roig M, Farré M, Segura J, Ventura R. Urinary metabolic profile of 19-norsteroids in humans: glucuronide and sulphate conjugates after oral administration of 19-nor-4-androstenediol. Rapid Commun Mass Spectrom 2008;22:3035–42.

[11] Badoud F, Boccard J, Schweizer C, Pralong F, Saugy M, Baume N. Profiling of steroid metabolites after transdermal and oral administration of testosterone by ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. J Steroid Biochem Mol Biol 2013;138:222–35.

[12] Gómez C, Pozo OJ, Geyer H, Marcos J, Thevis M, Schänzer W, et al. New potential markers for the detection of boldenone misuse. J Steroid Biochem Mol Biol 2012;132:239–46.

[13] Boccard J, Badoud F, Grata E, Ouertani S, Hanafi M, Mazerolles G, et al. A steroidomic approach for biomarkers discovery in doping control. Forensic Sci Int 2011;213:85–94.

[14] Sobel AE, Drekter IJ, Natelson S. Estimation of small amounts of cholesterol as the pyridine cholesteryl sulfate. J Biol Chem 1936;115:381–90.

[15] Fieser LF. Naphthoquinone antimalarials. XVI. Water-soluble derivatives of alcoholic and unsaturated compounds. J Am Chem Soc 1948;70:3232–7.

[16] Sobel AE, Spoerri PE. Steryl sulfates. I. Preparation and properties. J Am Chem Soc 1941;63:1259–61.

[17] Dusza JP, Joseph JP, Bernstein S. Steroid conjugates IV. The preparation of steroid sulfates with triethylamine-sulfur trioxide. Steroids 1968;12:49–61.

[18] Dusza JP, Joseph JP, Bernstein S. The preparation of estradiol-17β sulfates with triethylamine-sulfur trioxide. Steroids 1985;45:303–15.

[19] Dusza JP, Joseph JP, Bernstein S. A fusion method for the preparation of steroid sulfates. Steroids 1985;45:317–23.

[20] Mumma RO. Preparation of sulfate esters. Lipids 1966;1:221–3.

[21] Joseph JP, Dusza JP, Bernstein S. Steroid conjugates I. The use of sulfamic acid for the preparation of steroid sulfates. Steroids 1966;7:577–87.

[22] Desoky AY, Hendel J, Ingram L, Taylor SD. Preparation of trifluoroethyl- and phenyl-protected sulfates using sulfuryl imidazolium salts. Tetrahedron 2011;67:1281–7.

[23] Liu Y, Lien I-FF, Ruttgaizer S, Dove P, Taylor SD. Synthesis and protection of aryl sulfates using the 2,2,2-trichloroethyl moiety. Org Lett 2004;6:209–12.

[24] Martin SF, Dodge JA. Efficacious modification of the Mitsunobu reaction for inversions of sterically hindered secondary alcohols. Tetrahedron Lett 1991;32:3017–20.

[25] Marian M, Matkovics B, Vargha SJ. Steroids. XIV. Preparation of steroid sulfate salts. Acta Phys Chem 1971;17:85–9.

[26] McKenna J, Norymberski JK. 773. Steroid sulphates. Part I. Some solvolytic reactions of the salts of steroid sulphates. J Chem Soc 1957:3889–93.

[27] Keevil BG. Novel liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for measuring steroids. Best Pract Res Clin Endocrinol Metab 2013;27:663–74.

[28] Tomšíková H, Aufartová J, Solich P, Nováková L, Sosa-Ferrera Z, Santana-Rodríguez JJ. High-sensitivity analysis of female-steroid hormones in environmental samples. TrAC Trends Anal Chem 2012;34:35–58.

[29] Gomes RL, Meredith W, Snape CE, Sephton MA. Conjugated steroids: analytical approaches and applications. Anal Bioanal Chem 2009;393:453–8.

[30] Richmond V, Murray AP, Maier MS. Synthesis and acetylcholinesterase inhibitory activity of polyhydroxylated sulfated steroids: Structure/activity studies. Steroids 2013;78:1141–7.

