Supporting Information

# Energy-resolved fragmentation aiding the structure elucidation of steroid biomarkers

Christopher C. J. Fitzgerald [a], Christopher Bowen [b], Madysen Elbourne [c], Adam Cawley [d], and Malcom D. McLeod [a*]

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

[b] Mass Spectrometry Business Unit, Shimadzu Scientific Instruments (Australasia), Rydalmere, New South Wales, 2116, Australia

[c] Centre for Forensic Science, University of Technology Sydney, Broadway, New South Wales, 2007, Australia

[d] Australian Racing Forensic Laboratory, Racing NSW, Sydney, New South Wales, 2000, Australia.

*Corresponding Author email: malcom.mcleod@anu.edu.au

# Contents

# S1 Supplementary Figures, Tables and Schemes

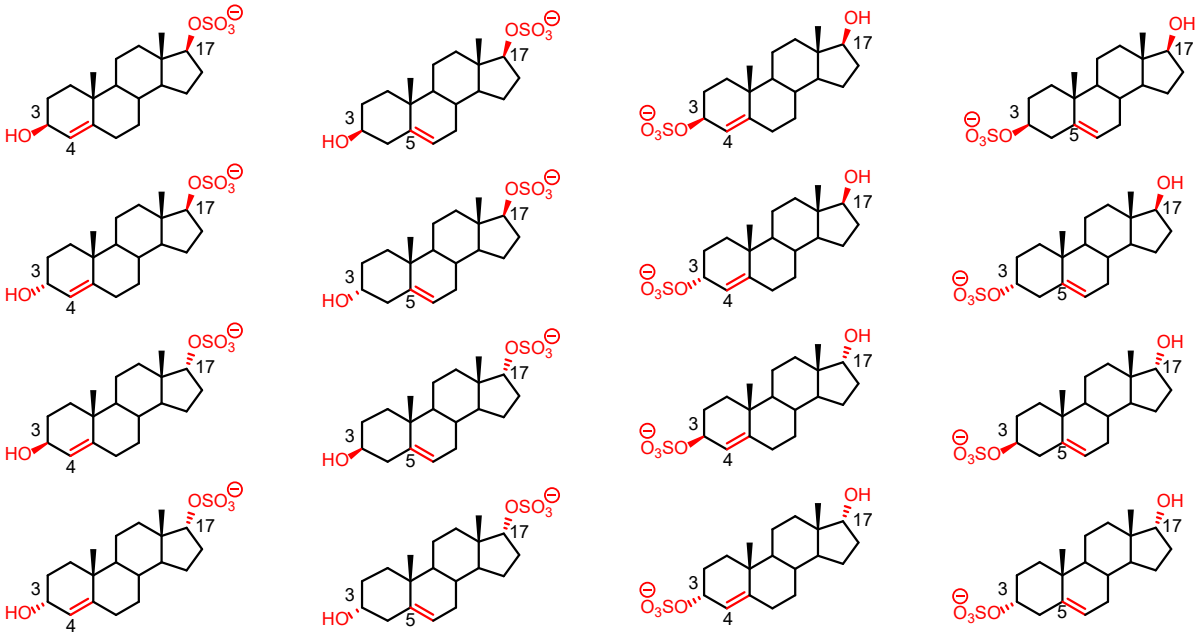


**Figure S1: The sixteen possible $C_{19}$ androstenediol isomers considered as potential candidates for the unknown metabolite.**

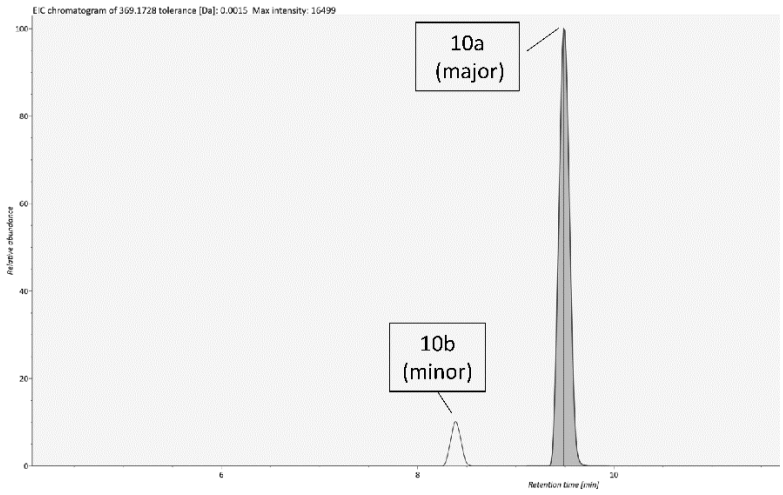



**Figure S2: extracted ion chromatograms of compounds 10a and 10b. The average area ratio between the two compounds was 9 : 1 in favour of the major isomer 10a. This aligns with the ratio that was observed in $^1$H NMR of 13: 1.**

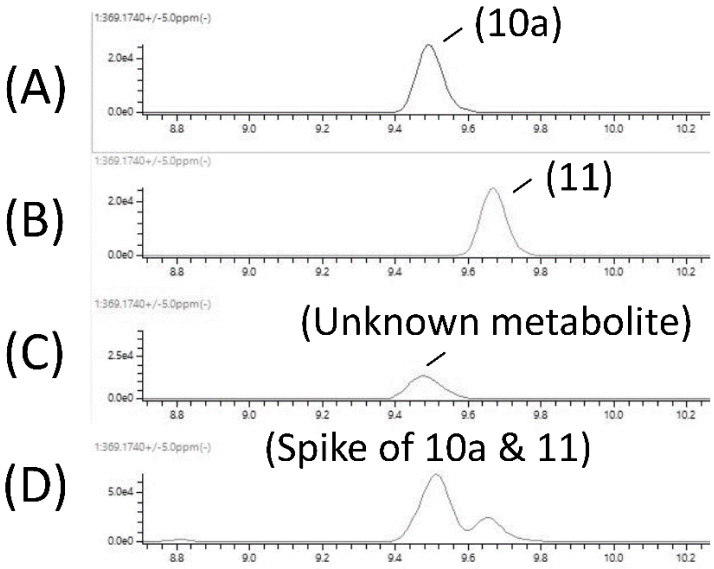



**Figure S3: Spike of compounds 10a and 11 to equine urine 12h after administration of testosterone propionate. (A) Compound 10a in stripped equine urine. (B) Compound 11 in stripped equine urine. (C) The unknown urinary metabolite in equine urine +12 hours after testosterone propionate administration. (D) Spike of compounds 10a and 11 in in equine urine +12 hours after testosterone propionate administration.**

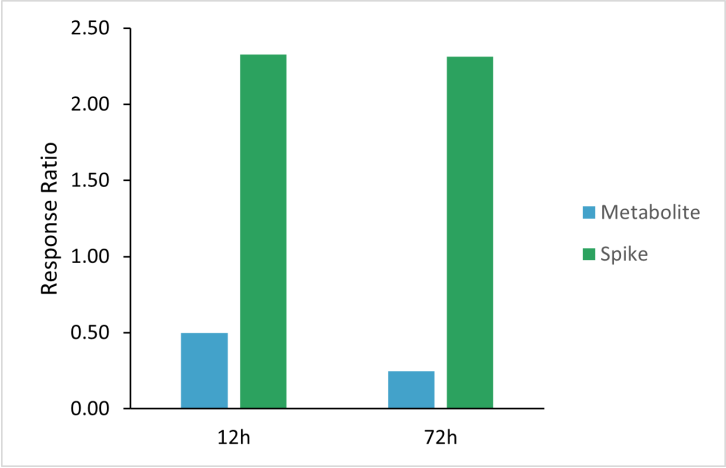



**Figure S4: The response ratios of the unknown urinary metabolite (Metabolite) and the same sample spiked with compound (10a) (Spike) at two different time points (+12 hours and +72 hours) after the administration of testosterone propionate. The response ratio was taken using peak area derived from extracted ion chromatograms of the metabolite and the stable isotope labelled internal standard epiandrosterone [$^{18}O_3$]-sulfate prepared in a previous study.[1]**

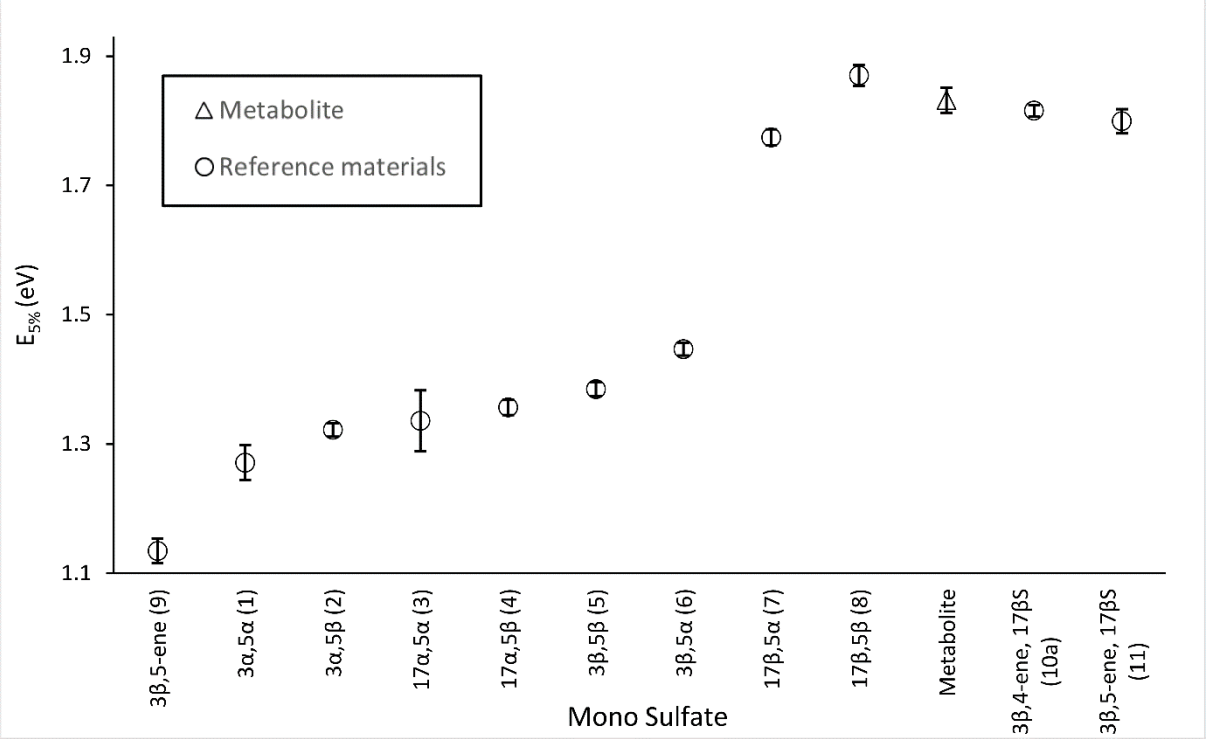



**Figure S5: The threshold energy ($E_{5\%}$) of 17β-oriented conjugates (10a) and (11) against all other synthesised compounds 1-9 and the urinary metabolite (Metabolite). Error bars represent standard deviation of replicate $E_{5\%}$ measurements ($n$ = 3).**

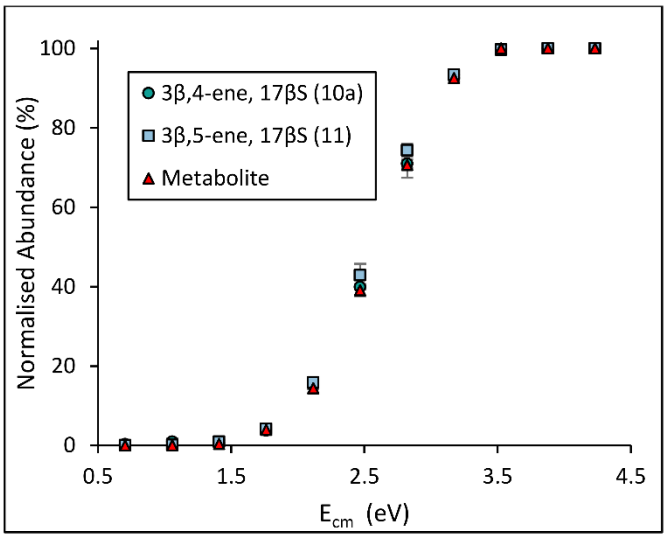



**Figure S6: Breakdown curves of the two synthesised 17β-oriented conjugates (10a) and (11), and the urinary metabolite (Metabolite). Error bars represent standard deviation of replicate normalised abundance measurements ($n$ = 3).**

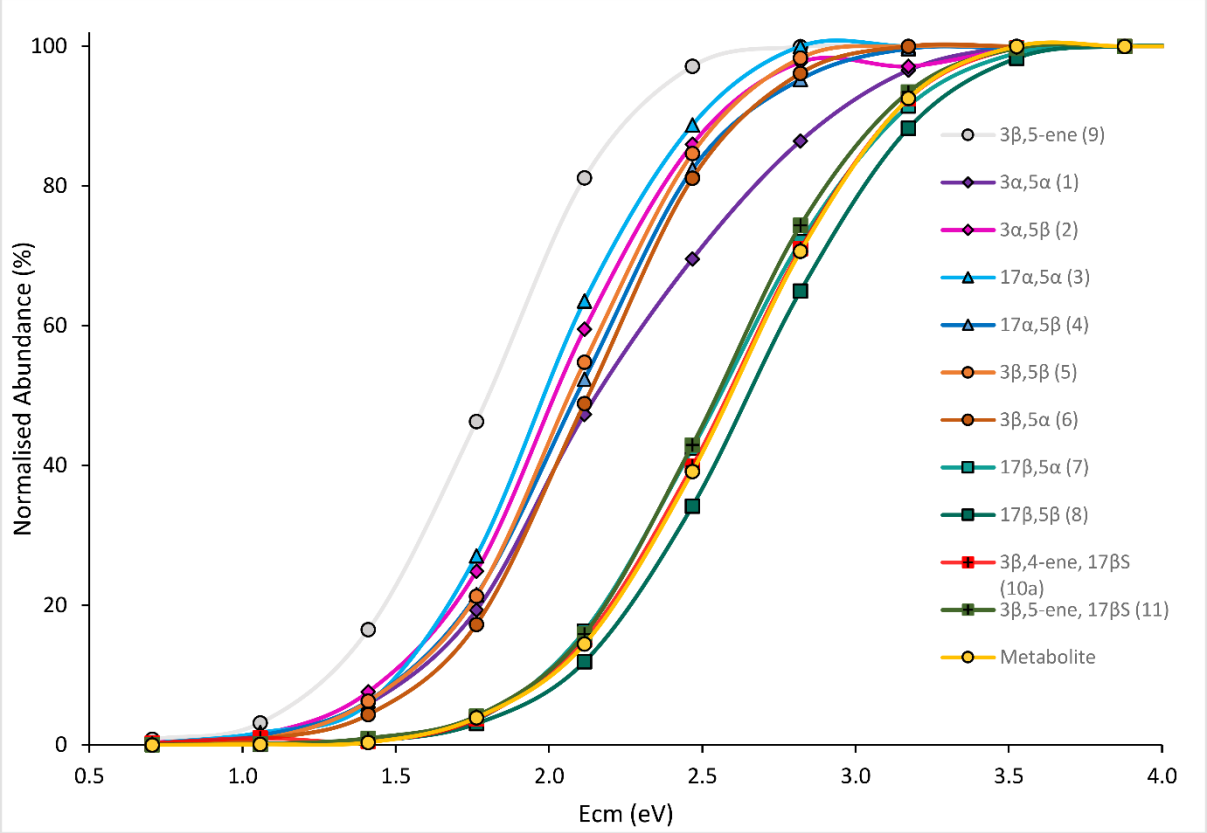
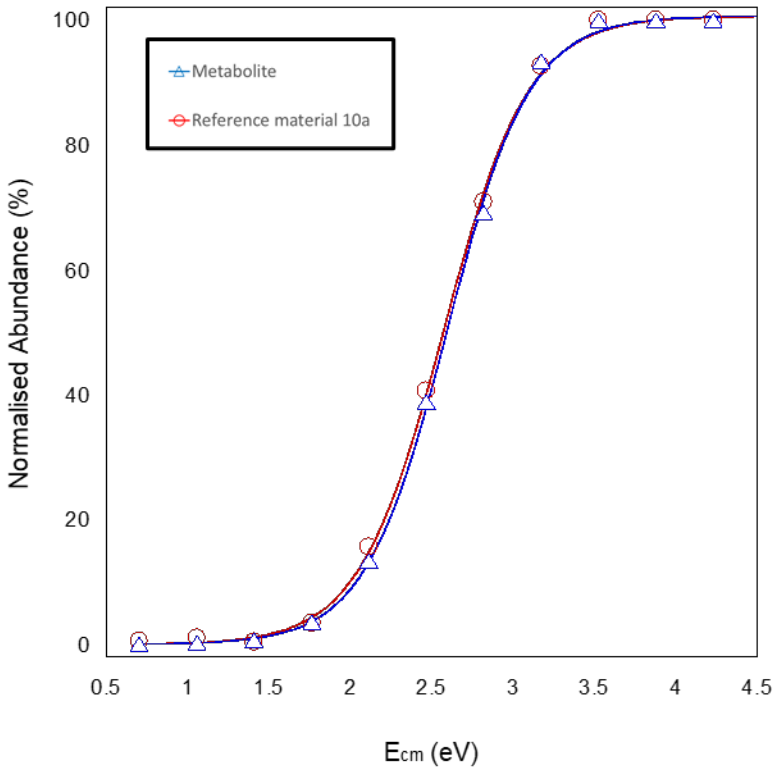



**Figure S7: Energy profiles of all synthesised compounds and the unknown urinary metabolite. Note: smoothed lines are not fitted to Eq.1, however, are present to highlight groupings between molecules.**




| y = m1/(1+(exp((m2-m0)*m3))) | | |
|---|---|---|
| | Value | Error |
| m1 | 100.6 | 0.44232 |
| m2 | 2.5742 | 0.0083941 |
| m3 | 3.8376 | 0.10664 |
| Chisq | 11.829 | NA |
| R | 0.99978 | NA |

| y = m1/(1+(exp((m2-m0)*m3))) | | |
|---|---|---|
| | Value | Error |
| m1 | 100.66 | 0.49938 |
| m2 | 2.5954 | 0.0093295 |
| m3 | 3.9474 | 0.12533 |
| Chisq | 15.118 | NA |
| R | 0.99972 | NA |

**Figure S8: Example of data gathered from single and separate injections of the metabolite and reference material 10a, plotted together. Fitting results to Eq. 1 are shown in the right, m1, m2, m3 correspond to the $BR_i$, $E_{1/2}$, & $b_i$ parameters of the model, respectively.**

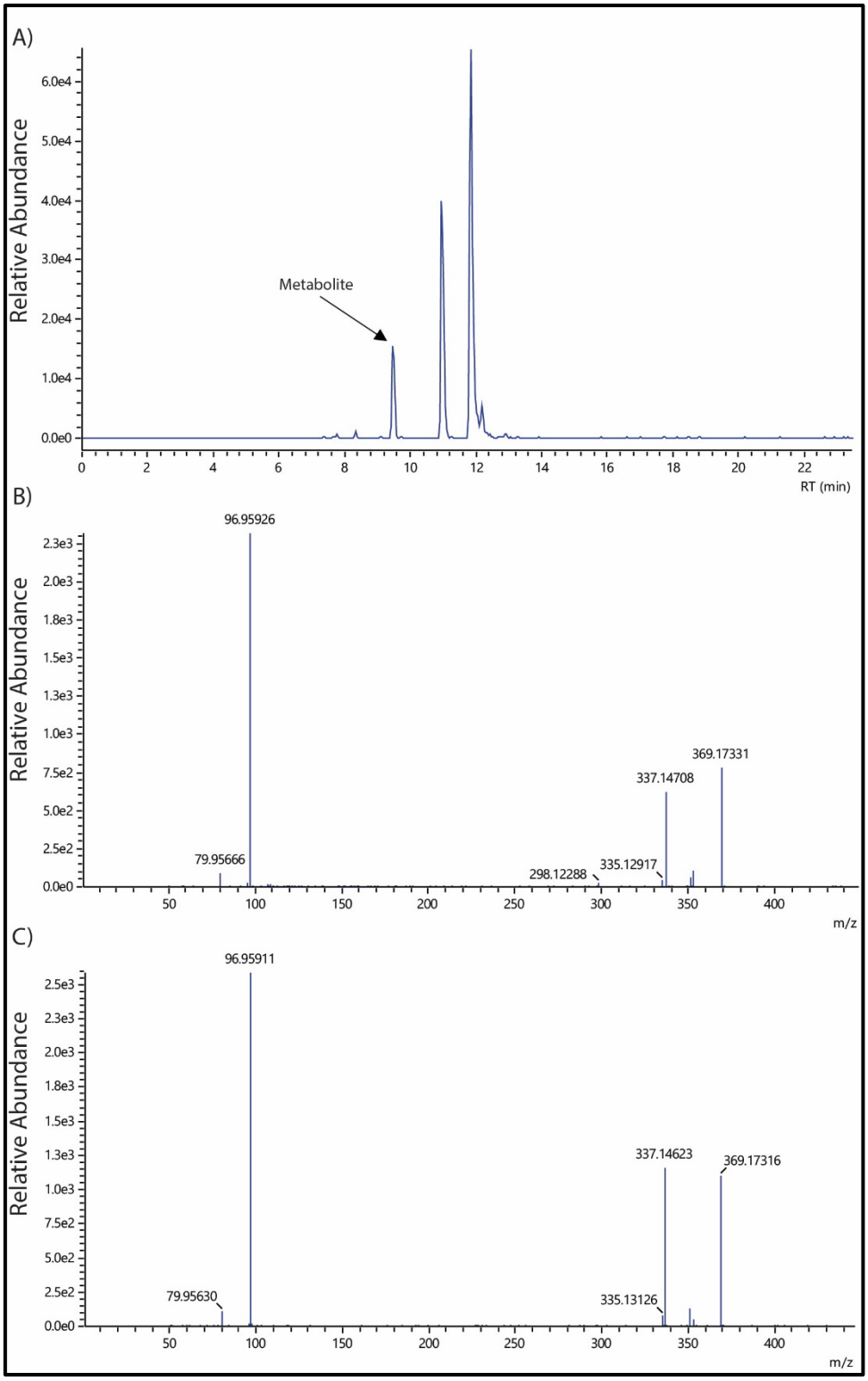



**Figure S 9: (A)Extracted ion chromatogram (m/z 369.1730) targeting the unknown metabolite (RT 9.49 min) derived from the sulfate fraction of post-administration urine (12 hours). Targeted MS/MS spectrum (CE 40) of (B) the unknown metabolite in equine urine and (C) the reference material Androst-4-ene-3β,17β-diol, 17-sulfate (10a).**

**Table S1: Retention times of synthesised library of mono-sulfates 1-9 (*n* = 3, for metabolite *n* = 6, taken from the 12h and 72h post administration urine samples in triplicate). All RTs are derived from the EIC. Chromatographic separation between all sulfate conjugation site epimers is distinct, with average separations of 1 & 6 (0.86 minutes), 2 & 5 (0.74 minutes), 3 & 7 (0.56 minutes), and 4 & 8 (0.68 minutes).**

| Compound | Average RT [min] | RSD (%) |
|---|---|---|
| 3α,5α (**1**) | 11.86 | 0.06 |
| 3α,5β (**2**) | 11.72 | 0.00 |
| 17α,5α (**3**) | 11.62 | 0.00 |
| 17α,5β (**4**) | 12.59 | 0.00 |
| 3β,5β (**5**) | 10.98 | 0.00 |
| 3β,5α (**6**) | 11.00 | 0.06 |
| 17β,5α (**7**) | 11.06 | 0.00 |
| 17β,5β (**8**) | 11.91 | 0.00 |
| 3β,5-ene (**9**) | 9.67 | 0.07 |
| Metabolite | 9.49 | 0.13 |

**Table S2: Average model fitting to Eq.1 for each compound, *n* =3. Good fits were indicated by $R^2$ values and relatively low $\chi^2$. SE indicates standard error for the given parameter. All modelling was done using KaleidaGraph® 4.5 program from Synergy software. Data presented is from a single day run of all the molecules.**