[31] Ikegawa S, Nagae K, Mabuchi T, Okihara R, Hasegawa M, Minematsu T, et al. Synthesis of 3- and 21-monosulfates of [2,2,3β,4,4-$^{2}H_5$]-tetrahydrocorticosteroids in the 5β-series as internal standards for mass spectrometry. Steroids 2011;76:1232–40.

[32] Kirk DN, Rajagopalan MS. 18-Substituted steroids. Part 12. Synthesis of aldosterone 21-sulphate, and an improved general procedure for preparing steroid sulphates. J Chem Soc Perkin Trans 1 1987:1339–42.

[33] Schänzer W, Opfermann G, Donike M. 17-Epimerization of 17α-methyl anabolic steroids in humans: metabolism and synthesis of 17α-hydroxy-17β-methyl steroids. Steroids 1992;57:537–50.

[34] Bi H, Massé R, Just G. Studies on anabolic steroids. 9. Tertiary sulfates of anabolic 17α-methyl steroids: synthesis and rearrangement. Steroids 1992;57:306–12.

[35] Bi H, Massé R. Studies on anabolic steroids—12. Epimerization and degradation of anabolic 17β-sulfate-17α-methyl steroids in human: Qualitative and quantitative GC/MS analysis. J Steroid Biochem Mol Biol 1992;42:533–46.

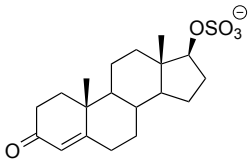
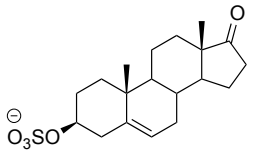
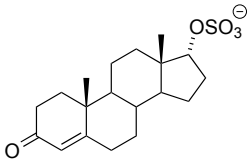
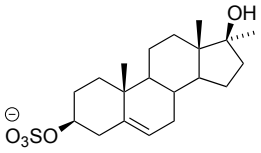
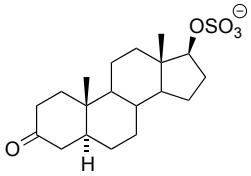
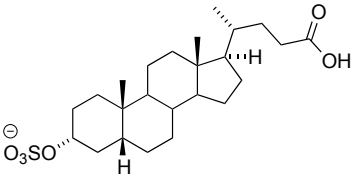
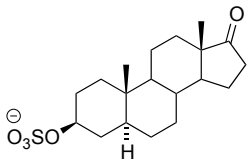
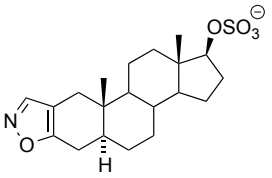
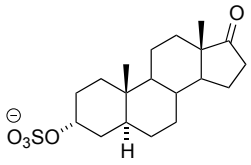
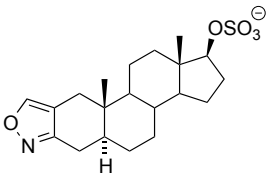
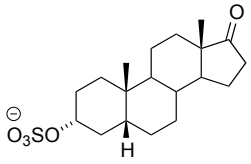
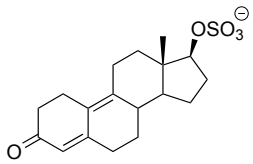
[36] Edlund PO, Bowers L, Henion J. Determination of methandrostenolone and its metabolites in equine plasma and urine by coupled-column liquid chromatography with ultraviolet detection and confirmation by tandem mass spectrometry. J Chromatogr B Biomed Sci App 1989;487:341–56.

[37] Tiidus PM, Enns DL. Point:Counterpoint: Estrogen and sex do/do not influence post-exercise indexes of muscle damage, inflammation, and repair. J Appl Physiol 2009;106:1010–2.

[38] Sanaullah, Bowers LD. Facile synthesis of [16,16,17-$^{2}H_{3}$]-testosterone, -epitestosterone and their glucuronides and sulfates. J Steroid Biochem Mol Biol 1996;58:225–34.