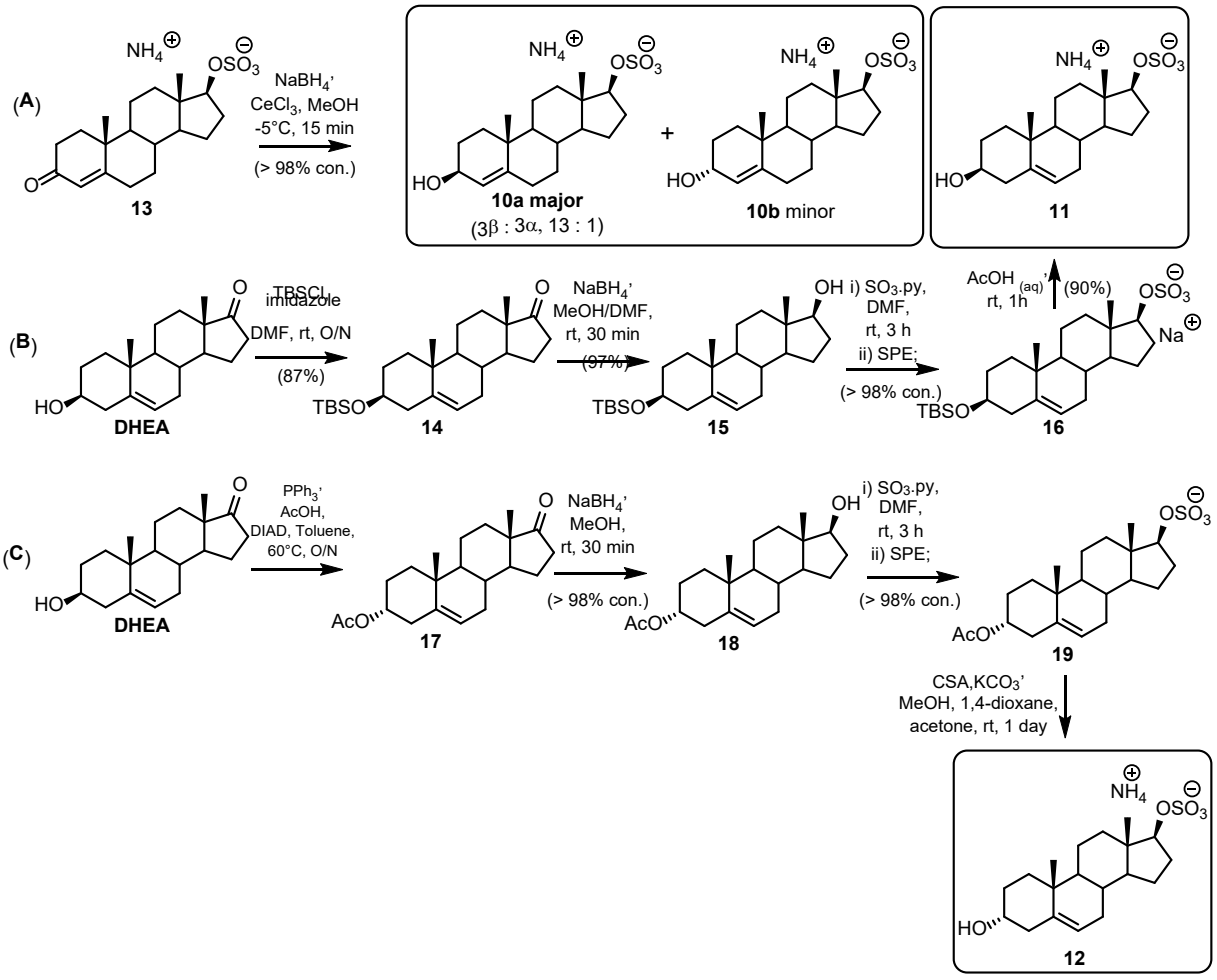
| Compound | $R^2$ | Chi Sq ($\chi^2$) | $BR_i$ | SE | $E_{1/2}$ | SE | $b_i$ | SE | $E_{5\%}$ | Stdev |
|---|---|---|---|---|---|---|---|---|---|---|
| 3β,5-ene (**9**) | 0.9997 | 6 | 100 | 0.3 | 1.79 | 0.01 | 4.49 | 0.09 | 1.13 | 0.03 |
| 3α,5α (**1**) | 0.9987 | 29 | 100 | 0.6 | 2.19 | 0.01 | 3.21 | 0.12 | 1.27 | 0.01 |
| 3α,5β (**2**) | 0.9997 | 7 | 100 | 0.3 | 2.02 | 0.01 | 4.20 | 0.09 | 1.32 | 0.05 |
| 17α,5α (**3**) | 0.9991 | 21 | 100 | 0.5 | 1.99 | 0.01 | 4.49 | 0.17 | 1.34 | 0.01 |
| 17α,5β (**4**) | 0.9998 | 5 | 100 | 0.3 | 2.09 | 0.01 | 4.02 | 0.08 | 1.36 | 0.01 |
| 3β,5β (**5**) | 0.9997 | 8 | 100 | 0.3 | 2.07 | 0.01 | 4.29 | 0.09 | 1.38 | 0.01 |
| 3β,5α (**6**) | 0.9999 | 2 | 100 | 0.2 | 2.13 | 0.003 | 4.34 | 0.06 | 1.45 | 0.01 |
| 17β,5α (**7**) | 0.9997 | 7 | 100 | 0.3 | 2.56 | 0.01 | 3.76 | 0.08 | 1.77 | 0.02 |
| 17β,5β (**8**) | 0.9998 | 6 | 100 | 0.3 | 2.65 | 0.01 | 3.77 | 0.07 | 1.87 | 0.02 |
| Metabolite | 0.9993 | 19 | 101 | 0.5 | 2.59 | 0.01 | 3.91 | 0.14 | 1.83 | 0.02 |
| 3β,4-ene, 17βS (**10a**) | 0.9997 | 9 | 101 | 0.4 | 2.58 | 0.01 | 3.87 | 0.10 | 1.82 | 0.01 |
| 3β,5-ene, 17βS (**11**) | 0.9997 | 9 | 100 | 0.4 | 2.54 | 0.01 | 3.96 | 0.10 | 1.80 | 0.03 |

**Table S3: Retention times of all synthesised mono-sulfate diol compounds 9-12 (*n* =3) and unknown metabolite. All RT are derived from the EIC. The epimers 10a and 10b have an average chromatographic separation of 1.13 minutes.**

| Compound | Average RT [min] | RSD (%) |
|---|---|---|
| Metabolite | 9.49 | 0.13 |
| Androst-5-ene-3β,17β-diol, 3-sulfate (**9**) | 9.67 | 0.07 |
| Androst-4-ene-3β,17β-diol, 17-sulfate (**10a**) | 9.49 | 0.02 |
| Androst-4-ene-3α,17β-diol, 17-sulfate (**10b**) | 8.38 | 0.07 |
| Androst-5-ene-3β,17β-diol, 17-sulfate (**11**) | 9.53 | 0.03 |
| Androst-5-ene-3α,17β-diol, 17-sulfate (**12**) | 11.42 | 0.14 |

## Synthesis of androstenediol 17β-sulfate isomers

Synthesis of the four isomers, androst-4-ene-3β,17β-diol 17-sulfate (**10a**), androst-4-ene-3α,17β-diol 17-sulfate (**10b**), androst-5-ene-3β,17β-diol 17-sulfate **(11**) and androst-5-ene-3α,17β-diol 17-sulfate (**12**) as their ammonium salts, was performed before finding a match. Briefly, sulfation of testosterone afforded testosterone sulfate (**13**) which was then reduced to the final steroid diol monosulfate (**10**) as a mixture of 3β (**10a**) and 3α (**10b**) epimers (in a ratio of 13:1, respectively), Scheme 1A. Protection of DHEA afforded the TBS protected DHEA (**14**) which was then reduced to give the steroid diol (**15**). Sulfation then afforded the TBS protected steroid diol mono-sulfate (**16**) which was then deprotected to give the final steroid diol mono-sulfate (**11**). Lastly, epimerisation under Mitsunobu conditions afforded acetate (**17**), reduction to steroid diol monoacetate (**18**) was followed by sulfation to (**19**) and then deprotection to give the steroid diol mono-sulfate (**12**)




**Scheme S1: The synthesis of 17β-sulfated steroids 10a, 10b, 11 and 12.**

# S2 Additional Methodology

## Chemicals, materials, and reagents

Chemicals and solvents including sodium borohydride ($NaBH_4$), dihydrotestosterone (17β-hydroxy-5α-androstan-3-one), and sulfur trioxide pyridine complex ($SO_3$•Py), were purchased from Sigma–Aldrich (Castle Hill, Australia). Androsterone (3α-hydroxy-5α-androstan-17-one), etiocholanolone (3α-hydroxy-5β-androstan-17-one), epiandrosterone (3β-hydroxy-5α-androstan-17-one), and testosterone (17β-hydroxyandrost-4-en-3-one) were obtained from Steraloids (Newport RI, USA). Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) was obtained from BDH (Poole, UK). Epitestosterone (17α-hydroxyandrost-4-en-3-one) was synthesised from testosterone using literature methods.[2] If not otherwise specified all other free steroid precursors to the final steroid monosulfate reference materials were synthesised by the authors with identity and purity determined by NMR analysis. Dimethylformamide, 99.8% was purchased from Chem-Supply (Gillman, Australia) and methanol-d4, 99.8% D from Cambridge Isotope Laboratories (Andover, MA, USA). MilliQ water was used in all aqueous solutions during sample treatment. LiChrosolv LC-MS grade water and methanol, and ammonium formate (99.995 %) from Merck (Bayswater, Australia) was used in the liquid chromatography mobile phases. Aqueous ammonia solution was obtained from ChemSupply (Gillman, Australia). Formic acid was obtained from Ajax Chemicals (Auburn, Australia). Solid-phase extraction (SPE) was performed using Waters (Rydalmere, Australia) Oasis weak anion exchange (WAX) 6 cc cartridges (PN 186004647), Oasis WAX 3 cc cartridges (PN 186002492) or Sep-Pak Vac C18 3 cc cartridges (PN 186004619). All UHPLC samples were filtered using 0.2 μm spin filters from PhaseSep (Doncaster East, Australia). Weighing of internal standards and reference compounds was performed on an Ultra-micro balance UMX2 from Mettler Toledo (Port Melbourne, Australia), with a readability of ±0.0001 mg. The internal standards nandrolone sulfate, cholanediol bis(sulfate), and stable isotope labelled (SIL) epiandrosterone [$^{18}O_3$]-sulfate, were prepared as the corresponding ammonium salts, by the authors and were subjects of a previous publication.[1]

## Animal Administration and sample collection

Animal Administration was approved by both Charles Sturt University (Wagga Wagga, NSW, Australia) and Racing NSW Animal Care and Ethics Committees. Testoprop® (testosterone propionate, 250 mg) was administered by intramuscular injection in the neck, opposite to the sampling jugular catheter to a thoroughbred gelding (635 kg, 14-year-old). Urine samples were collected at -72, -48, -24, 0, 2, 4, 6, 8, 12, 24, 36, 48, 72 hours and then daily to 28 days after administration. Samples were stored at -20 °C at the Australian Racing Forensic Laboratory (ARFL) or the Australian National University. These samples were taken as a subject of a previous publication. [1]

## Equine urine sample preparation

The method has been reported previously.[1] For samples containing the unknown metabolite; an aliquot of urine at either time points +12 h or +72 h post administration of testosterone propionate was used. For reference materials, stripped equine was used, which comprised of the flow through fraction of urine from and un-doped horse that was passed through a C18 SPE cartridge.

## General procedure for urine sample preparation

An aliquot of urine (metabolite) or stripped urine (reference materials) (0.6 mL) was mixed with phosphate buffer (0.3 mL, 100 mM, pH 5.4) and centrifuged (1100 x *g*, 5 min) to pellet solids. The

supernatant (0.750 mL) was fortified with a mixture of analytical internal standards (60 µL, 2500 ng/mL, of nandrolone sulfate, cholanediol bis(sulfate) and epiandrosterone [$^{18}O_3$]-sulfate) and/or reference materials (60 µL, 2500 ng/mL), giving a final concentration equivalent to 300 ng/mL original urine volume (0.5 mL) per standard. The supernatant was then loaded onto a Waters™ Oasis WAX SPE cartridge (3 cc), that was pre-conditioned with methanol (2 mL) and water (2 mL). Samples were washed with NaOH (2 mL, 0.1 M), phosphate buffer (2 mL, 100mM, pH 7.4), and Milli-Q water (2 mL) before elution with a mixture of ethyl acetate: methanol: diethyl amine (25:25:1 v/v/v, 3 mL) into clean 10 mL glass tubes. All samples were then evaporated to dryness under reduced pressure at 40 °C and stored at -20 °C until analysis. For analysis, the dry samples were re-dissolved with MeOH: water (100 µL, 10 % v/v), filtered using 0.2 µm spin filters and stored at 5°C until analysis. This resulted in a sample approximately 5 x the concentration of the original urine sample (0.5 mL).

**Instrumentation**

For compound characterisation, $^{1}H$ NMR and $^{13}C$ NMR spectra were recorded in deuterated chloroform ($CDCl_3$), deuterated methanol ($CD_3OD$), or deuterium monoxide ($D_2O$), using a Bruker Avance 400 MHz, 600MHz, or 700 MHz spectrometer at 298 K. Chemical shifts are reported in parts per million (ppm) downfield shift from TMS (δ=0), as follows: chemical shift (δ) (multiplicity, coupling constant(s) J (Hz), relative integral, where multiplicity is defined as, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, or combinations of the foregoing. The signal due to the residual protonated solvent (i.e., $CHCl_3$) or $^{13}C$ was used as an internal reference. Low resolution mass spectrometry (LRMS) using negative or positive electrospray ionisation (ESI) was performed on a Micromass ZMD ESI-Quad. High resolution mass spectrometry (HRMS) was performed on a Waters LCT Premier XE mass spectrometer or a Thermo-Fischer Scientific Orbitrap Elite™ Hybrid Ion Trap-Orbitrap mass spectrometer. Infrared spectra were recorded on a Perkin-Elmer 1800 Series FTIR spectrometer. Melting points were measured on an SRS Opti-melt MPA 100 automated melting point system and are uncorrected. Reactions were monitored by analytical thin layer chromatography (TLC) performed on aluminium-backed 0.2 mm thick silica gel 60 F254 plates as supplied by Merck. Eluted plates were visualised by staining using a solution of sulfuric acid: methanol (5% v/v), followed by heating. Flash chromatographic separations were carried out following protocols defined by Still et al.[3] with silica gel 60 (40–63 µm) as the stationary phase and analytical reagent (AR) or HPLC-grade solvents as indicated. Tetrahydrofuran (THF), methanol, and dichloromethane were dried using a glass contour solvent purification system based on technology originally described by Grubbs et al.[4] Optical rotations were performed on a Rudolph Research Analytical, Autopol I Automatic Polarimeter (589 nm fixed wavelength, 10 mm cell).

## S3 General procedures for synthesis

**General procedure 1 for small scale steroid sulfation reaction.**

A solution of $SO_3$•py (30 mg, 188 mmoL) was added to a solution of free steroid (5 mg) in *N,N*-dimethylformamide (0.5 mL) and the resulting solution was capped and stirred at room temperature for 3 h. The reaction was then quenched with water (10 mL) and subjected to SPE according to general procedures 2 and 3.

**General procedure 2 for the small-scale steroid conjugate purification by WAX SPE.** Weak anion exchange (WAX) solid phase extraction (SPE) was used to separate a steroid conjugate from any unreacted steroid following a reaction. The procedure was adapted from the literature.[3–5] A WAX SPE cartridge (6 cc, Waters Oasis®) was preconditioned with methanol (5 mL) followed by water (15 mL), under positive pressure of nitrogen. The conjugate/free steroid mixture (1 mg $mL^{-1}$, water) was then loaded onto the cartridge, which was then washed with formic acid in water (2% v/v, 15 mL), then water (15 mL) and then methanol (15 mL) to elute any free steroid. Finally, the steroid conjugate was eluted using aqueous ammonia solution in methanol (5% v/v, 15 mL). The eluted fraction was concentrated *in vacuo* to yield the steroid sulfate as the corresponding ammonium salt.

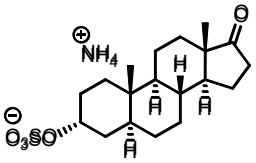
**General procedure 3 for small scale de-salting of steroid conjugate by C18 SPE.** This C18 SPE was used to separate steroid conjugates from excess ammonium salt following a reaction or after extraction with WAX SPE. The procedure was adapted from the literature.[3–5] A C18 SPE cartridge (3 cc) was preconditioned with methanol (3 mL) followed by water (3 mL), under positive pressure of nitrogen. The reaction mixture (1 mg $mL^{-1}$, water) was then loaded onto the cartridge, which was then washed with aqueous ammonia solution (2% v/v, 2 mL), and then water (3 mL). The sulfate conjugate was eluted with methanol (9 mL) and then concentrated *in vacuo* to yield the steroid sulfate as the corresponding ammonium salt.

**General procedure 4 for determining conversion by $^1$H NMR analysis**

This step was used to calculate the ratio of steroid sulfate conjugate relative to free steroid or steroid diol remaining in the reaction mixture after a conjugation or small-scale reduction reaction. The procedure employed a modified WAX SPE (general procedure 2) protocol, washing with formic acid in water (2% v/v, 15 mL), water (15 mL), before elution with aqueous ammonia solution in methanol (5% v/v, 15 mL). This fraction resulted in a mixture of product and any unreacted material. A $^1$H NMR spectrum was obtained and integration of a suitable signal (typically C3-H or C17-H) from both the starting steroid and steroid product was used to determine the percent conversion of sulfation or reduction.

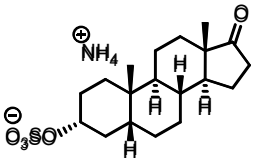
# S4 Synthesis of steroid mono-sulfate library

Androsterone-3-sulfate, ammonium salt (1)



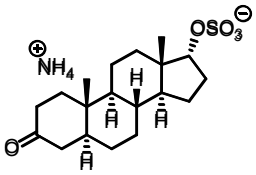
Androsterone (5.00 mg, 17.2 µmol) was reacted according to the general procedure 1 for small scale sulfation. This gave the title compound as a colourless solid (> 98% conversion). $^1$H NMR (400 MHz, $CD_3OD$) δ 4.60 (t, *J* = 2.9 Hz, 1H), 2.42 (dd, 1H), 2.14 – 1.89 (m, 3H), 1.83 (m, 1H), 1.77 – 1.44 (m, 9H), 1.42 – 1.18 (m, 6H), 1.06 (m, 1H), 0.87 (d, *J* = 2.9 Hz, 6H), 0.81 (m, 1H); LRMS (-ESI): *m/z* 369.3 (100%, $[C_{19}H_{29}O_5S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$) 369.1738, found 369.1730. Spectroscopic data and spectra was found to match the literature for the compound.[3]

Etiocholanolone 3-sulfate, ammonium salt (2)



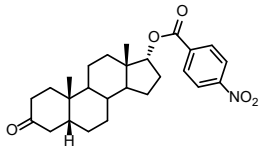
Etiocholanolone (5.00 mg, 17.2 µmol) was reacted according to the general procedure 1 for small scale sulfation. This yielded the title compound as a colourless solid (> 98% conversion). $^1$H NMR (400 MHz, $CD_3OD$) δ 4.29 (m, 1H, C3-H), 2.43 (dd, J = 19.2, 8.6 Hz, 1H), 2.09 (dt, *J* = 19.3, 8.9 Hz, 1H, C16-H), 2.02–1.19 (m, 19H), 1.05 (m, 1H), 0.99 (s, 3H), 0.87 (s, 3H); LRMS (-ESI): *m/z* 369.3 (100%, $[C_{19}H_{29}O_5S]^-$; HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$), 369.1737, found 369.1730. Spectroscopic data and spectra was found to match the literature for the compound.[3]

Epidihydrotestosterone 3-sulfate, ammonium salt (3)



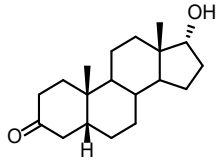
*Epidihydrotestosterone* (5.00 mg, 17.2 µmol) was reacted according to the general procedure 1 for small scale sulfation. This yielded the title compound as a colourless solid (> 98% conversion). $^1$H NMR (400 MHz, $CD_3OD$) δ 4.33 (d, *J* = 5.7 Hz, 1H), 2.48 (td, *J* = 14.7, 6.6 Hz, 1H), 2.36 (t, *J* = 14.4 Hz, 1H), 2.28 – 1.89 (m, 5H), 1.81 – 1.31 (m, 13H), 1.20 (m, 1H), 1.07 (s, 3H), 1.01 (m, 1H), 0.77 (s, 3H); LRMS (-ESI): *m/z* 369.2 (100%, $[C_{19}H_{29}O_5S]^-$; HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$) 369.17412, found 369.17353. Spectroscopic data and spectra was found to match the literature for the compound.[3]

17α-[(4-Nitrobenzoyl)oxy]-5β-androstane-3-one (15)



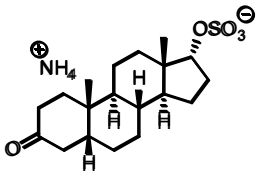
Diethyl azodicarboxylate (DEAD, 44 µL, 0.25 mmol) was added dropwise to a stirring solution of 5β-Androstane-17β-ol-3-one (30 mg, 0.1 mmol), 4-nitrobenzoic acid (42 mg, 0.25 mmol), triphenyl phosphine (70 mg, 0.25 mmol), in toluene (5 mL). The reaction was then heated to 80°C under an atmosphere of nitrogen and left to stir for 3 days. The reaction was then quenched with water (15 mL) and extracted with ethyl acetate (45 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude was then subject to silica flash chromatography (30% EtOAc : Petroleum spirits (40-60°C)), giving the title compound as a colourless solid (20.8 mg, 47 %). $^1$H NMR (600 MHz, $CDCl_3$) δ 8.34–8.28 (m, 2H), 8.23–8.18 (m, 2H), 5.10 (d, *J* = 6.3, 0.9 Hz, 1H), 2.79–2.68 (m, 1H), 2.41–2.27 (m, 2H), 2.17 (m, 1H), 2.09–1.99 (m, 2H), 1.98–1.81 (m, 2H), 1.74 (m, 1H), 1.69–1.60 (m, 2H), 1.49–1.37 (m, 4H), 1.37–1.18 (m, 6H), 1.15 (m, 1H), 1.05 (s, 3H), 0.86 (s, 3H); $^{13}$C NMR (151 MHz, $CDCl_3$) δ 213.13, 164.41, 150.66, 136.25, 130.78, 123.73, 83.94, 50.75, 45.55, 44.24, 42.48, 40.91, 37.33, 37.09, 36.01, 35.19, 32.53, 30.39, 26.72, 26.36, 24.99, 22.82, 20.75, 16.92 (two peaks overlapping); LRMS (+ESI): *m/z* 462.2 (60%, $[C_{26}H_{33}NO_5Na]^+$); HRMS (+ESI) *m/z* 462.2256 calcd. for $[C_{26}H_{33}NO_5Na]^+$ ([M+Na]$^+$), found 462.2266.

17α-Hydroxy-5β-androstane-3-one (16)



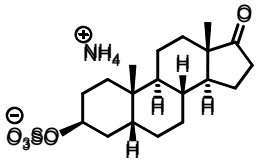
Sodium hydroxide (450 µL, 5M) was added to a stirring solution of 17α-[(4-nitrobenzoyl)oxy]-5β-androstane-3-one (5.8 mg, 13.2 µmol) in methanol (2 mL), and left at room temperature for 16 hours. The methanol was then taken off under reduced pressure, and the crude was then dissolved in 10 mL of water and then purified according to general procedure 3 for small scale purification using C18 giving the title compound as a colourless solid (3.8 mg, 13.1 µmol, 99 % isolated yield). $^1$H NMR (600 MHz, $CDCl_3$) δ 3.75 (m, 1H), 2.68 (m, 1H), 2.35 (m, 1H), 2.20 – 2.10 (m, 2H), 2.10 – 1.98 (m, 2H), 1.94 – 1.74 (m, 3H), 1.69 – 1.36 (m, 10H), 1.31 – 1.12 (m, 4H), 1.03 (s, 3H), 0.69 (s, 3H); $^{13}$C NMR (151 MHz, $CDCl_3$) δ 213.38, 80.11, 48.97, 45.60, 44.57, 42.51, 40.83, 37.39, 37.33, 35.94, 35.18, 32.67, 31.75, 26.74, 26.31, 22.83, 20.92, 17.22 (one peak overlapping); LRMS (+ESI): *m/z* 313.2 (100%, $[C_{19}H_{30}O_2Na]^+$); HRMS (+ESI) *m/z* 313.2143 calcd. for $[C_{19}H_{30}O_2Na]^+$ ([M+Na]$^+$), found 313.2157.

17α-Hydroxy-5β-androstane-3-one 17-sulfate, ammonium salt (4)



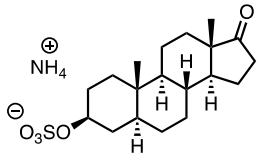
17α-Hydroxy-5β-androstane-3-one (5.00 mg, 17.2 μmol) was reacted according to the general procedure 1 for small scale sulfation. This yielded the title compound as a colourless solid (> 98% conversion, with a ratio of 6.7 : 1 of the 17α : 17β epimers). Data is reported for the major 17α isomer where appropriate. $^{1}$H NMR (600 MHz, $CD_3OD$) δ 4.34 (d, *J* = 5.8 Hz, 1H), 2.87 (t, *J* = 14.2 Hz, 1H), 2.49 (td, *J* = 14.8, 5.7 Hz, 1H), 2.25–2.04 (m, 3H), 2.03–1.88 (m, 4H), 1.89–1.72 (m, 3H), 1.68–1.45 (m, 5H), 1.41 (m, 1H), 1.36–1.12 (m, 4H), 1.05 (s, 3H), 0.78 (s, 3H); $^{13}$C NMR (151 MHz, $CD_3OD$) δ 216.31, 87.98, 50.78, 46.34, 46.13, 43.27, 41.57, 38.40, 37.99, 37.22, 36.15, 32.96, 31.27, 27.77, 27.20, 25.53, 23.02, 21.79, 17.26. LRMS (-ESI): *m/z* 369.2 (100%, $[C_{19}H_{29}O_5S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$) 369.17412, found 369.17353. Spectroscopic data and spectra was found to match the literature for the compound.[3]

Epietiocholanolone 3-sulfate, ammonium salt (5)



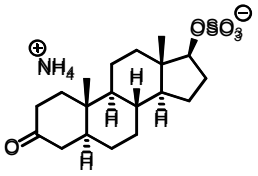
Epietiocholanolone (5.00 mg, 17.2 μmol) was reacted according to the general procedure 1 for small scale sulfation. This yielded the title compound as a colourless solid (> 98% conversion). $^{1}$H NMR (400 MHz, $CD_3OD$) δ 4.66 (m, 1H), 2.43 (dd, *J* = 19.2, 8.7 Hz, 1H), 2.07 (m, 1H), 2.02–1.90 (m, 3H), 1.88–1.61 (m, 5H), 1.63–1.33 (m, 8H), 1.35–1.13 (m, 4H), 1.01 (s, 3H), 0.87 (s, 3H); $^{13}$C NMR (151 MHz, $CD_3OD$) δ 224.22, 76.95, 52.69, 41.49, 38.36, 36.79, 36.60, 36.01, 32.95, 32.39, 31.50, 27.40, 26.67, 26.24, 24.22, 22.74, 21.47, 14.18, one carbon peak is overlapping or obscured; LRMS (-ESI): *m/z* 369.3 (100%, $[C_{19}H_{29}O_5S]^-$; HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$), 369.1731, found 369.1730.

Epiandrosterone 3-sulfate (6)



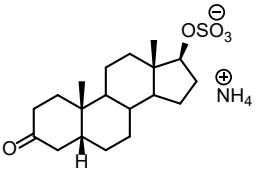
Epiandrosterone (5.00 mg, 17.2 μmol) was reacted according to the general procedure 1 for small scale sulfation. This yielded the title compound as a colourless solid (> 98 % conversion). $^{1}$H NMR (400 MHz, $CD_3OD$): δ 4.25 (m, 1H), 2.43 (dd, *J* = 19.1, 8.8 Hz, 1H), 2.14 – 1.90 (m, 3H), 1.87 – 1.15 (m, 15H), 1.12 – 0.97 (m, 2H), 0.88 (m, 6H), 0.76 (m, 1H); LRMS (-ESI): *m/z* 369.3 (100%, $[C_{19}H_{29}O_5S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$) 369.1738, found 369.1730. Spectroscopic data and spectra was found to match the literature for the compound.[3]

Dihydrotestosterone 3-sulfate, ammonium salt (7)



Dihydrotestosterone (5.00 mg, 17.2 μmol) was reacted according to the general procedure 1 for small scale sulfation. This yielded the title compound as a colourless solid (> 98% conversion). $^{1}$H NMR (400 MHz, $CD_3OD$) δ 4.22 (t, J = 8.5 Hz, 1H), 2.49 (td, *J* = 14.8, 6.7 Hz, 1H), 2.37 (t, *J* = 14.4 Hz, 1H), 2.28–1.92 (m, 5H), 1.80–1.12 (m, 13H), 1.07 (s, 3H), 1.07 (m, 1H), 0.83 (s, 3H), 0.77 (m, 1H); LRMS (-ESI): *m/z* 369.3 (100%, $[C_{19}H_{29}O_5S]^-$; HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M-NH_4]^-$) 369.17412, found 369.17353. Spectroscopic data and spectra was found to match the literature for the compound.[3]

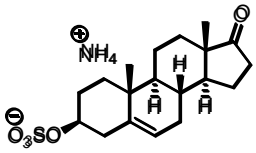
17β-Hydroxy-5β-androstane-3-one 17-sulfate, ammonium salt (8)



17β-Hydroxy-5β-androstane-3-one (11.2 mg, 38.6 μmol) was reacted according to the general procedure 1 for small scale sulfation. This yielded the title compound as a colourless solid (> 98% conversion). $^{1}$H NMR (400 MHz, $CD_3OD$) δ 4.26 (t, *J* = 8.5 Hz, 1H), 2.85 (t, *J* = 14.3 Hz, 1H), 2.47 (td, *J* = 14.6, 5.4 Hz, 1H), 2.18 (m, 1H), 2.13 – 2.06 (m, 2H), 2.01 – 1.87 (m, 3H), 1.85 – 1.69 (m, 2H), 1.66 – 1.60 (m, 2H), 1.58 – 1.50 (m, 3H), 1.49 – 1.15 (m, 7H), 1.05 (s, 3H), 0.82 (s, 3H).;$^{13}$C NMR (176 MHz, $CD_3OD$) δ 216.64, 88.15, 51.53, 46.03, 44.07, 43.23, 41.83, 38.28, 37.97, 37.94, 36.82, 36.12, 29.21, 27.66, 26.37, 24.34, 22.99, 21.78, 12.13; LRMS (-ESI): *m/z* 369.2 (100%, $[C_{19}H_{29}O_5S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M-NH_4]^-$), 369.1736 found 369.1727.

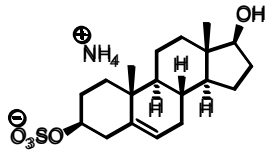
## S5 Synthesis of steroid diol mono-sulfate reference materials

Dehydroepiandrosterone 3-sulfate, ammonium salt (20)



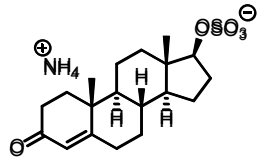
DHEA (5.00 mg, 17.3 μmol) was reacted according to the general procedure 1 for small scale sulfation. This yielded the title compound as a colourless solid (> 98% conversion). $^1$H NMR (400 MHz, $CD_3OD$) δ 5.44 (dt, *J* = 5.2, 1.9 Hz, 1H), 4.14 (tt, *J* = 11.5, 4.8 Hz, 1H), 2.57 (ddd, *J* = 13.3, 5.0, 2.4 Hz, 1H), 2.51 – 2.30 (m, 2H), 2.18 – 1.88 (m, 4H), 1.84 – 1.47 (m, 9H), 1.41 – 1.22 (m, 2H), 1.14 (m, 1H), 1.07 (s, 3H), 0.90 (s, 3H).; LRMS (-ESI): *m/z* 367.2 (100%, $[C_{19}H_{27}O_5S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{27}O_5S]^-$ ($[M\text{-}NH_4]^-$) 367.1579, found 367.1584. Spectroscopic data and spectra was found to match the literature for the compound.[5]

Androst-5-ene-3β,17β-diol 3-sulfate, ammonium salt, ammonium salt (9)



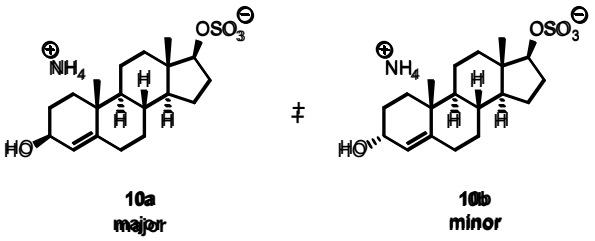
Procedure taken from Pranata, Fitzgerald, Mcleod, et.al. (2019).[5] DHEAS (5.00 mg, 17.3 μmol) was reacted with $NaBH_4$ (10 mg, 0.26 mmol) in MeOH (1 mL). The reaction was left stirring at room temperature for 2 hours. The mixture was then quenched with water (10 mL) and adjusted to a pH of 7 with aqueous HCl solution. Following this the mixture was subjected to SPE purification according to general procedures 2 and 3. This yielded the title compound as a colourless solid (> 98% conversion). $^1$H NMR (400 MHz, $CD_3OD$) δ 5.39 (d, *J* = 1.9 Hz, 1H), 4.13 (tt, *J* = 11.5, 4.7 Hz, 1H), 3.58 (t, *J* = 8.6 Hz, 1H), 2.54 (ddd, *J* = 13.3, 5.1, 2.3 Hz, 1H), 2.34 (m, 1H), 2.12 – 1.81 (m, 5H), 1.71 – 1.41 (m, 7H), 1.28 (m, 1H), 1.18 – 1.07 (m, 2H), 1.05 (s, 3H), 1.03 – 0.87 (m, 2H), 0.75 (s, 3H); LRMS (-ESI): *m/z* 368.8 (100%, $[C_{19}H_{29}O_5S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$) 369.1736, found 369.1733. Spectroscopic data and spectra was found to match the literature for the compound.[5]

Testosterone sulfate, ammonium salt (13)



Testosterone (5.00 mg, 17.3 μmol) was reacted according to the general procedure 1 for small scale sulfation. This gave the title compound as a colourless solid (> 98% conversion). $^1$H NMR (400 MHz, $CD_3OD$) δ 5.71 (s, 1H), 4.23 (t, *J* = 7.9 Hz, 1H), 2.56–2.41 (m, 2H), 2.36–1.96 (m, 5H), 1.90 (ddt, *J* = 11.6, 5.7, 2.9 Hz, 1H), 1.82–1.56 (m, 5H), 1.56–1.25 (m, 2H), 1.24 (s, 3H), 1.19 (m, 1H), 1.08 (s, 3H), 0.86 (s, 3H); LRMS (-ESI): *m/z* 366.8 (100%, $[C_{19}H_{27}O_5S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$) 367.1579, found 367.1580. Spectroscopic data and spectra was found to match the literature for the compound.[5]

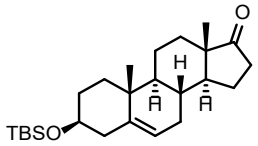
Androst-4-ene-3β,17β-diol 17-sulfate, ammonium salt (10)




**10a** major **10b** minor

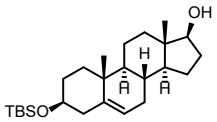
The procedure was adapted from Pranata et.al. (2019).[5] Testosterone sulfate, ammonium salt (5 mg, 13.0 μmol) was reacted with sodium borohydride (25 mg, 0.66 mmol) and cerium (III) chloride (25 mg, 0.10 mmol), stirring in methanol (1 mL) at -5 °C for 15 min. The reaction was then quenched by the addition of cold saturated aqueous citric acid solution (8-10 mL) and adjusted to pH 4. The resulting solution was then subject to purification by the general procedures 2 & 3 for small scale steroid conjugate purification by C18 SPE. This gave the title compound (10a) as a colourless solid (> 98% conversion, with a ratio of 13 : 1 of the 3β : 3α epimer). Spectral data reported for the major isomer (**10a**).$^{1}$H NMR (400 MHz, $CD_3OD$) δ 5.25 (s, 1H), 4.21 (t, *J* = 8.4 Hz, 1H), 4.06 (m, 1H), 2.30–2.09 (m, 2H), 2.06–1.22 (m, 14H), 1.15 (m, 1H), 1.08 (s, 3H), 1.05–0.84 (m, 2H), 0.83 (s, 3H), 0.76 (m, 1H); $^{13}$C NMR (101 MHz, $CD_3OD$) δ 147.81, 124.95, 88.09, 68.47, 56.17, 51.58, 43.87, 38.47, 37.90, 37.20, 36.80, 33.88, 33.22, 29.90, 29.11, 24.35, 21.63, 19.39, 12.08; LRMS (-ESI): *m/z* 368.8 (100%, $[C_{19}H_{29}O_5S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$) 369.1736, found 369.1732. Spectroscopic data and spectra was found to match the literature for the compound.[5]

3β-(*Tert*-butyldimethylsilyloxy)-androst-5-en-17-one (14)



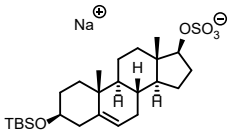

This procedure was adapted from Hosoda, Fukushima, and Fishman, (1973).[6] Dehydroepiandrosterone (500 mg. 1.73 mmol), imidazole (283 mg, 4.15 mmol) and *tert*-butyldimethylsilyl chloride (389 mg, 2.57 mmol) were dissolved in anhydrous DMF (10 mL). The reaction was left to stir for 10 minutes at room temperature. The resulting cloudy solution was diluted with water and extracted with ethyl acetate (3x 25 mL)/ The combined organic extract was washed with brine and dried over anhydrous $MgSO_4$ and filtered. The solvent was removed under reduced pressure to give a crude colourless solid, which was then purified by silica flash column chromatography. (25 % ETOAc : Petroleum spirits (40-60°C)), to afford the title compound as a colourless solid (673 mg, 87 %). Rf: 0.6 (25% ETOAc : Petroleum spirits (40-60°C)), m.p. 148°C, $^{1}$H NMR (700 MHz, $CDCl_3$) δ 5.34 (m, 1H), 3.48 (tt, *J* = 11.0, 4.8 Hz, 1H), 2.46 (dd, *J* = 19.10, 8.98 Hz, 1H), 2.34–2.01 (m, 4H), 2.00–1.60 (m, 8H), 1.56–1.39 (m, 2H), 1.34-1.21 (m, 2H), 1.07 (m, 1H), 1.02 (s, 3H), 0.98 (m, 1H), 0.90–0.88 (m, 12H), 0.06 (s, 6H) ; $^{13}$C NMR (176 MHz, $CDCl_3$) δ 221.36, 141.95, 120.55, 51.96, 50.48, 47.71, 42.93, 37.46, 36.88, 36.00, 32.16, 31.67, 31.61, 30.98, 26.07, 22.03, 20.50, 19.61, 18.40, 13.69, -4.44, 3 peaks doubled; LRMS (+ESI): *m/z* 827.6 (100%, $[2M+Na]^+$). HRMS (+ESI) *m/z* calcd. for $[C_{25}H_{42}O_2SiNa]^+$ ($[M+Na]^+$) 425.2852, found 425.2880. Spectroscopic data and spectra was found to match the literature for the compound.[6]

3β-(*Tert*-butyldimethylsilyloxy)-androst-5-en-17β-ol (15)



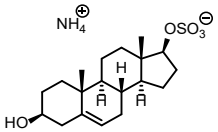

Procedure adapted from Pranata, Fitzgerald, Mcleod, et.al. (2019).[5] 3β-(*Tert*-butyldimethylsilyloxy)-androst-5-en-17-one (500 mg, 1.24 mmol) and $NaBH_4$ (95 mg, 2.48 mmol) were dissolved in MeOH:THF (1:1 v/v). The solution was stirred for 45 minutes at room temperature. The reaction mixture was quenched by the addition of water (50 mL) and exacted with ethyl acetate (3x30 mL). The organic extracts were combined and washed with brine and dried over anhydrous $MgSO_4$ then filtered. The solvent was removed under reduced pressure to give a colourless solid, which was purified by flash silica column chromatography (30% EtOAc : Petroleum spirits (40-60°C)). This gave the title compound as a colourless solid (600 mg, 97%). Rf = 0.2 (30% EtOAc : Petroleum spirits (40-60°C)); m.p. 140-145°C; $^1$H NMR (700 MHz, $CDCl_3$ δ 5.31 (dt, *J* = 5.5, 1.9 Hz, 1H), 3.64 (t, *J* = 9.0 Hz, 1H), 3.48 (tt, *J* = 11.0, 4.7 Hz, 1H), 2.35 – 1.93 (m, 4H), 1.89 – 1.67 (m, 3H), 1.65 – 1.37 (m, 7H), 1.34 – 1.19 (m, 2H), 1.08 (ddd, *J* = 17.3, 10.7, 4.2 Hz, 2H), 1.01 (s, 3H), 0.94(m, 1H), 0.89 (s, 9H), 0.75 (s, 3H), 0.06 (s, 6H); $^{13}$C NMR (176 MHz, $CDCl_3$) δ 141.82, 120.99, 82.08, 51.52, 50.49, 42.97, 42.89, 37.57, 36.83, 36.76, 32.22, 32.13, 31.68, 30.69, 26.09, 23.60, 20.81, 19.63, 18.41, 11.10, -4.43, 3 peaks doubled; LRMS (+ESI): *m/z* 831.7 (100%, $[2M+Na]^+$), HRMS (+ESI) *m/z* calcd. for $[C_{25}H_{45}O_2Si]^+$ ($[M+H]^+$) 405.3189, found 405.3188. Spectroscopic data and spectra was found to match the literature for the compound.[6]

3β-(*Tert*-butyldimethylsilyloxy)-androst-5-en-17β-ol 17-sulfate, sodium salt (16)



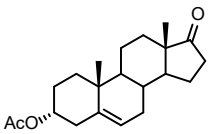

This procedure was adapted from Hungerford, McLeod et. al. (2006).[7] 3β-(*Tert*-butyldimethylsilyloxy)-androst-5-en-17β-ol (60 mg, 0.148 mmol), activated molecular sieves (4Å) and anhydrous DMF (1 mL) and anhydrous pyridine (1.5 mL, 18.5 mmol) were stirred under an atmosphere of nitrogen for 16 hours at room temperature. After which time, a solution of anhydrous sulfur trioxide pyridine complex ($SO_3$•Py) in DMF (0.4 mol/L, 3 mL) was added dropwise. The reaction mixture was stirred at room temperature for a further 6 hours, and 2 further aliquots of anhydrous $SO_3$•Py in DMF (0.4 mol/L, 1 mL) were added at the 1-hour and 2-hour timepoints. After 6 hours, full consumption of the reagents was indicated by TLC (1 : 1, EtOAc : Petroleum spirits (40-60°C)). The reaction mixture was quenched with 50 mL of saturated $NaHCO_3$ (aq) solution and extracted with EtOAc (3x30 mL). The organic extracts were combined and washed with brine and dried over anhydrous $MgSO_4$ and filtered. The solvent was removed under reduced pressure to give a residue, excess DMF was removed by trituration with cold n-hexane (10 mL, -77°C (acetone and dry ice)) to give the title compound as a colourless solid (20.7 mg, 28%). Rf = 0.8 (7:2:1, EtOAc : MeOH : $H_2O$); m.p. 198-202°C; $^1$H NMR (600 MHz, $CD_3OD$) δ 5.33 (d, *J* = 5.1 Hz, 1H), 4.23 (t, *J* = 9.1 Hz, 1H), 3.49 (m, 1H), 2.31–2.10 (m, 4H), 2.05–1.94 (m, 2H), 1.86 (m, 1H), 1.83–1.69 (m, 2H), 1.68–1.41 (m, 4H), 1.40–1.13 (m, 4H), 1.09 (s, 1H), 1.03 (s, 3H), 0.96 (m, 1H), 0.90 (s, 9H), 0.82 (s, 3H), 0.09–0.04 (m, 6H).; $^{13}$C NMR (151 MHz, $CD_3OD$) δ 246.51 (Si-CH3), 142.47, 122.17, 88.13, 74.09, 52.15, 51.80, 43.95, 43.73, 38.54, 37.84, 37.76, 33.18, 32.57, 29.14, 26.37, 24.42, 21.68, 19.89, 19.01, 11.99 4 peaks doubled, overlapping or obscured; LRMS (-ESI): *m/z* 483.2 (100%, $[M-Na]^-$), HRMS (+ESI) *m/z* calcd. for $[C_{25}H_{43}O_5SiS]^-$ ($[M-Na]^-$) 483.2600, found 483.2598.

Androst-5-ene-3β,17β-diol 17-sulfate, ammonium salt (11)



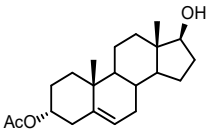

This procedure was adapted from Hungerford, McLeod et. al. (2006).[7] 3β-(*Tert*-butyldimethylsilyloxy)-androst-5-en-17β-ol 17-sulfate, sodium salt (5mg, 12.1 μmol) was added to a solution of aqueous acetic acid (2.00 mL, 14.3 mM) at stirred at room temperature for an hour. The reaction mixture was quenched by the addition of water (10 mL), the resulting solution was extracted using weak anion exchange SPE as follows. The 6cc WAX cartridge was first conditions by MeOH (6 mL), then by milliQ water (6 mL). the reaction mixture was then loaded and then washed with aqueous formic acid (6 mL, 3% v/v) followed by MilliQ water (6 mL). The product was eluted with ammonium hydroxide in MeOH (6 mL, 5 % v/v). The eluent was then concentrated under reduced pressure and reconstituted in MilliQ water (6 mL). This was then purified using the standard procedure for C18 SPE, to remove excess ammonium salts. This gave the title compound as a colourless solid (4.2 mg, 89 %). Rf = 0.7 (7:2:1, EtOAc : MeOH : $H_2O$); $^1$H NMR (600 MHz, $CD_3OD$) δ 5.34 (dd, *J* = 4.6, 2.7 Hz, 1H), 4.23 (dd, *J* = 9.1, 7.8 Hz, 1H), 3.40 (m, 1H), 2.30 – 2.11 (m, 4H), 2.00 (dtd, *J* = 12.5, 4.6, 2.7 Hz, 2H), 1.88 (dt, *J* = 13.2, 3.5 Hz, 1H), 1.83 – 1.70 (m, 2H), 1.67 – 1.42 (m, 6H), 1.33 (m, 1H), 1.20 (td, *J* = 12.9, 4.4 Hz, 1H), 1.11 (m, 1H), 1.04 (s, 3H), 0.97 (m, 1H), 0.82 (s, 3H): $^{13}$C NMR (151 MHz, $CD_3OD$) δ 142.35, 122.20, 88.12, 72.42, 52.18, 51.81, 43.74, 43.02, 38.55, 37.86, 37.77, 33.19, 32.57, 32.31, 29.16, 24.42, 21.70, 19.89, 11.99. LRMS (-ESI): *m/z* 369.2 (100%, $[M\text{-}NH_4]^-$), HRMS (+ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M\text{-}NH_4]^-$) 369.1732, found 369.1744.

3α-Acetoxyandrost-5-en-17-one (17)



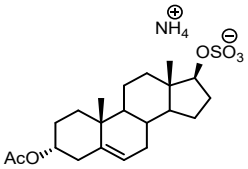

This compound was synthesised for the use in another unpublished work by Dr. Komba Thomas. Diisopropyl azodicarboxylate (785 mL, 4.0 mmol) was added dropwise to a solution of DHEA (1 g, 3.5 mmol), acetic acid (228 mL, 4.0 mmol), and $PPh_3$ (1.1 g, 4.0 mmol) in THF (20 mL) at 0 $^0$C under $N_2$. The resulting mixture was stirred at room temperature overnight, then solvated in $CH_2Cl_2$, washed with aqueous 2 M HCl, saturated $NaHCO_3$ and brine solutions, dried over anhydrous $Na_2SO_4$, and concentrated under reduced pressure. The crude residue was purified by silica gel flash column chromatography (hexane/EtOAc, 7:1) to give a white solid which was triturated from MeOH (633 mg, 55%). m.p. 166-168$^o$C; $[\alpha]_D$=22 (*c*=0.1 in $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): δ 5.30 (m, 1H), 4.99 (t, *J* = 3.0 Hz, 1H), 2.52 – 2.42 (m, 2H), 2.23 (dt, *J* = 15.3, 2.5 Hz, 1H), 2.15 – 2.03 (m, 2H), 2.01 (s, 3H), 1.94 (m, 1H), 1.85 (ddd, *J* = 12.8, 4.2, 2.8 Hz, 1H), 1.81 – 1.60 (m, 6H), 1.58 – 1.21 (m, 5H), 1.21 – 1.08 (m, 1H), 1.03 (s, 3H), 0.89 (s, 3H); $^{13}$C NMR (101 MHz, $CDCl_3$): δ 170.97, 138.88, 121.48, 70.53, 67.22, 51.95, 50.16, 47.69, 37.25, 36.39, 35.99, 33.57, 31.58, 31.55, 30.86, 26.23, 21.99, 21.57, 20.18, 19.07, 13.68; FTIR: $\upsilon_{max}$= 2940 (m, =C-H), 1723 (s, C=O), 1236 and 1018 (s, C-O) cm$^{-1}$; HRMS (EI): *m/z* calcd for $C_{19}H_{26}O$: 270.1978 [*M*-$AcOH_2$]$^+$; found: 270.1966.

3α-Acetoxyandrost-5-en-17β-ol (18)



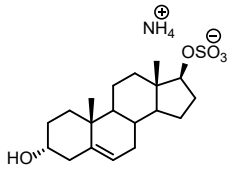

Procedure taken from Pranata, Fitzgerald, Mcleod, et.al. (2019).[5] 3α-Acetoxyandrost-5-en-17-one (10.00 mg, 30.03 μmol) was reacted with $NaBH_4$ (10 mg, 0.26 mmol) in MeOH (1 mL). The reaction was left stirring at room temperature for 2 hours. The mixture was then quenched with water (10 mL) and adjusted carefully to a pH of 7 with aqueous HCl solution. Following this the mixture was subjected to SPE purification according to general procedures 2 and 3. This yielded the title compound as a colourless solid (> 98% conversion). $^1$H NMR (700 MHz, $CDCl_3$) δ 5.27 (m, 1H), 4.99 (t, *J* = 3.0 Hz, 1H), 3.65 (t, *J* = 8.6 Hz, 1H), 2.48 (dp, *J* = 15.3, 3.2 Hz, 1H), 2.22 (dt, *J* = 15.3, 2.6 Hz, 1H), 2.08 (m, 1H), 2.01 (s, 3H), 2.00 – 1.96 (m, 1H), 1.87 – 1.83 (m, 1H), 1.80 – 1.72 (m, 2H), 1.66 – 1.57 (m, 3H), 1.53 – 1.37 (m, 4H), 1.31 – 1.25 (m, 2H), 1.13 – 1.06 (m, 2H), 1.03 (s, 3H), 1.02 – 0.96 (m, 1H), 0.77 (s, 3H); $^{13}$C NMR (176 MHz, $CDCl_3$): δ 171.01, 138.73, 121.93, 82.10, 70.69, 51.53, 50.21, 42.88, 37.23, 36.76, 36.45, 33.68, 32.04, 31.60, 30.70, 26.30, 23.58, 21.60, 20.52, 19.10, 11.11; LRMS (+ESI): *m/z* 355.2 (100%, $[C_{21}H_{32}O_3Na]^+$); HRMS (+ESI) *m/z* calcd. for $[C_{21}H_{32}O_3Na]^+$ ($[M+Na]^+$) 355.2249, found 355.2249.

3α-Acetoxyandrost-5-en-17β-ol 17-sulfate, ammonium salt (19)




3α-Acetoxyandrost-5-en-17β-ol (5.00 mg, 15.1 μmol) was reacted according to the general procedure 1 for small scale sulfation. This gave the title compound as a colourless solid (> 98% conversion).$^1$H NMR (700 MHz, $CD_3OD$) δ 5.28 (m, 1H), 4.95 (t, *J* = 3.1 Hz, 1H), 4.24 (t, *J* = 8.5 Hz, 1H), 2.54 (m, 1H), 2.23 – 2.14 (m, 2H), 2.01 (m, 1H), 1.99 (s, 3H), 1.86 – 1.28 (m, 14H), 1.21 (td, *J* = 13.1, 4.2 Hz, 1H), 1.06 (s, 3H), 0.83 (s, 3H); $^{13}$C NMR (176 MHz, $CD_3OD$): δ 172.69, 139.92, 122.85, 88.13, 72.31, 52.25, 51.66, 43.74, 38.24, 37.86, 37.27, 34.68, 33.07, 32.53, 29.15, 27.08, 24.40, 21.38, 21.22, 19.36, 12.00; LRMS (-ESI): *m/z* 411.2 (100%, $[C_{21}H_{31}O_6S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{21}H_{31}O_6S]^-$ ($[M]^-$) 411.1841, found 411.1843.

Androst-5-ene-3α,17β-diol 17-sulfate, ammonium salt (12)




3α-Acetoxyandrost-5-en-17β-ol 17-sulfate, ammonium salt (3 mg, 7.04 μmol) was added to a stirring solution of MeOH (1 mL) and aqueous sodium hydroxide solution (2M, 0.5 mL), and left over night at room temperature. After which time the reaction was quenched with critic acid (2 mL, 5% v/v) and extracted with ethyl acetate (3 x 10 mL), washed with brine (5 mL), and dried over anhydrous magnesium sulfate. The organic fraction was then concentrated under reduced pressure and reconsistuted with 10 mL of MilliQ water. It was then purified by SPE as per general procedures 2 and 3. This gave the title compound as its ammonium salt with a >98% conversion.$^{1}$H NMR (700 MHz, $CD_3OD$) δ 5.31 (m, 1H), 4.23 (t, *J* = 8.6 Hz, 1H), 3.96 (m, 1H), 2.52 (d, *J* = 14.9 Hz, 1H), 2.18 (m, 1H), 2.07 (d, *J* = 14.9 Hz, 1H), 2.02 – 1.96 (m, 3H), 1.81 – 1.71 (m, 3H), 1.70 – 1.57 (m, 3H), 1.57 – 1.42 (m, 3H), 1.37 – 1.28 (m, 2H), 1.20 (td, *J* = 13.2, 4.2 Hz, 1H), 1.10 (td, *J* = 11.8, 4.7 Hz, 1H), 1.08 – 1.03 (m, 1H), 1.04 (s, 3H), 0.83 (s, 3H); $^{13}$C NMR (176 MHz, $CD_3OD$): 140.30, 123.05, 88.18, 67.94, 52.24, 51.60, 43.74, 40.32, 38.37, 37.90, 33.98, 33.14, 32.63, 29.62, 29.17, 24.42, 21.38, 19.35, 12.01 ; LRMS (-ESI): *m/z* 369.1 (100%, $[C_{19}H_{29}O_5S]^-$); HRMS (-ESI) *m/z* calcd. for $[C_{19}H_{29}O_5S]^-$ ($[M]^-$) 369.1736, found 369.1742.

# S6 References


(1) Fitzgerald, C. C. J.; Hedman, R.; Uduwela, D. R.; Paszerbovics, B.; Carroll, A. J.; Neeman, T.; Cawley, A.; Brooker, L.; McLeod, M. D. Profiling Urinary Sulfate Metabolites With Mass Spectrometry. *Front. Mol. Biosci.* **2022**, *9*, 829511. https://doi.org/10.3389/fmolb.2022.829511.

(2) McKinney, A. R.; Cawley, A. T.; Young, E. B.; Kerwick, C. M.; Cunnington, K.; Stewart, R. T.; Ambrus, J. I.; Willis, A. C.; McLeod, M. D. The Metabolism of Anabolic-Androgenic Steroids in the Greyhound. *Bioanalysis* **2013**, *5* (7), 769–781. https://doi.org/10.4155/bio.13.40.

(3) Waller, C. C.; McLeod, M. D. A Simple Method for the Small Scale Synthesis and Solid-Phase Extraction Purification of Steroid Sulfates. *Steroids* **2014**, *92*, 74–80. https://doi.org/10.1016/j.steroids.2014.09.006.

(4) Ma, P.; Kanizaj, N.; Chan, S.-A.; Ollis, D. L.; McLeod, M. D. The Escherichia Coli Glucuronylsynthase Promoted Synthesis of Steroid Glucuronides: Improved Practicality and Broader Scope. *Org. Biomol. Chem.* **2014**, *12* (32), 6208–6214. https://doi.org/10.1039/C4OB00984C.

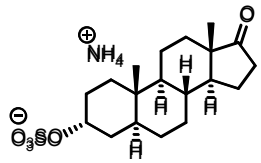
(5) Pranata, A.; Fitzgerald, C. C.; Khymenets, O.; Westley, E.; Anderson, N. J.; Ma, P.; Pozo, O. J.; McLeod, M. D. Synthesis of Steroid Bisglucuronide and Sulfate Glucuronide Reference Materials: Unearthing Neglected Treasures of Steroid Metabolism. *Steroids* **2019**, *143*, 25–40. https://doi.org/10.1016/j.steroids.2018.11.017.

(6) Hosoda, H.; Fukushima, D. K.; Fishman, J. Convenient, High Yeild Conversion of Androst-5-Ene-3β,17β-Diol to Dehydroisoandrosterone. *J. Org. Chem.* **1973**, *38* (24), 4209–4211.

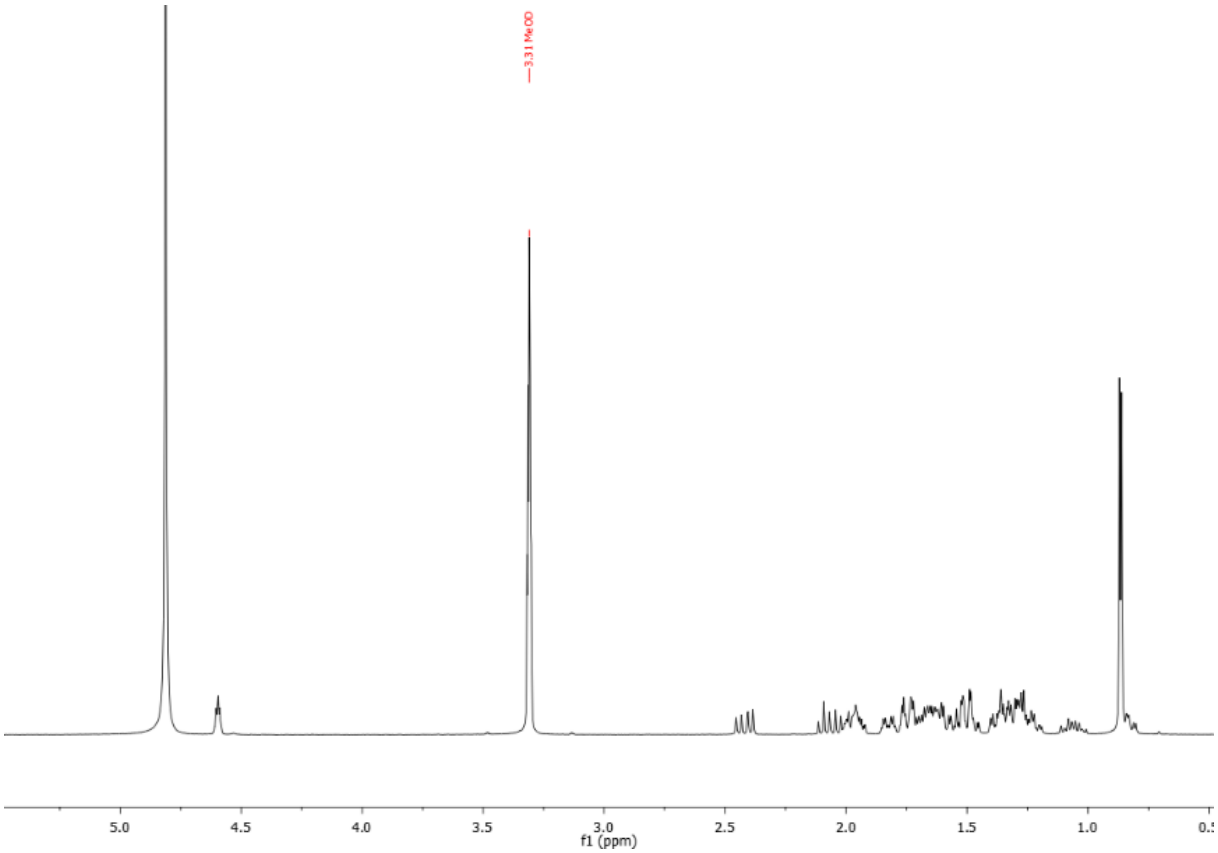
(7) Hungerford, N. L.; McKinney, A. R.; Stenhouse, A. M.; McLeod, M. D. Selective Manipulation of Steroid Hydroxyl Groups with Boronate Esters: Efficient Access to Antigenic C-3 Linked Steroid–Protein Conjugates and Steroid Sulfate Standards for Drug Detection. *Org. Biomol. Chem.* **2006**, *4* (21), 3951–3959. https://doi.org/10.1039/B610499A.

# S7 Spectroscopic data for synthesised compounds

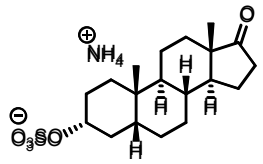
Androsterone 3-sulfate, ammonium salt (1)



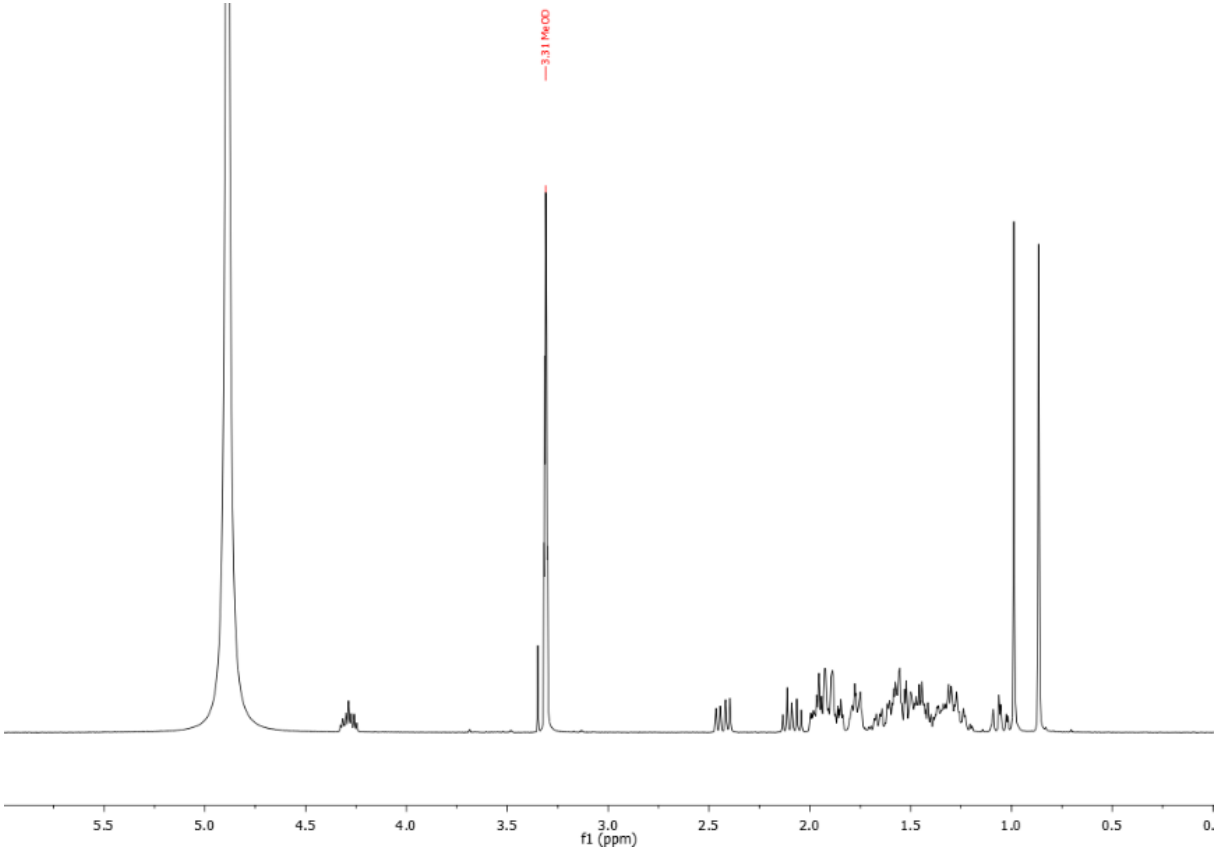
$^{1}$H-NMR (400MHz) $CD_3OD$



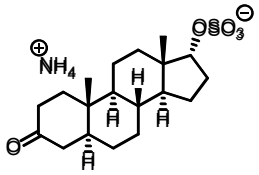
Etiocholanolone 3-sulfate, ammonium salt (2)



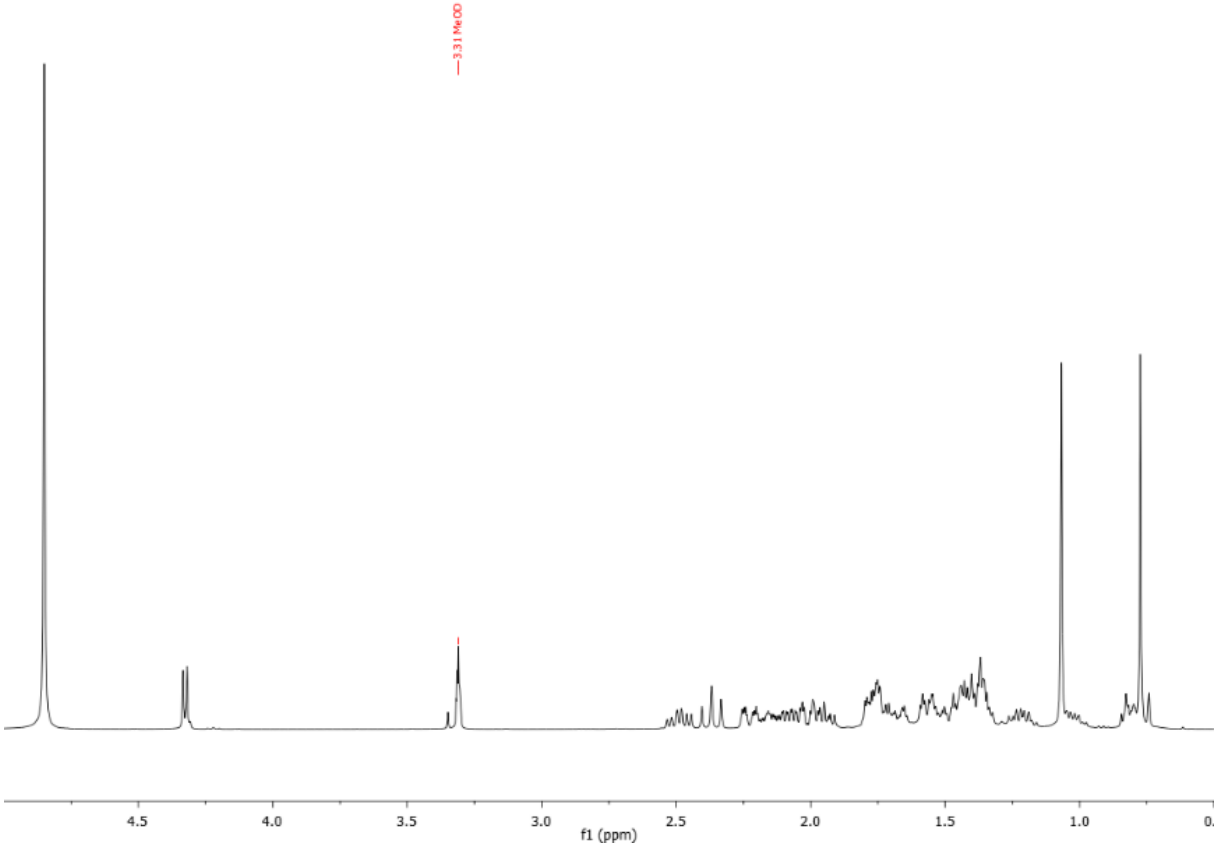
$^{1}$H-NMR (400MHz) $CD_3OD$



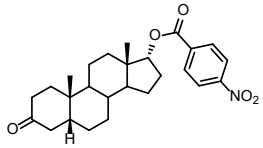
Epidihydrotestosterone 3-sulfate, ammonium salt (3)



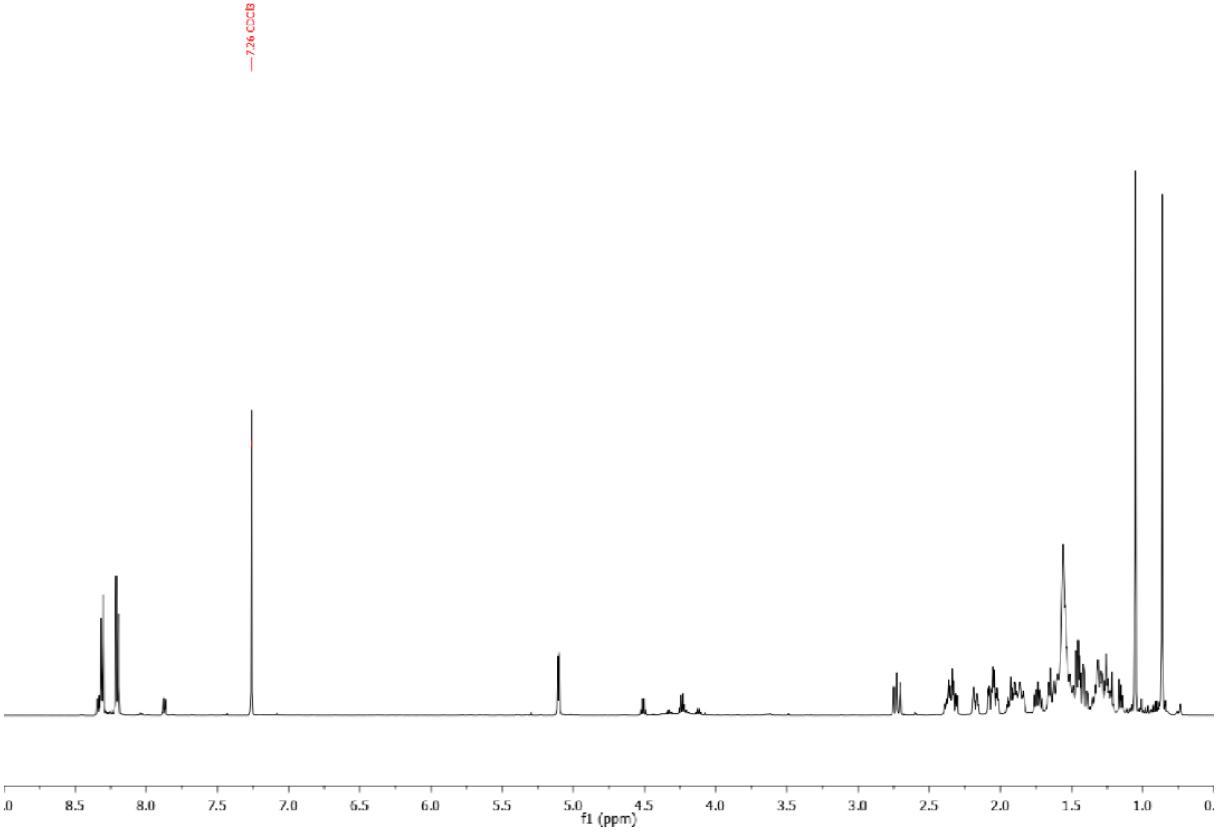
$^{1}$H-NMR (400MHz) $CD_3OD$



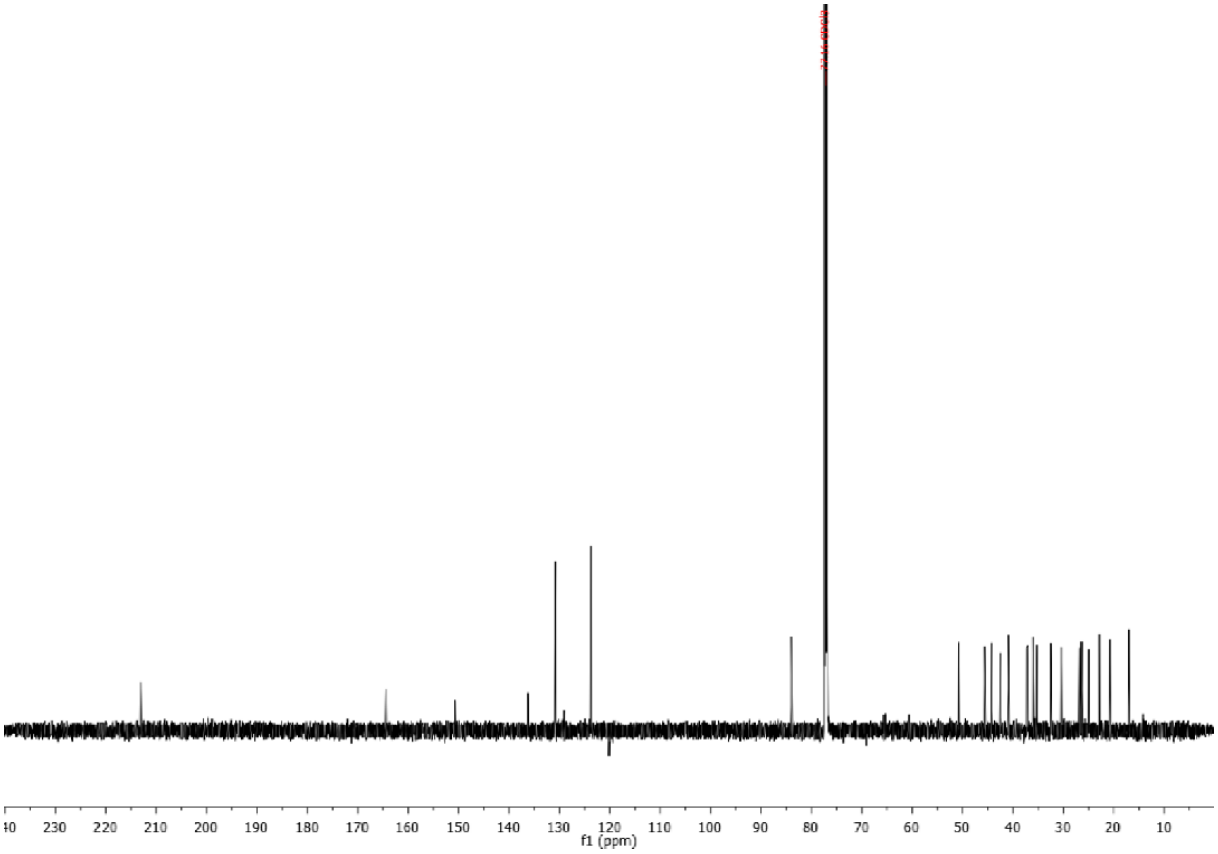
17α-[(4-Nitrobenzoyl)oxy]-5β-androstan-3-one (20)



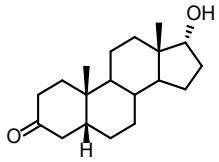

$^{1}$H-NMR (600MHz) $CDCl_3$



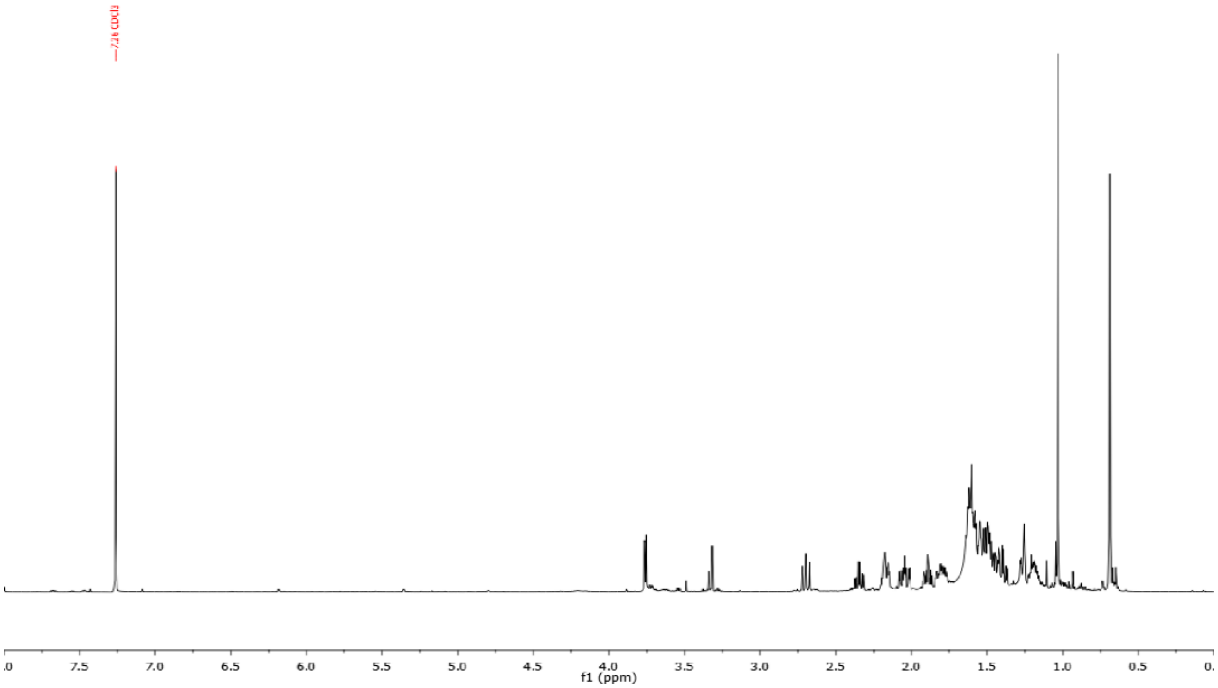
$^{13}$C-NMR (151 MHz) $CDCl_3$



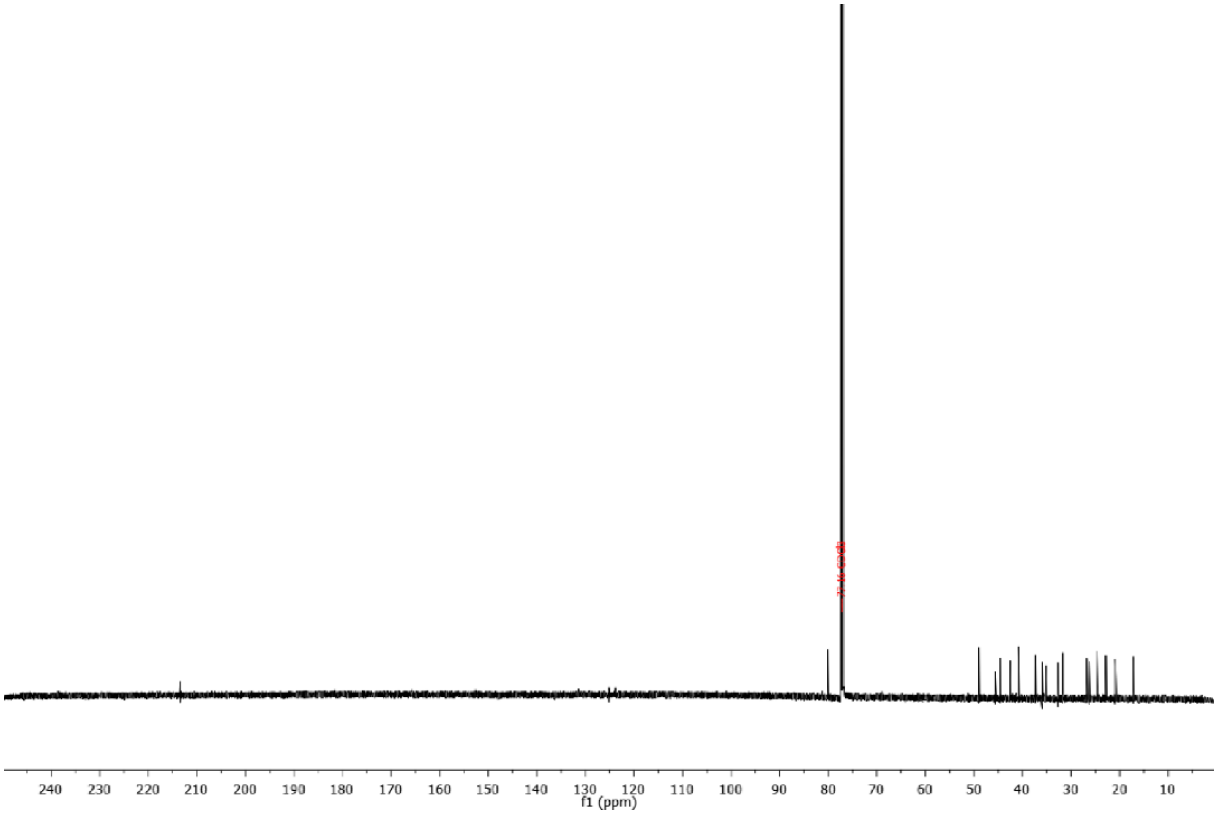
17α-Hydroxy-5β-androstan-3-one (21)



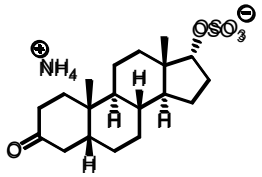

$^{1}$H-NMR (600MHz) $CDCl_3$



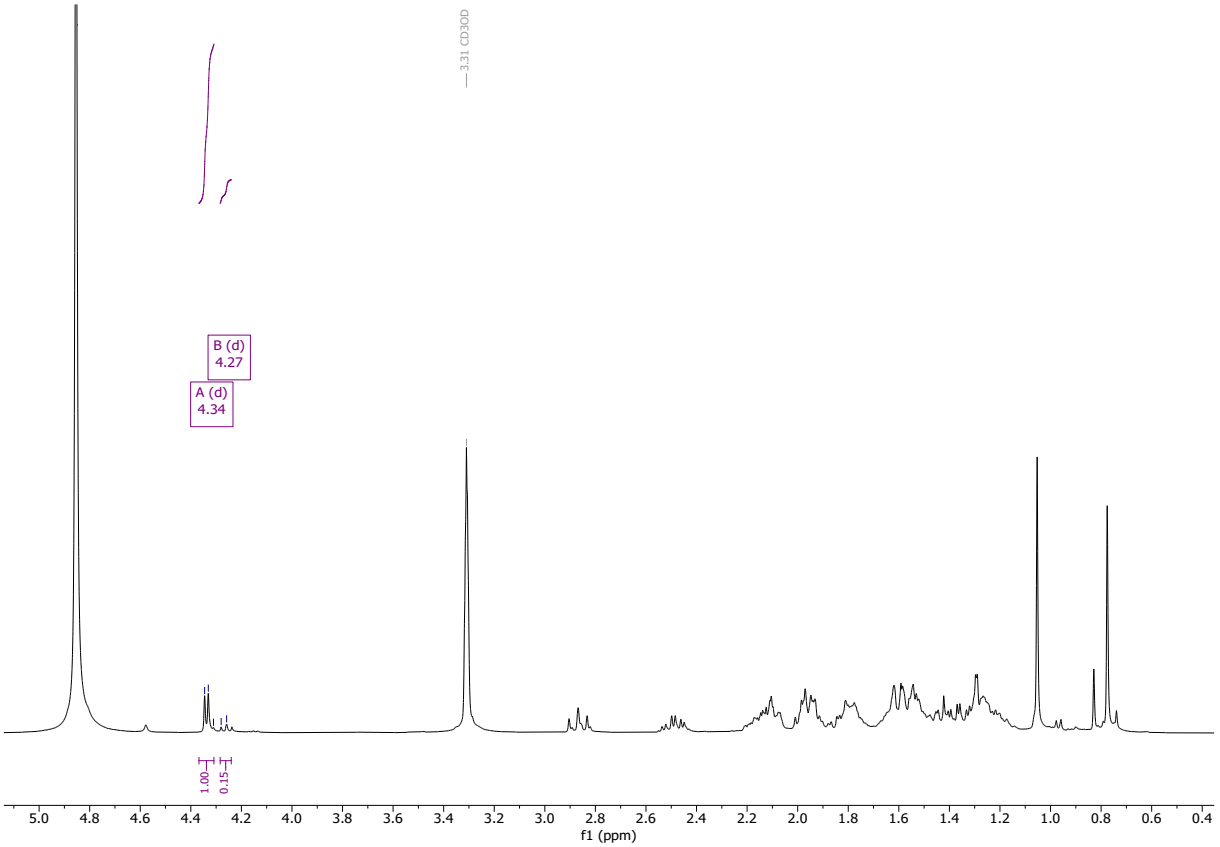
$^{13}$C-NMR (151 MHz) $CDCl_3$



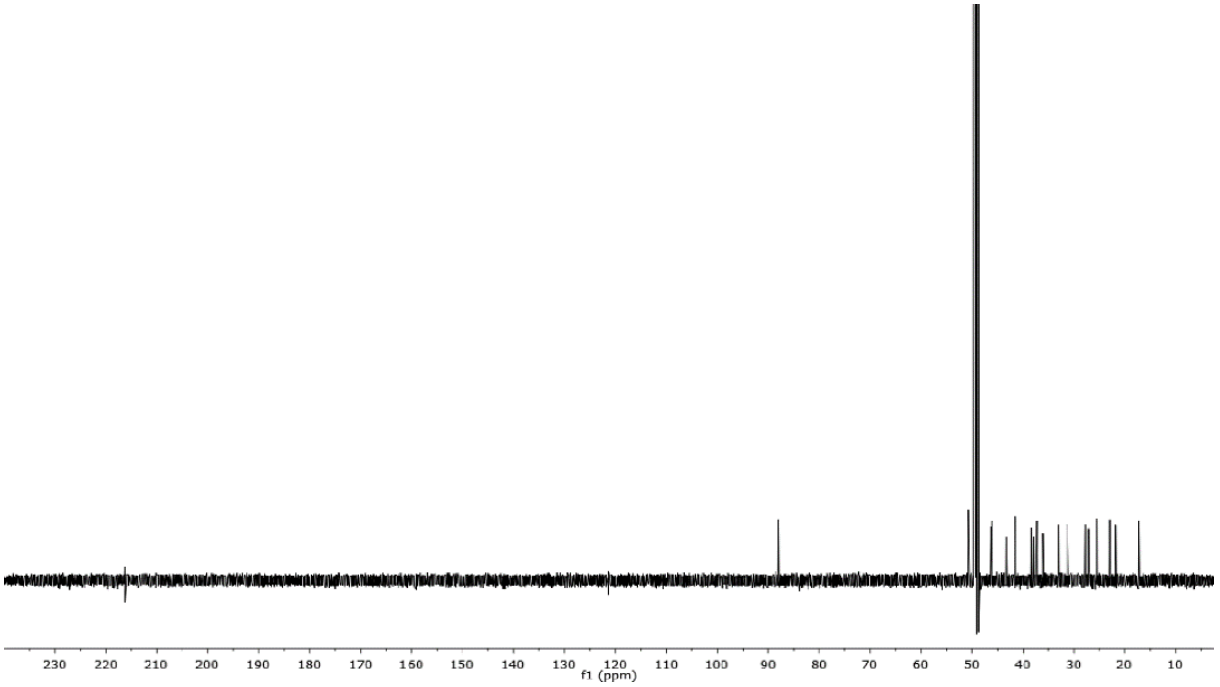
17α-Hydroxy-5β-androstane-3-one 17-sulfate, ammonium salt (4)



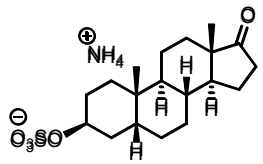

$^{1}$H-NMR (700MHz) $CD_3OD$



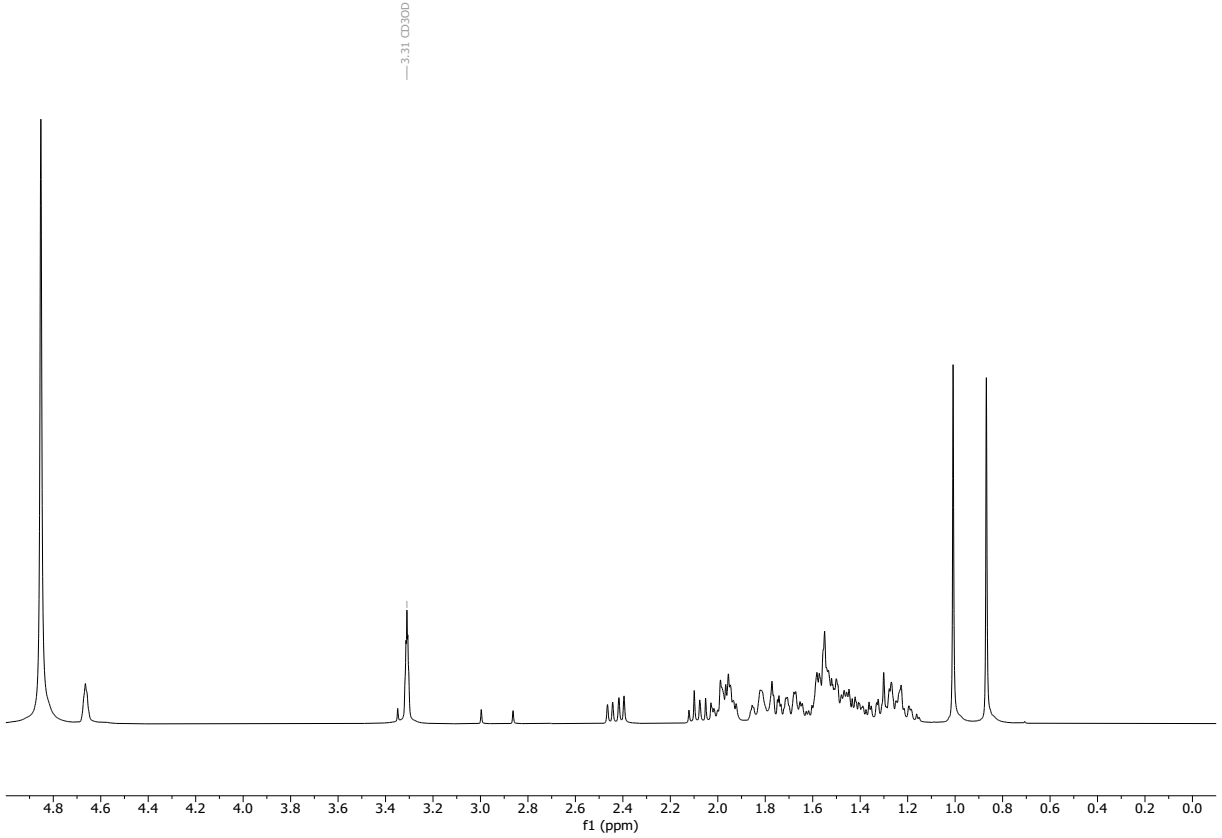
$^{13}$C-NMR (151 MHz) $CD_3OD$



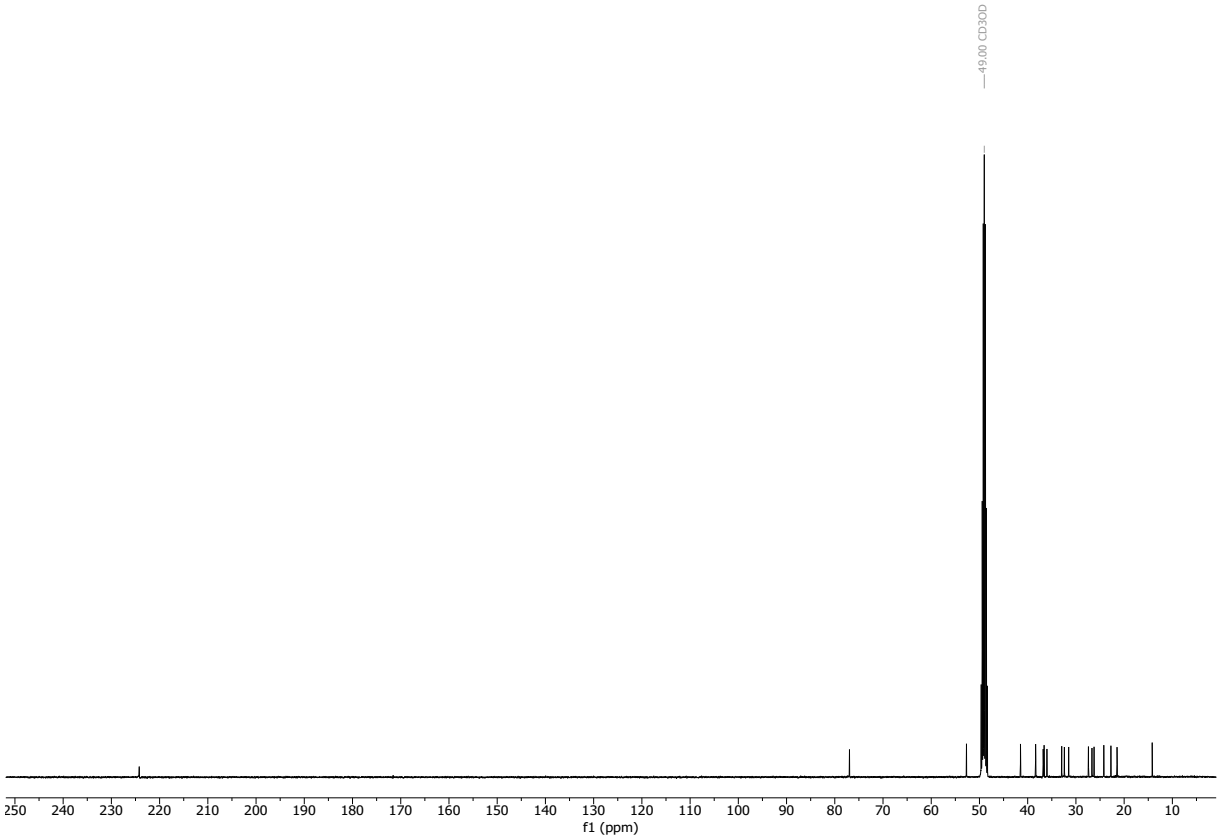
Epietiocholanolone 3-sulfate, ammonium salt (5)



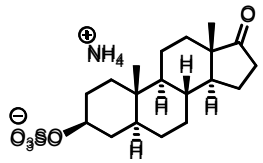
$^{1}$H-NMR (400MHz) $CD_3OD$



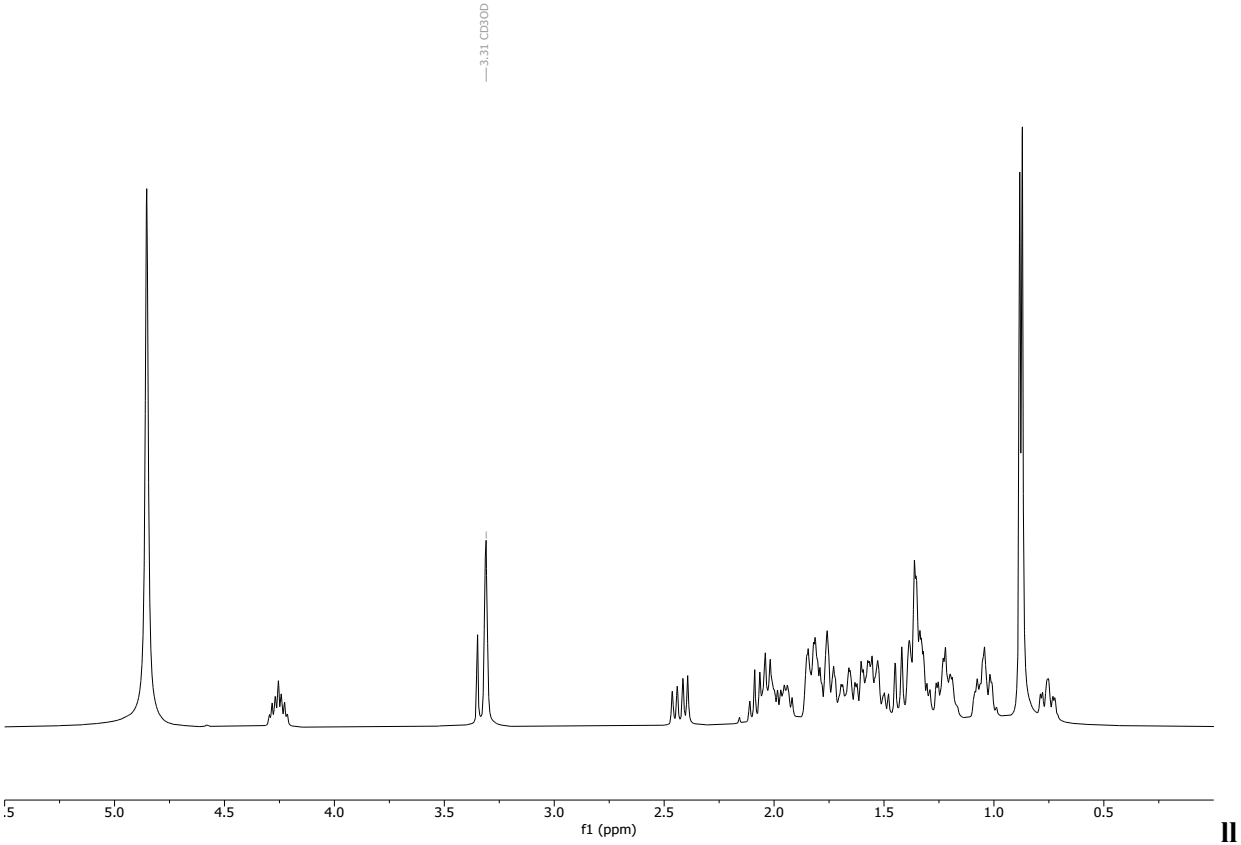
$^{13}$C-NMR (176 MHz) $CD_3OD$



Epiandrosterone 3-sulfate, ammonium salt (6)

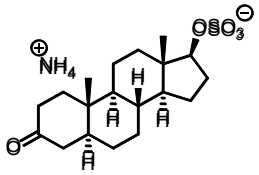



$^{1}$H-NMR (400MHz) $CD_3OD$

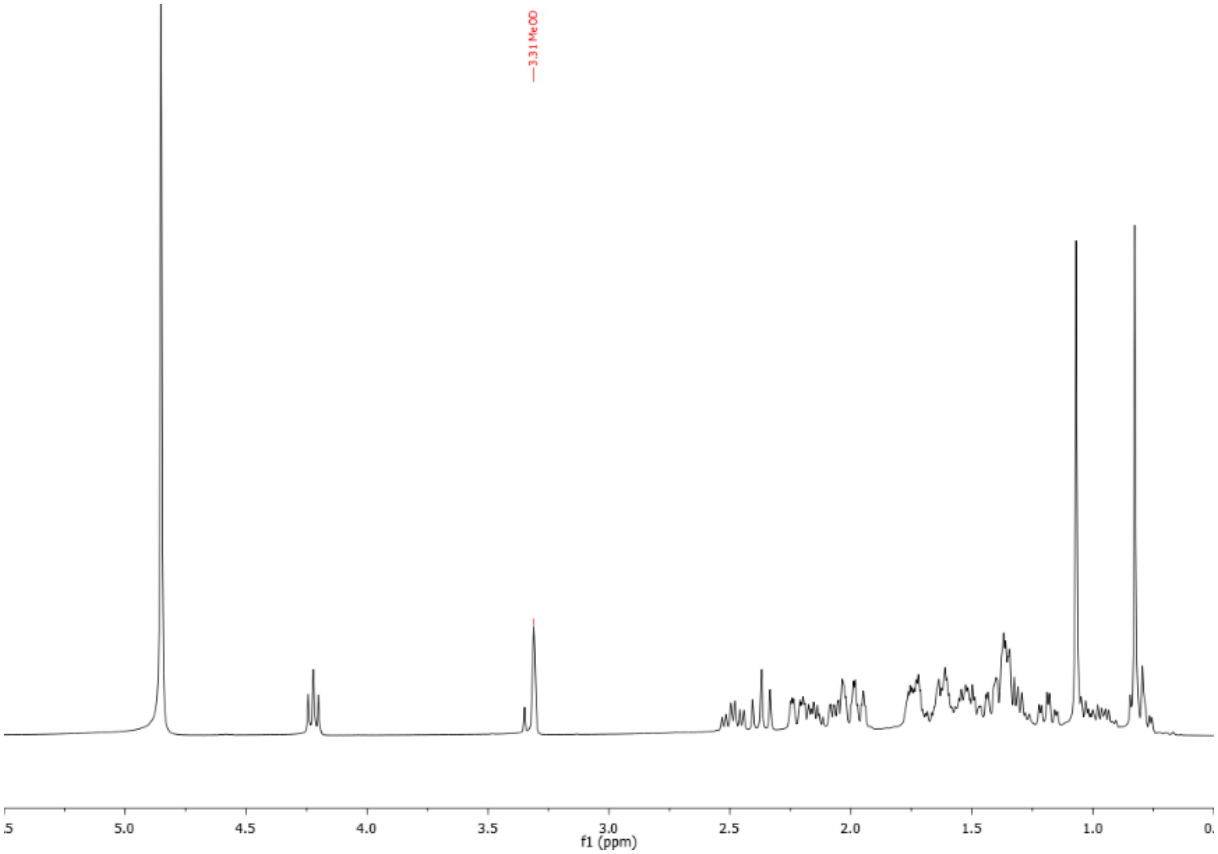



II

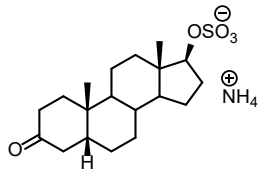
Dihydrotestosterone 3-sulfate, ammonium salt (7)



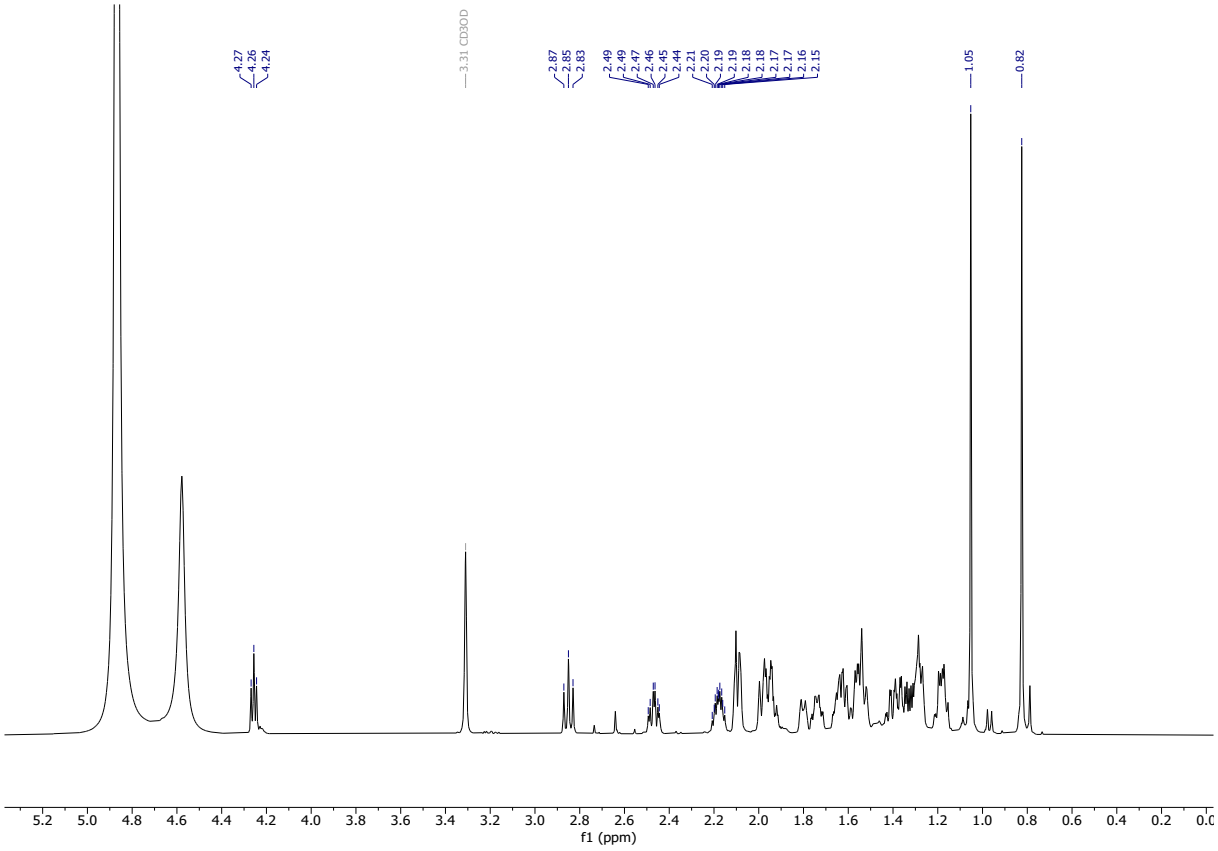
$^{1}$H-NMR (400MHz) $CD_3OD$



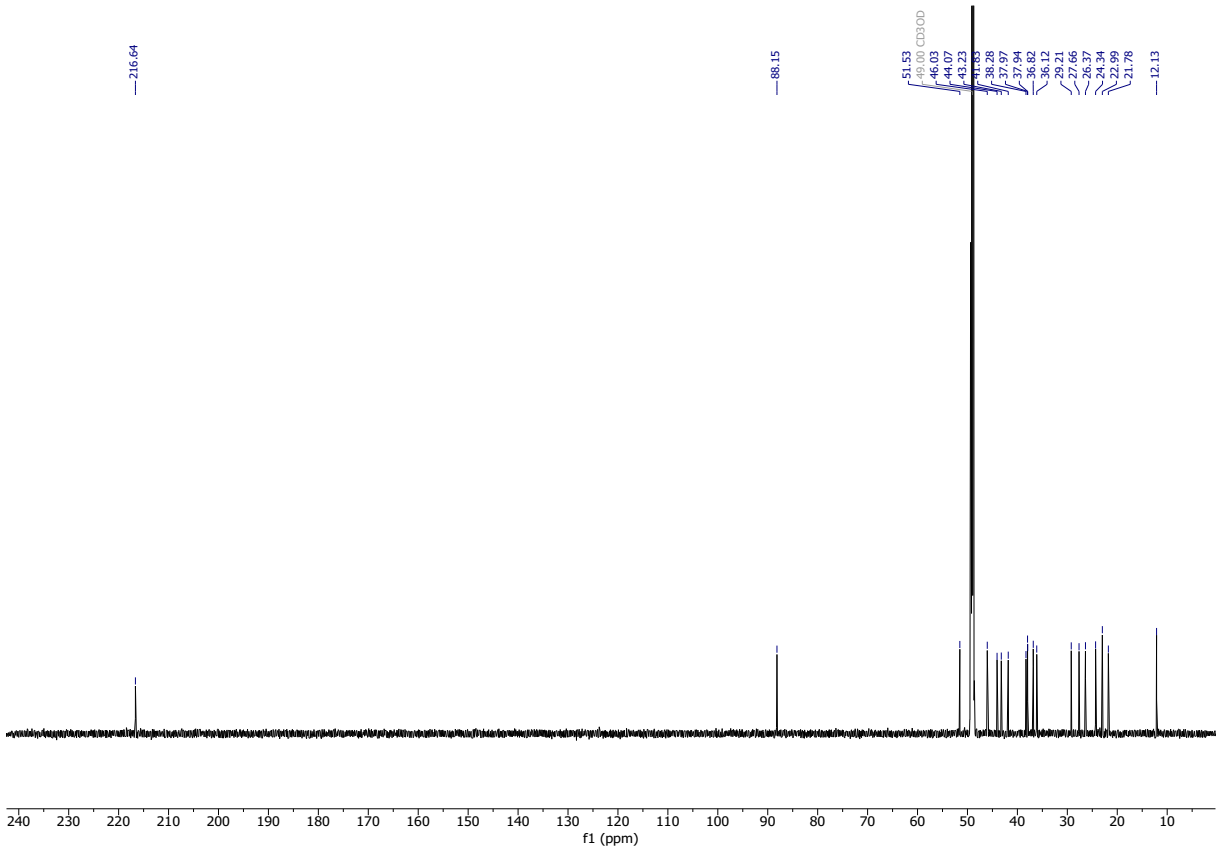
17β-Hydroxy-5β-androstan-3β-one 17-sulfate, ammonium salt (8)



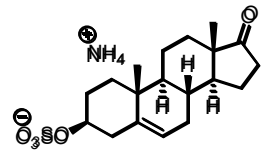

$^{1}$H-NMR (400MHz) $CD_3OD$



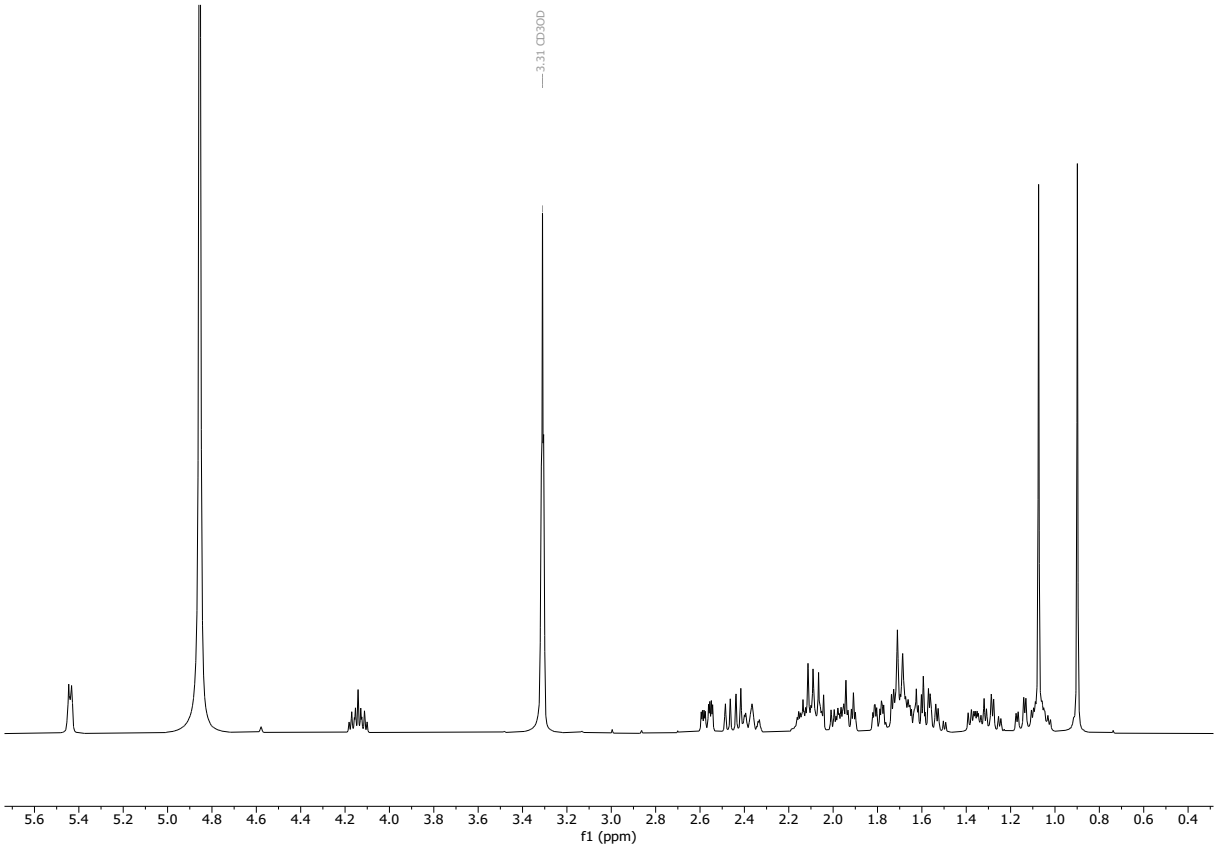
$^{13}$C-NMR (101 MHz) $CD_3OD$



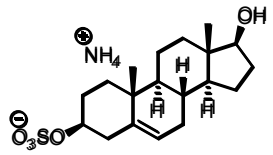
Dehydroepiandrosterone 3-sulfate, ammonium salt (22)



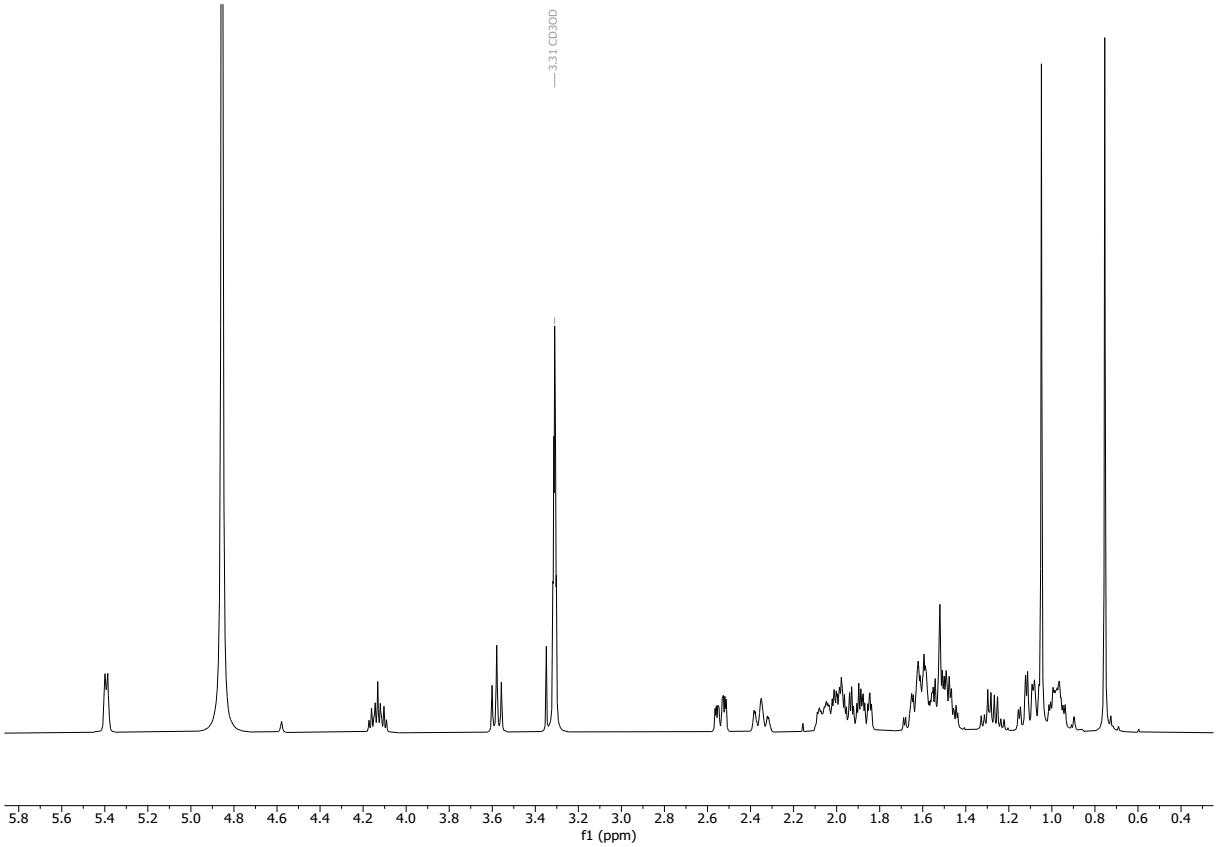
$^{1}$H-NMR (400MHz) $CD_3OD$



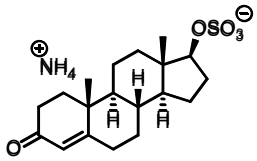
Androst-5-ene-3β,17β-diol 3-sulfate, ammonium salt, ammonium salt (9)



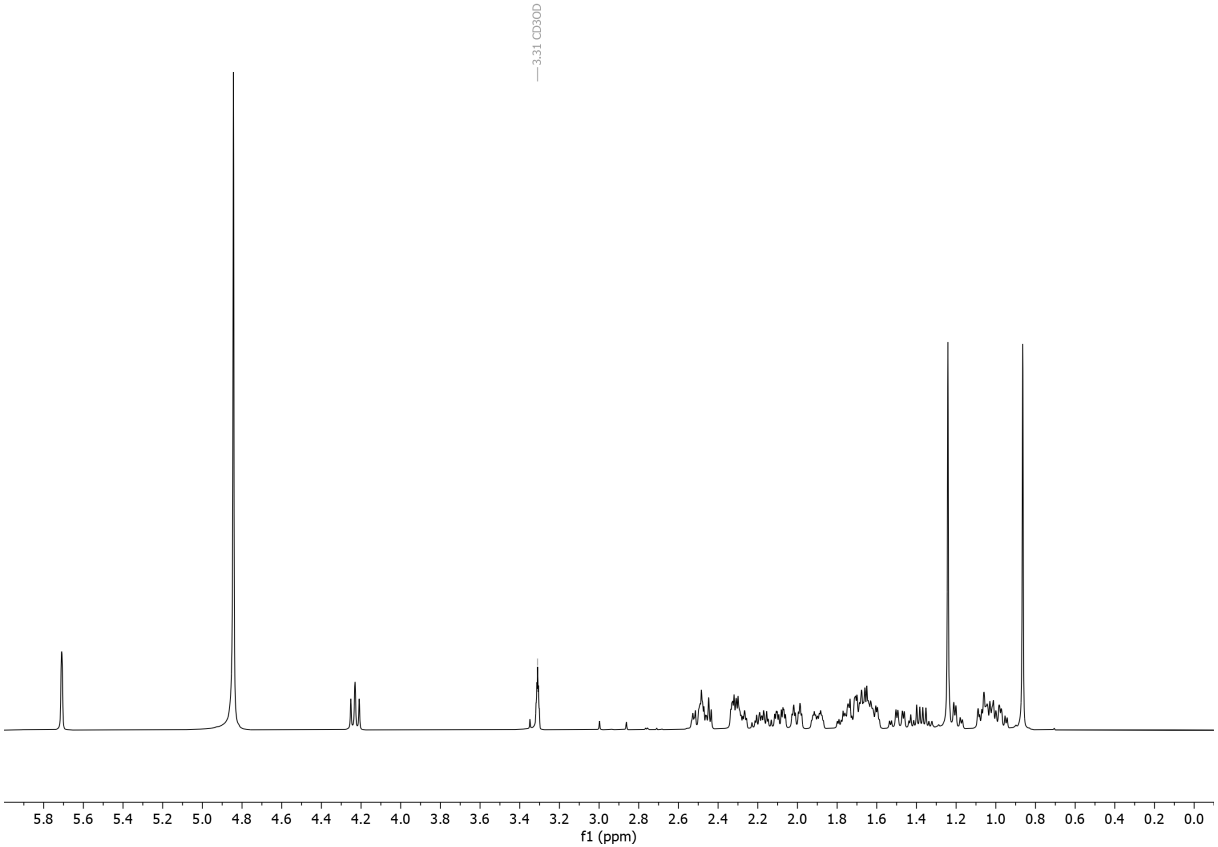

$^{1}$H-NMR (400MHz) $CD_3OD$



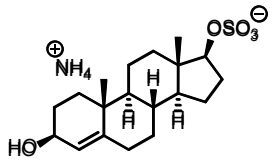
Testosterone sulfate, ammonium salt (13)



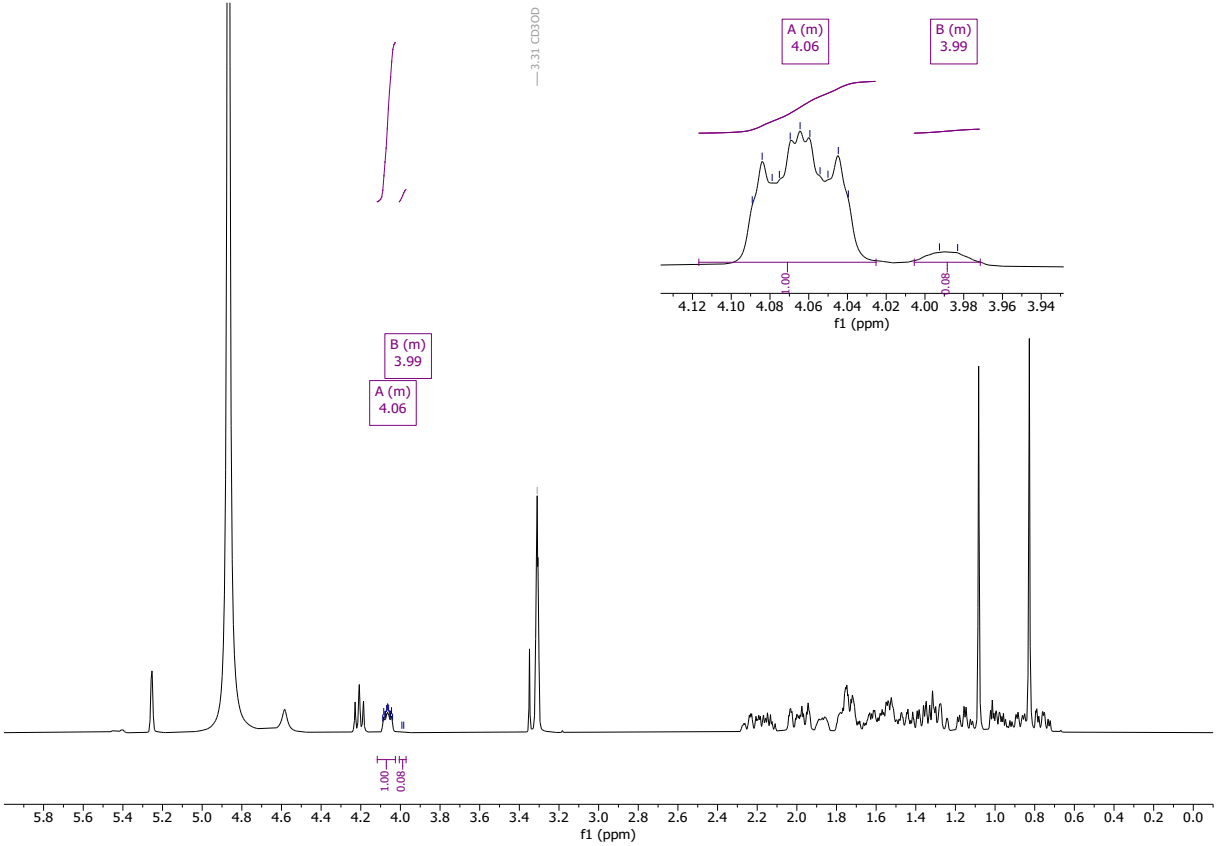

$^{1}$H-NMR (400MHz) $CD_3OD$



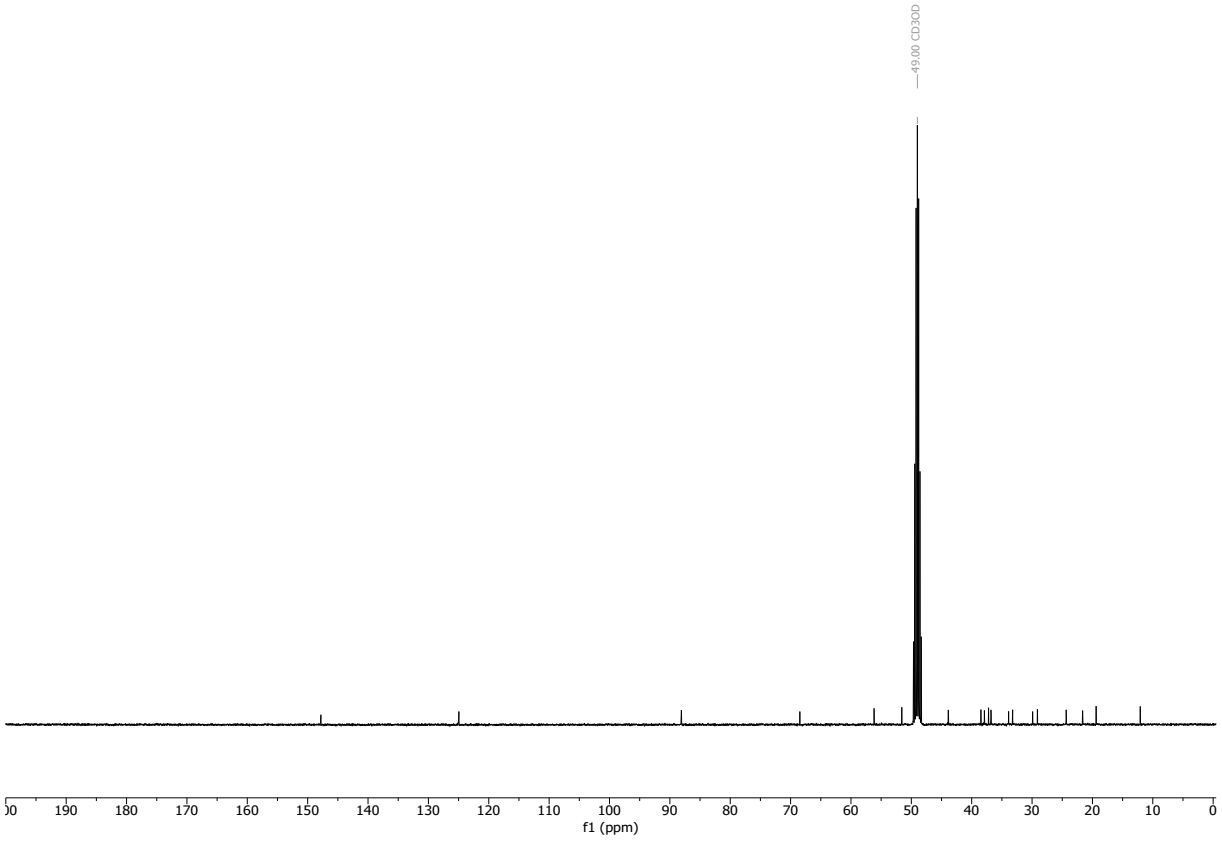
Androst-4-ene-3β,17β-diol 17-sulfate, ammonium salt (10)



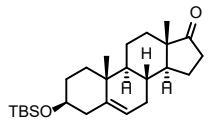

$^{1}$H-NMR (400MHz) $CD_3OD$



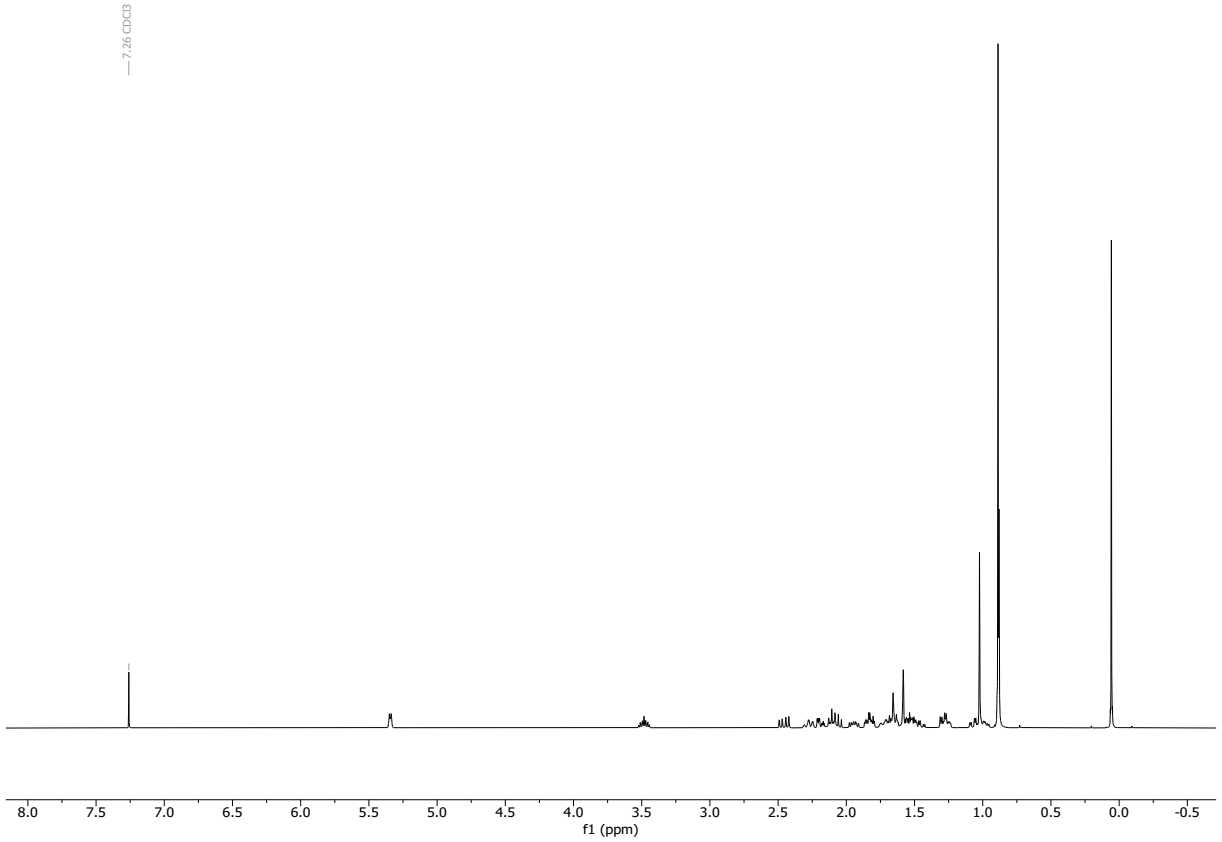
$^{13}$C-NMR (101 MHz) $CD_3OD$



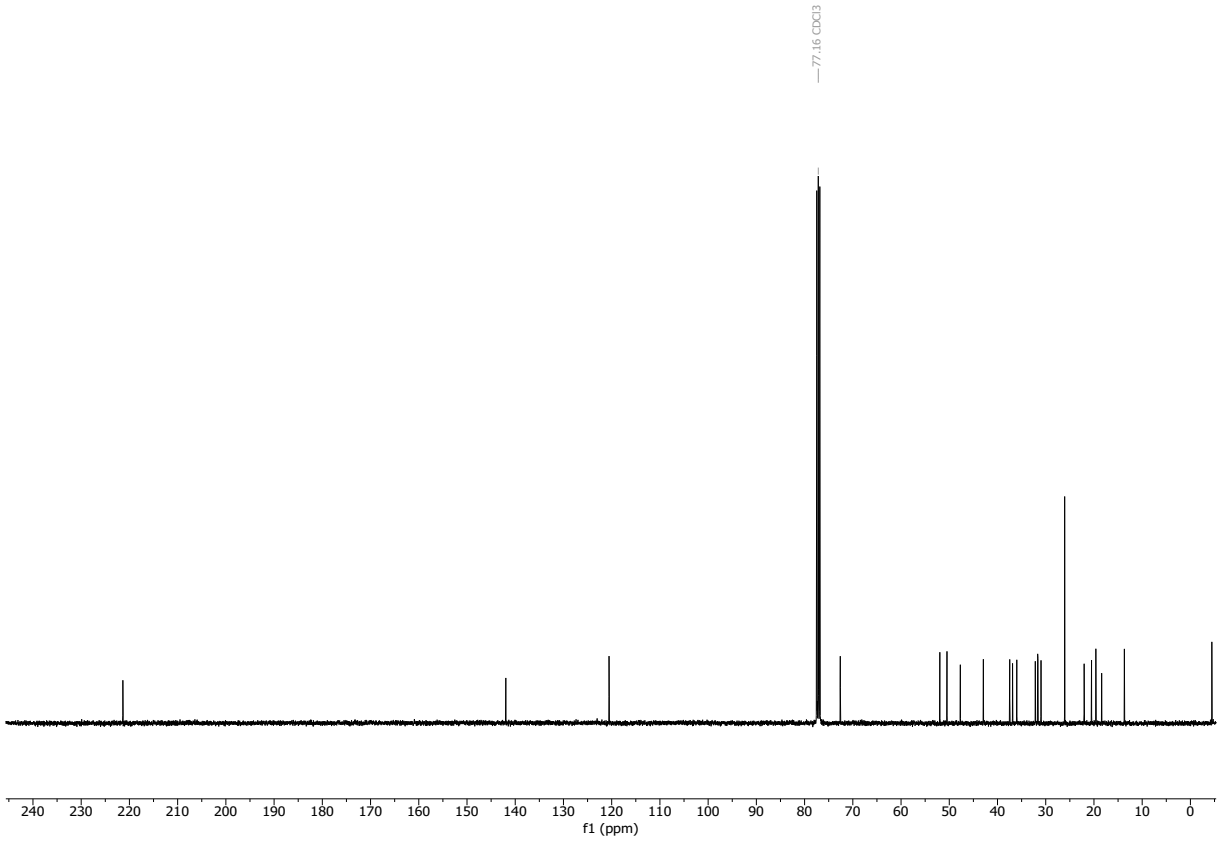
3β-(*Tert*-butyldimethylsilyloxy)-androst-5-en-17-one (14)



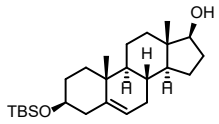

$^{1}$H-NMR (700MHz) $CDCl_3$



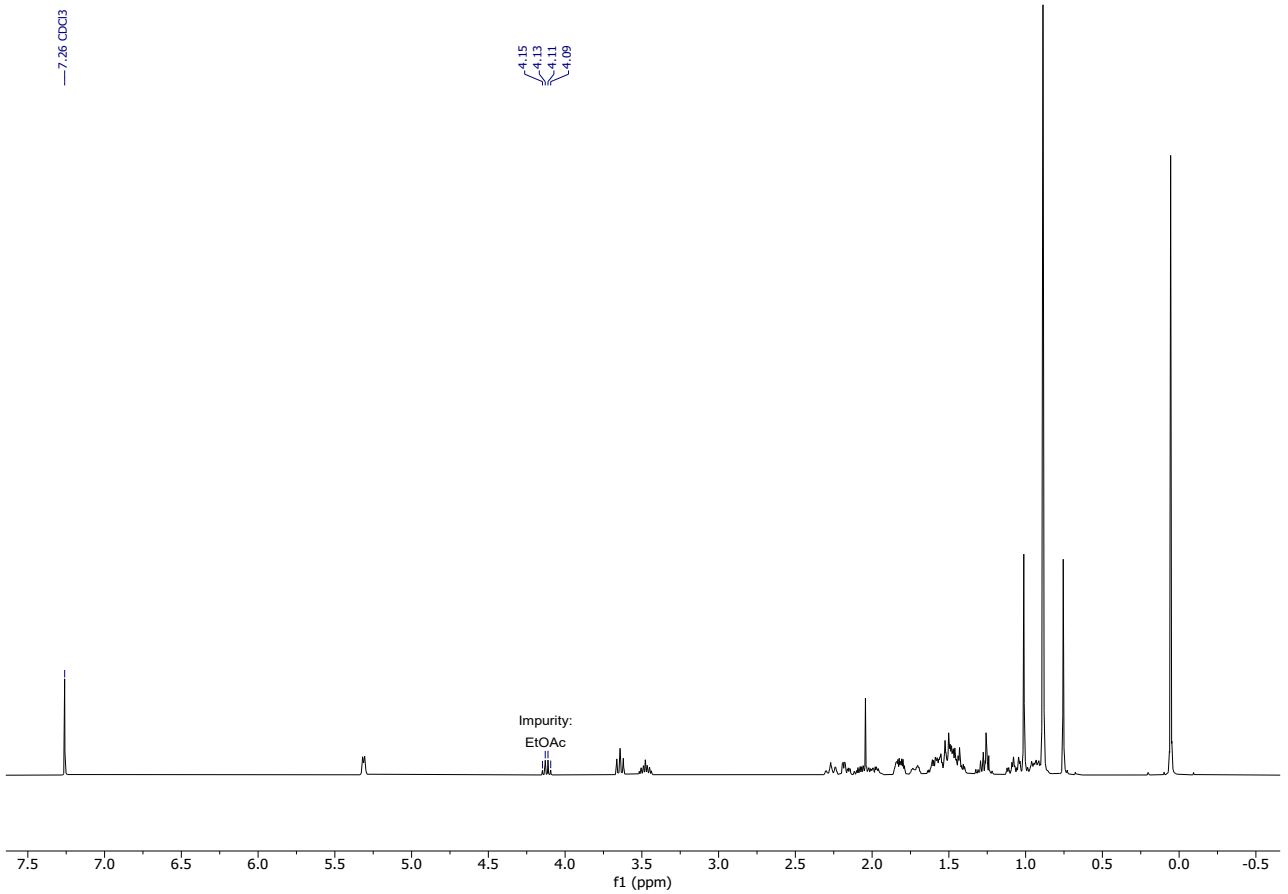
$^{13}$C-NMR (176 MHz) $CDCl_3$



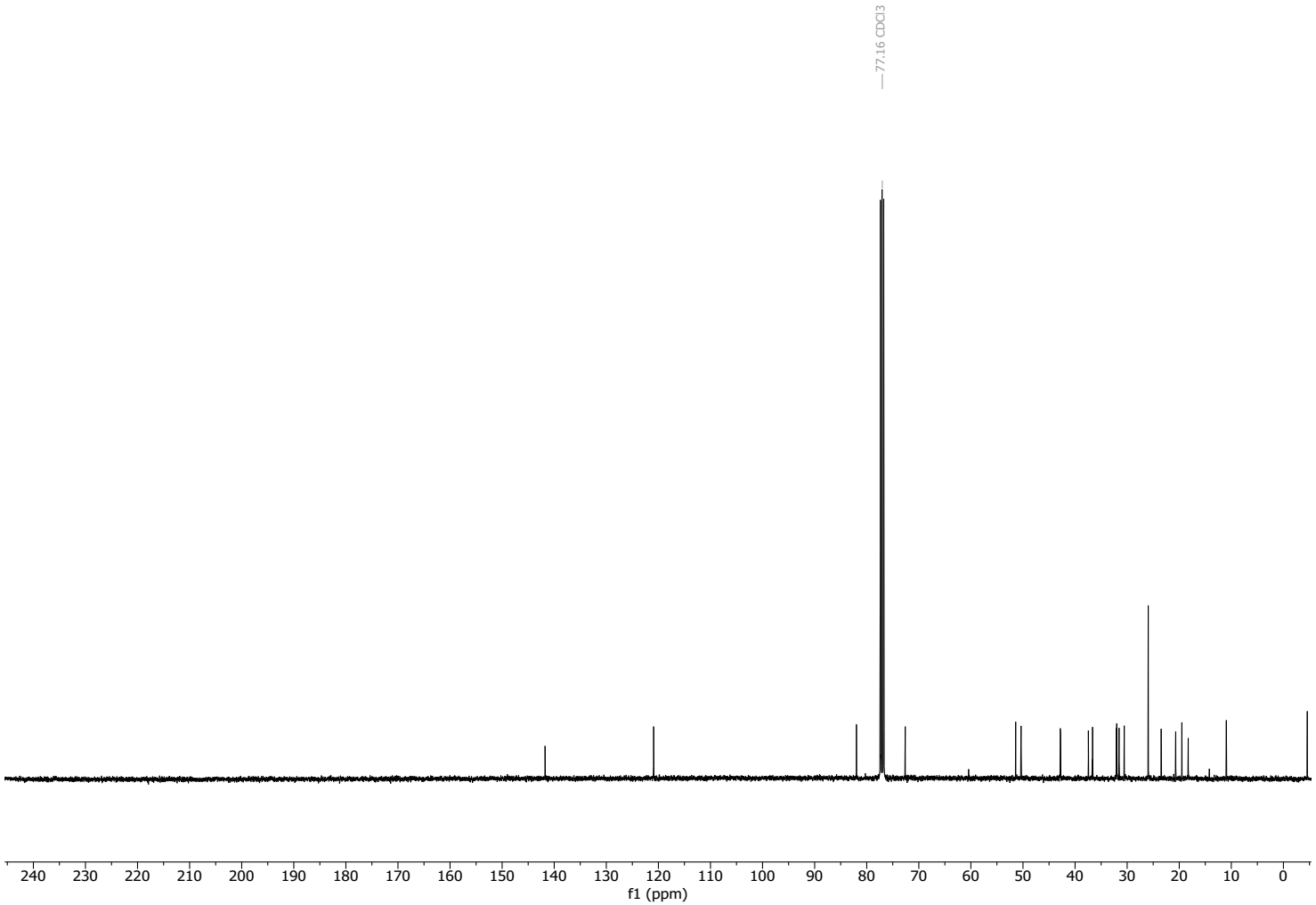
3β-(*Tert*-butyldimethylsilyloxy)-androst-5-en-17β-ol (15)



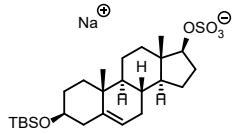

$^{1}$H-NMR (700MHz) $CDCl_3$



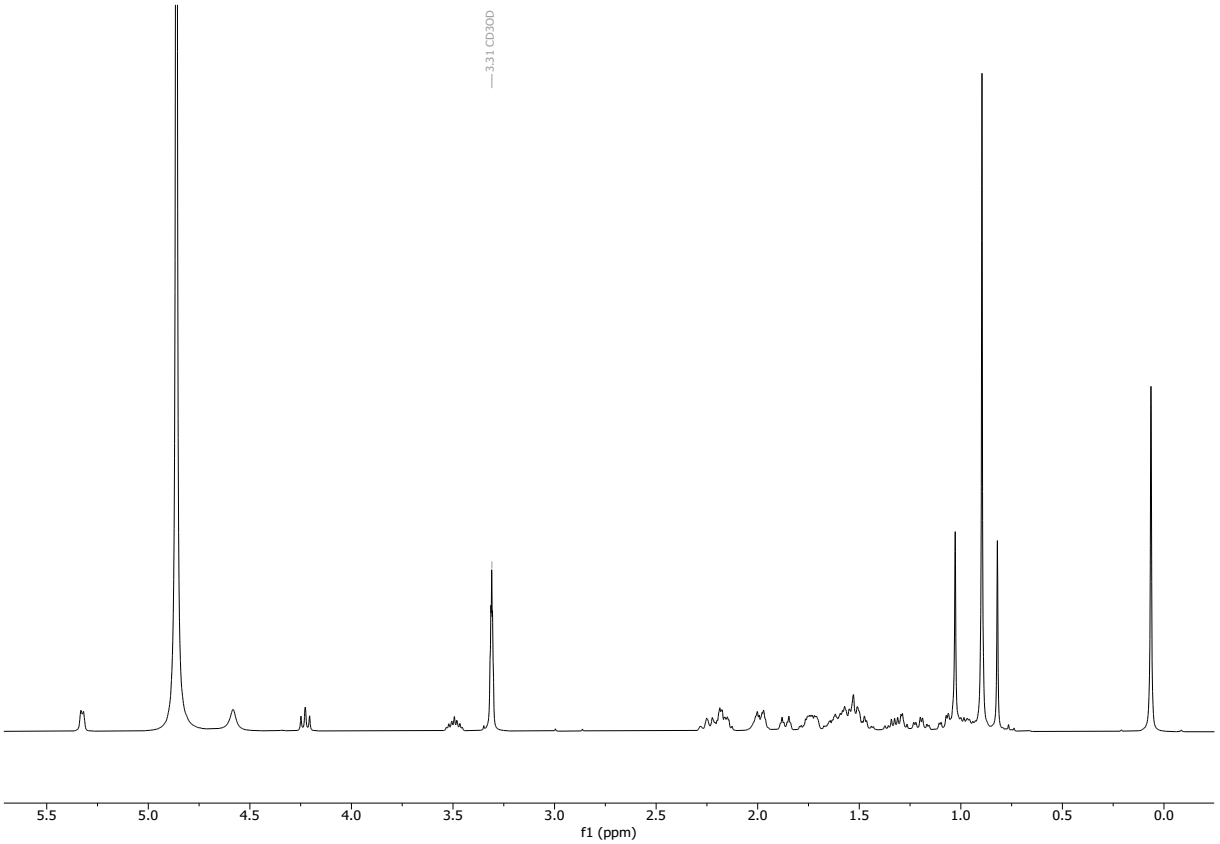
$^{13}$C-NMR (176 MHz) $CDCl_3$



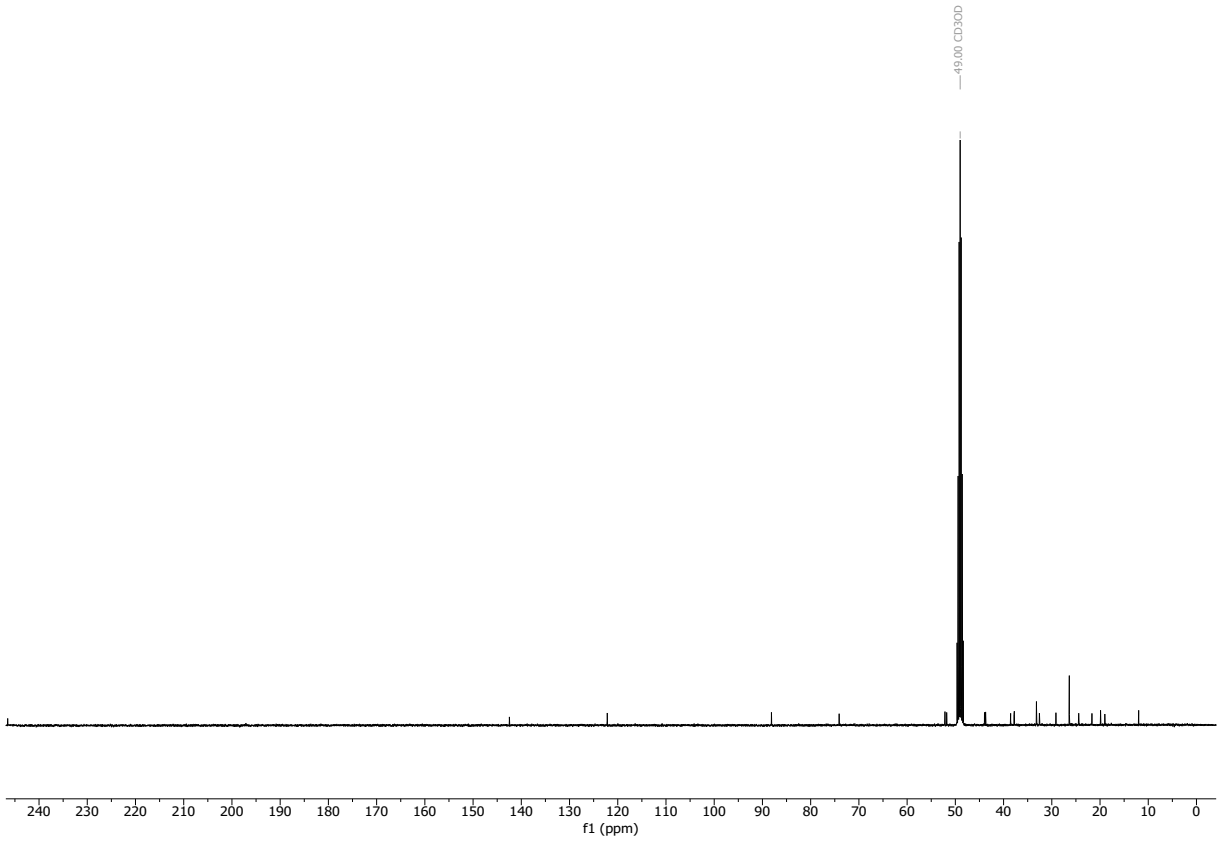
3β-(*Tert*-butyldimethylsilyloxy)-androst-5-en-17β-ol 17-sulfate, sodium salt (16)



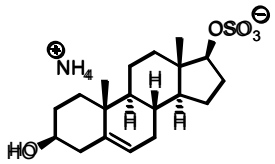

$^{1}$H-NMR (600MHz) $CD_3OD$



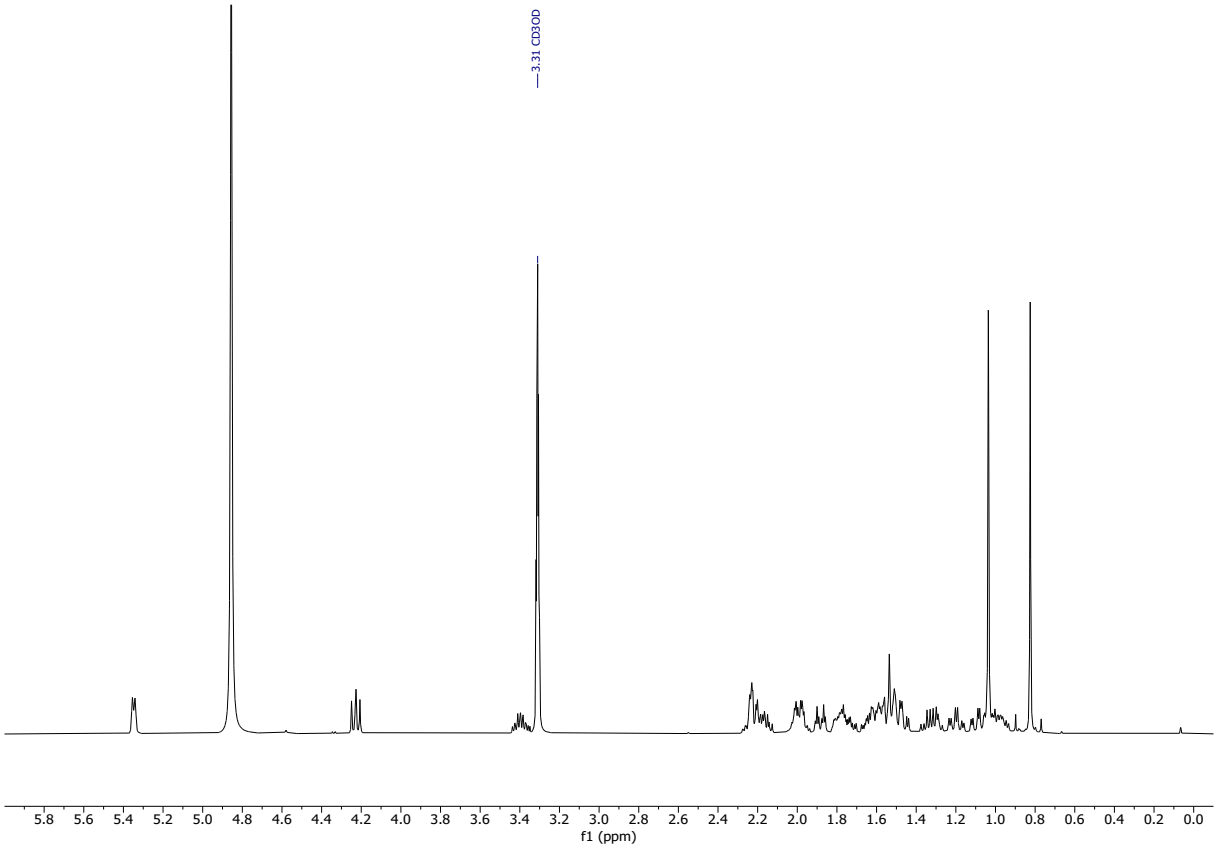
$^{13}$C-NMR (151 MHz) $CD_3OD$



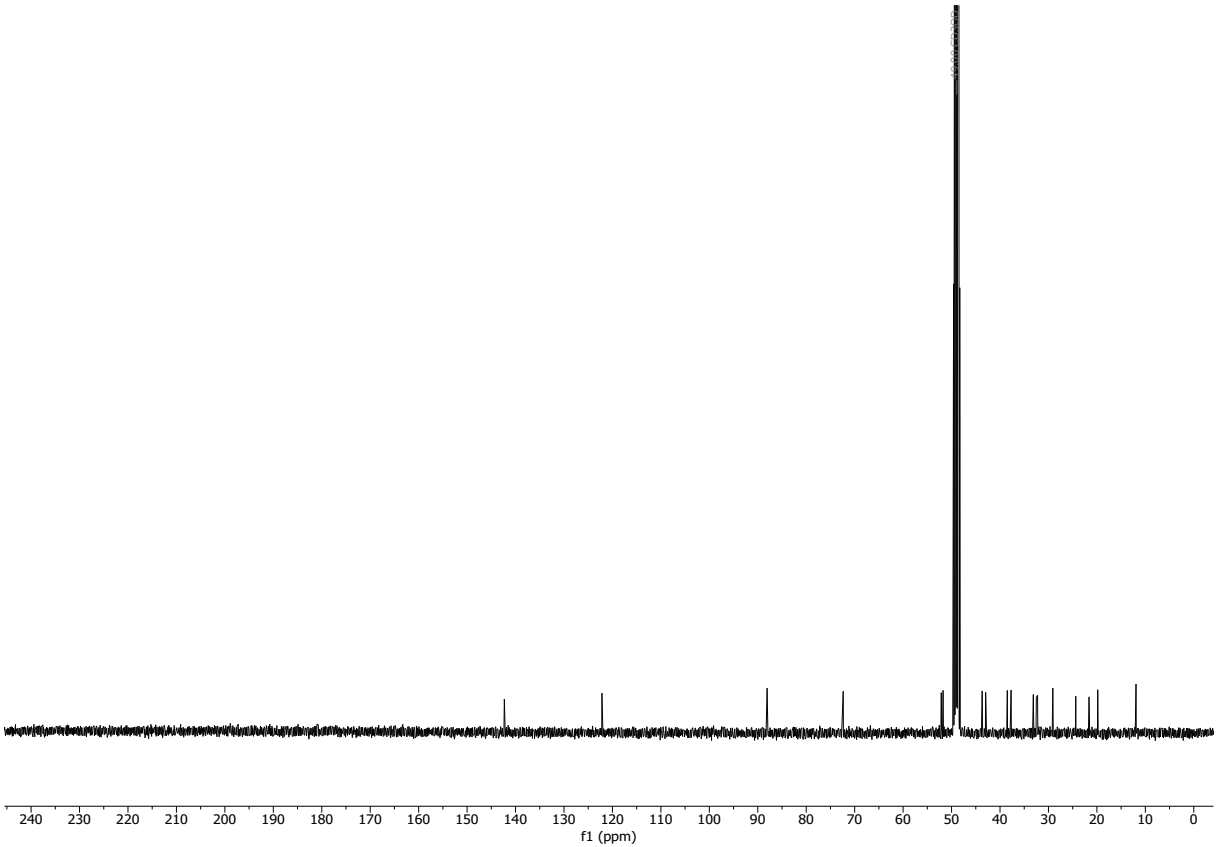
Androst-5-ene-3β,17β-diol 17-sulfate, ammonium salt (11)



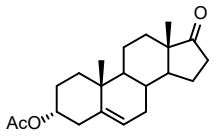

$^{1}$H-NMR (600MHz) $CD_3OD$



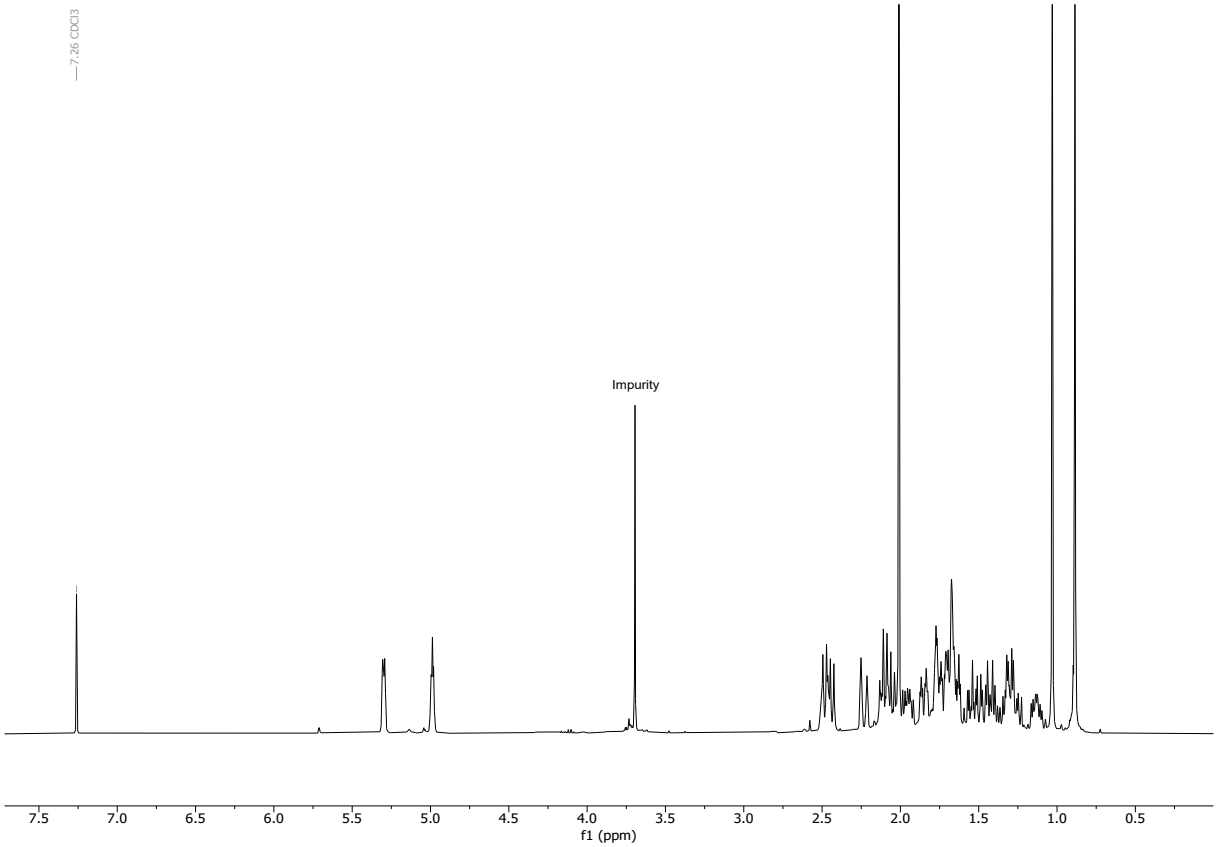
$^{13}$C-NMR (151 MHz) $CD_3OD$



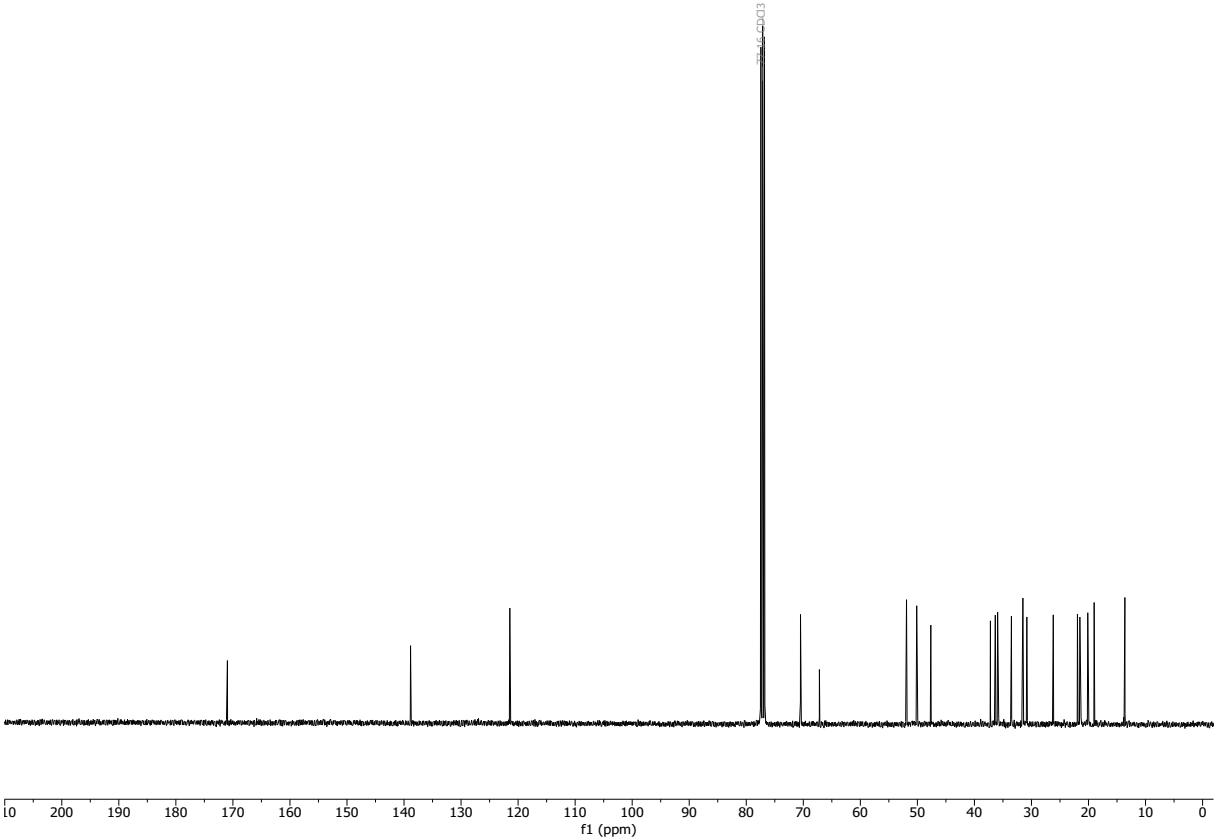
3α-Acetoxyandrost-5-en-17-one (17)



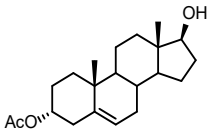

$^{1}$H-NMR (400MHz) $CDCl_3$



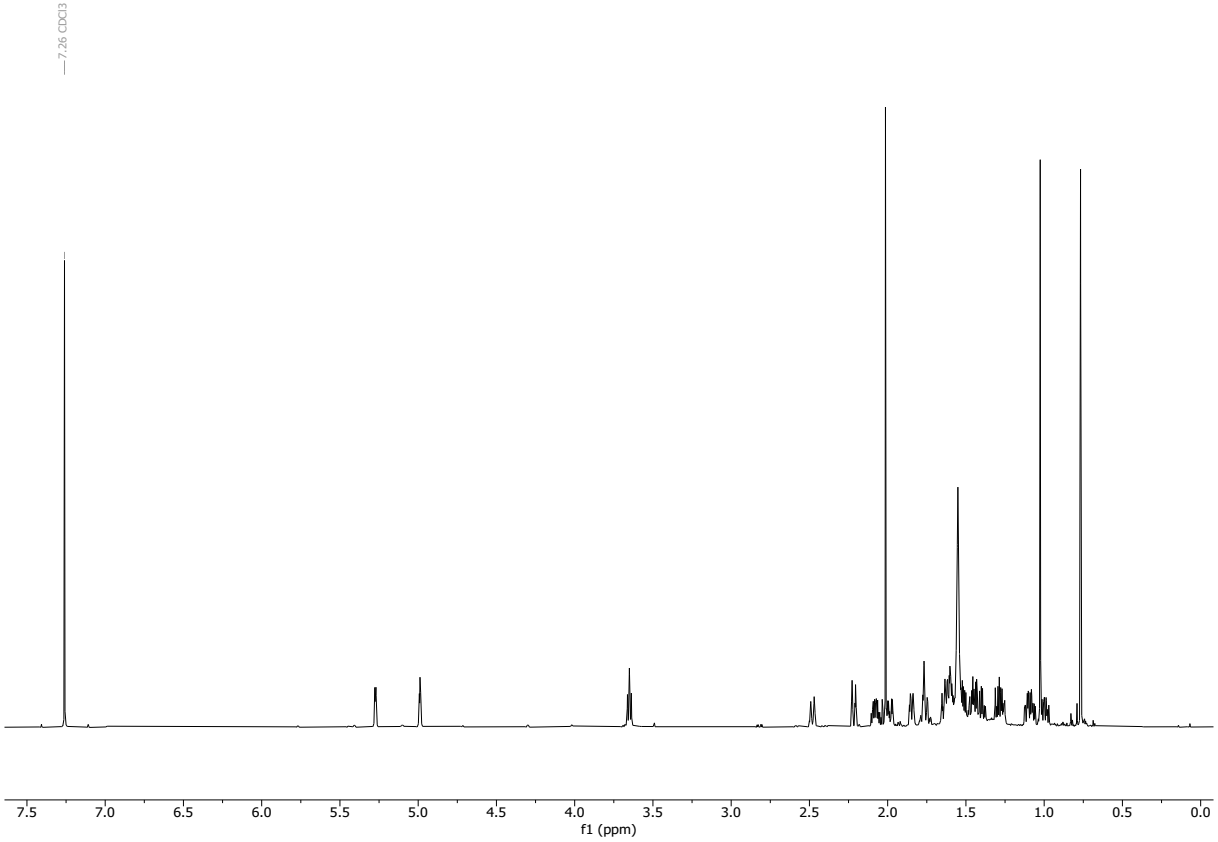
$^{13}$C-NMR (101 MHz) $CDCl_3$



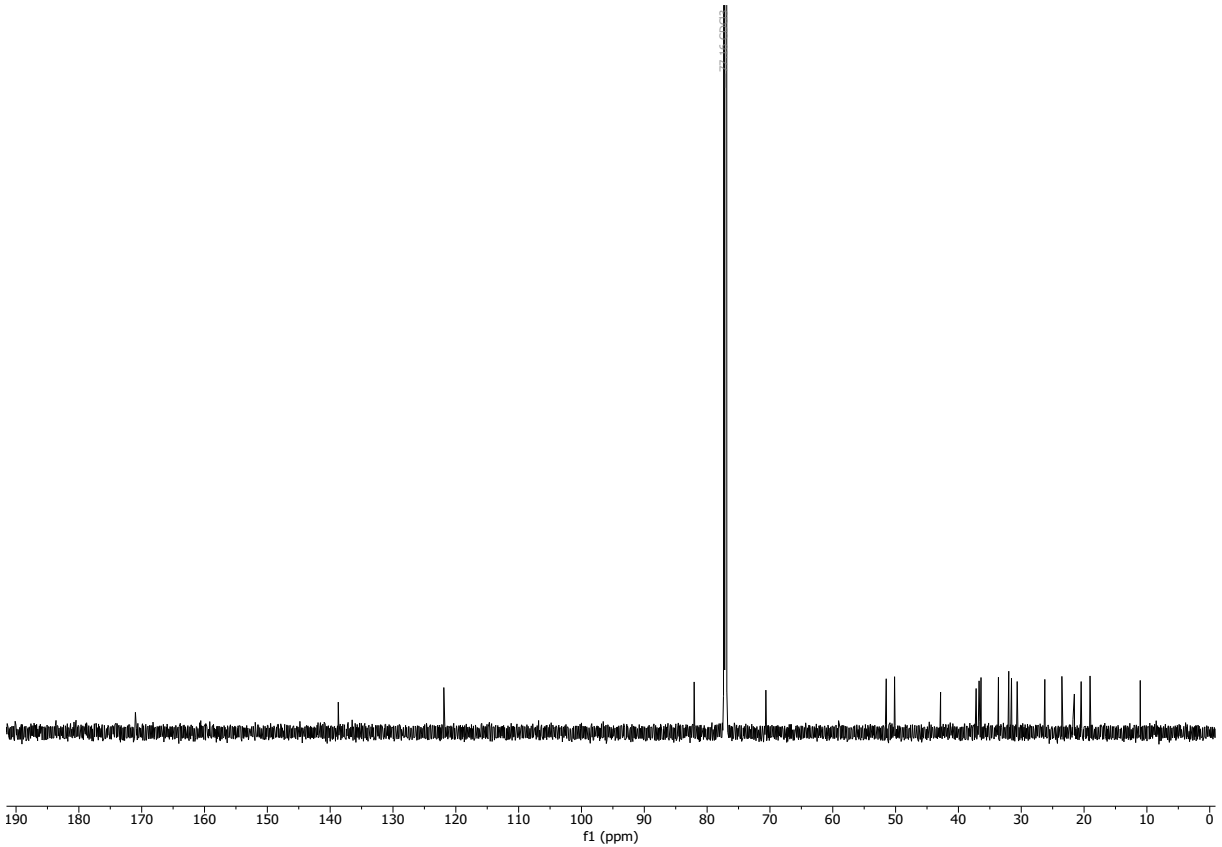
3α-Acetoxyandrost-5-en-17β-ol (18)



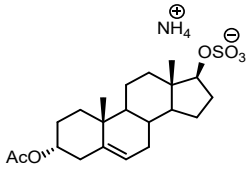

$^{1}$H-NMR (700MHz) $CDCl_3$



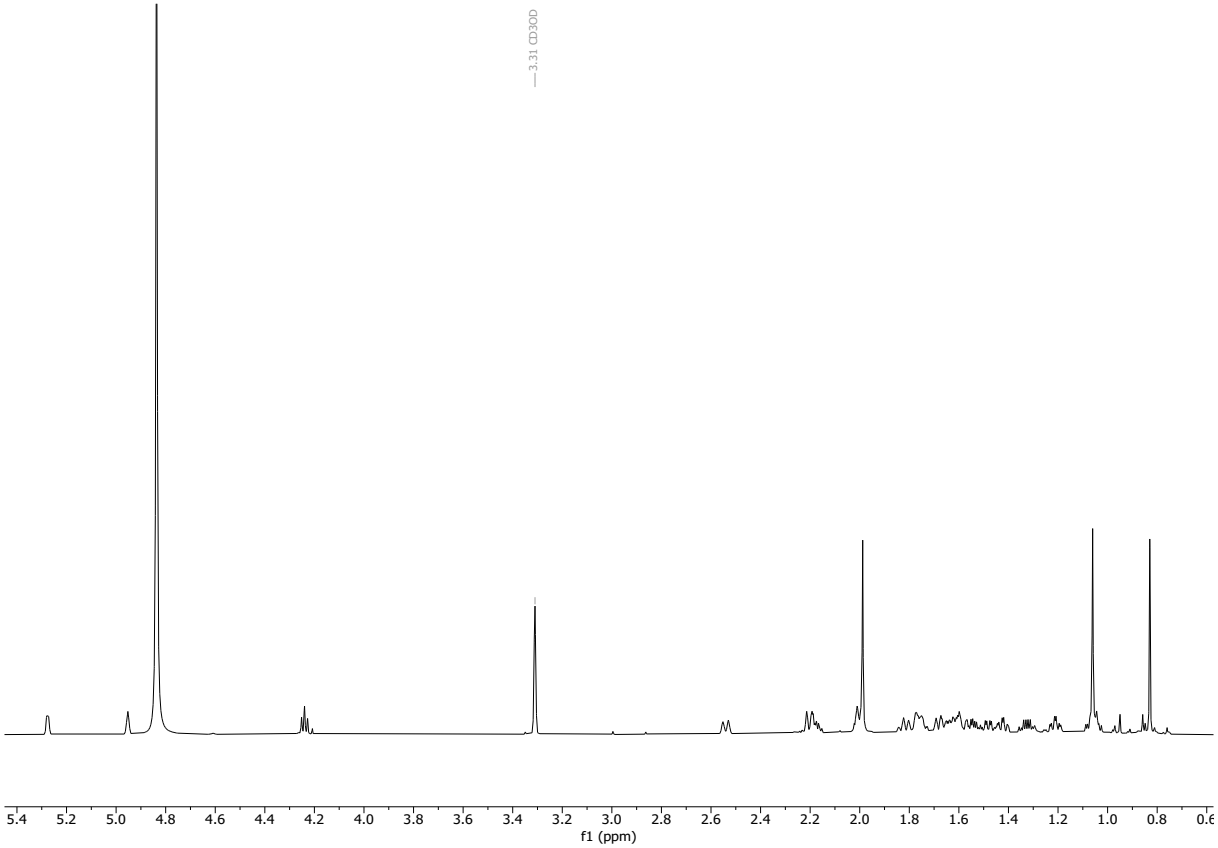
$^{13}$C-NMR (176 MHz) $CDCl_3$



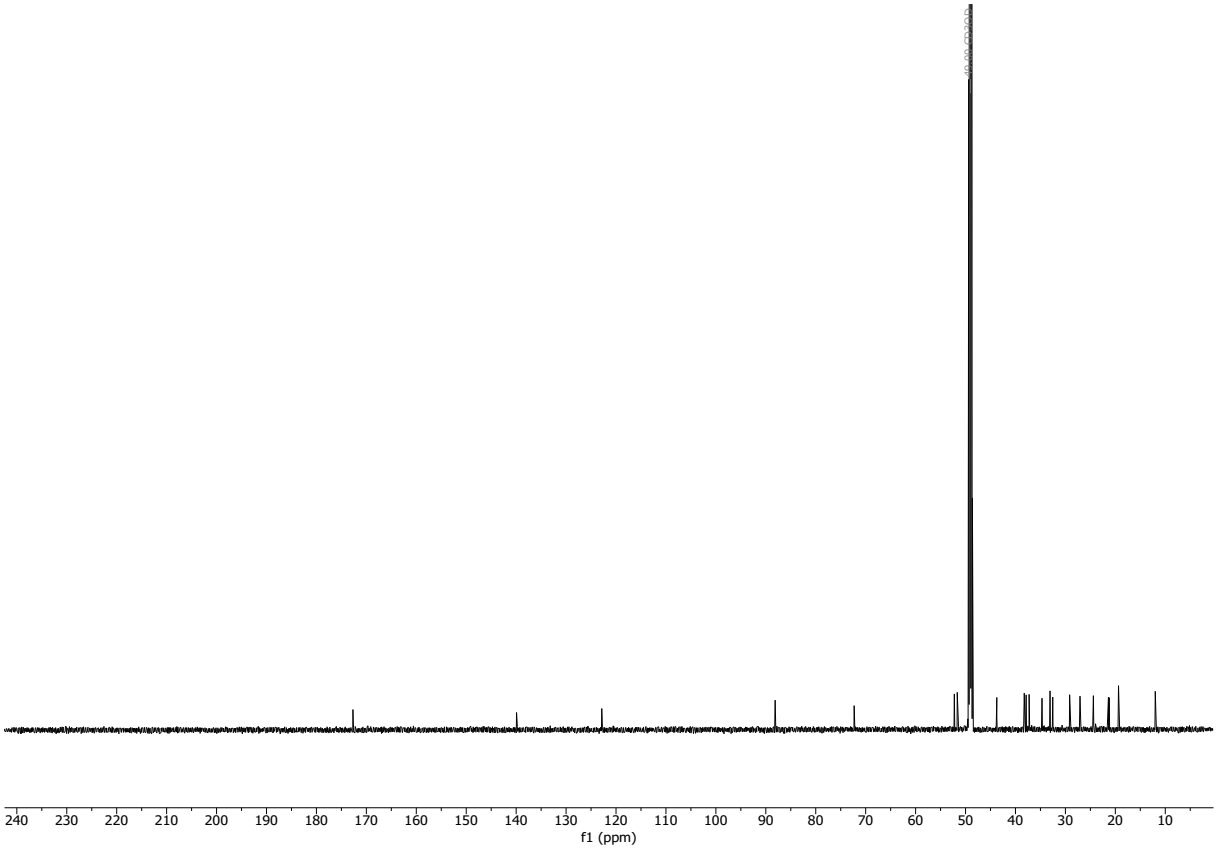
3α-Acetoxyandrost-5-en-17β-ol 17-sulfate, ammonium salt (19)



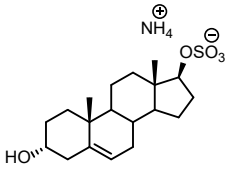

$^{1}$H-NMR (700MHz) $CD_3OD$



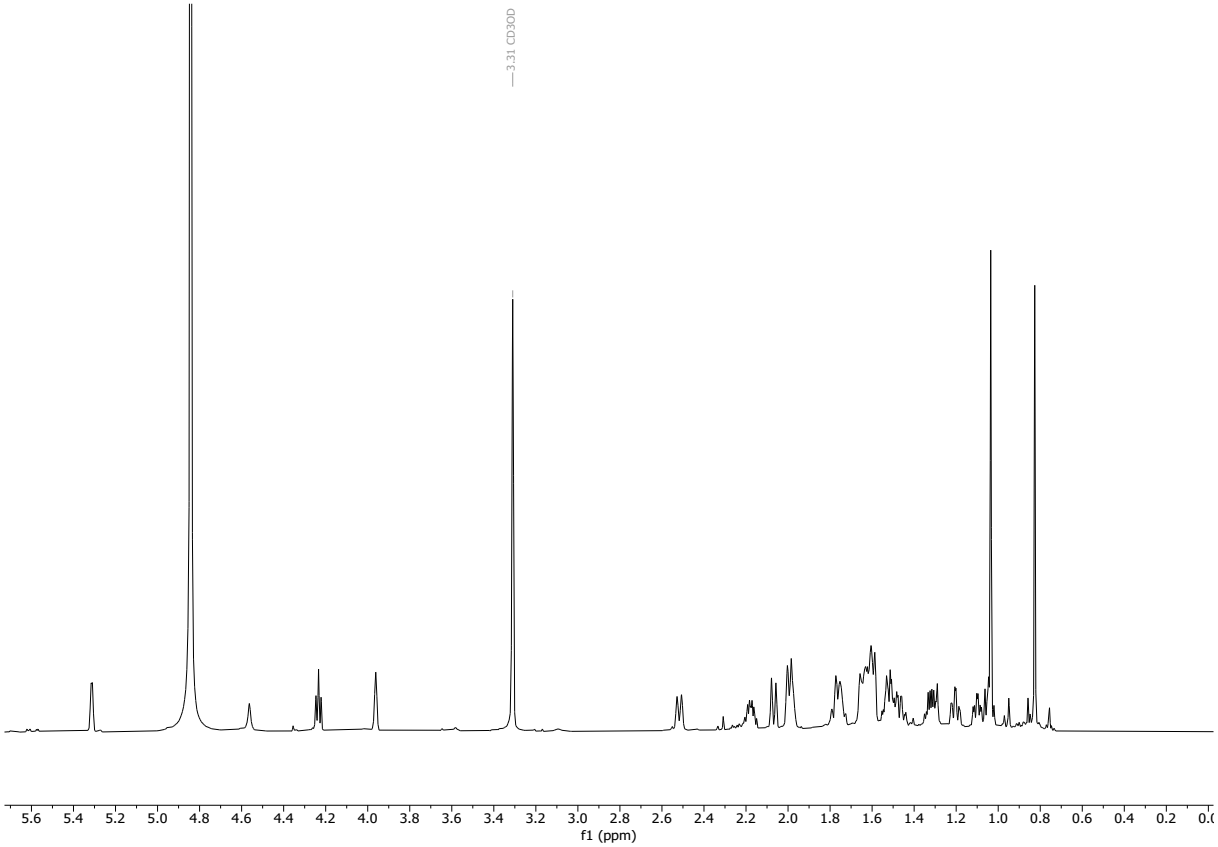
$^{13}$C-NMR (176 MHz) $CD_3OD$



Androst-5-ene-3α,17β-diol 17-sulfate, ammonium salt (12)




$^{1}$H-NMR (700MHz) $CD_3OD$



$^{13}$C-NMR (176 MHz) $CD_3OD$