Tables

Table 1. Synthesis of steroid sulfates using SPE purification

Entry[a]	Steroid sulfate	Conversion (%)[b] (Yield %)[c]	Entry[a]	Steroid sulfate	Conversion (%)[b] (Yield)[c]
1a[d,e] **1b**[d,e,f] **1c**[d,e,f]		>98 (94) (40) (43)	**7a**[d,e] **7b**[d,e,f]		>98 (63)
2a[d,e] **2b**[d,e,f]		>98 (41)	**8a**[d,e]		>98
3a[d,e] **3b**[d,e,f]		97 (21)	**9a**[d,e] **9b**[d,e,f]		>98 (76)
4a[d,e] **4b**[d,e,f]		>98 (54)	**10a**[d,e]		>98
5a[d,e] **5b**[d,e,f]		>98 (42)	**11a**[d,e]		>98
6a[d,e] **6c**[d,e,f]		>98 (55)	**12a**[d,e]		>98

[a] Steroid sulfate prepared as: **a**, ammonium salt; **b**, triethylammonium salt; or **c**, pyridinium salt.

[b] Conversion based on 400 MHz ^{1}H NMR integration.

[c] Isolated yield of pure steroid sulfate.

[d] The 400 or 600 MHz ^{1}H NMR and ESI MS is provided in the supporting information.

[e] The 100 or 150 MHz ^{13}C NMR spectrum is provided in the supporting information.

[f] Conditions: steroid (1 equiv), $SO_3.NEt_3$/SO_3.py (1.1–1.6 equiv), pyridine, RT, 2–20 h.

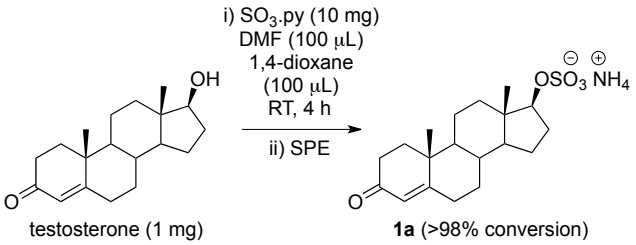
Scheme legends

Scheme 1. Small scale synthesis and purification of steroid sulfates.

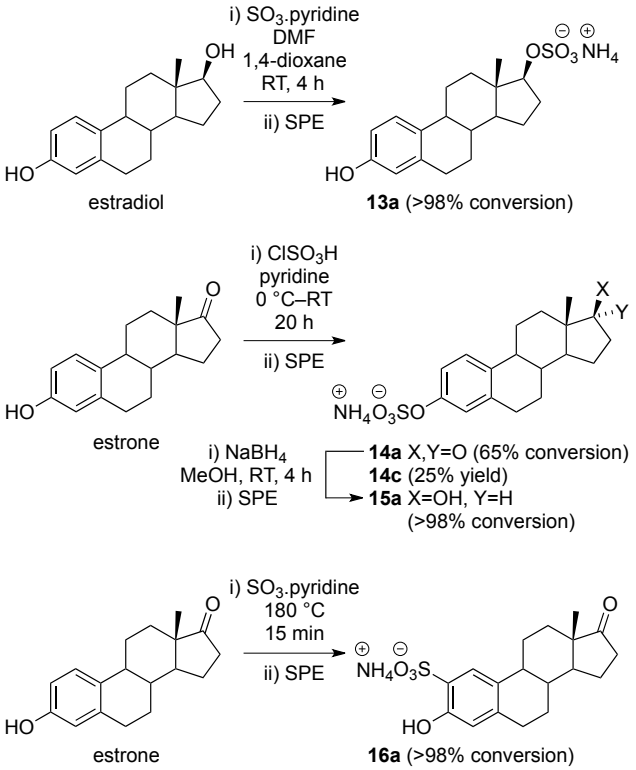
Scheme 2. Small scale sulfation of estrogenic steroids.

Graphics

Scheme 1:



Scheme 2:



Graphical abstract: