Mechanism of the Third Oxidative Step in the Conversion of Androgens to Estrogens by Cytochrome P450 19A1 Steroid Aromatase

Supporting Information

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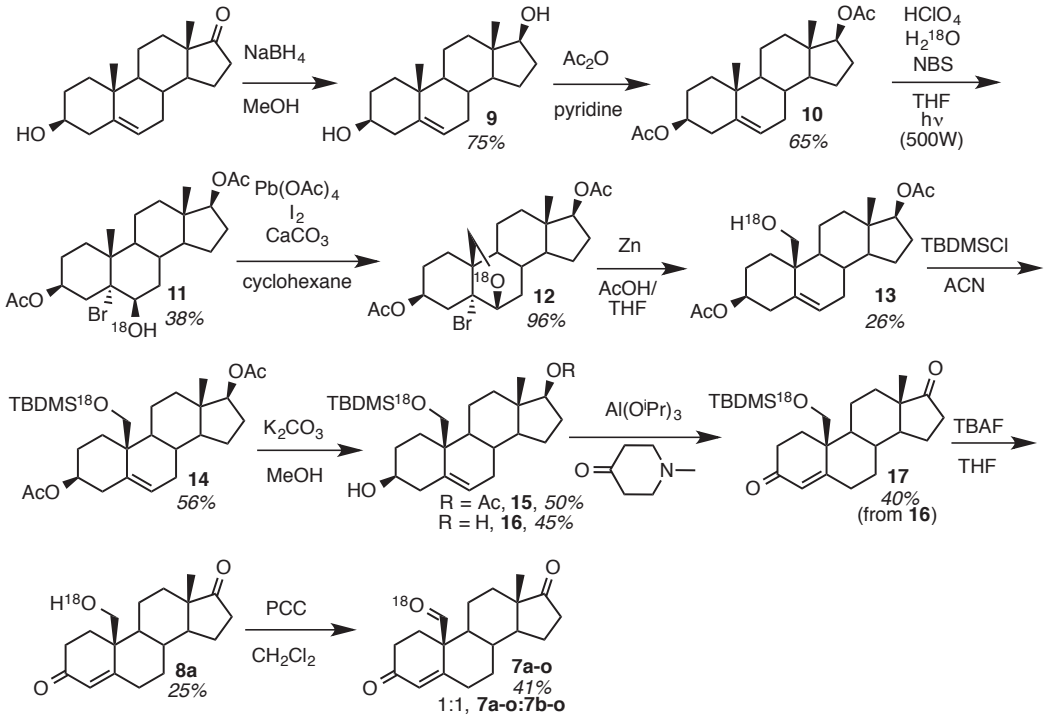


Figure S1: Outline of synthesis of 19-CH^{18}O androstenedione (**7a-o**).

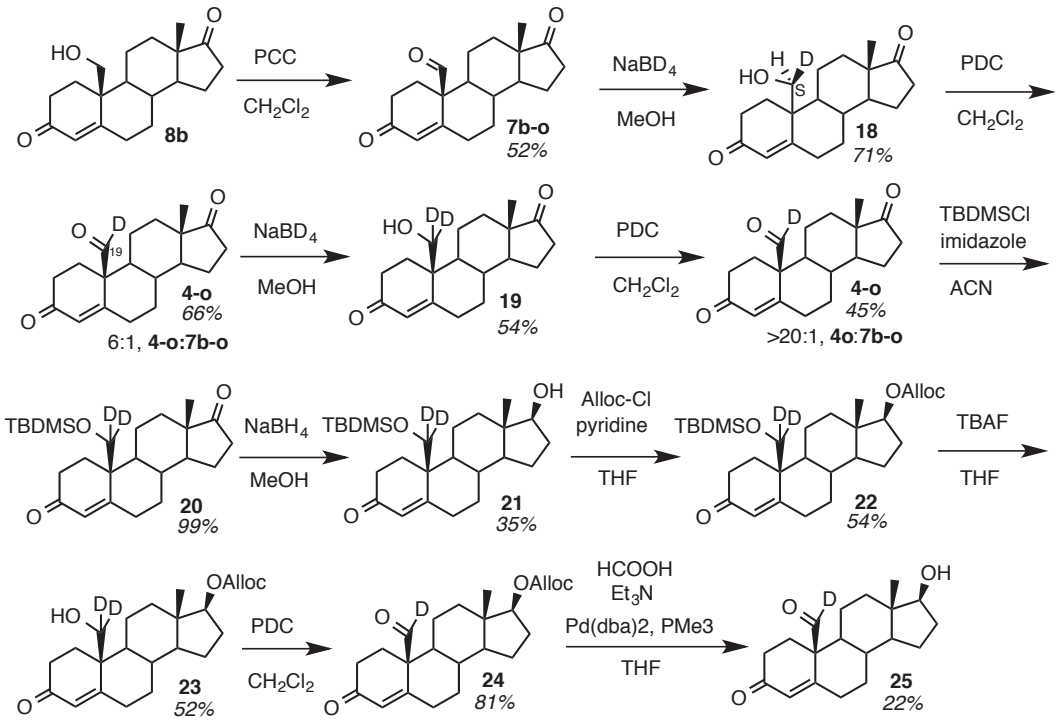
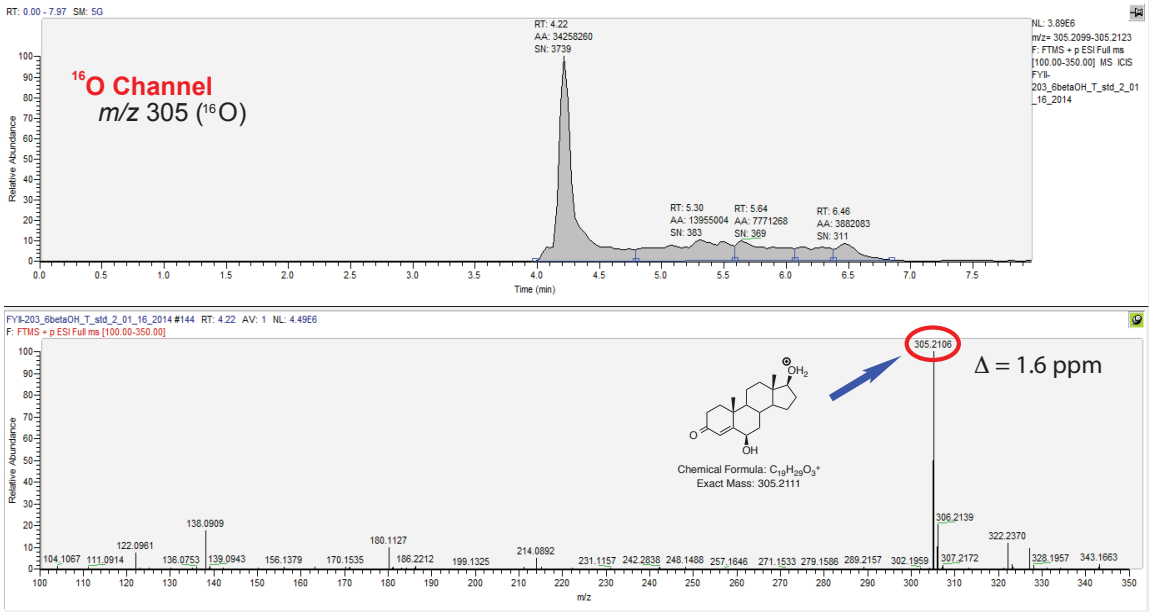


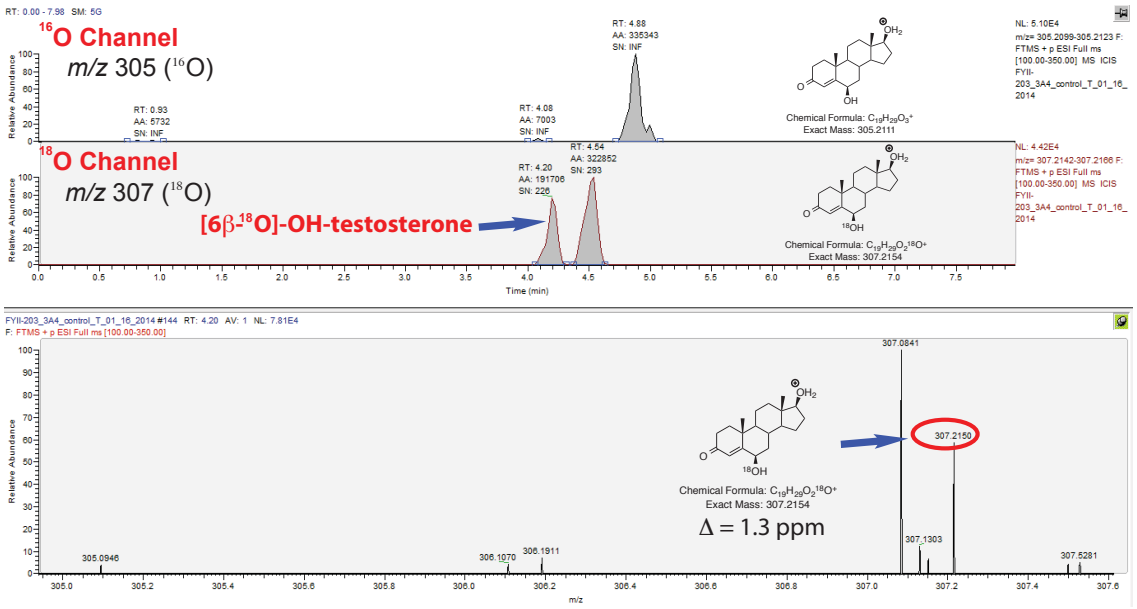
Figure S2: Outline of syntheses of 19-C^{2}HO androstenedione (**4-o**) and 19-C^{2}HO testosterone (**25**).

Figure S3: Incorporation of label from $^{18}O_2$ into 6β-hydroxytestosterone by P450 3A4.

(A) Commercially available 6β-hydroxytestosterone standard (*m/z* 305.2111) was used to establish the t_R at 4.2 min (*m/z* 305.2111 Area = 34258260, retention time = 4.22 min):



(B) Incubation of P450 3A4 with testosterone in the presence of $^{18}O_2$, showing the 6β-hydroxytestosterone product with incorporation of one atom of ^{18}O (>98%). The t_R 4.2 min peak (*m/z* 307.2154) matched the t_R of the standard for 6β-hydroxytestosterone (*m/z* 307.2154 Area = 191706, retention time = 4.20 min):



Note: the two channels were set at the same sensitivity in all of the $^{16}O/^{18}O$ traces in the Supporting Information.

A duplicate P450 3A4 control incubation for testosterone data; note t_R shift (to 4.46 min) for 6β-hydroxytestosterone on a different date (1_15_2014) (*m/z* 307.2154 Area = 243358, retention time = 4.46 min):

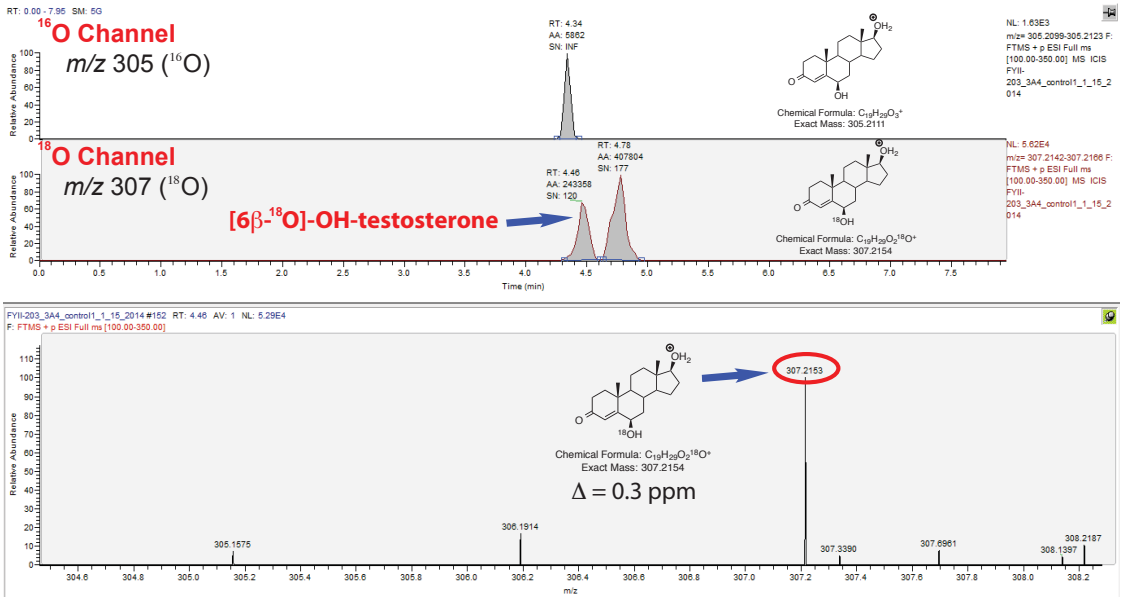
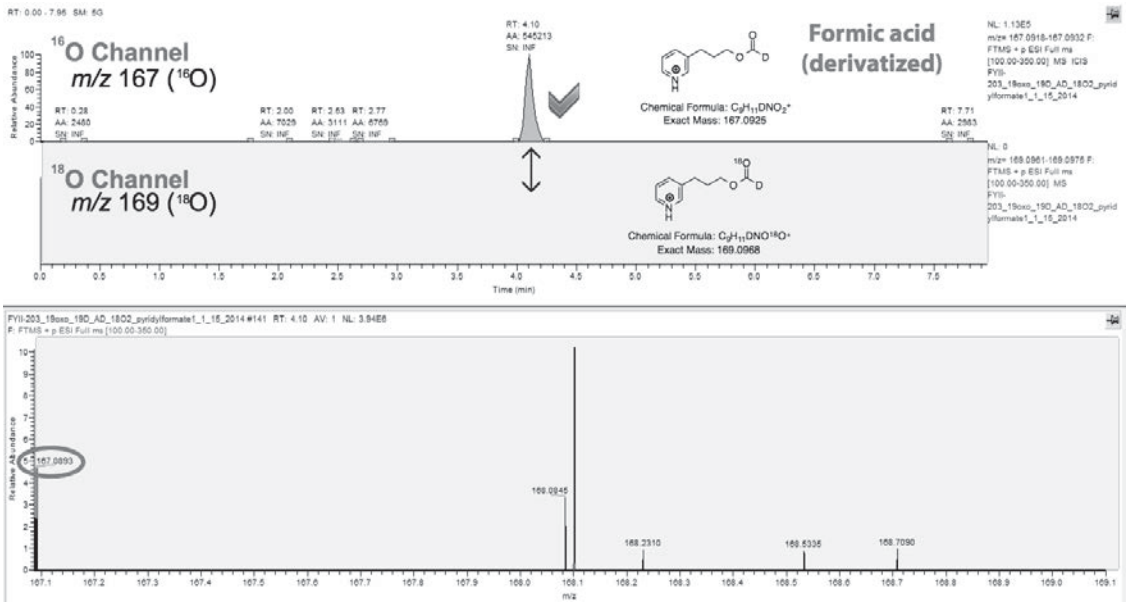
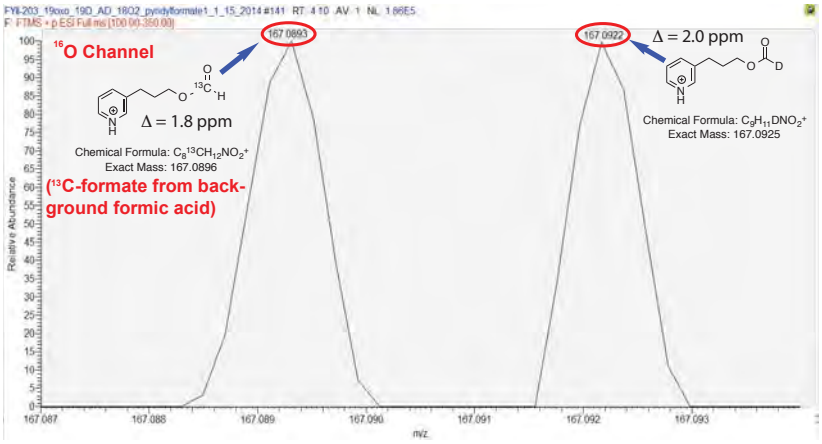
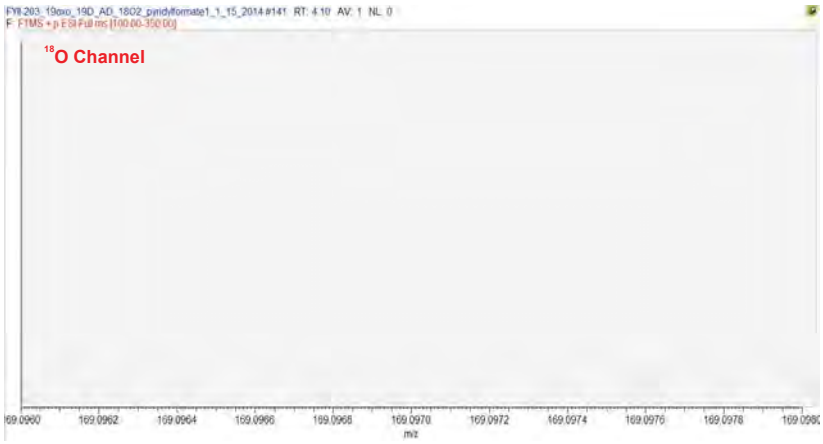


Figure S4: Lack of incorporation of label from $^{18}O_2$ into formic acid in the aromatization of 19-C^{2}HO androstenedione by P450 19A1 (*m/z* range: 167.084-169.100) (*m/z* 167.0925 Area = 545213):

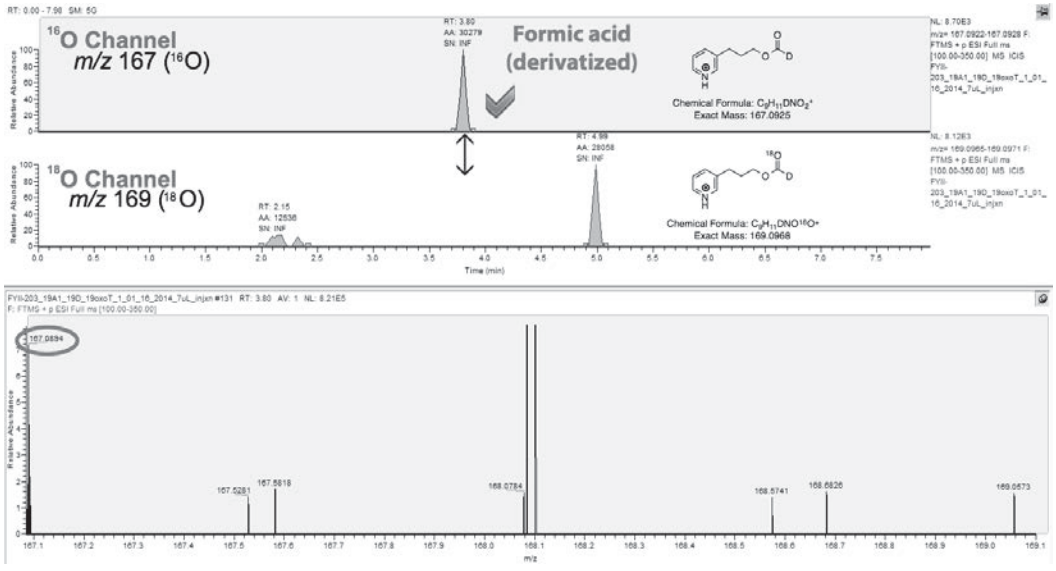


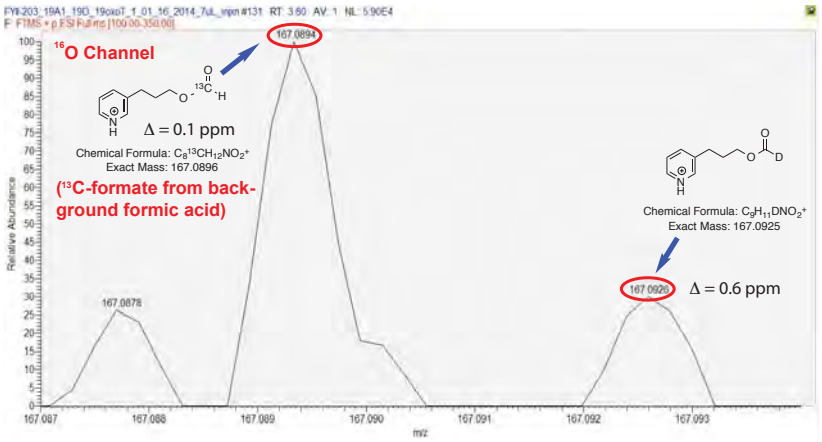




(As in other cases, the two channels were set at the same sensitivity.)

Figure S5: Lack of incorporation of label from $^{18}O_2$ into formic acid in the aromatization of 19-C^{2}HO testosterone by P450 19A1 (*m/z* range: 167.084-169.100) (*m/z* 167.0925 Area = 30279):




16O Channel
167.0894
Δ = 0.1 ppm
Chemical Formula: C8 13CH12NO2+
Exact Mass: 167.0896
(13C-formate from back-ground formic acid)
Chemical Formula: C9H11DNO2+
Exact Mass: 167.0925
167.0926
Δ = 0.6 ppm
167.0878
Relative Abundance
m/z

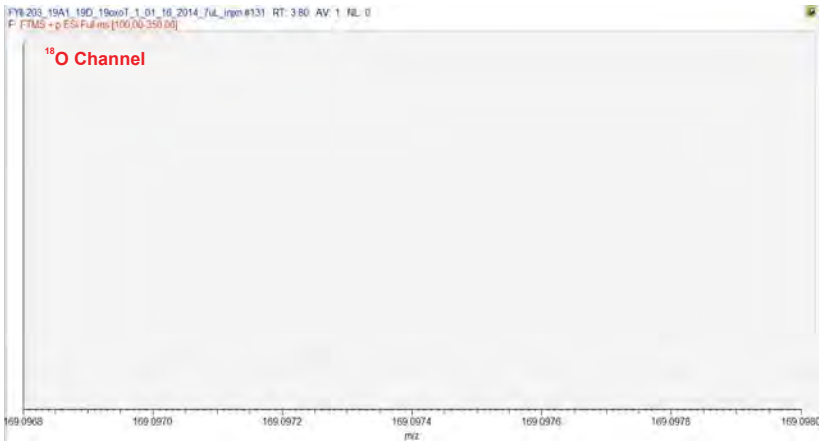
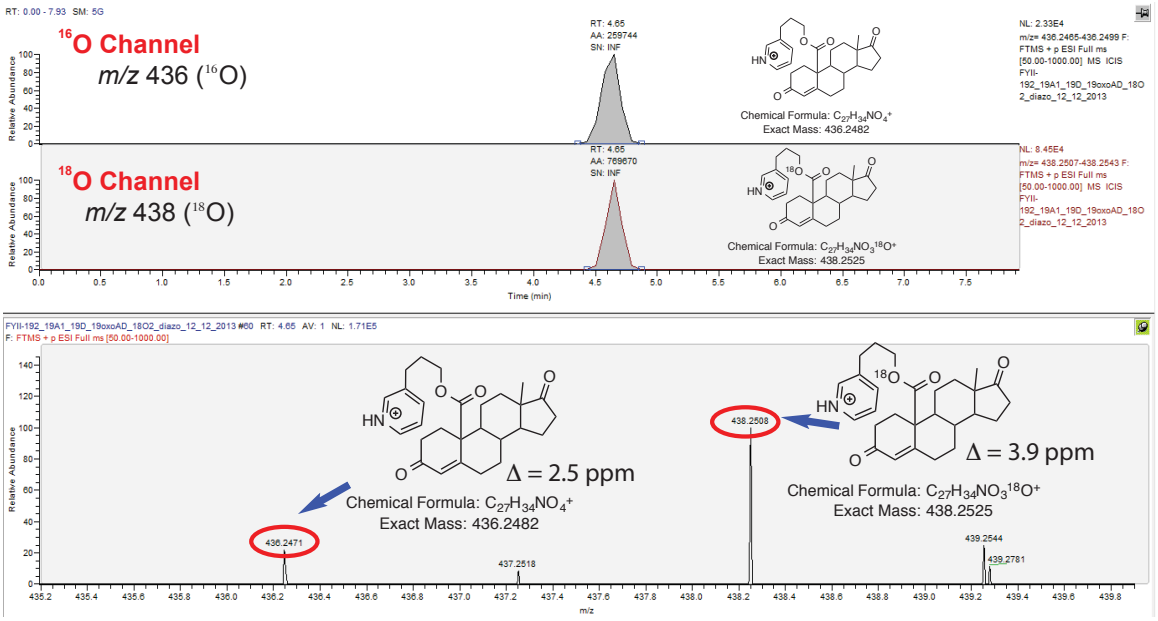
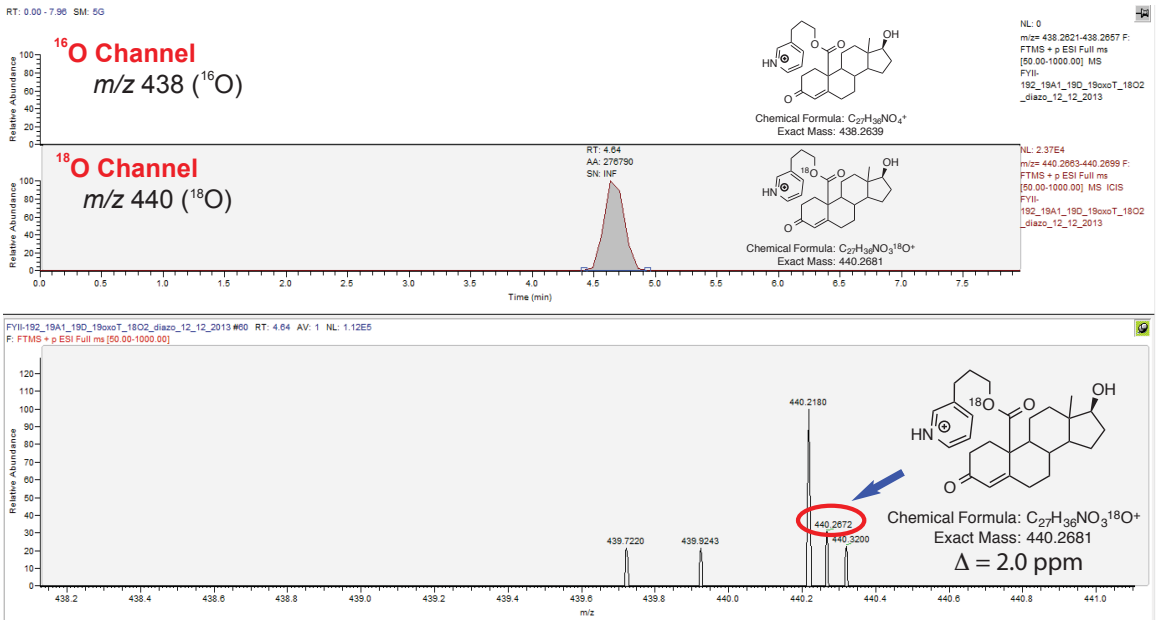

18O Channel
m/z

Figure S6: Mass spectra of (3-pyridine)-3-propyl ester derivatives of 19-CO_2H androstenedione and 19-CO_2H testosterone formed from 19-C^2HO androstenedione and 19-C^2HO testosterone by P450 19A1.

(A) 19-CO_2H androstenedione (*m/z* 436.2482 Area = 259744 and *m/z* 438.2525 Area = 769670):

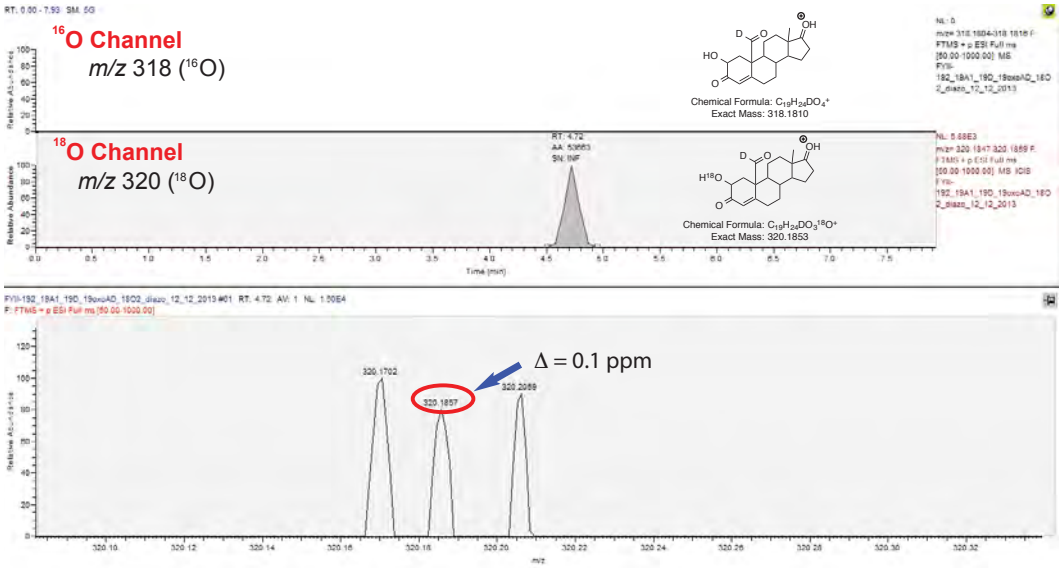


(B) 19-CO_2H testosterone (*m/z* 440.2681 Area = 276790):


RT: 0.00 - 7.96 SM: 5G
16O Channel
m/z 438 (16O)
Chemical Formula: C27H36NO4+
Exact Mass: 438.2639
NL: 0
m/z= 438.2621-438.2657 F: FTMS + p ESI Full ms [50.00-1000.00] MS FYII-192_19A1_19D_19oxoT_18O2_diazo_12_12_2013
18O Channel
m/z 440 (18O)
RT: 4.64
AA: 276790
SN: INF
Chemical Formula: C27H36NO3 18O+
Exact Mass: 440.2681
NL: 2.37E4
m/z= 440.2663-440.2699 F: FTMS + p ESI Full ms [50.00-1000.00] MS ICIS FYII-192_19A1_19D_19oxoT_18O2_diazo_12_12_2013
Relative Abundance
Time (min)

FYII-192_19A1_19D_19oxoT_18O2_diazo_12_12_2013 #60 RT: 4.64 AV: 1 NL: 1.12E5
F: FTMS + p ESI Full ms [50.00-1000.00]
440.2180
440.2672
440.3200
439.7220
439.9243
Chemical Formula: C27H36NO3 18O+
Exact Mass: 440.2681
Δ = 2.0 ppm
Relative Abundance
m/z

(C) Oxygenated aldehyde product mass is *m/z* 320.1853 [for 19-C^{2}HO androstenedione + ^{18}O] (*m/z* 320.1853 Area = 53663, retention time = 4.72 min):.



(D) Oxygenated aldehyde product mass is *m/z* 322.20091 [19-C^{2}HO testosterone + ^{18}O] (*m/z* 322.2009 Area = 1210425, retention time = 4.82 min):

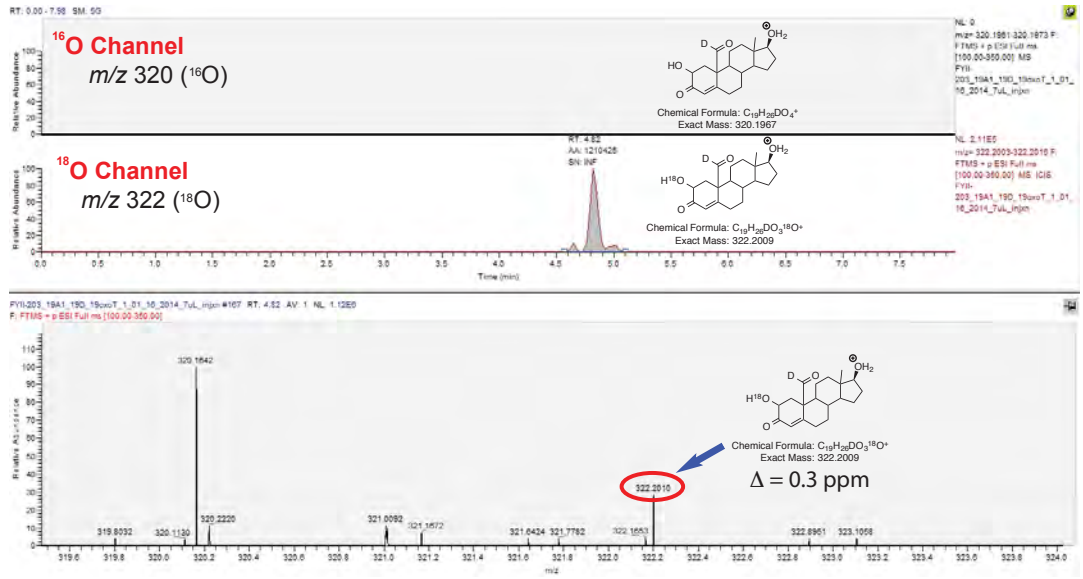
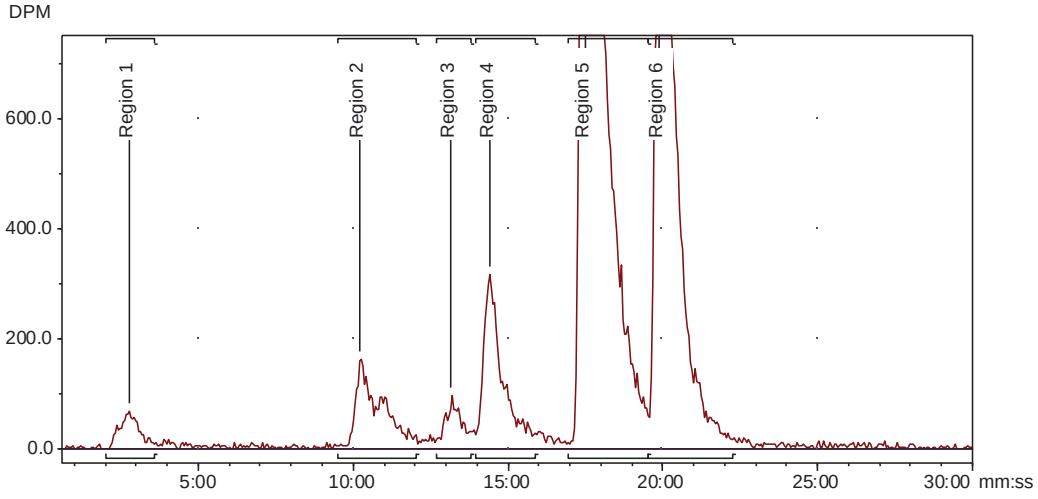


Figure S7: HPLC of products of [4-^{14}C]-androstenedione and [4-^{14}C]-testosterone formed by P450 19A1.

(A) [4-^{14}C]-Androstenedione products (from 110 min reaction incubation time). Assignments based on co-elution with injected standards, using UV-detection (240 nm). Region 1: androstenedione19-oic acid, Region 2: 19-OH androstenedione, Region 3: X (unidentified), Region 4: 19-oxoandrostenedione, Region 5: androstenedione, Region 6: estrone:



(B) [4-^{14}C]-Testosterone products (from 110 min reaction incubation time). Region 1: testosterone 19-oic acid and (based on co-elution with standards by UV-detection at 240 nm): Region 2: 19-OH testosterone, Region 6: 19-oxotestosterone, Region 7: testosterone, Region 8: 17β-estradiol:

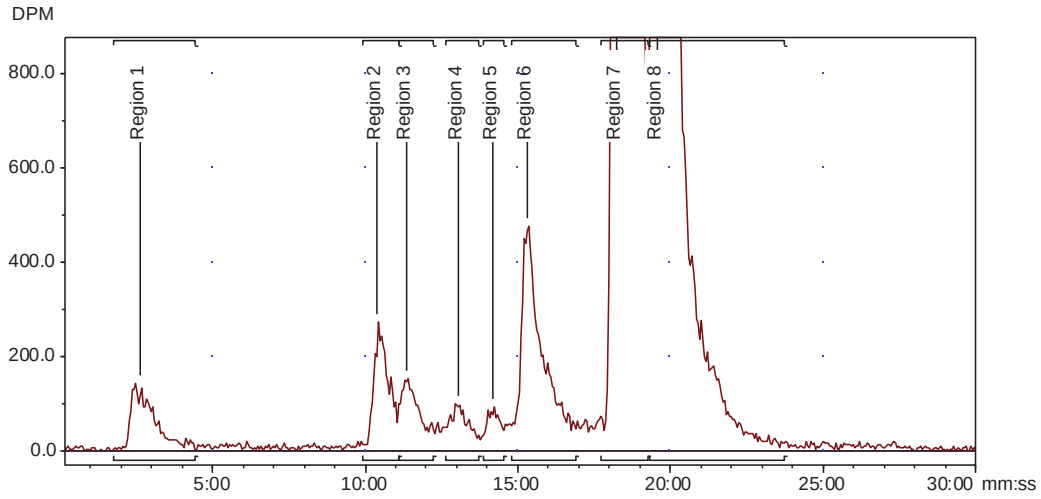
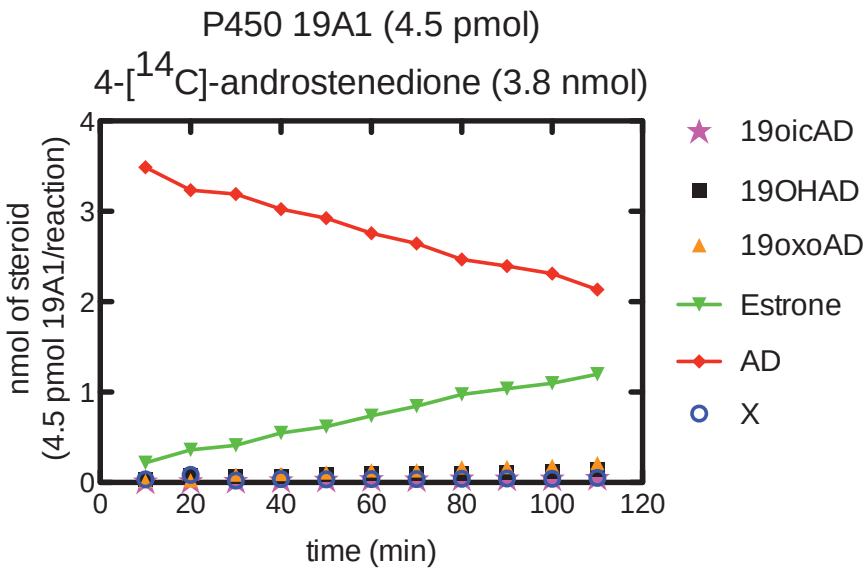


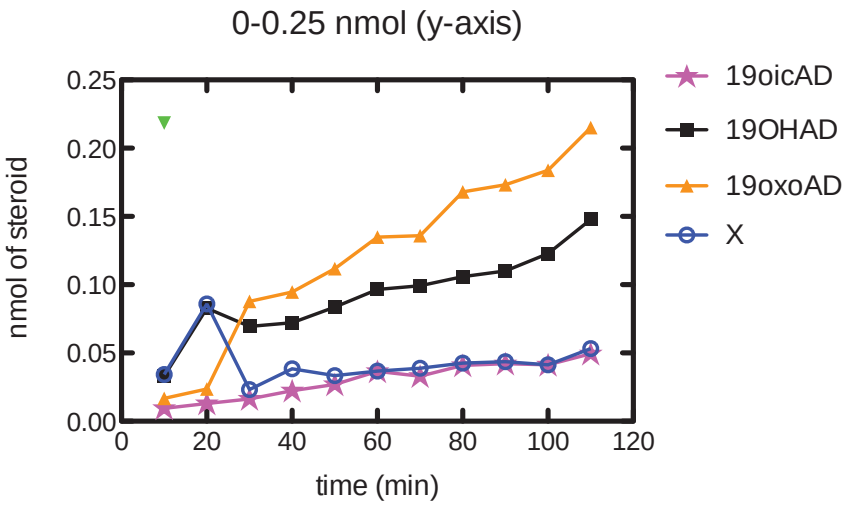
Figure S8: Kinetic course of formation of products of [4-^{14}C]-androstenedione and [4-^{14}C]-testosterone formed by P450 19A1.

(A) Androstenedione

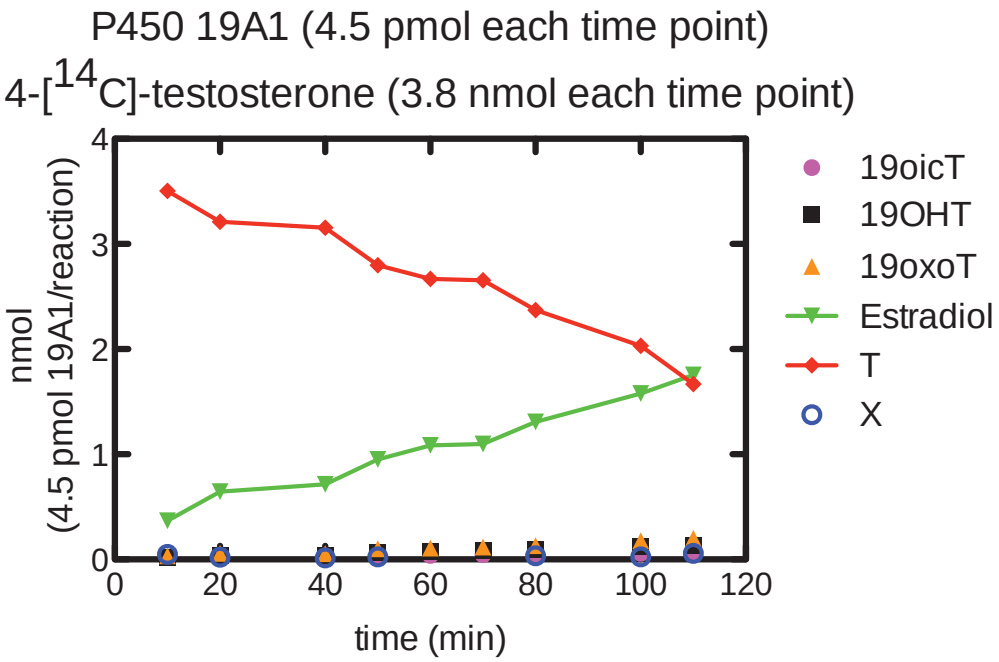
AD: androstenedione; T: testosterone; X: monooxygenated product of AD 19-oxo-AD or 19-oxo-T.



Expanded plot:



(B) Testosterone



Expanded plot:

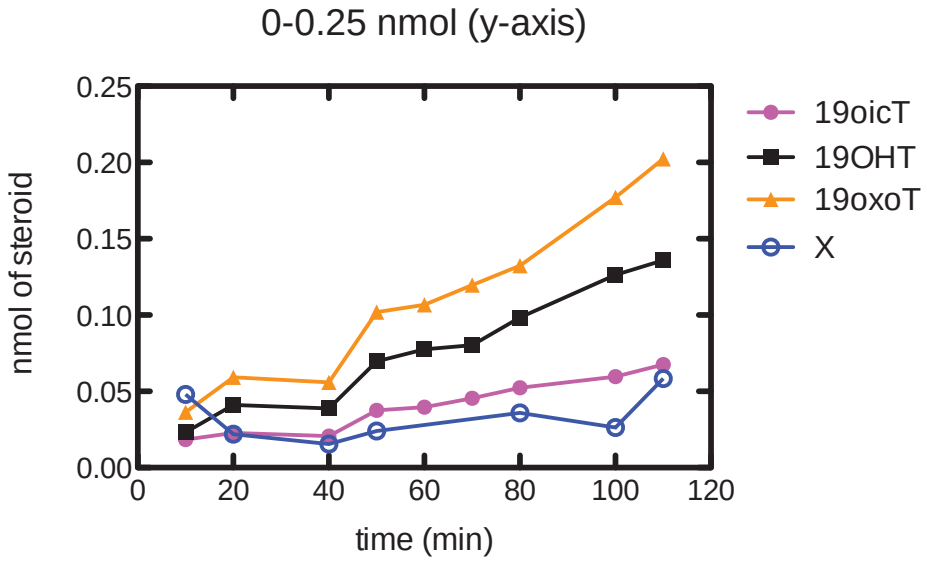
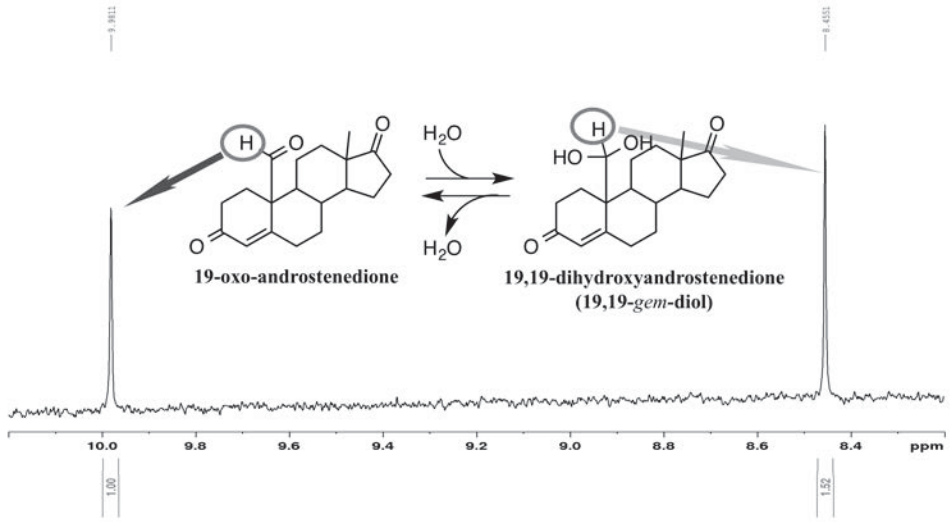


Figure S9: ^{1}H-NMR spectra of 19-oxo-androstenedione and 19-oxotestosterone (each 4 μM); equilibria of aldehyde and *gem*-diol forms (baseline corrected δ 10.2-8.20 ppm region).

(A) 19-CHO androstenedione



(B) 19-CHO testosterone

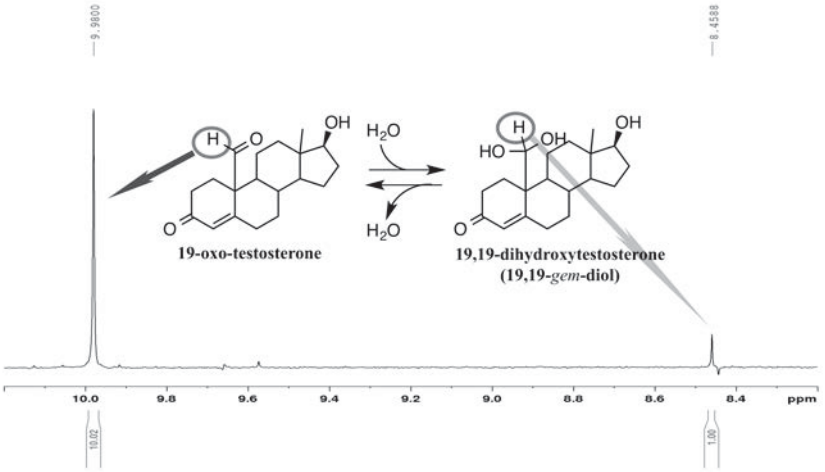
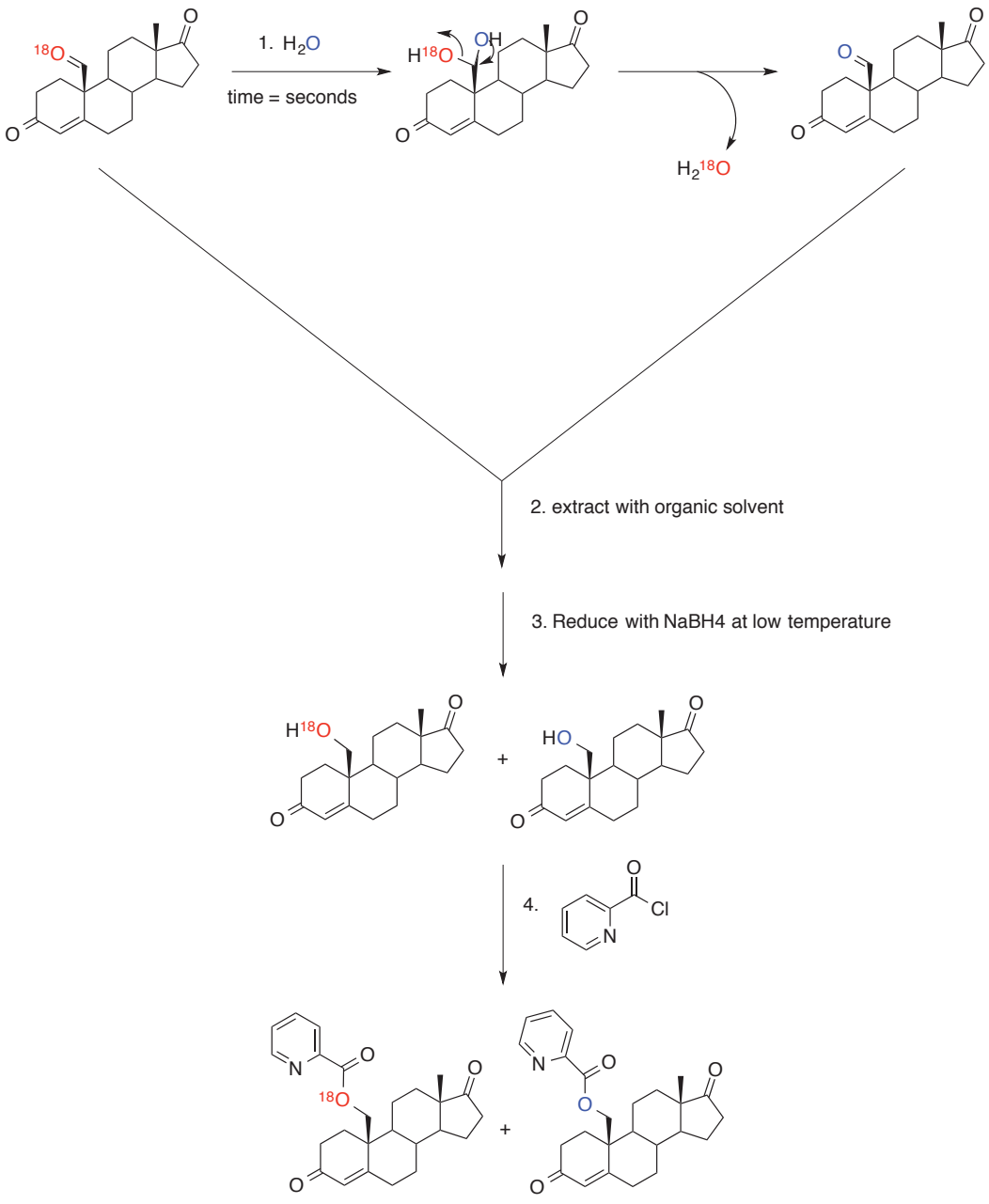


Figure S10: (A) Scheme for kinetic analysis of androstenedione 19-CH^{18}O hydration and dehydration using ^{18}O exchange.



(B) Procedure:

1. Add pH 7.4 potassium phosphate (50 mM) solution (1.0 mL) to ^{18}O-labeled 19-oxoAD (1.00:1.04, $^{18}O/^{16}O$ ratio based on MS) in CH_3CN (15 μL of a 500 μM solution), mix with vortex device for 1 s*
2. Quench with the addition of MTBE (2 mL)
3. Extract MTBE layer and dry with $MgSO_4$ (~200 mg)
4. Dry organic layer (N_2 stream)
5. Add 0.5 mL CH_3OH
6. Cool to -78 °C, add 10 μL of $NaBH_4$ solution (5 mg/mL in CH_3OH)
7. Mix with vortex device for 5 s
8. Quench with addition of H_2O (1 mL)
9. Extract with EtOAc (1.5 mL)**
10. Concentrate (N_2 stream), to LC-MS (low-resolution MS)

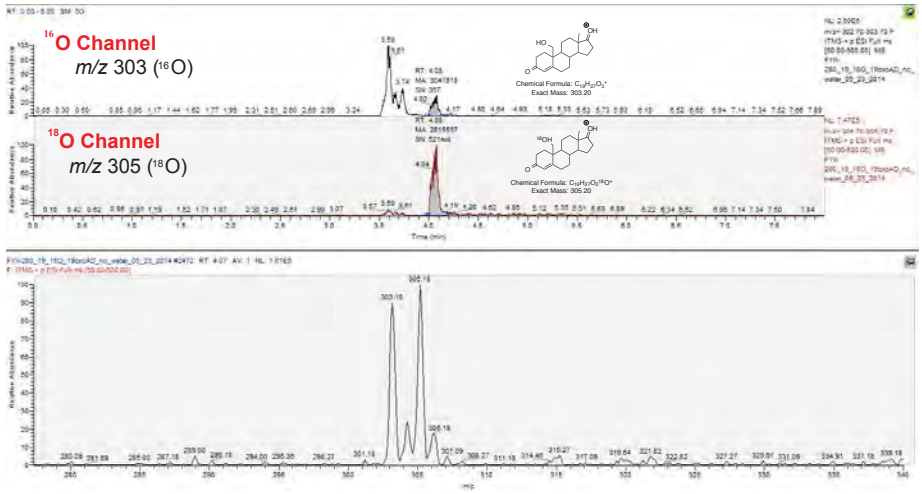
-For assays without derivatization with pyridine-2-carbonyl chloride, the parent ions were fragmented with 35 eV (*m/z* 303 for no ^{18}O, *m/z* 305 for ^{18}O-incorporation, corresponding to 19-hydroxyandrostenedione and [19-^{18}O]-19-hydroxyandrostenedione, respectively).

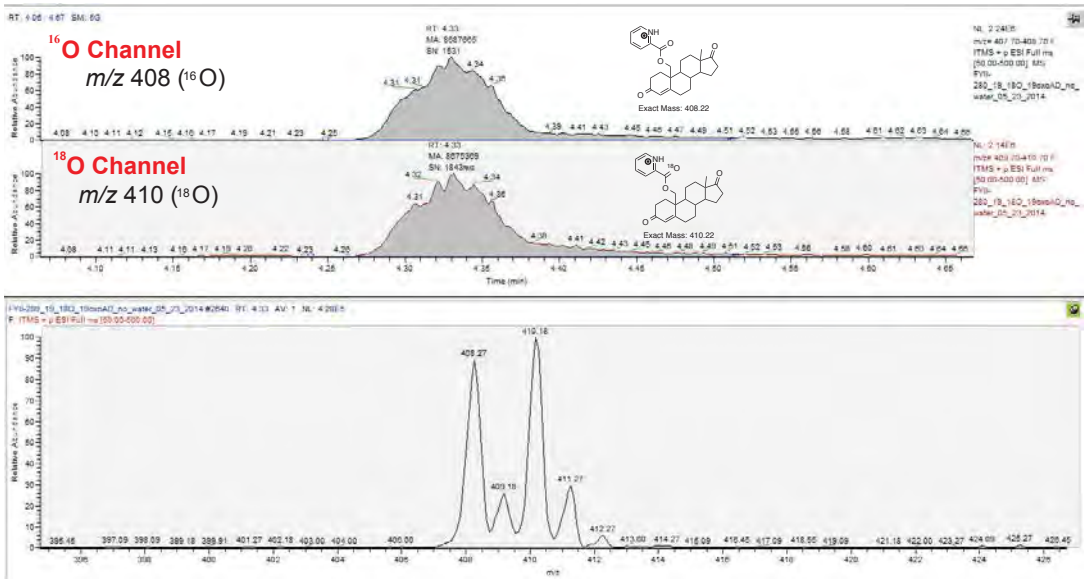
*When the concentration of the steroid in the water solution was increased to 500 μM, the ^{18}O label remained intact even after 1 hour of shaking at room temperature. Therefore, it was necessary to decrease the concentration of the steroid to <10 μM to observe the exchange of the ^{18}O atom. In addition, if the exchange was performed at 10 °C instead of

room temperature (at low concentration), the ratio of ^{18}O:^{16}O would fluctuate as the time increased, suggesting poor solubility at lower temperatures.

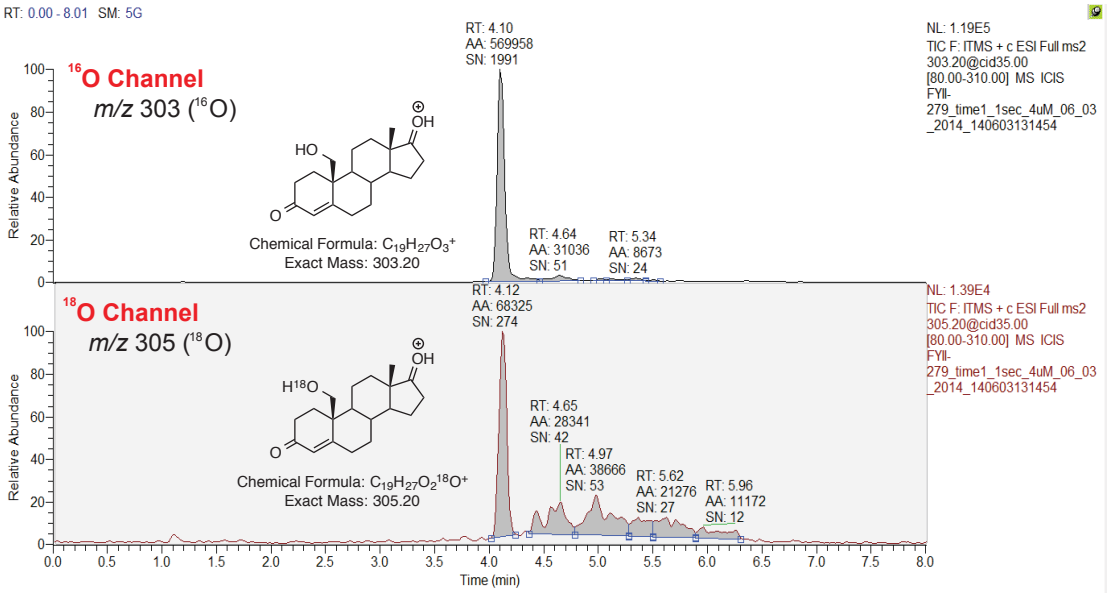
**Alternative: dry EtOAc layer (19-OHAD) with $MgSO_4$ (~200 mg), filter, and concentrate (N_2 stream). Add 20 μL of pyridine-2-carbonyl chloride solution (10 mg/mL in THF), mix with vortex device, and dry, to increase sensitivity for LC-MS.

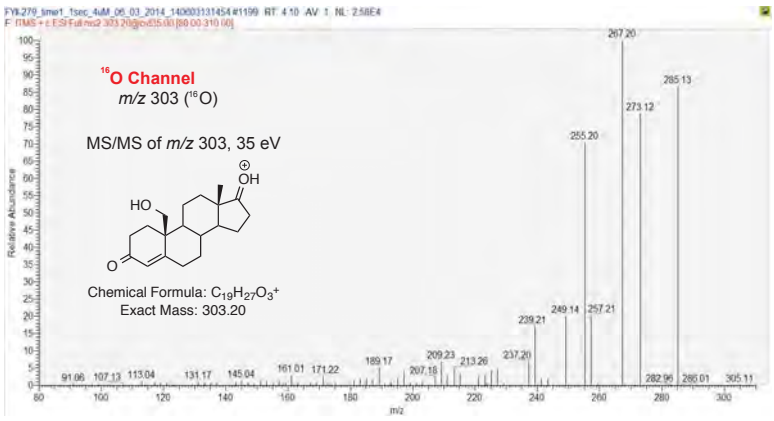
(C) Mass spectra generated with no water added (*m/z* 303.2 Area = 3047818, *m/z* 305.2 Area = 2815557)::

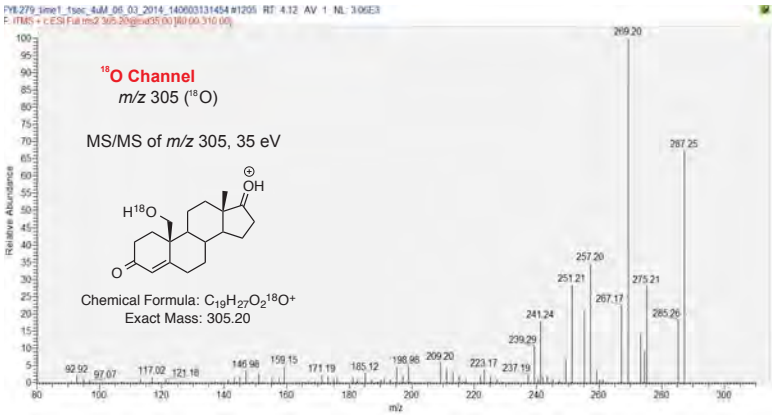




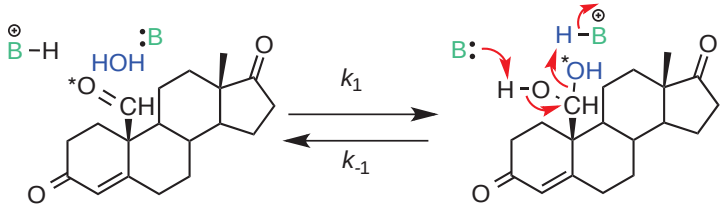
(D) Mass spectra after addition of water (1 s time point) (*m/z* 303.2 Area = 569958, *m/z* 305.2 Area = 68325):







(E)



$K_{eq} = k_{-1}/k_1$ NMR (D_2O)

Add steroid to H_2O, extract at varying t, derivatize & measure loss of *O (MS) (k_{obs})

$K_{eq} = k_{-1}/k_1$

$k_{obs} = k_1 + k_{-1}$

$k_1 = k_{obs} - k_{-1}$

$k_1 = k_{obs} - k_1 * K_{eq}$

$k_1 + k_1 * K_{eq} = k_{obs}$

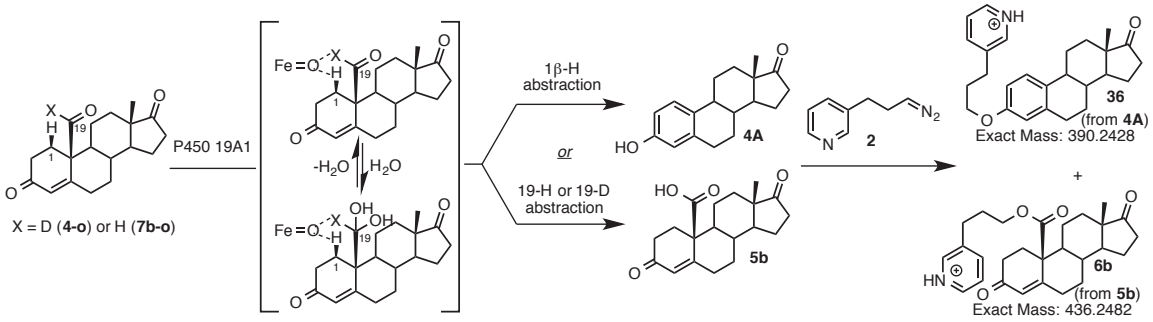
$k_1(1 + K_{eq}) = k_{obs}$

$k_1 = k_{obs}/(1 + K_{eq})$

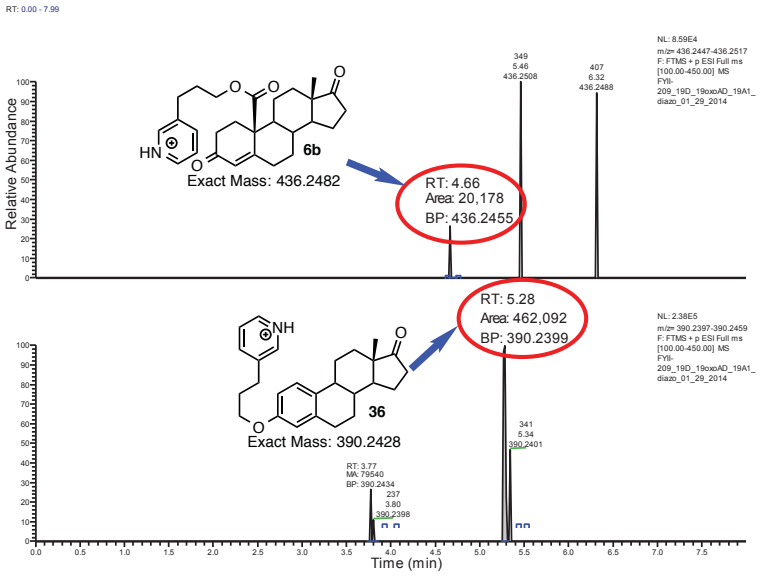
In the case of androstenedione, $K_{eq} \approx 1$ and $t_{1/2} < 1$ s, so $k_{obs} > 1$ s^{-1} and thus $k_{hydration}$ and $k_{dehydration} > 0.5$ s^{-1} (in the absence of enzyme).

Figure S11. Metabolic switching. Incubation of P450 19A1 with 19-C^2HO androstenedione (**4-o**) or 19-oxoandrostenedione (**7b-o**).

(A)



(B) P450 19A1 incubation with [19-^{2}H]-19-oxoandrostenedione (**4-o**) substrate:



(C) P450 19A1 incubation with 19-oxoandrostenedione (**7b-o**) substrate:

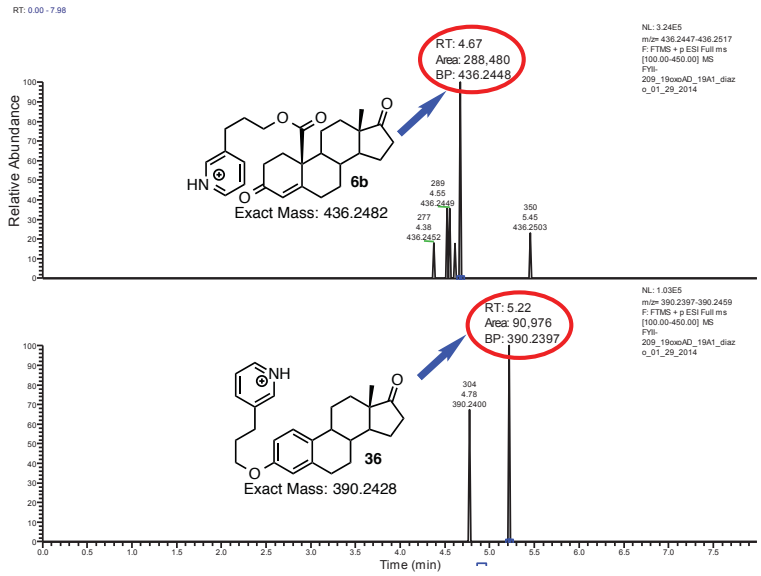


Figure S11. Metabolic switching experiments. Incubation of P450 19A1 with [19-^{2}H]-19-oxoandrostenedione (**4-o**) or 19-oxoandrostenedione (**7b-o**). (A) Scheme of incubation followed by derivatization with diazo compound **2**. (B) and (C) Ion chromatograms from incubations with **4-o** or **7b-o** scanned for masses of pyridine propyl ester (**6b**) and phenol ether (**36**) (m/z 436.2482 and m/z 390.2482) derived from 19-oic acid product (**5b**) and estrogen product (**4A**), respectively. Mass tolerance window was 8 ppm. (B) Incubation of P450 19A1 with [19-^{2}H]-19-oxoandrostenedione (**4-o**) substrate. (C) Incubation of P450 19A1 with 19-oxoandrostenedione (**7b-o**) substrate. Pyridinepropyl ester **6b**: retention time of 4.67 or 4.66 min. Phenol propylpyridyl ether **36**: retention time of 5.22 or 5.28 min. These retention times matched the standards in separate LC-MS runs. BP: Base Peak, RT: Retention Time.

$[\mathbf{36/6b}]_H$ = Product ratio (**36/6b**) with **7b-o** as substrate

$[\mathbf{36/6b}]_D$ = Product ratio (**36/6b**) with **4-o** as substrate

$k_H/k_D = [\mathbf{36/6b}]_H/[\mathbf{36/6b}]_D = [3.2/0.044] = 73$

Metabolic switching experiments.

Incubations were run in 1 mL total volume and the final concentrations of substrate, P450 19A1, and reductase were 300 μM, 5 nM, and 170 nM, respectively. 1,2-***α***-Didodecanoyl-*sn*-glycero-3-phosphocholine (0.3 μg/mL), reductase (2.4 μM), and purified P450 19A1 (700 pM) were added to a test tube in a volume of 70 μL and allowed to sit at room temperature for 5 min. Water (750 μL) was added followed by phosphate buffer (pH 7.4, 1 M, 100 μL). The substrate was added (i.e. either (**7b-o**, 3 mM, 10 μL) or (**4-o**, 5 mM, 6 μL)) and the incubation was initiated with the addition of NADPH generating system (Final concentrations: glucose-6-phosphate (7 mM), glucose-6-phosphate dehydrogenase (2 μg/mL), $NADP^+$ (18 μM)). The incubation was shaken at 50 rpm at 37 °C. After 10 min, the reaction was terminated with the addition of CH_2Cl_2 (3 mL). The mixture was cooled to 0 °C and HCl (3 M, 400 μL) was added and resulting mixture was vortexed for 5 seconds. The organic layer was extracted and dried with $MgSO_4$ and the solid was filtered. Diazo reagent solution (compound **2** in diethyl ether, 2 mL, cf. Section *5. Enzyme Incubations* under sub-heading: *Diazo reagent (**2**) preparation*, p. S-139) was added to the filtrate and dried down. The samples were dissolved in 60 μL MeOH and analyzed by LC-MS.

Abbreviations:

≈

AD: androstenedione

Alloc: alloxycarbonyl

BP: base peak

CAM: ceric ammonium molybdate

DBA: dibenzylideneacetone

DHEA: dehydroepiandrosterone

DMSO: dimethyl sulfoxide

ESI: electrospray ionization

EtOAc: ethyl acetate

FT-MS: Fourier-transform mass spectrometry

HRMS: high-resolution mass spectrometry

LTQ: linear trap quadrupole

MTBE: methyl *tert*-butyl ether

PCC: pyridinium chlorochromate

t_R: retention time

T: testosterone

TBAF: *tert*-butyl ammonium fluoride

TBDMS: *tert*-butyl dimethylsilyl

TEA: triethylamine

THF: tetrahydrofuran

UPLC: ultra-performance liquid chromatography

Materials and Methods

1. *Instrumentation*

UV/Vis spectra were recorded using an OLIS/Aminco DW-2a spectrophotometer (On-Line Instrument Systems, Bogart, GA).

For radioactivity analysis of incubations with [4-^{14}C]-labeled androstenedione or testosterone, a β-RAM detector (IN-US Systems, Tampa, FL) in-line with a UV detector (SpectroMonitor 3200 variable wavelength detector) from Thermo Separation Products was used and the data was analyzed with Laura software version 4.0.4.101. A Hitachi Pump L-7100 HPLC was used, with a manual injection setup.

NMR spectra were acquired in the Vanderbilt facility on Bruker instruments equipped with a 5 mm Z-gradient TCI Cryo-probe for the 600 (^{1}H)/150 (^{13}C) MHz instrument (14.1 Tesla Bruker magnet, Bruker AV-II console) or a 5 mm Z-gradient broadband inverse (BBFO) probe for the 600 (^{1}H)/150 (^{13}C) MHz instrument (9.4 Tesla Oxford magnet, DRX-400 console). The NMR spectra were processed on Topspin software. NMR solvent chemical shifts were referenced for $CDCl_3$ (δ 7.26 ppm for ^{1}H NMR, 77.16 ppm for ^{13}C NMR), CD_3CN (1.94 ppm for ^{1}H NMR, 118.26 ppm for ^{13}C NMR), and D_2O (4.79 ppm for ^{1}H NMR) according to literature precedents.[1]

^{1}H NMR of 19-oxoandrostenedione and 19-oxotestosterone was done in pD 7.8 D_2O. Potassium phosphate buffer (pH 7.8, 0.1 M, 3.0 mL, solution A) was azeotroped with acetone using reduced pressure, and the salts were reconstituted in D_2O (3 mL). Steroid (1 mg) was reconstituted in D_2O (potassium phosphate) solution (0.7 mL). This mixture was diluted 10-fold in the potassium phosphate (50 mM) D_2O solution (solution A). The steroid concentration had to be diluted in the D_2O solution in order for the *gem*-diol methine (CH at C19) proton to appear. If the steroid was saturated in the D_2O solvent (~2 mg/mL), the *gem*-diol methine, then no *gem*-diol methine signal was visible.

High-resolution mass spectrometry (HRMS) was done with a Waters SYNAPT (Waters, Milford, MA) (tuned to ~15,000 resolution) or a Thermo (ThermoFisher, Waltham, MA) LTQ XL Orbitrap (100,000 resolution setting) instrument in the FTMS analyzer mode. Data acquisition was done under Profile Mode (Orbitrap). Mass spectra were analyzed using QualBrowser version 2.0.7 software. The ppm range was analyzed within 4 ppm of mass accuracy.

An LTQ-XL hybrid ion trap-Orbitrap mass spectrometer was used to measure the exact masses (HRMS) of the formate ester products in the electrospray (ESI)-positive mode. The mass spectrometer was calibrated with Thermo Scientific Pierce LTQ ESI Positive Ion Calibration Solution (Fisher catalog #88322). The tube lens voltage was adjusted to 125 V when calibrating in order to obtain the *m*/*z* peak at 138.066190. The mass spectrometer was tuned with a 1 mM solution of 3-(pyridin-3-yl)-propyl formate (derivative of formic acid used in experiments) in CH_3OH by direct injection. An Acquity

UPLC was connected in line with the mass spectrometer. The sequence of the runs for the mass spectrometer was run using XCalibur software. Under the LTQ Tune Plus program, the FTMS analyzer was set to 1 microscan and the injection time was adjusted to 20 ms. The method for the instrument settings was adjusted to 100,000 resolution and the scan range for the masses are indicated on the ion chromatograms but in general were changed to either: (1) *m*/*z* 100-350 for the 3-(pyridin-3-yl)-propyl formate masses, which were between *m*/*z* 166-170 (e.g. compounds **3a**, **3b**, **3c**), or (2) *m*/*z* 50-1000 or 100-450 for steroids, which ranged from *m*/*z* 270 to 304 (e.g. compounds **7a-o**, **8a**, **4A**), and derivatized steroid 19-oic esters (e.g. compounds **6a**, **6b**) and propylpyridyl ethers (e.g. compound **36**) were *m*/*z* 435-442 and 390-393).

The LTQ1 machine was used for the 19-[^{18}O]-oxo androgen exchange experimental measurements in the ESI positive mode.

2. Materials

Silica gel (cat. No. 02826-5, 32-63u, FLASH grade, 60A, for flash column chromatography) was purchased from Dynamic Adsorbents (Norcross, GA). 19-Hydroxyandrostenedione (CAS:510-64-5, cat. No. WS32425) and DHEA (CAS: 53-43-0, cat. No. WS50937) were purchased from Waterstonetech LLC (Carmel, IN). Tetrahydrofuran (THF) with butylated hydroxytoluene (BHT, 250 ppm) stabilizer was used (Sigma 178810). 3-(Pyridin-3-yl)propan-1-amine was purchased from Pharmabridge (Doylestown, PA). ^{18}O-Oxygen lecture bottles and a compressed gas regulator with CGA inlet size 180 were purchased from SigmaAldrich (St. Louis, MO). TLC plates with fluorescent indicator (F_{254}) were purchased from SigmaAldrich (Z293024). Preparative

TLC plates with 2000 μm thickness and fluorescent indicator (Z513059) were purchased from SigmaAldrich. 1,2-α-Didodecanoyl-*sn*-glycero-3-phosphocholine (CAS: [3436-44-0]) was from Sigma. 3,17-Dioxo-androst-4-ene-19-oic acid (CAS: 4757-95-3) was purchased from Santa Cruz Biotechnology (Dallas, TX). Pyridine-2-carbonyl chloride hydrochloride (product number 075735) was purchased from Matrix Scientific (Columbia, SC). Radioactive steroids (in C_2H_5OH) were purchased from Perkin Elmer: [4-^{14}C]-androstenedione (48 mCi/mmol) and [4-^{14}C]-testosterone (59 mCi/mmol). *tert*-Butyldimethylsilyl chloride was purchased from Oakwood Chemical (West Columbia, SC).

For mass spectrometry assays, an ACQUITY® UPLC BEH C18 octadecylsilane (1.7 μm, 2.1 mm × 100 mm) column (Waters) was used (23 °C, No. 186002352, serial no. 017139310255 02) equipped with a guard column (23 °C, VanGuard™ no. 186003975) was used. For radioactive assays analyzed by HPLC, an octadecylsilyl (C_{18}) Phenomenex 150 mm × 4.6 mm Prodigy™ 3 μm ODS-3 100 Å column (23 °C, number: 00F-4222-E0) was used.

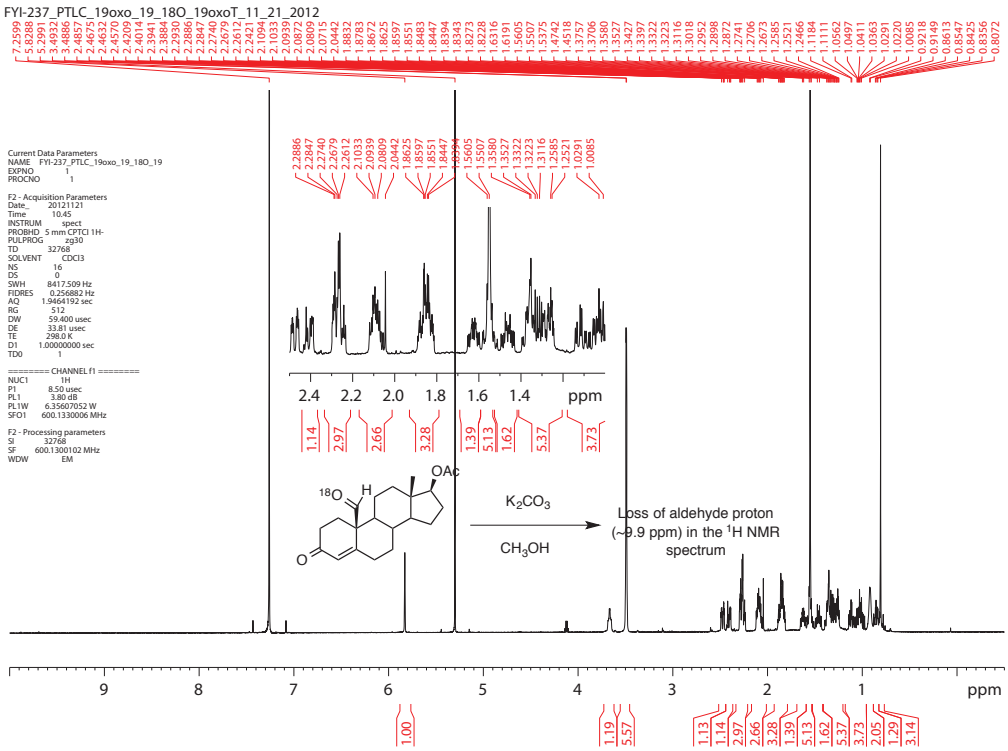
TLC plates with fluorescent indicator (F_{254}) were purchased from SigmaAldrich (Z293024). TLC analysis was visualized by a UV lamp or after charring with ceric ammonium molybdate (CAM/Hanessian's) stain:[2] ammonium molybdate (4.8% w/v), ceric ammonium molybdate (0.2% w/v), H_2SO_4 (6% v/v) in H_2O.

3. *Enzymes*

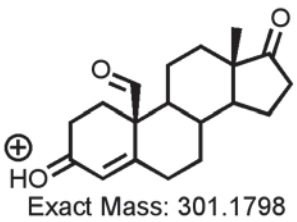
Recombinant human P450 19A1 was expressed in *Escherichia coli* and purified as described previously.[3] Recombinant *E. coli* rat NADPH-P450 reductase was prepared as described.[4]

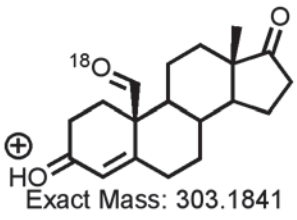
*4. Chemical synthesis: Compounds **9** to **35** (including HRMS and NMR spectra)*

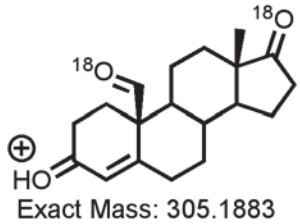
General: For the 19-oxotestosterone syntheses, an Alloc-protecting group on the 17-hydroxy position was necessary. In preliminary studies, deprotection of the 17-acetate (from 17-acetoxy-19-oxo-testosterone) in the final step using K_2CO_3 in CH_3OH yielded a compound with the loss of the aldehyde peak in the ^1H NMR spectrum of the product (~9.9 ppm) when using an acetate protecting group on the 17-position.

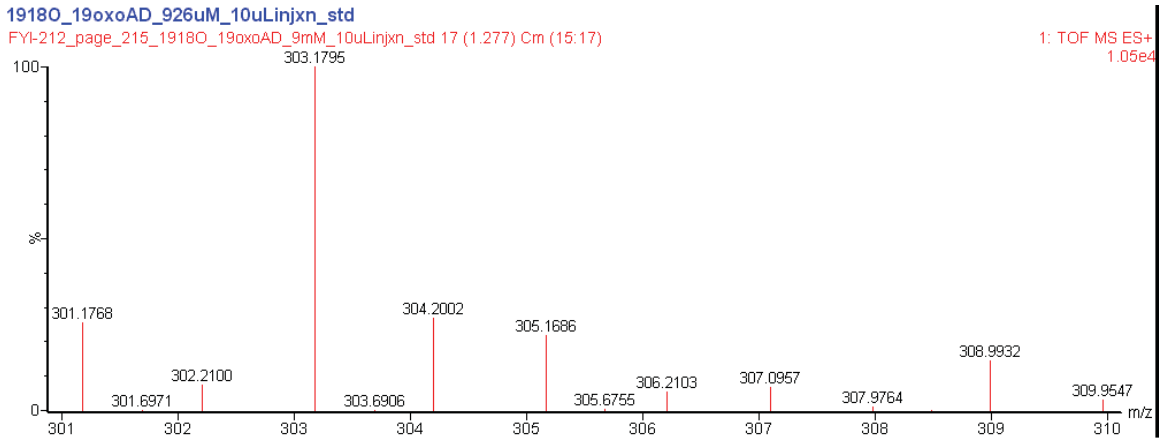


When synthesizing [19-^{18}O]-19-oxoandrostenedione, the protection of the 17-position was necessary as the acetate because ^{18}O-incorporation was observed in the 17-oxo position when DHEA-3-acetate was used in the bromohydrin formation step (HRMS spectra were recorded on the SYNAPT mass spectrometer):









For ^{18}O-labeled compounds, the ^{13}C chemical shift of the carbon directly attached to the oxygen is known to shift upfield slightly (~0.05 ppm); therefore, for comparison, the ^{13}C chemical shifts of the carbon attached to the ^{18}O-atom are reported to four decimal places in some of the ^{18}O-labeled compounds.[5]

For the synthesis of 19-deutero-19-oxo androgens, pyridinium dichromate (PDC) was used to oxidize the 19-deutero-19-hydroxy androgen precursor because of a higher isotope effect with the PDC reagent (comparing deuterium enrichment in the Akhtar[6] report and the Numazawa[7] report).

In order to increase the amount of deuterated isotopomer in the 19-deutero-19-oxo androgen product, a 19,19-dideutero-19-hydroxy androgen was used as the final precursor before oxidation with PDC.

When deprotecting the Alloc-group in the synthesis of 19-deutero-19-oxotestosterone (**25**), initial conditions with $Pd(PPh_3)_4$ for the Tsuji-Trost type deallylation resulted in

difficulties of removing triphenylphosphine oxide. Therefore, conditions were optimized to use a mixture of $Pd(DBA)_2$ and PMe_3 as the palladium source.

The diazo reagent was designed so that the $-N_2$ group was not on the benzylic position, because the formate group is cleaved in ESI positive mode when 2-nitrobenzyl formate was used in the preliminary studies.

Katritzky's reagent was used to access the monosubstituted urea.[8]

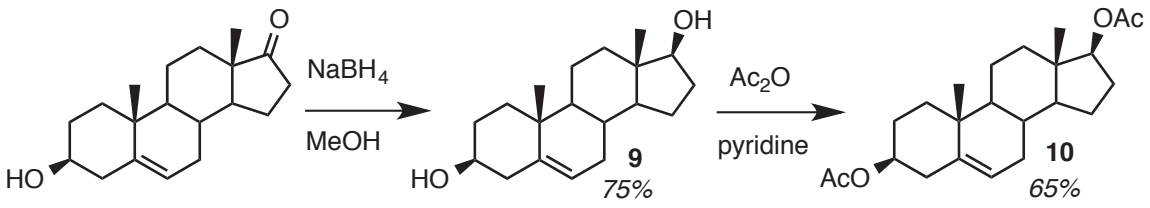
Kirchner *et al*. protocol was followed for the transformation of urea (**32**) to the nitrosourea compound (**33**), and in turn, to the diazo compound.[9]

**SAFETY*:

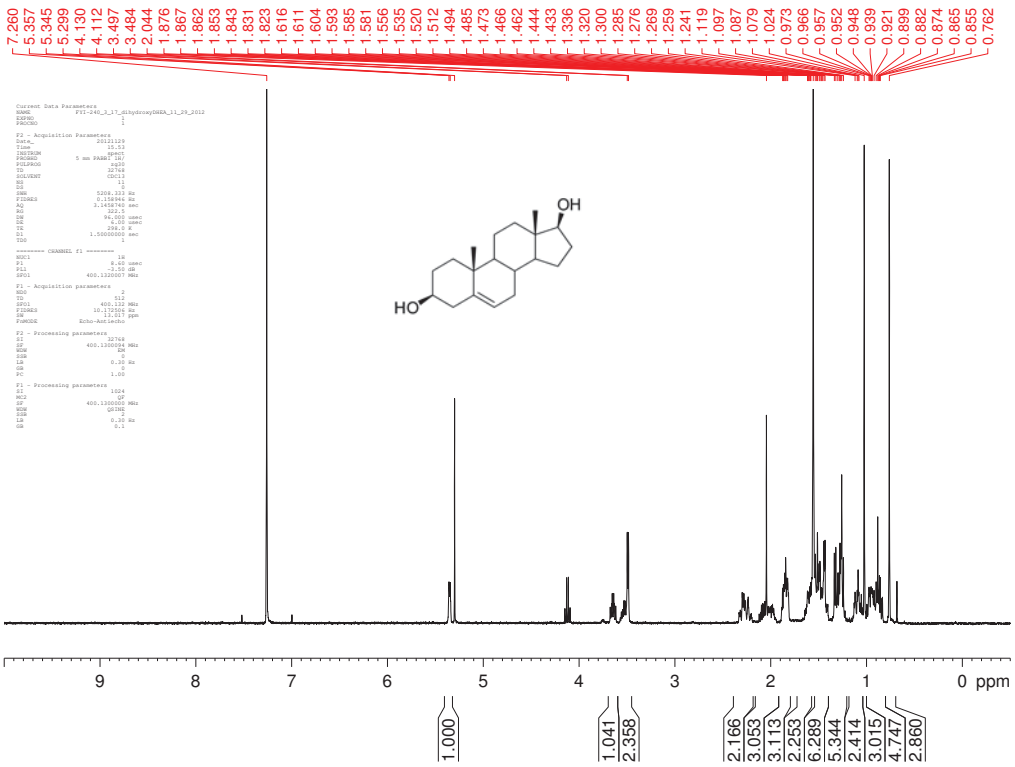
N-Nitrosourea compounds are carcinogenic. Extra safety precautions were taken when handling the *N*-nitrosourea (**33**) to minimize exposure: ventilated hoods were used when working with these compounds and as with any chemicals, a labcoat, eye protection, and double gloves were used.

Diazo compounds are explosive. When synthesizing diazo compound **2**, the use of glassware with ground glass joints were avoided. The temperature the compound was exposed to never reached above room temperature. The compound was not synthesized on a large scale.

Individual syntheses (Supporting Information Figures S1, S2):



4.1. Androst-5-en-3β,17β-diol (9). $NaBH_4$ (0.5 g, 13.2 mmol) was added to a stirring solution of DHEA (2.0 g, 6.9 mmol) in CH_3OH (50 mL) and the reaction was stirred for 30 min at 23 °C. The reaction mixture was concentrated *in vacuo* and filtered through a column of silica gel (50% hexanes/EtOAc (v/v) to 100% EtOAc) to afford androst-5-en-3β,17β-diol (**9**) (1.5 g, 75%). The crude material could be used (without column filtration with silica gel) for the next step (i.e. diacetylation at the 3- and 17-hydroxy positions). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.36-5.35 (m, 1H), 3.67-3.62 (m, 1H), 3.56-3.49 (m, 1H), 2.33-2.20 (m, 2H), 2.12-1.96 (m, 2H), 1.88-1.82 (m, 1H), 1.64-1.40 (m, 8H), 1.33-1.24 (m, 2H), 1.14-1.04 (m, 2H), 1.02 (s, 3H), 1.00-0.91 (m, 2H), 0.76 (s, 3H); HRMS (*m*/*z*): calcd for $C_{19}H_{31}O_2$, $[M+H]^+$, 291.2319; found, 291.2318 (Δ 0.3 ppm).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0.865
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0.762
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9
8
7
6
5
4
3
2
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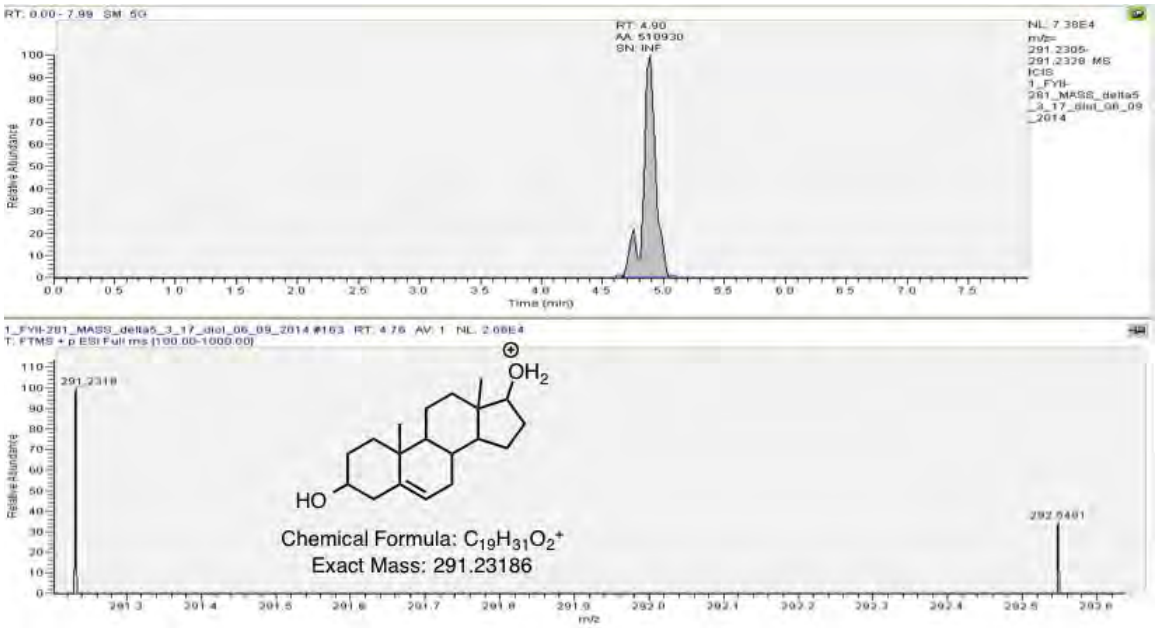
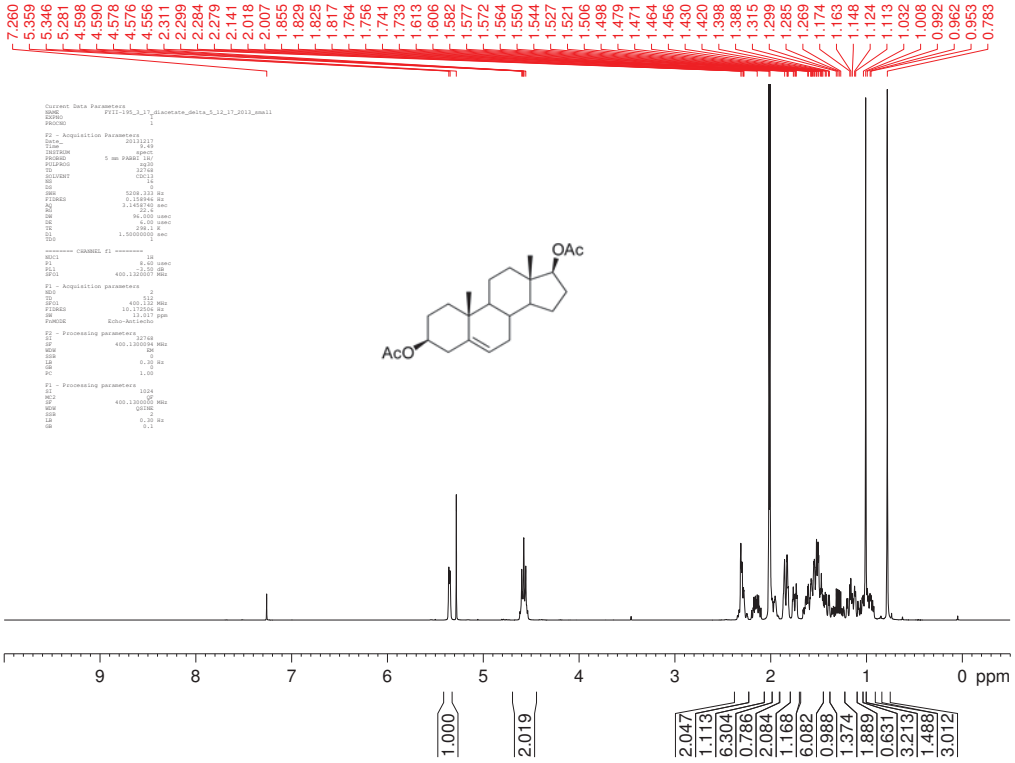


4.2. Androst-5-en-3β,17β-diyl diacetate (10). Androst-5-en-3β,17β-diol (**9**) (5.0 g, 17 mmol) was dissolved in pyridine (10 mL) and acetic anhydride (40 mL) and 4-dimethylaminopyridine (500 mg) were added. The resulting solution was stirred under reflux for 10 h. The reaction mixture was diluted with EtOAc (100 mL) and washed with HCl (1 M, 100 mL) and subsequently with $NaHCO_3$ (sat. aqueous solution, 3 × 100 mL). The organic layer was concentrated *in vacuo* and the resulting crude material was purified by flash column chromatography (100% hexanes to 50% hexanes/EtOAc, v/v) to afford the diacetate (**10**) (4.0 g, 11 mmol, 65%) as a white solid. R_f 0.80 (hexanes:EtOAc, 4:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38-5.37 (m, 1H, H-6), 4.62-4.58 (m, 2H, H-3, H-17), 2.34-2.30 (m, 2H), 2.22 (s, 3H), 2.20-2.16 (m, 1H), 2.04 (s, 3H), 2.03 (s, 3H), 1.99-1.97 (m, 2H), 1.89-1.84 (m, 2H), 1.79-1.74 (m, 1H), 1.66-1.41 (m, 7H), 1.41-1.30 (m, 1H), 1.23-1.03 (m, 1H), 1.01 (s, 3H), 1.00-0.95 (m, 1H), 0.80 (s, 3H); ^{13}C NMR (100

MHz, $CDCl_3$) δ 170.8, 170.1, 166.2, 139.5, 122.0, 82.5, 73.6, 50.8, 49.8, 42.2, 37.9, 36.8, 31.5, 31.2, 27.5, 27.3, 23.4, 21.9, 21.1, 20.9, 20.3, 19.1, 11.7; HRMS (*m*/*z*): calcd for $C_{23}H_{38}NO_4$, $[M+NH_4]^+$, 392.2795; found, 392.2801 (Δ 1.5 ppm).



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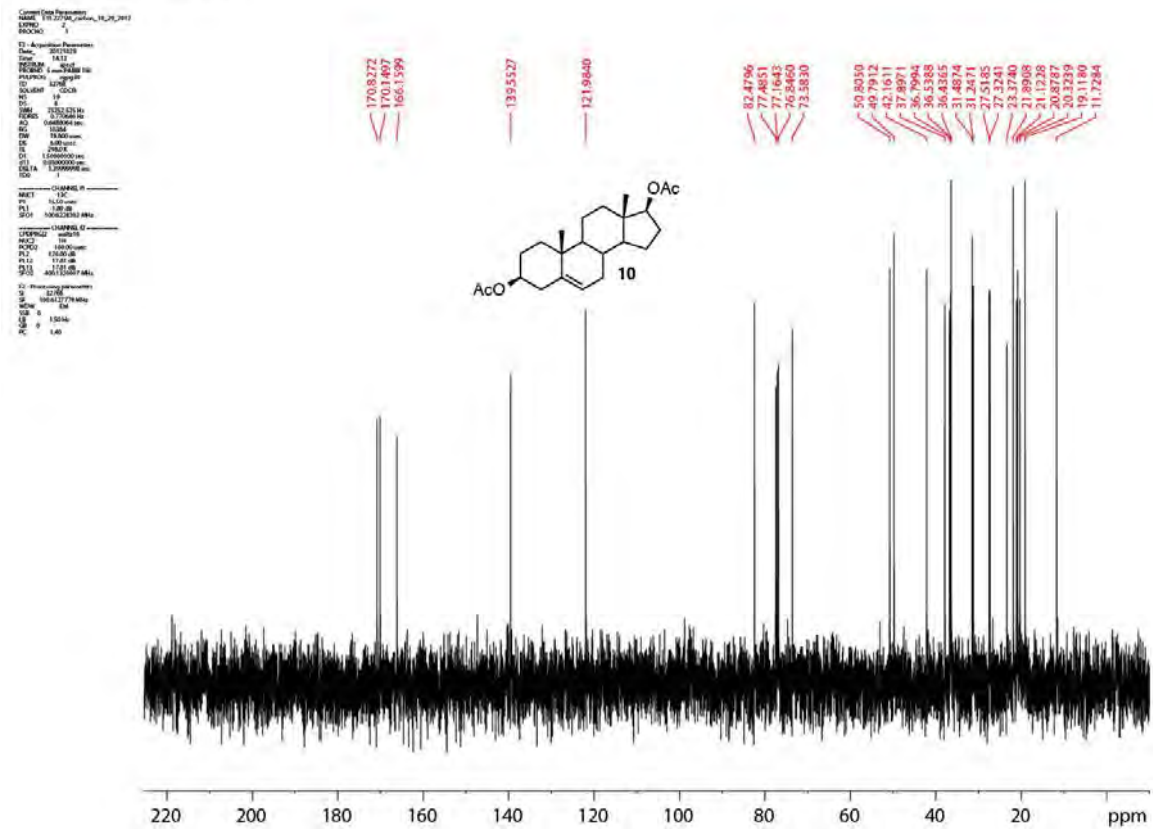
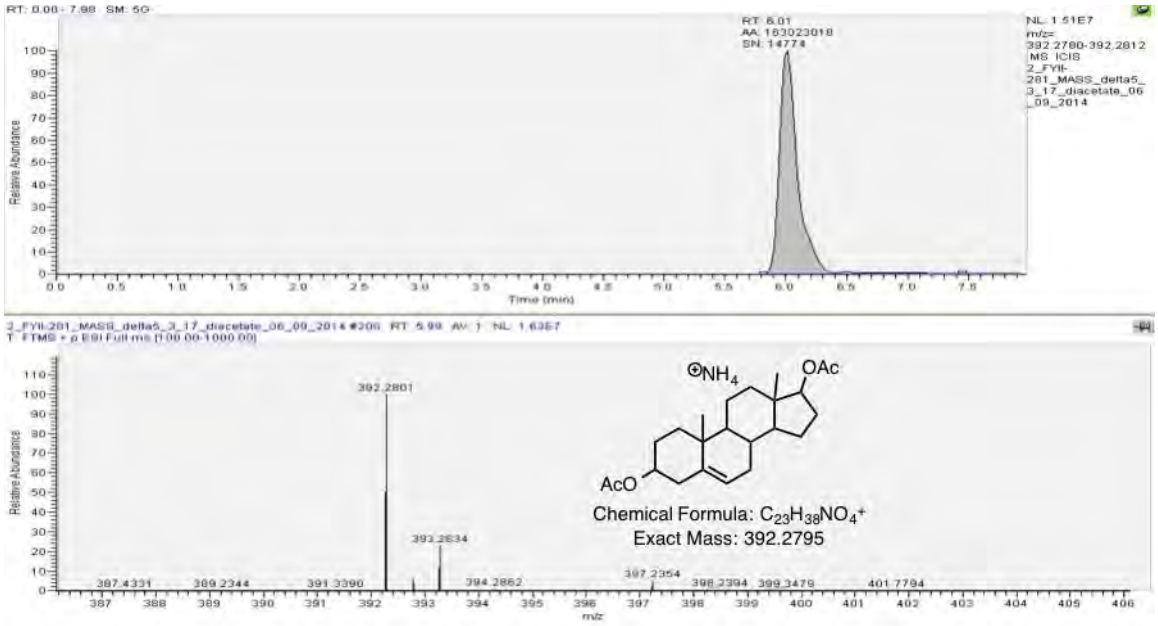
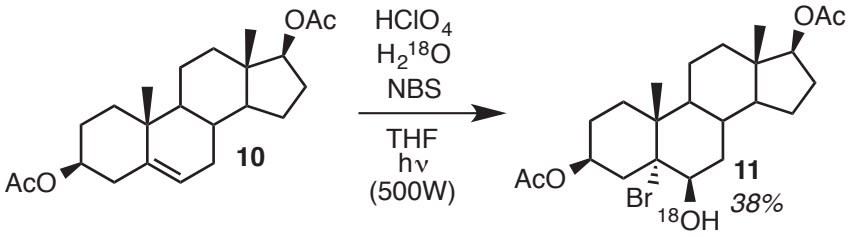


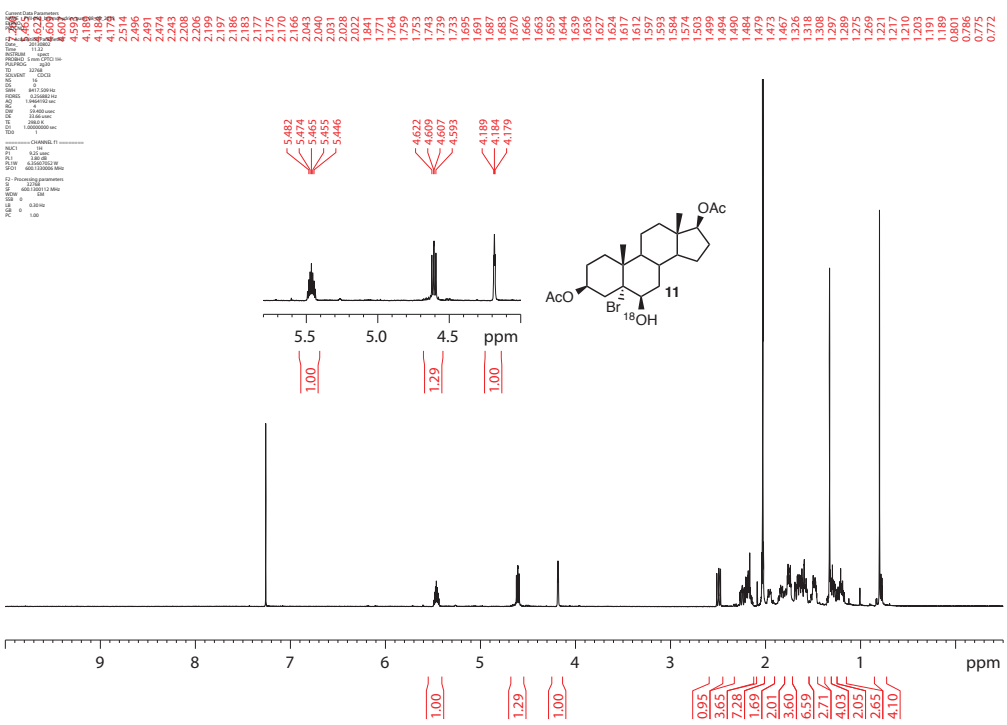




4.3. [6-^{18}O]-Androst-5-en-3β,17β-diyl diacetate 5,6-bromohydrin (11). To a solution of the above diacetate (**10**) (2.60 g, 6.95 mmol), *N*-bromosuccinimide (1.24 g, 6.95 mmol), $H_2{}^{18}O$ (1.2 mL) in THF was added, plus $HClO_4$ (0.05 mL, 10% in THF, v/v), and the reaction was stirred at 23 °C for 5 h. The reaction mixture was directly loaded on a silica gel column and filtered. The isolated material was concentrated *in vacuo* and purified via flash column chromatography (100% hexanes to 50% hexanes/EtOAc, v/v) to afford the bromohydrin (**11**) (1.25 g, 2.65 mmol, 38%). R_f 0.11 (hexanes:EtOAc, 4:1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.49-5.44 (m, 1H, H-3), 4.61 (dd, J_1 = 9.1, J_2 = 7.9 Hz, 1H, H-17), 4.18 (apparent t, J = 2.9 Hz, 1H, H-6), 2.49 (dd, J_1 = 13.6, J_2 = 10.6 Hz, 1H), 2.24 (ddd, J_1 = 14.4, J_2 = 12.7, J_3 = 3.7 Hz, 1H), 2.21-2.14 (m, 2H), 2.03 (s, 3H),

1.98-1.94 (m, 1H), 1.87-1.80 (m, 2H), 1.79-1.73 (m, 3H), 1.69-1.56 (m, 6H), 1.52-1.46 (m, 2H), 1.35-1.27 (m, 2H), 1.34 (s, 3H), 1.27-1.17 (m, 3H), 0.80 (s, 3H), 0.80-0.77 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.3, 170.6, 86.4 (C-5), 82.8 (CH, C-17), 75.7 (CH), 72.2 (CH, C-3), 50.3 (CH), 47.6 (CH), 42.9, 40.6, 38.5 (CH_2), 36.8 (CH_2), 35.2 (CH_2), 34.3 (CH_2), 30.6 (CH), 27.7 (CH_2), 26.4 (CH_2), 23.5 (CH_2), 21.5 (CH_3), 21.3 (CH_3), 20.9 (CH_2), 18.1 (CH_3, C-19), 12.4 (CH_3, C-18); HRMS (*m*/*z*): calcd for $C_{23}H_{39}NO_4$, $[M+NH_4]^+$, 490.2049; found, 490.2050 (Δ 0.2 ppm).

FYII-090_bromohydrin_pure_08_02_2013



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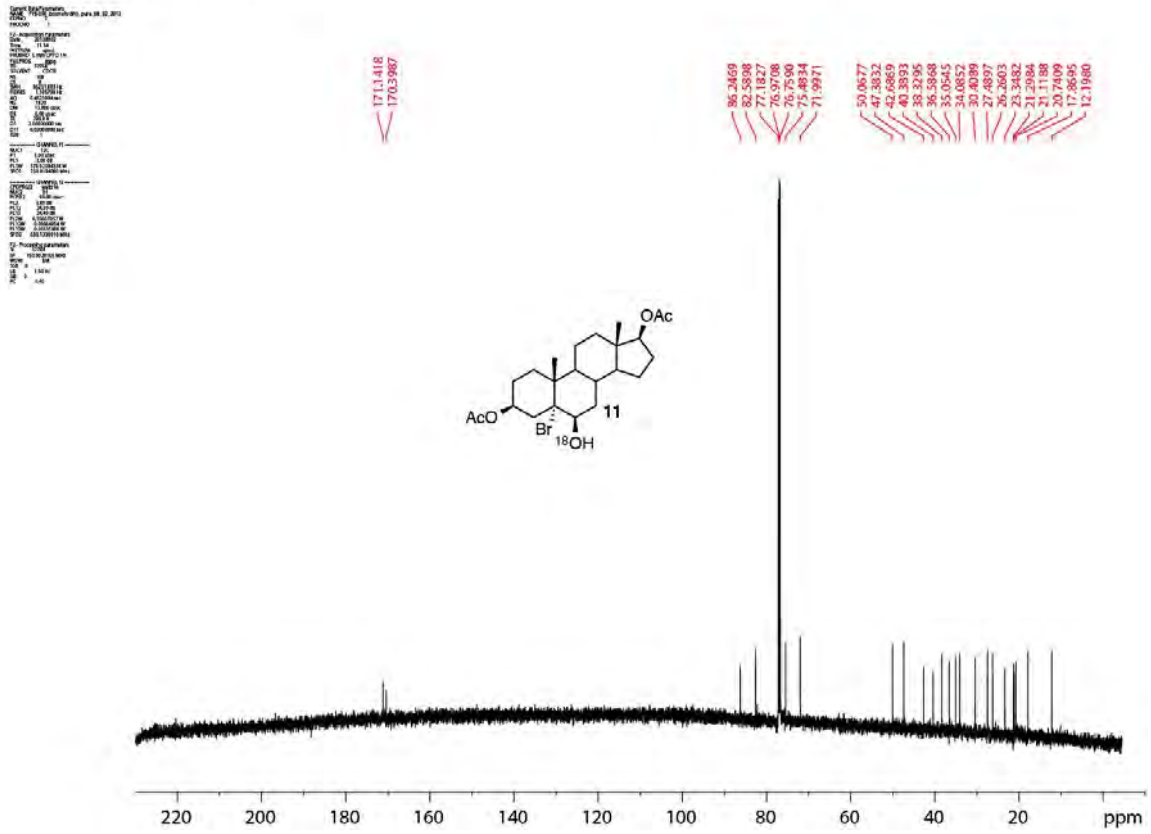

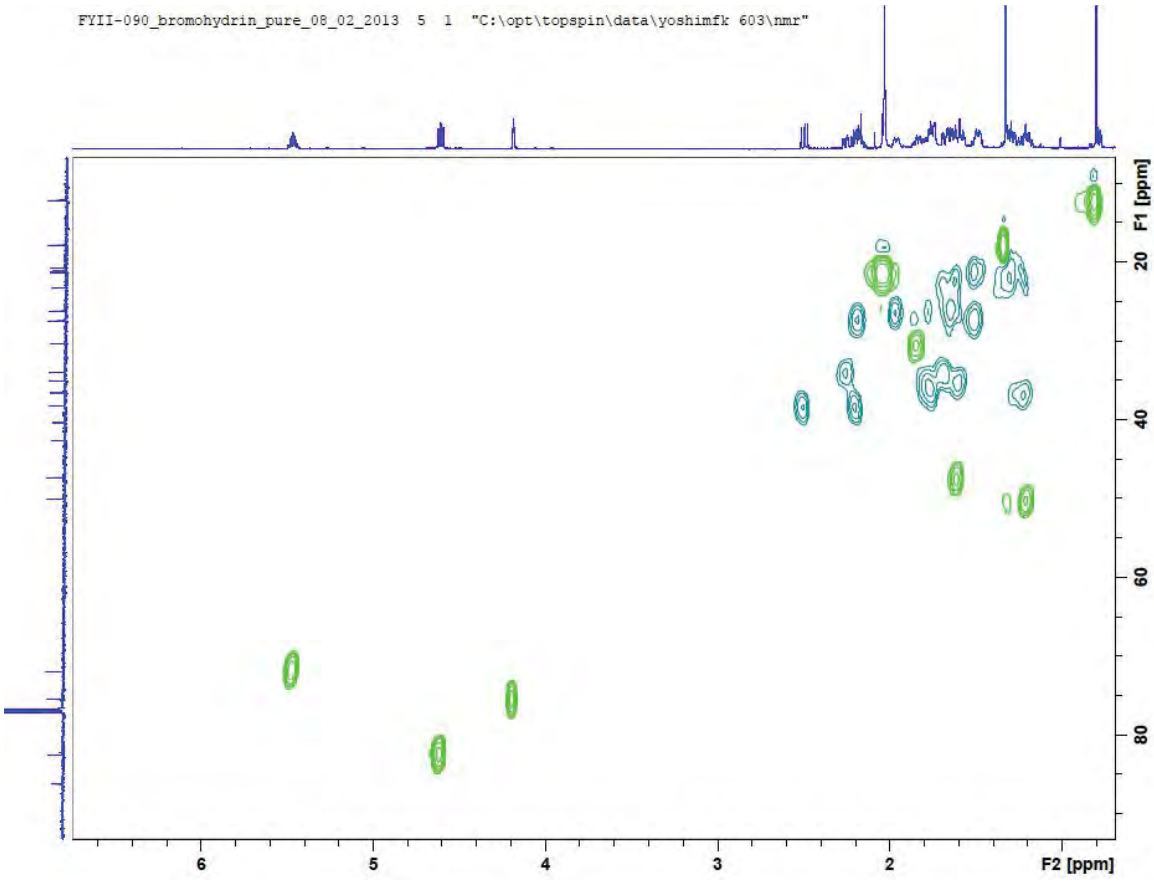
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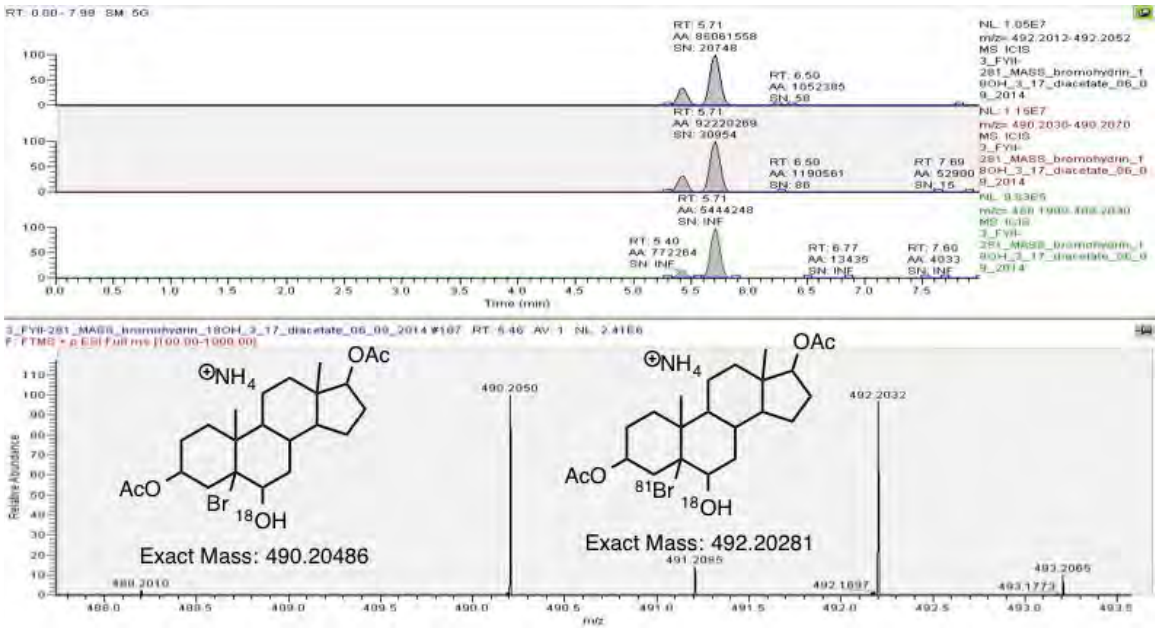
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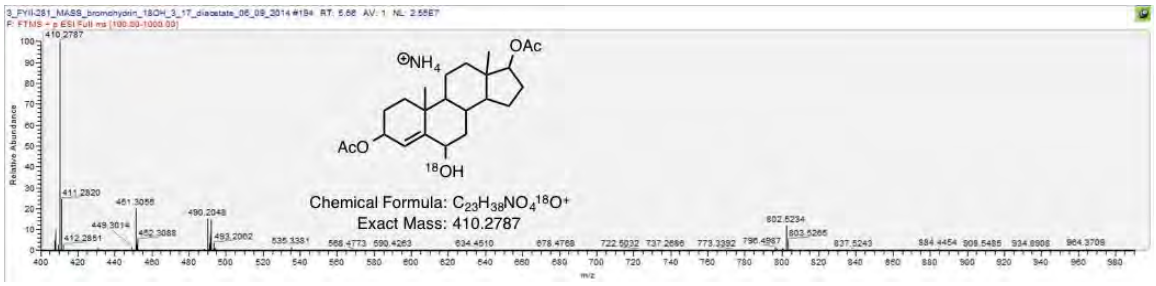
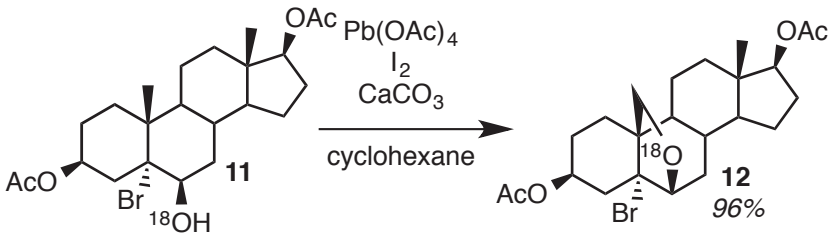
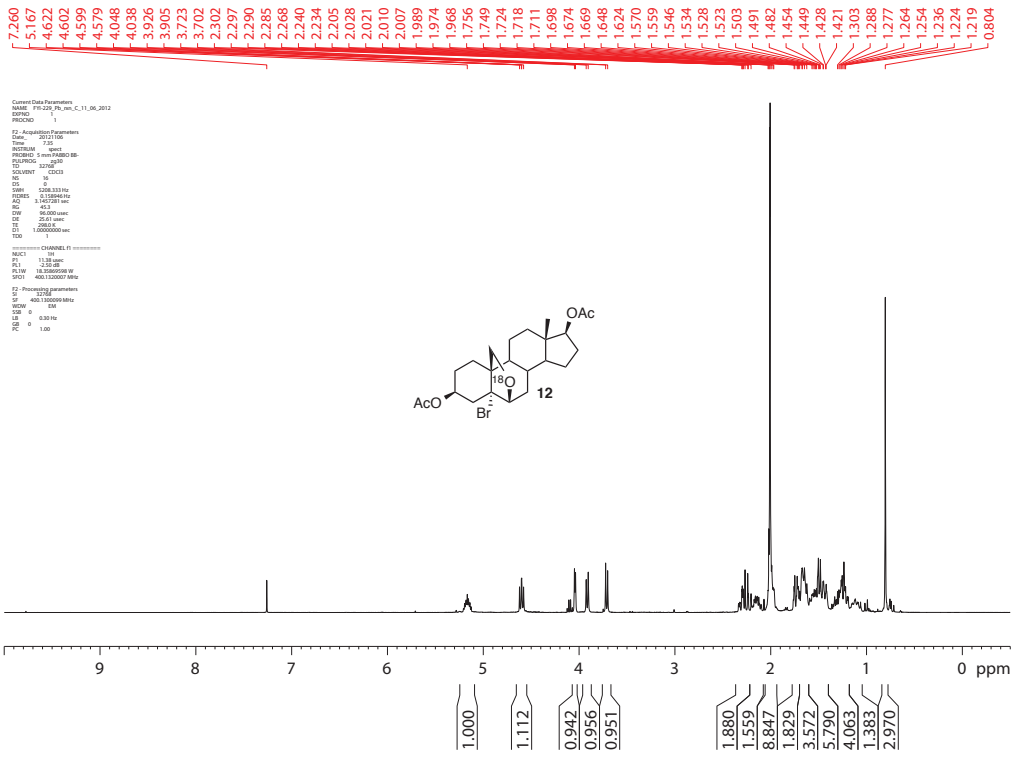
m/z





4.4. [19-^{18}O]-Androst-5-en-3β,17β-diyl diacetate oxacycle (12). The above bromohydrin (**11**) (1.25 g, 2.65 mmol), $Pb(OAc)_4$ (4.70 g, 10.6 mmol, 4 mol eq), $CaCO_3$ (1.85 g, 18.5 mmol, 7 mol eq), and I_2 (1.00 g, 3.97 mmol, 1.5 mol eq) were added to cyclohexane (50 mL). The flask was equipped with a reflux condenser and the reaction mixture was stirred at reflux in the presence of light (500 W). The reaction mixture was filtered through a silica gel column and purified via flash column chromatography (100% hexanes to 50% EtOAc/hexanes, v/v) to afford the oxacycle (**12**) (1.20 g, 2.54 mmol, 96%). R_f 0.22 (hexanes:EtOAc, 4:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.19-5.13 (m, 1H), 4.60 (dd, J_1 = 9, J_2 = 8 Hz, 1H, H-17), 4.04 (d, J = 4 Hz, 1H), 3.92 (d, J = 8 Hz, 1H), 3.71 (d, J = 8 Hz, 1H), 2.31 (ddd, J_1 = 13.9, J_2 = 4.7, J_3 = 1.8 Hz, 1H), 2.24 (dd, J_1 = 13.8, J_2 = 11.2, 1H), 2.20-2.10 (m, 1H), 2.01 (s, 3H), 2.00 (s, 3H), 1.77-1.70 (m, 1H), 1.70-1.60 (m, 3H), 1.60-1.41 (m, 5H), 1.38-1.19 (m, 4H), 1.17-1.06 (m, 1H), 0.80 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.6, 170.4, 82.4, 82.2, 74.3, 70.0, 67.6, 48.9, 48.8, 46.0,

43.4, 41.4, 36.8, 33.2, 27.7, 26.9, 23.4, 23.0, 22.2, 21.4, 21.2, 12.6; HRMS (*m*/*z*): calcd for $C_{23}H_{37}BrNO_4{}^{18}O$, $[M+NH_4]^+$, 488.1892; found, 488.1890 (Δ 0.4 ppm).



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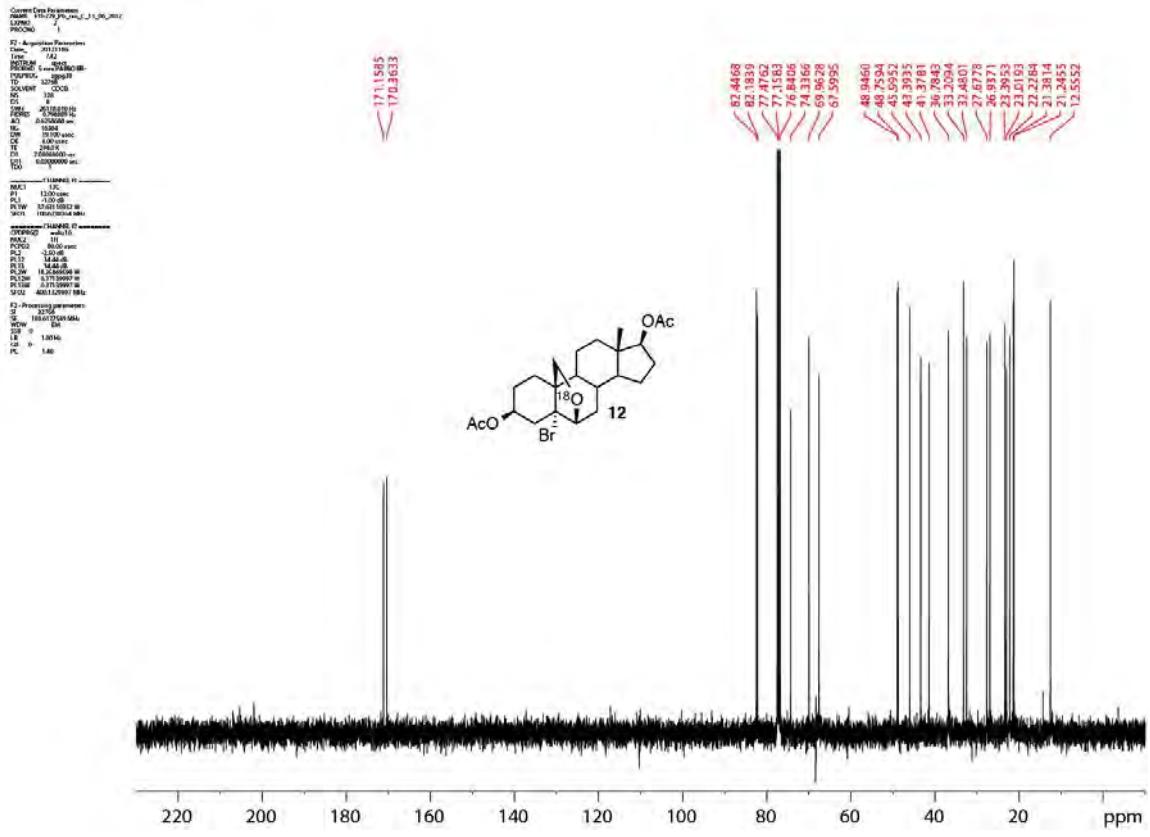
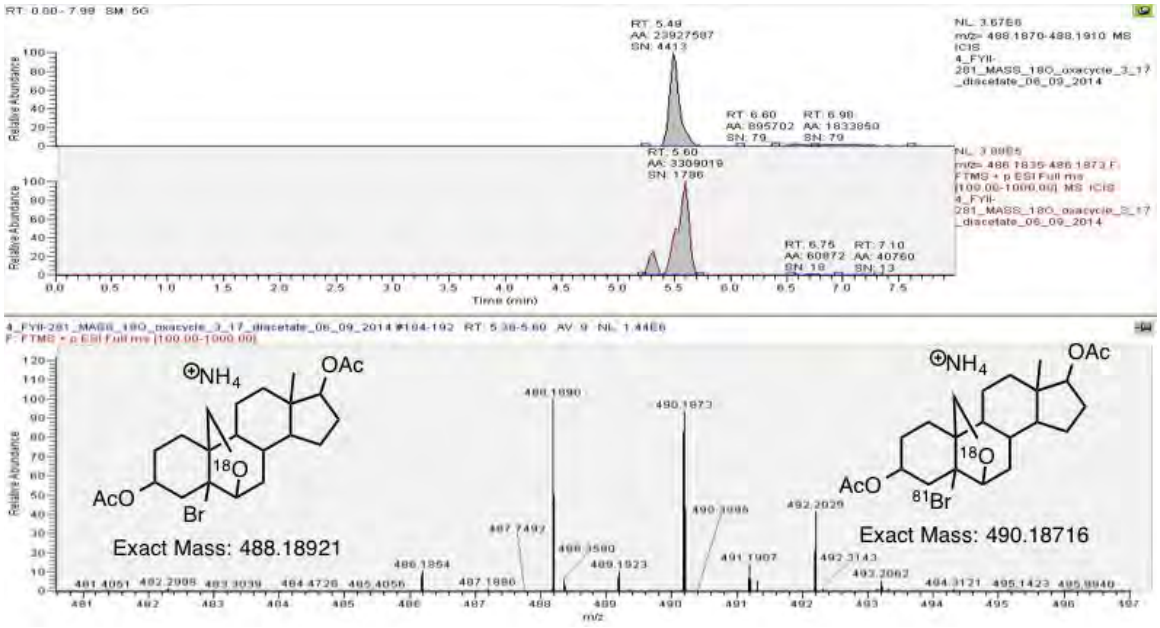
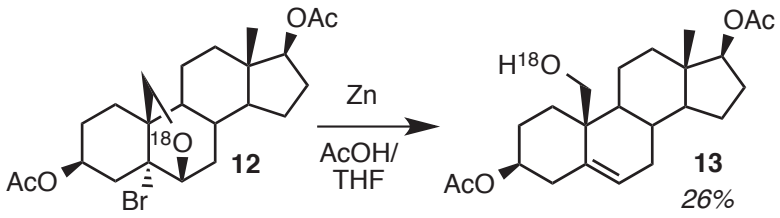
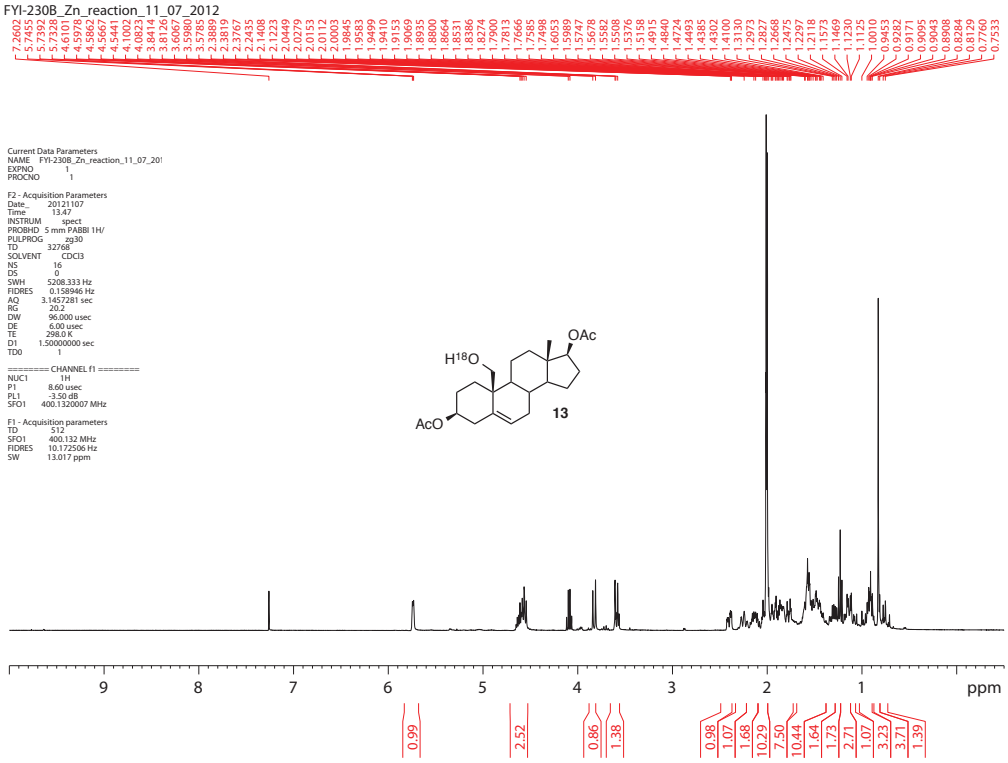


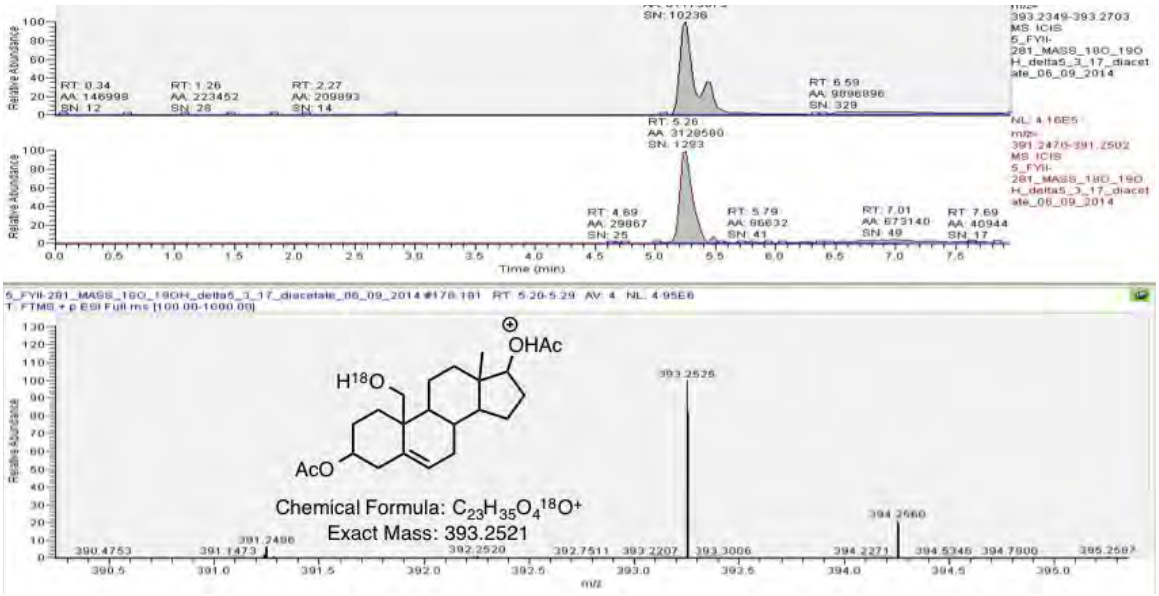


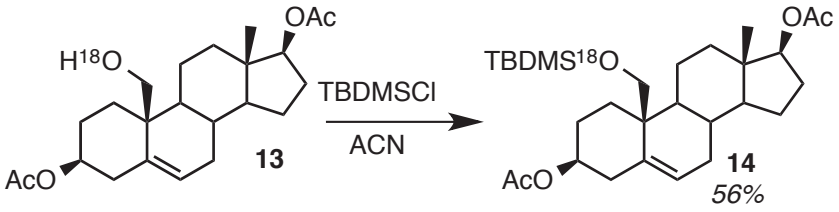


4.5. [19-^{18}O]-19-Hydroxyandrost-5-en-3β,17β-diyl diacetate (13). Zn dust (1.5 g) was added to a solution of oxacycle (**12**) (1.1 g, 2.3 mmol) and formic acid (0.05 mL) in THF (50 mL). The reaction was stirred at reflux for 30 min. The reaction mixture was filtered through a short pad of silica and purified via flash column chromatography (100% hexanes to 50% hexanes/EtOAc, v/v) to afford the alcohol (**13**) (230 mg, 0.59 mmol, 26%). R_f 0.08 (hexanes:EtOAc, 4:1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.34-5.33 (m, 1H), 4.58-4.53 (m, 2H), 2.32-2.24 (m, 2H), 2.16-2.10 (m, 1H), 1.99 (s, 3H), 1.98 (s, 3H),

1.97-1.93 (m, 1H), 1.85-1.81 (m, 2H), 1.75-1.72 (m, 1H), 1.63-1.42 (m, 6H), 1.43-1.36 (m, 1H), 1.31-1.20 (m, 7H), 1.18-1.06 (m, 2H), 1.04-1.00 (m, 1H), 0.99 (s, 3H), 0.97-0.92 (m, 1H), 0.85-0.80 (m, 5H), 0.76 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1, 170.4, 139.8, 122.2 (CH), 82.8 (CH), 73.9 (CH), 51.1 (CH), 50.0 (CH), 42.4, 38.1 (CH_2), 37.0 (CH_2), 36.8 (CH_2), 36.7, 31.7 (CH), 31.6 (CH_2), 31.5 (CH_2), 27.8 (CH_2), 27.6 (CH_2), 23.6, 22.7, 21.4 (CH_3), 21.1 (CH_3), 20.6 (CH_2), 19.4 (CH_3), 14.1 (CH_3), 12.0 (CH_3); HRMS (*m/z*): calcd for $C_{23}H_{35}O_4{}^{18}O$, $[M+H]^+$, 393.2521; found, 393.2525 (Δ 1.0 ppm).

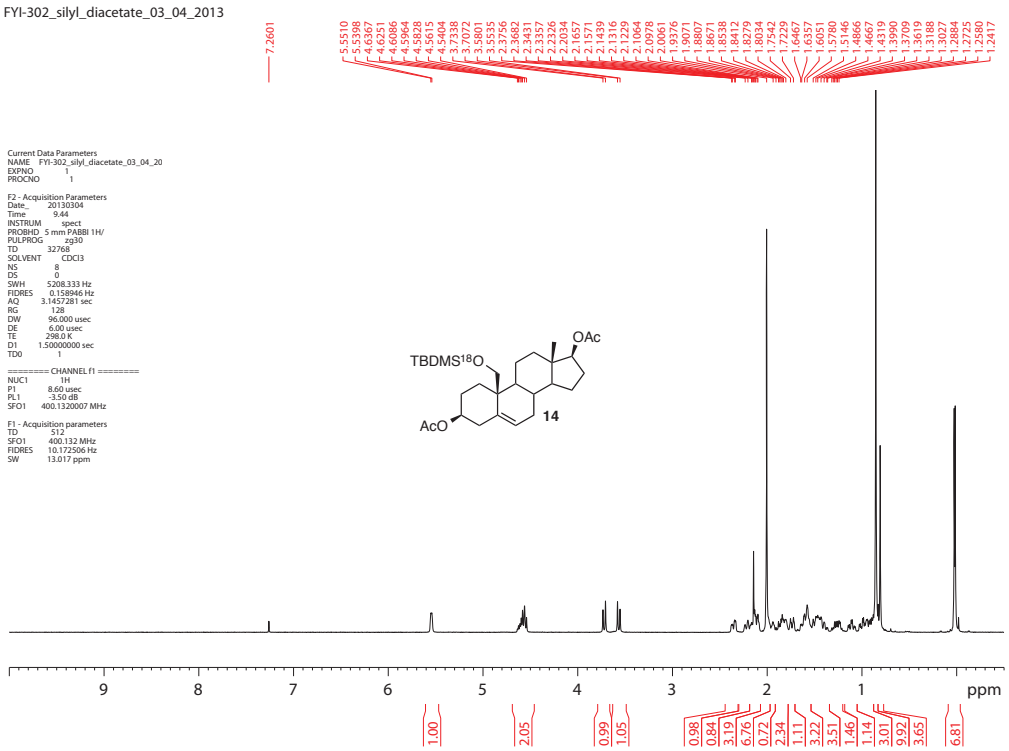






4.6. [19-^{18}O]-19-Hydroxy-TBDMS androst-5-en-3β,17β-diyl diacetate (14). A solution of TBDMSCl (1.00 g), imidazole (0.75 g), and the above alcohol (**13**) (230 mg, 0.59 mmol) were stirred in CH_3CN (50 mL) for 10 h. The reaction mixture was diluted with EtOAc (100 mL) and washed with H_2O (2 × 100 mL). The crude material was purified by flash column chromatography (100% hexanes to 50% hexanes:EtOAc v/v) to afford the silyl ether (**14**) (167 mg, 0.33 mmol, 56%). R_f 0.71 (hexanes:EtOAc, 4:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.55-5.54 (m, 1H), 4.64-4.54 (m, 2H), 3.72, 3.56 (ABq, J = 10.6 Hz, 2H), 2.38-2.33 (m, 1H), 2.23-2.10 (m, 4H), 2.01 (s, 6H), 1.95-1.71 (m, 4H), 1.65-1.56 (m, 3H), 1.51-1.36 (m, 3H), 1.32-1.21 (m, 1H), 1.14-1.08 (m, 1H), 1.03-0.88 (m, 3H), 0.85 (s, 9H), 0.81 (s, 3H), 0.03 (s, 3H), 0.02 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.3, 170.5, 135.8, 125.8 (CH), 82.9 (CH), 73.7 (CH), 63.1 (CH), 51.7 (CH),

50.4 (CH), 42.6, 41.5, 38.6, 37.2, 32.6 (CH), 31.2, 28.2, 27.7, 25.9, 23.6, 21.5, 21.4, 21.3, 18.2, 12.3, -5.50, -5.53.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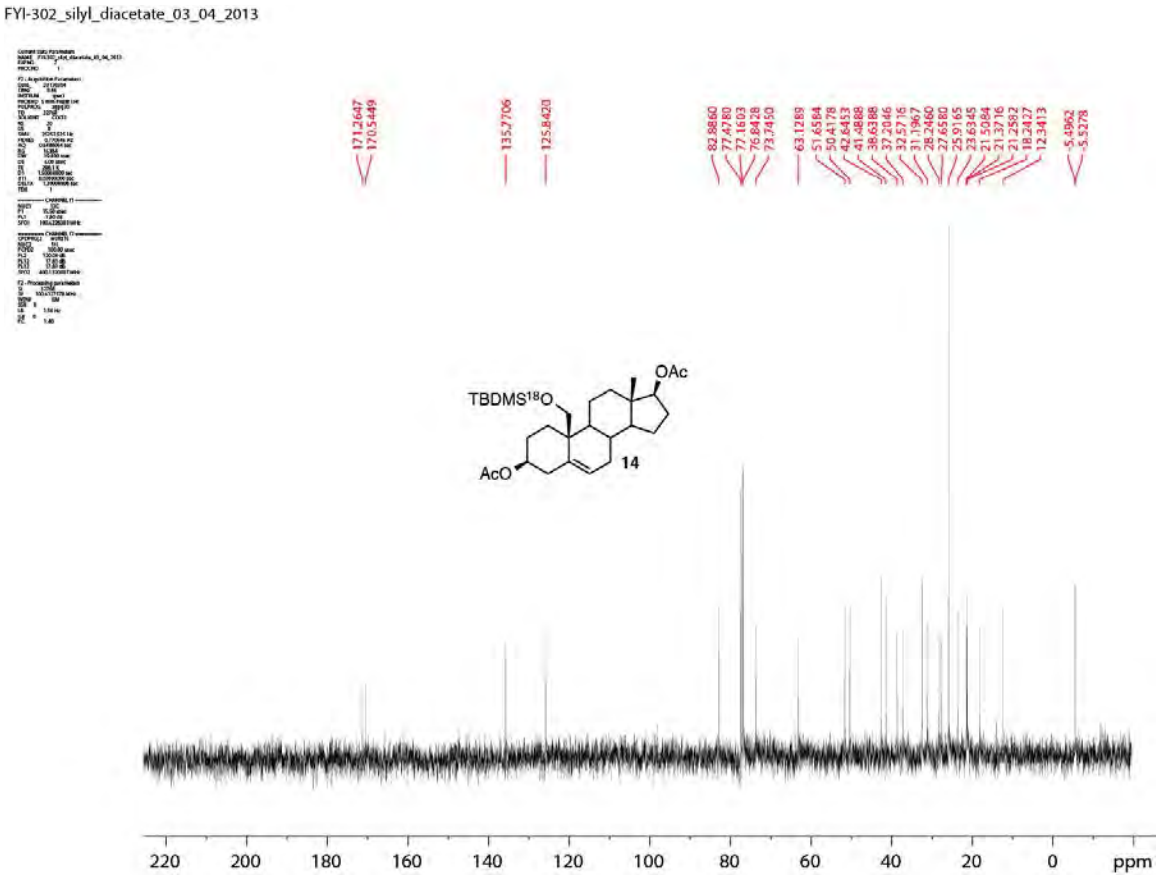
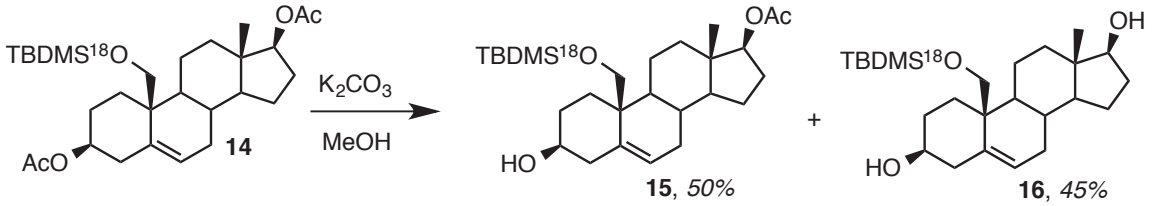
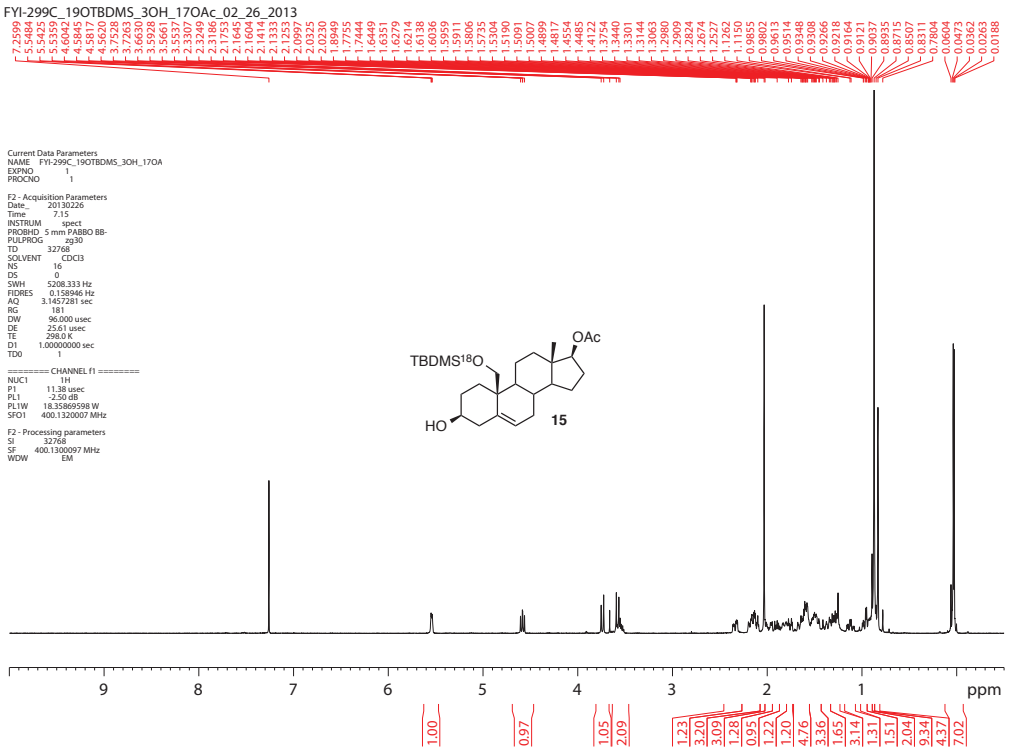




4.7. [19-^{18}O]-19-Hydroxy-TBDMS androst-5-en-3β-hydroxy-17βacetate (15) and [19-^{18}O]-19-Hydroxy-TBDMS androst-5-en-3β,17β-diol (16). K_2CO_3 (88 mg, 0.66 mmol, 0.53 mol eq) was added to a solution of the above diacetate (**14**) (630 mg, 1.24 mmol, 1 mol eq) in CH_3OH (100 mL). The reaction was stirred for 10 h at 23 °C and carefully monitored by TLC. The reaction mixture was concentrated under a stream of air in a water bath at 23 °C. The crude material was purified via flash column

chromatography (100% hexanes to 50% hexanes:EtOAc v/v, to 100% EtOAc) to afford the mono-alcohol (**15**) (153 mg, 0.33 mmol, 50%) and diol (**16**) (140 mg, 0.30 mmol, 45%).

[19-^{18}O]-19-Hydroxy-TBDMS androst-5-en-3β-hydroxy-17β-acetate (15): R_f 0.41 (hexanes:EtOAc, 1:1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.50 (m, 1H), 4.56 (apparent t, J = 8.9 Hz, 1H), 3.71, 3.54 (ABq, J = 10.6 Hz, 2H), 3.54-3.48 (m, 1H), 2.30 (ddd, J_1 = 10.8, J_2 = 4.6, J_3 = 2.3 Hz, 1H), 2.15-2.05 (m, 4H), 1.99 (s, 3H), 1.97-1.92 (m, 1H), 1.90-1.84 (m, 1H), 1.78-1.76 (m, 1H), 1.73-1.71 (m, 1H), 1.66-1.53 (m, 2H), 1.49-1.43 (m, 2H), 1.36-1.21 (m, 2H), 1.11-1.06 (m, 1H), 0.94-0.88 (m, 2H), 0.84 (s, 9H), 0.80 (s, 3H), 0.00 (s, 3H), -0.01 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.4, 136.9, 124.7 (CH), 82.9 (CH), 71.4 (CH), 63.2 (CH_2), 51.7 (CH), 50.5 (CH), 42.6 (CH_2), 41.4, 37.2 (CH_2), 32.8 (CH_2), 32.6 (CH), 32.0 (CH_2), 31.2 (CH_2), 27.6 (CH_2), 25.9 (CH), 23.6 (CH_2), 21.4 (CH_2), 21.2 (CH_3), 18.2, 12.3 (CH_3), -5.5, -5.6.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3.09
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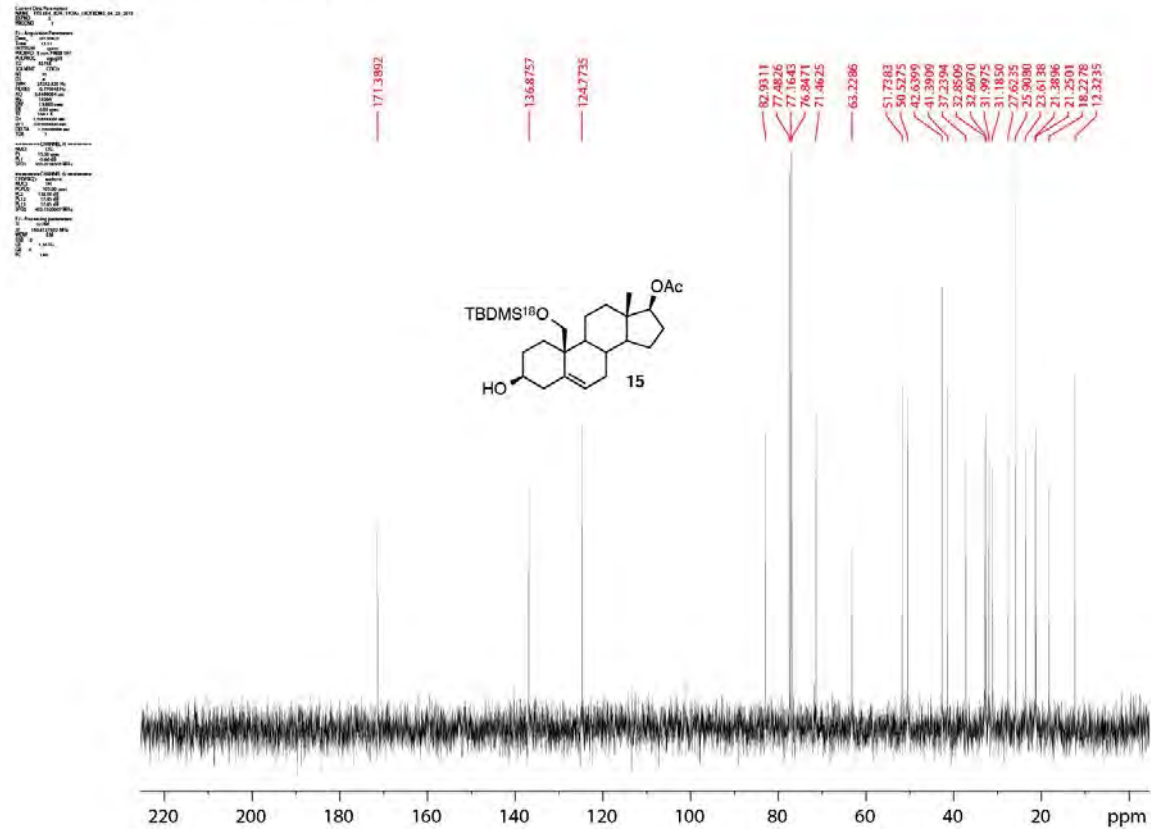
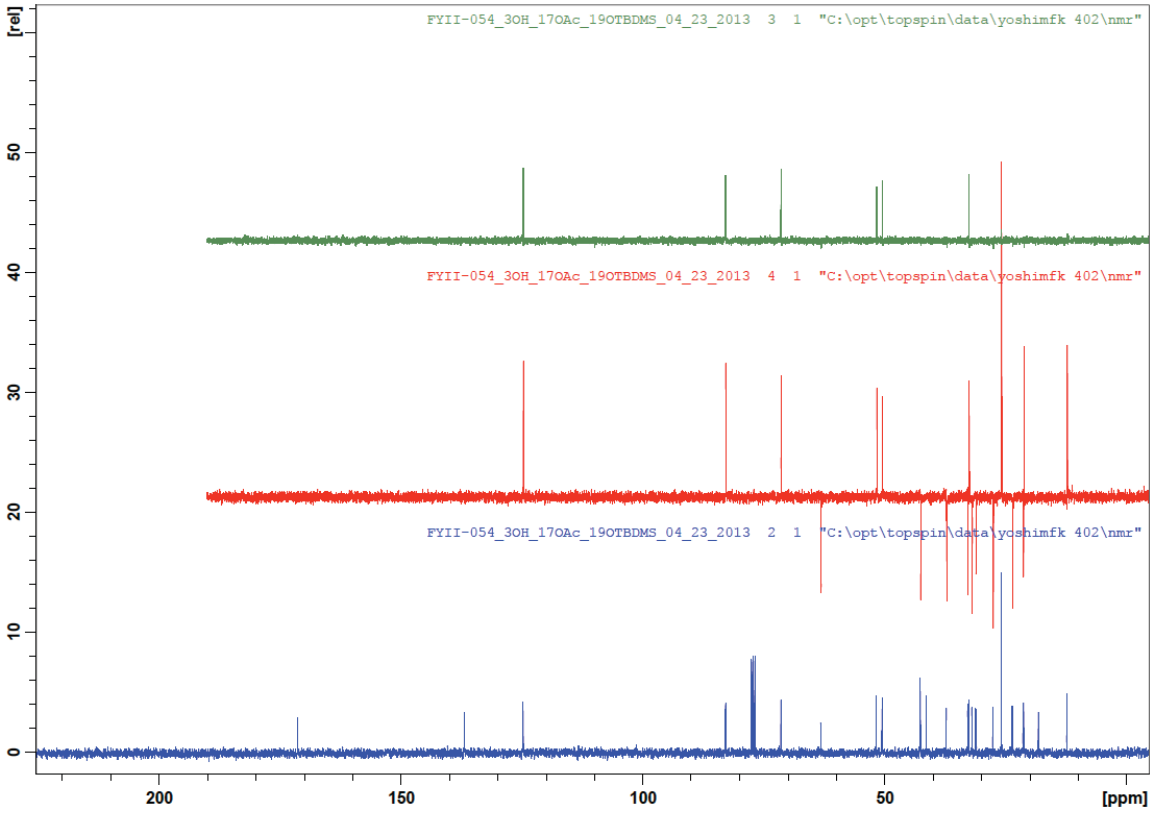
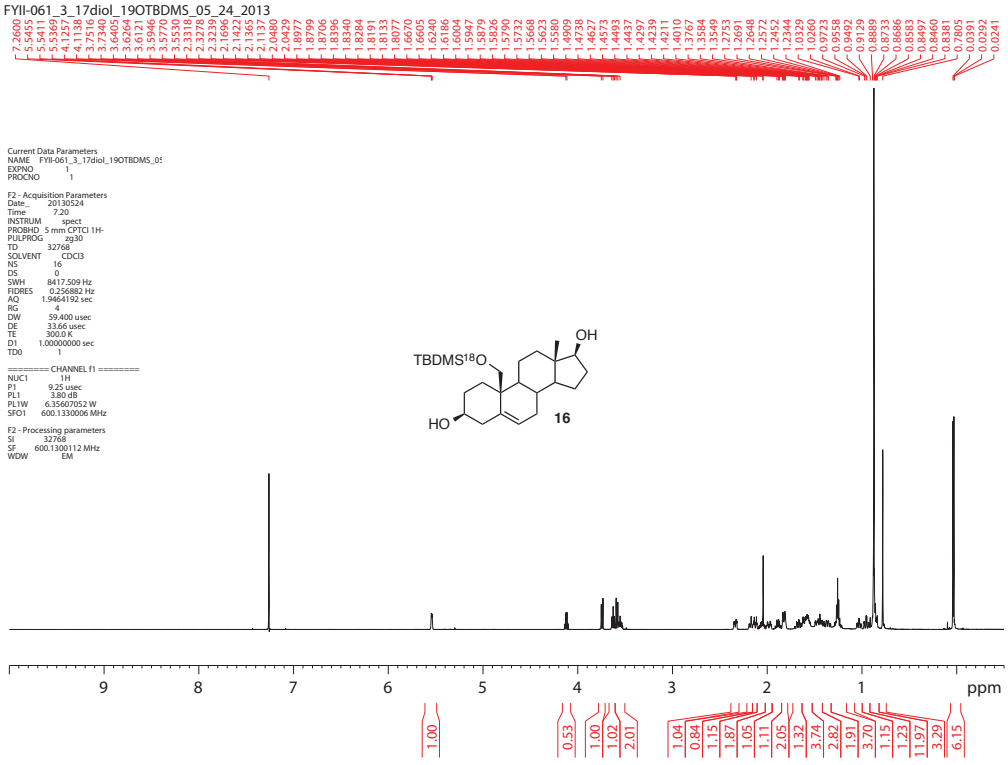
4.37
7.02

FYII-054_3OH_17OAc_19OTBDMS_04_23_2013





[19-^{18}O]-19-Hydroxy-TBDMS androst-5-en-3β,17β-diol (16): R_f 0.19 (hexanes:EtOAc, 1:1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.46-5.41 (m, 1H), 3.74 (d, J = 10.6 Hz, 1H), 3.63 (apparent t, J = 8.5 Hz, 1H), 3.59 (d, J = 10.6 Hz, 1H), 3.56-3.53 (m, 1H), 2.34 (ddd, J_1 = 13.0, J_2 = 4.7, J_3 = 2.4 Hz, 1H), 2.19-2.15 (m, 1H), 2.13 (dt, J_1 = 13.6, J_2 = 3.5 Hz, 1H), 2.09-2.02 (m, 1H), 1.98 (dtd, J_1 = 17.6, J_2 = 5.3, J_3 = 2.6 Hz, 1H), 1.88 (qd, J_1 = 10.8, J_2 = 5.5 Hz, 1H), 1.84-1.79 (m, 2H), 1.67 (qd, J_1 = 13.0, J_2 = 4.1 Hz, 1H), 1.63-1.55 (m, 3H), 1.50-1.41 (m, 2H), 1.41-1.33 (m, 2H), 1.29-1.21 (m, 2H), 1.03 (td, J_1 = 12.8, $_2$ = 4.2 Hz, 1H), 0.98-0.92 (m, 1H), 0.89-0.84 (m, 2H), 0.87 (s, 9H), 0.78 (s, 3H), -0.04 (s, 3H), -0.29 (s, 3H).



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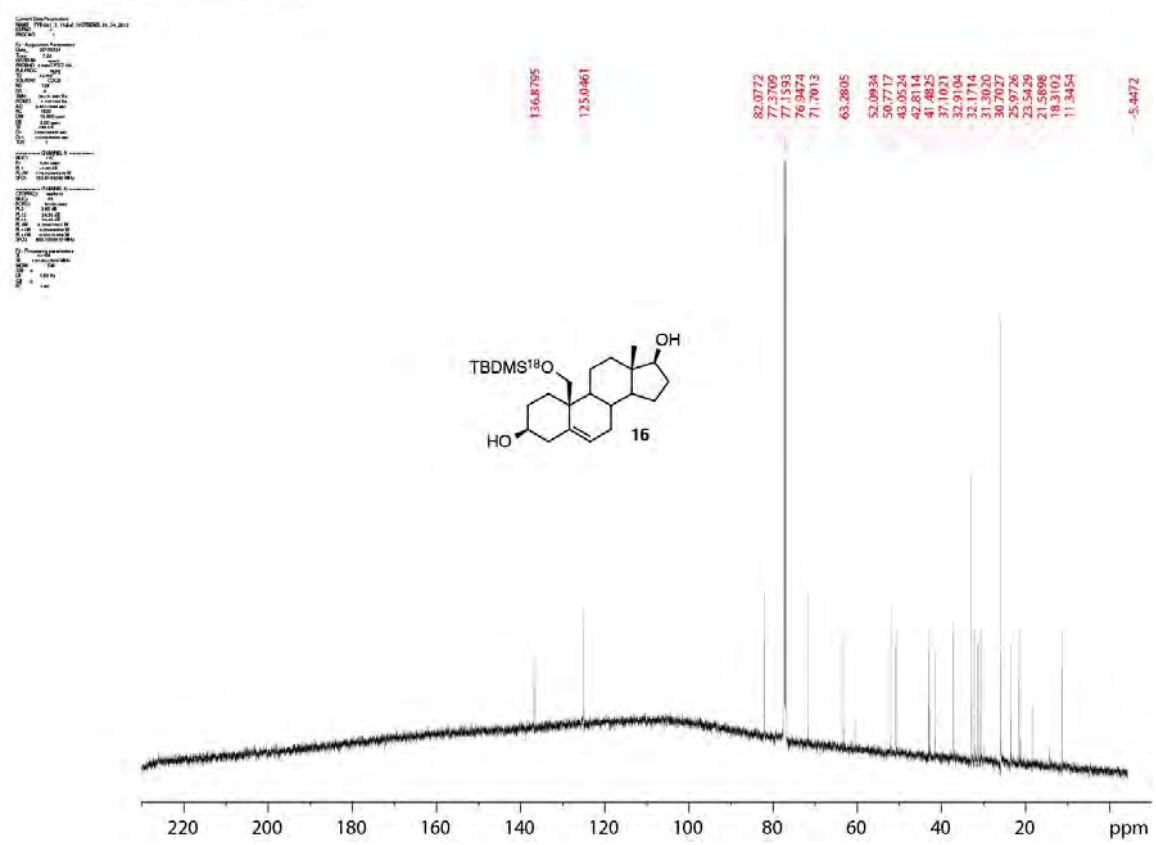
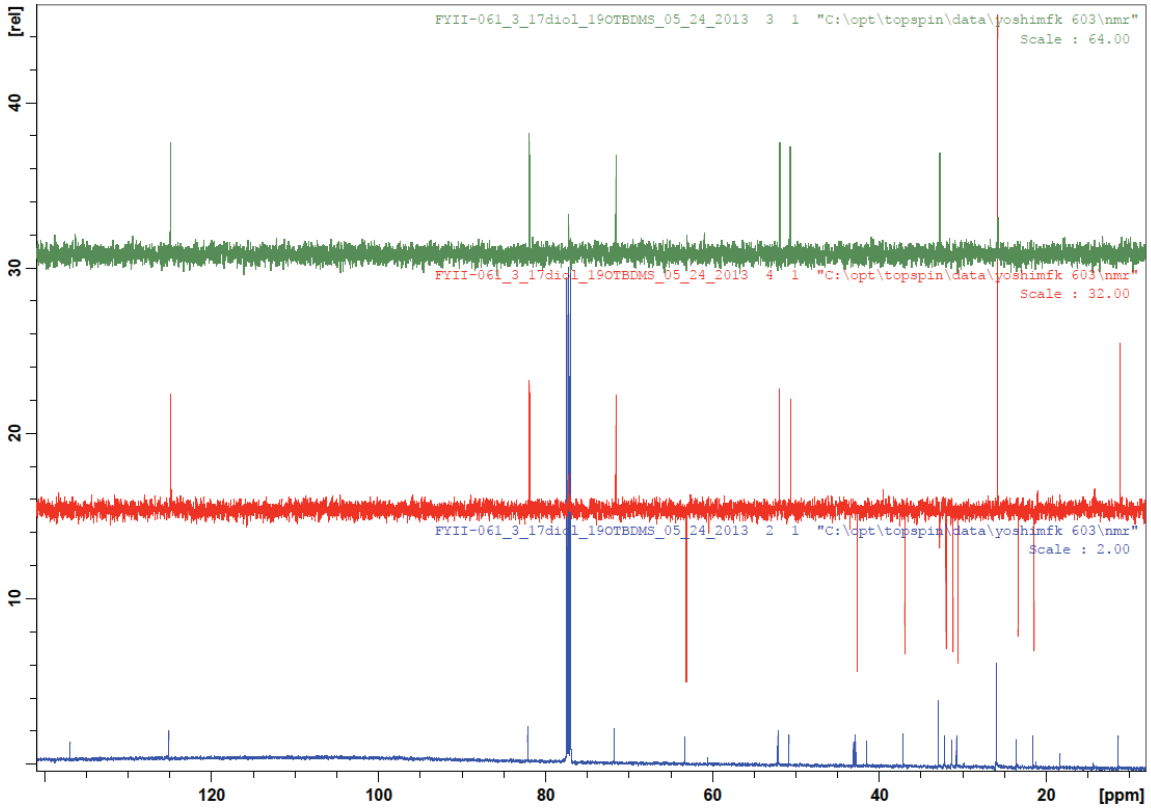
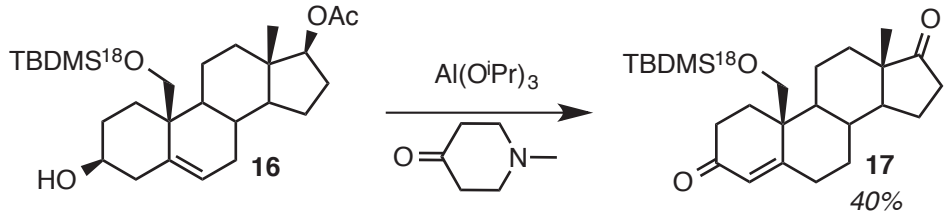
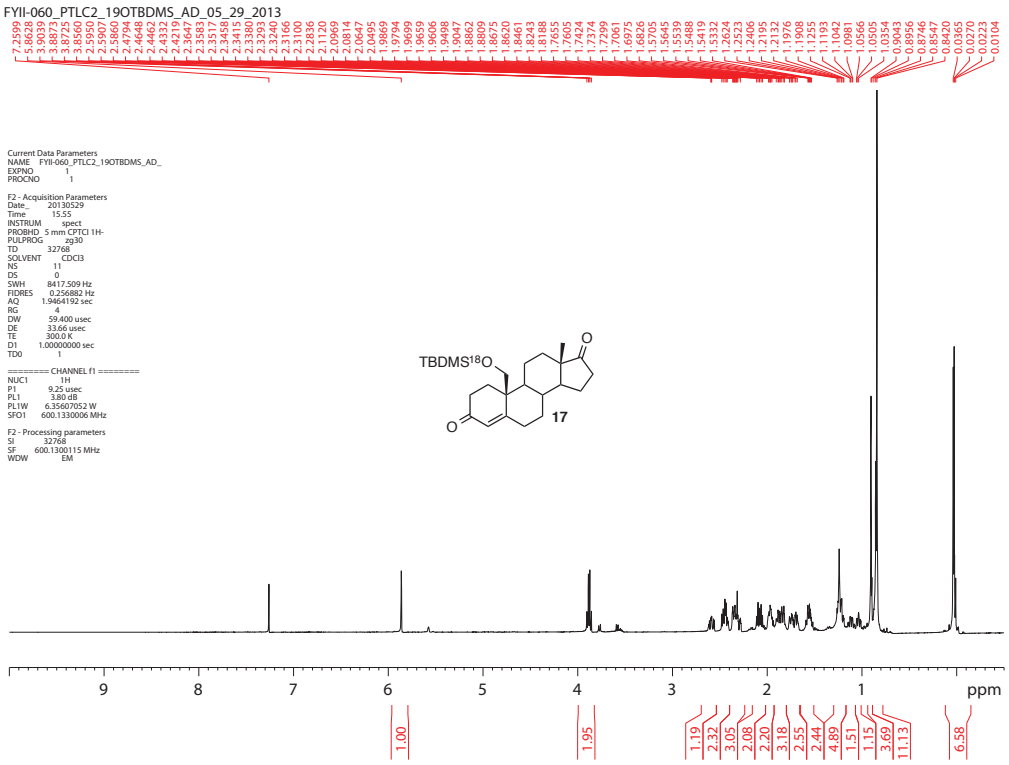






4.8. [19-^{18}O]-19-Hydroxy-TBDMS androst-4-en-3-one (17). A solution of *N*-methylpiperidone (2.0 mL, 16.3 mmol, 54 mol eq) and the above alcohol (**16**) (0.14 g, 0.30 mmol, 1.0 mol eq) in toluene (150 mL) was heated under reflux for 30 min in a round bottom flask equipped with a Dean-Stark trap and condenser. After 25 mL of solvent was condensed, aluminum (III) isopropoxide (600 mg, 2.9 mmol, 9.7 mol eq) was added to the reaction mixture and the reaction was heated at reflux for 2 h. The concentrated reaction mixture was purified via flash column chromatography to afford the diketone (**17**) (48 mg, 0.12 mmol, 40%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.86 (m, 1H), 3.90, 3.86 (ABq, J = 9.6 Hz, 2H), 2.62-2.56 (m, 1H), 2.48-2.41 (m, 2H), 2.36-2.28 (m, 3H), 2.11-2.05 (m, 2H), 1.99-1.94 (m, 2H), 1.90-1.82 (m, 3H), 1.77-1.68 (m, 2H), 1.59-1.52 (m, 2H), 1.26-1.19 (m, 4H), 1.26 (apparent qd, J_1 = 12.7, J_2 = 3.7 Hz, 1H), 1.06-1.01 (m, 1H), 0.90 (s, 3H), 0.84 (s, 9H), 0.04 (s, 3H), 0.03 (s, 3H); ^{13}C NMR ($CDCl_3$: 77.1576) (150 MHz, $CDCl_3$) δ 220.4, 199.9685, 167.6, 126.2, 65.9417 (CH_2, C19), 54.2, 51.4, 47.7, 43.7, 36.0, 35.8, 34.8, 33.7, 33.4, 31.8, 30.9, 25.9, 21.8 (CH_2), 21.0 (CH_2), 18.2, 14.0 (CH_3), -5.56, -5.64; HRMS (*m/z*): calcd for $C_{19}H_{27}O_2{}^{18}O$, $[M+H]^+$, 305.1997; found, 305.1990 (Δ 2.3 ppm).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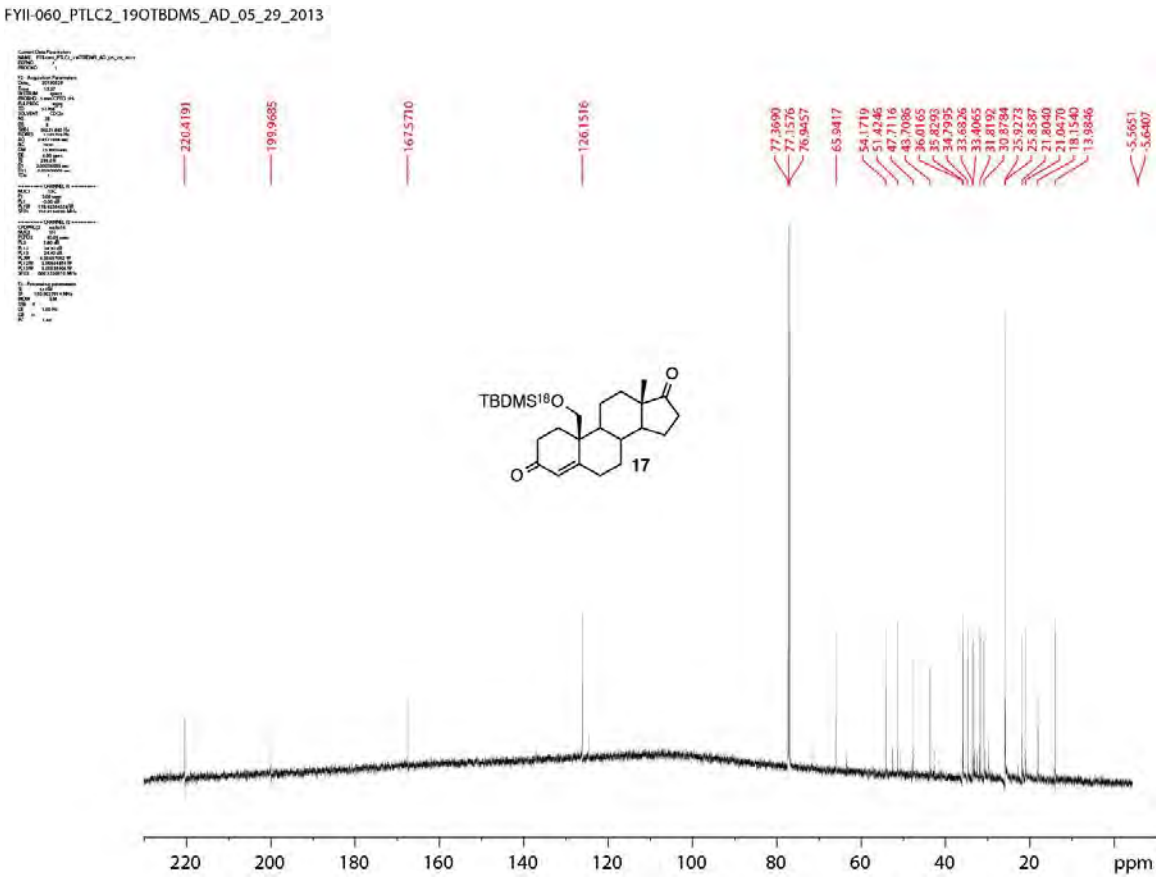
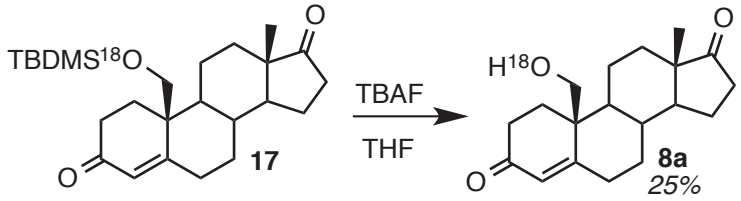
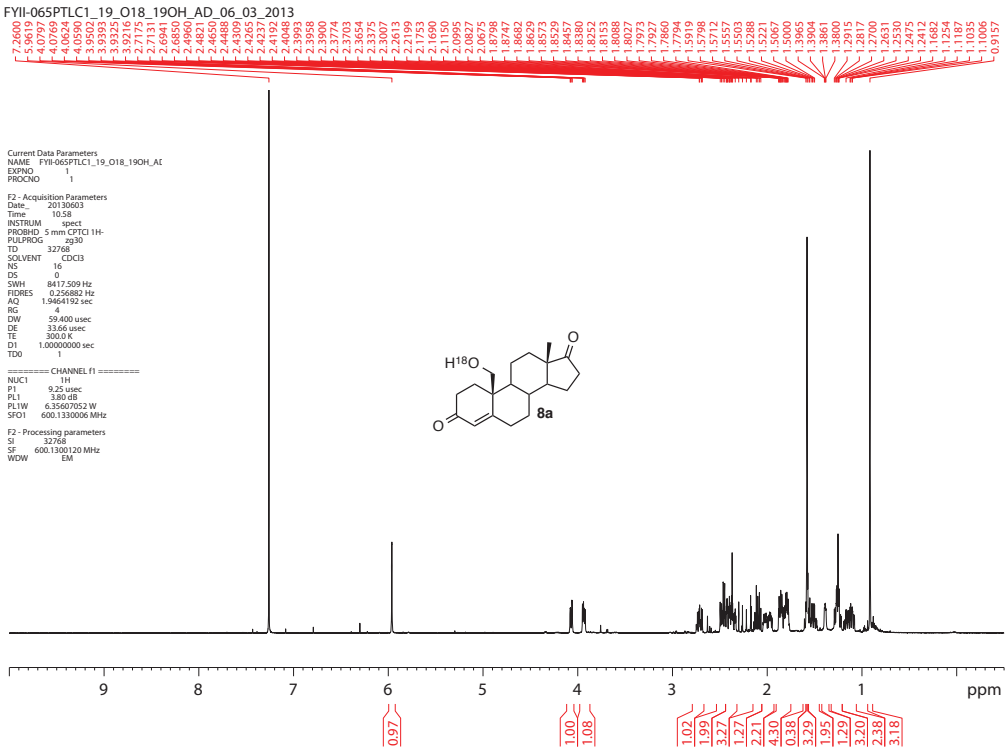
6.58





4.9. [19-^{18}O]-19-Hydroxyandrost-4-en-3,17-dione (8a). Tetrabutylammonium fluoride (0.05 mL of 1 M solution in THF, 0.05 mmol, 0.042 mol eq) was added to a solution of TBDMS ether (**17**) (48 mg, 0.12 mmol) in THF. The reaction was stirred for 1 hour and purified by flash column chromatography (100% hexanes to 40% hexanes/EtOAc, v/v) to afford the alcohol (**8a**) (10 mg, 0.03 mmol, 25%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.96 (m, 1H), 4.07 (dd, J_1 = 10.7, J_2 = 2.0 Hz, 1H), 3.94 (dd, J_1 = 10.6, J_2 = 6.5 Hz, 1H), 2.75-2.69 (m, 1H), 2.50-2.42 (m, 2H), 2.40-2.33 (m, 3H), 2.11 (dd, J_1 = 9.9, J_2 = 9.2 Hz, 1H), 2.04-

2.01 (m, 1H), 1.99-1.95 (m, 1H), 1.88-1.77 (m, 4H), 1.61-1.48 (m, 2H), 1.40-1.38 (m, 1H), 1.29-1.22 (m, 2H), 1.15 (qd, J_1 = 12.1, J_2 = 4.3 Hz, 1H), 1.10 (ddd, J_1 = 12.8, J_2 = 11.0, J_3 = 3.8 Hz, 1H), 0.91 (s, 3H); ^{13}C NMR ($CDCl_3$: 77.1603) (150 MHz, $CDCl_3$) δ 220.3, 199.8, 166.2, 127.3, 66.0828 (CH_2, C19), 54.2 (CH), 51.4 (CH), 47.7, 43.9, 36.0, 35.8, 35.1, 33.6, 33.5, 31.8 (CH_2), 31.0 (CH_2), 21.8 (CH_2), 21.0 (CH_2), 14.0 (CH_3).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RG 4
DW 59.400 usec
DE 33.66 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
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PL1W 6.35607052 W
SFO1 600.1330006 MHz
F2 - Processing parameters
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SF 600.1300120 MHz
WDW EM
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O
O
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6
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3
2
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ppm
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1.08
1.02
1.99
3.27
1.27
2.21
4.30
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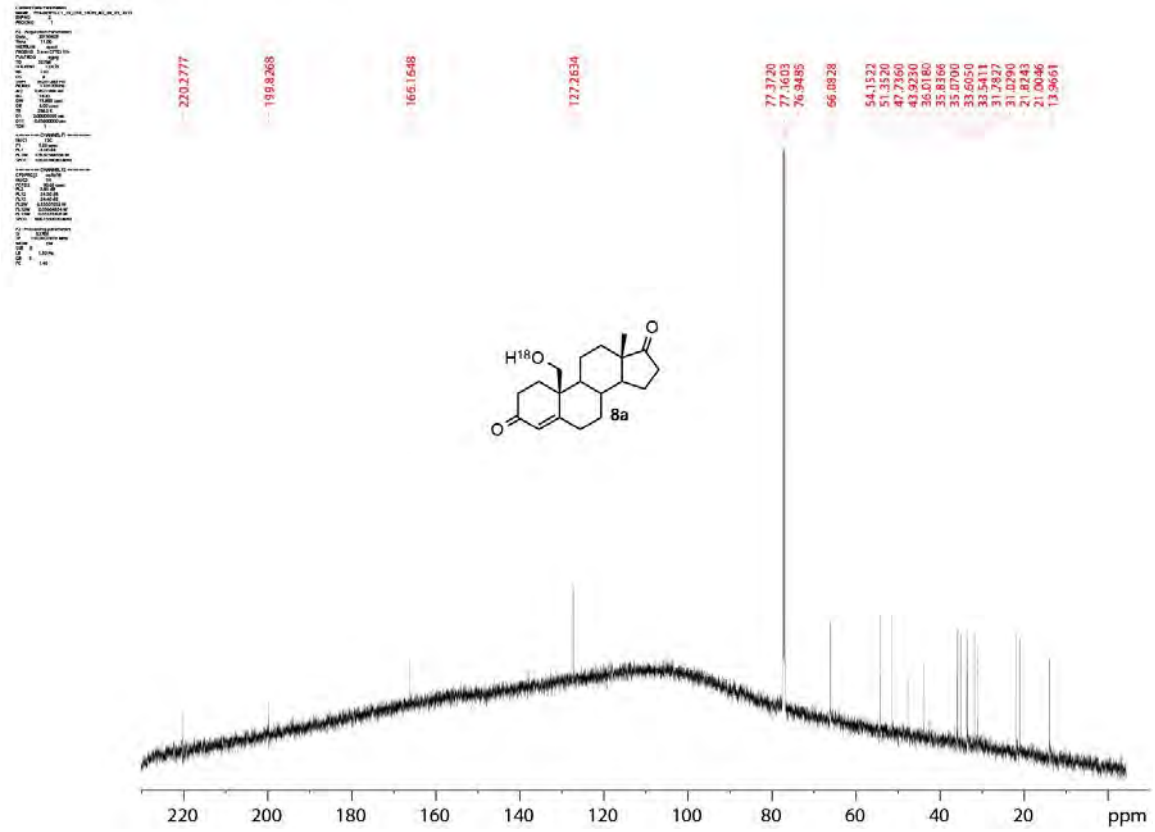
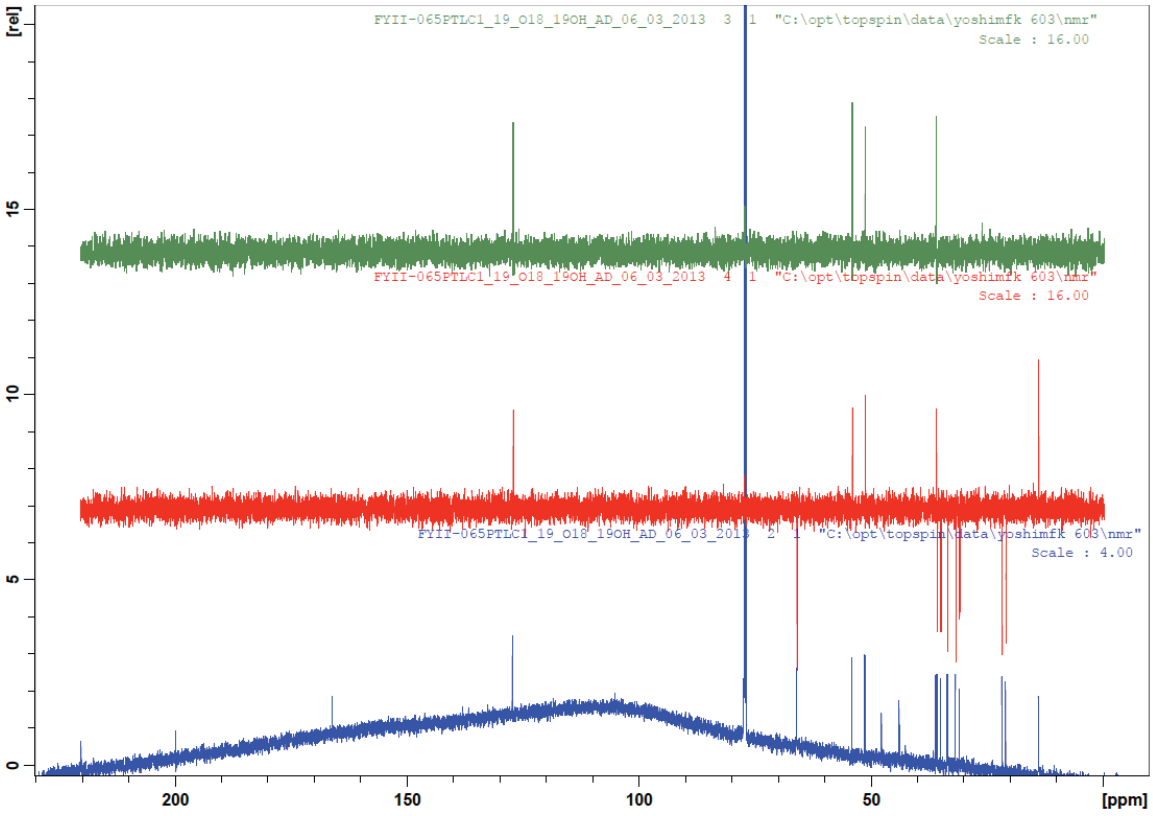
3.20
2.38
3.18

FYII-065PTLC1_19_O18_19OH_AD_06_03_2013

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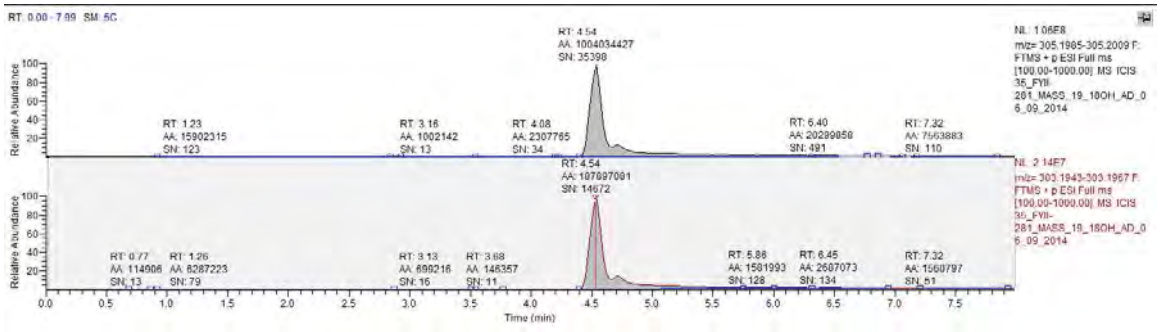
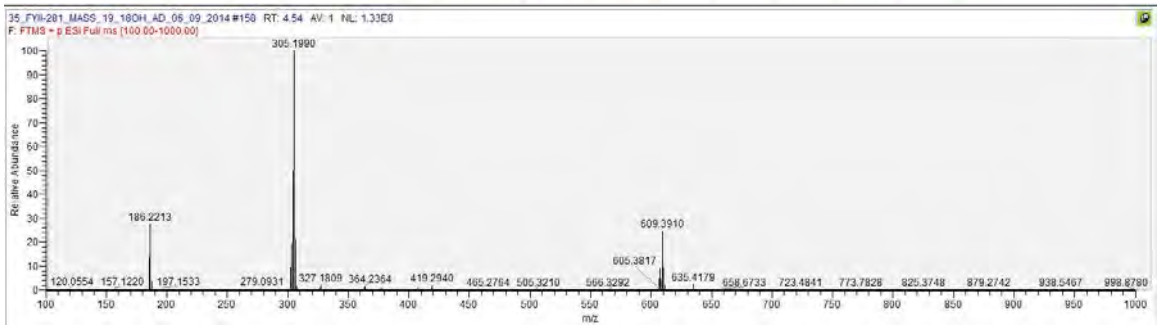

Relative Abundance
Time (min)


Relative Abundance
m/z
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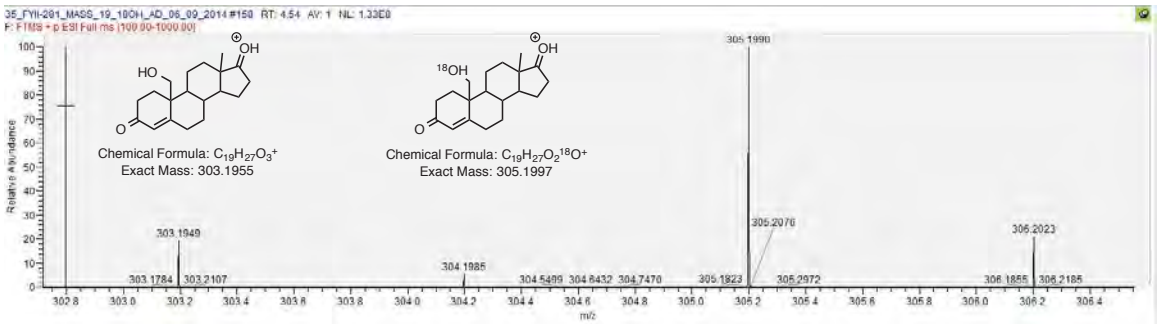
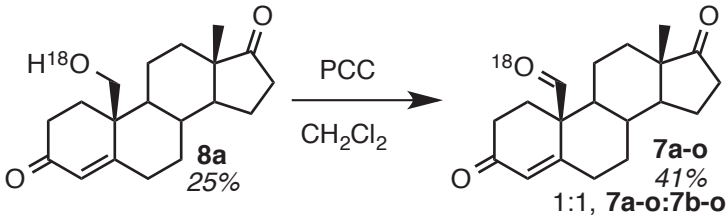
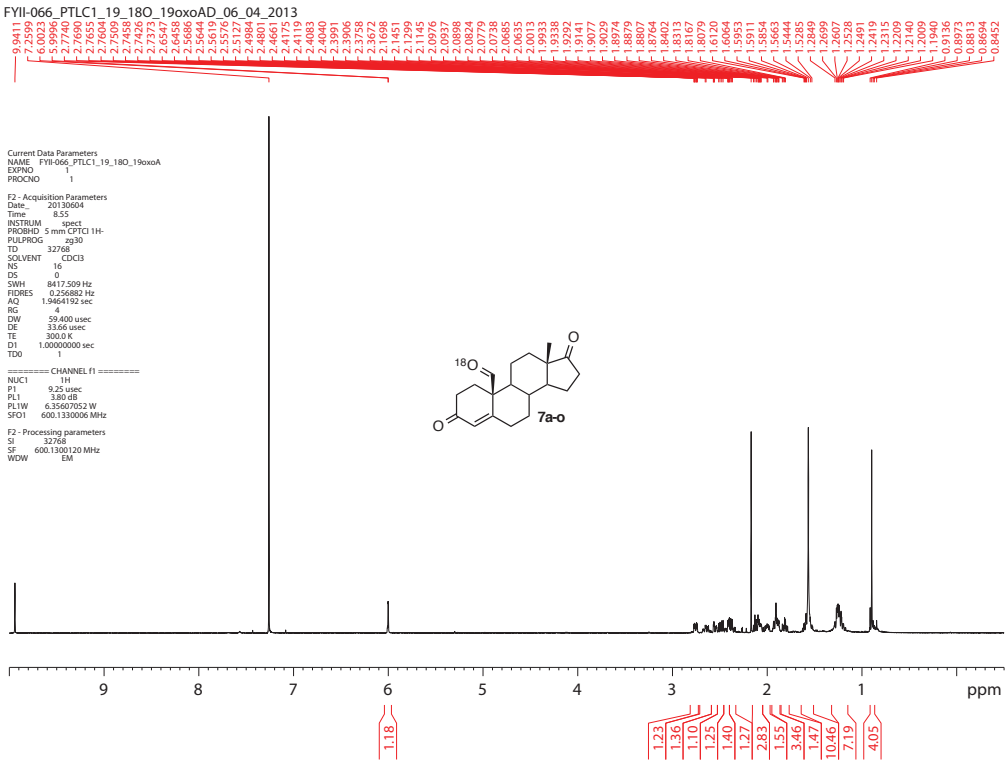
605.3817





4.10. [19-^{18}O]-19-Oxoandrostenedione (7a-o). Pyridinium chlorochromate (7 mg, 0.032 mmol, 1 mol eq) was added to a solution of [19-^{18}O]-19-hydroxyandrost-4-en-3,17-dione (**8a**) (10 mg, 0.033 mmol) in CH_2Cl_2 (3 mL) and the reaction was directly purified by preparative TLC (60% hexanes/EtOAc, v/v) to afford the aldehyde (**7a-o**) (4 mg, 0.013 mmol, 41%, 0.8:1.0, **7a-o** to **7b-o** by MS). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.94 (s, 1H), 6.00 (m, 1H), 2.76 (ddd, J_1 = 13.9, J_2 = 5.1, J_3 = 3.2 Hz, 1H), 2.69-2.60 (m, 1H), 2.76 (ddd, J_1 = 15.2, J_2 = 4.1, J_3 = 2.6 Hz, 1H), 2.49 (dd, J_1 = 19.4, J_2 = 8.4 Hz, 1H), 2.43 (ddd, J_1 = 16.8, J_2 = 4.7, J_3 = 2.6 Hz, 1H), 2.37 (ddd, J_1 = 17.0, J_2 = 14.0, J_3 = 5.2 Hz, 1H), 2.14-2.06 (m, 2H), 2.02-1.98 (m, 1H), 1.94-1.86 (m, 3H), 1.81 (td, J_1 = 13.9, J_2 = 5.4 Hz, 1H), 1.33-1.18 (m, 5H), 0.90 (s, 3H); ^{13}C NMR ($CDCl_3$: 77.1589) (150 MHz, $CDCl_3$) δ 219.8, 200.9548 (CH, C19, from ^{16}O) or 200.9089 (CH, C19, from ^{18}O), 197.8, 160.6, 127.8 (CH), 55.1, 53.7 (CH), 51.1 (CH), 47.5, 36.5 (CH), 35.8, 34.3, 33.8, 31.6, 30.4, 29.8, 21.7, 21.2, 13.8 (CH_3); HRMS (*m/z*): calcd for $C_{19}H_{25}O_2{}^{18}O$, $[M+H]^+$, 303.1841; found, 303.1841 (Δ <0.1 ppm).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TE 300.0 K
D1 1.00000000 sec
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ppm
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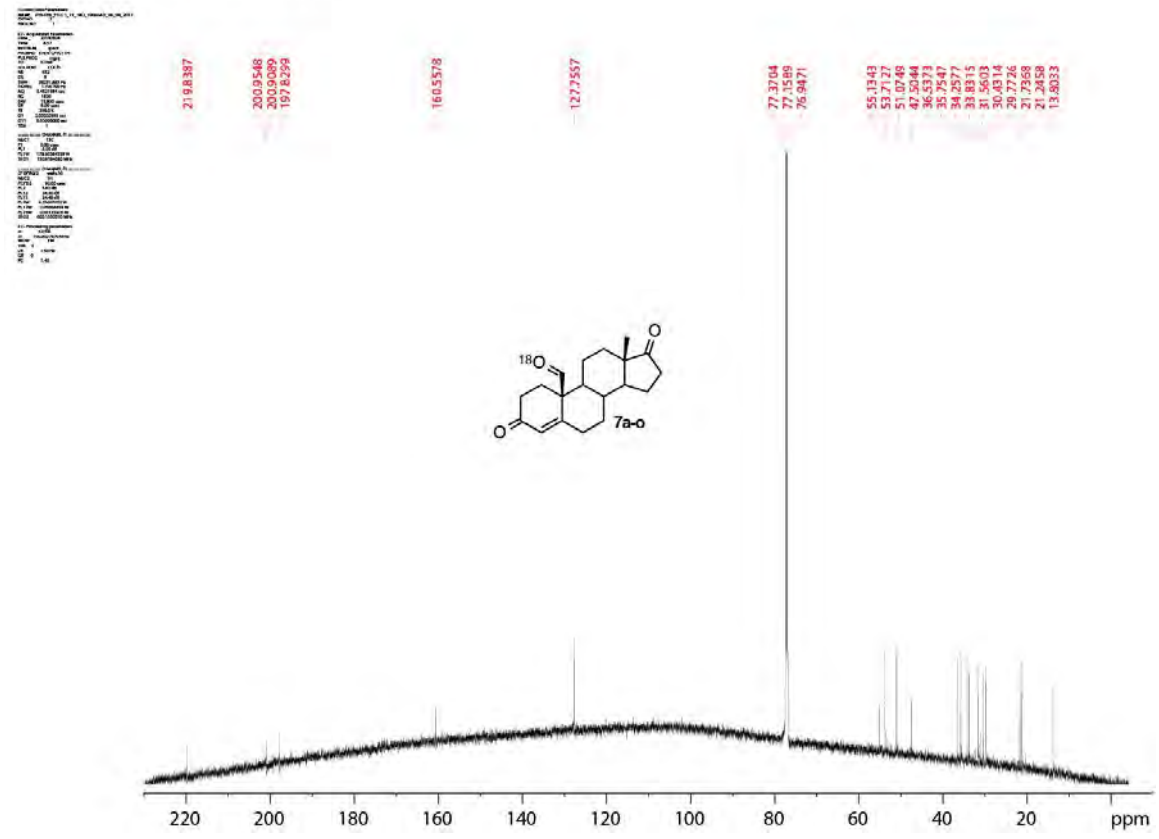
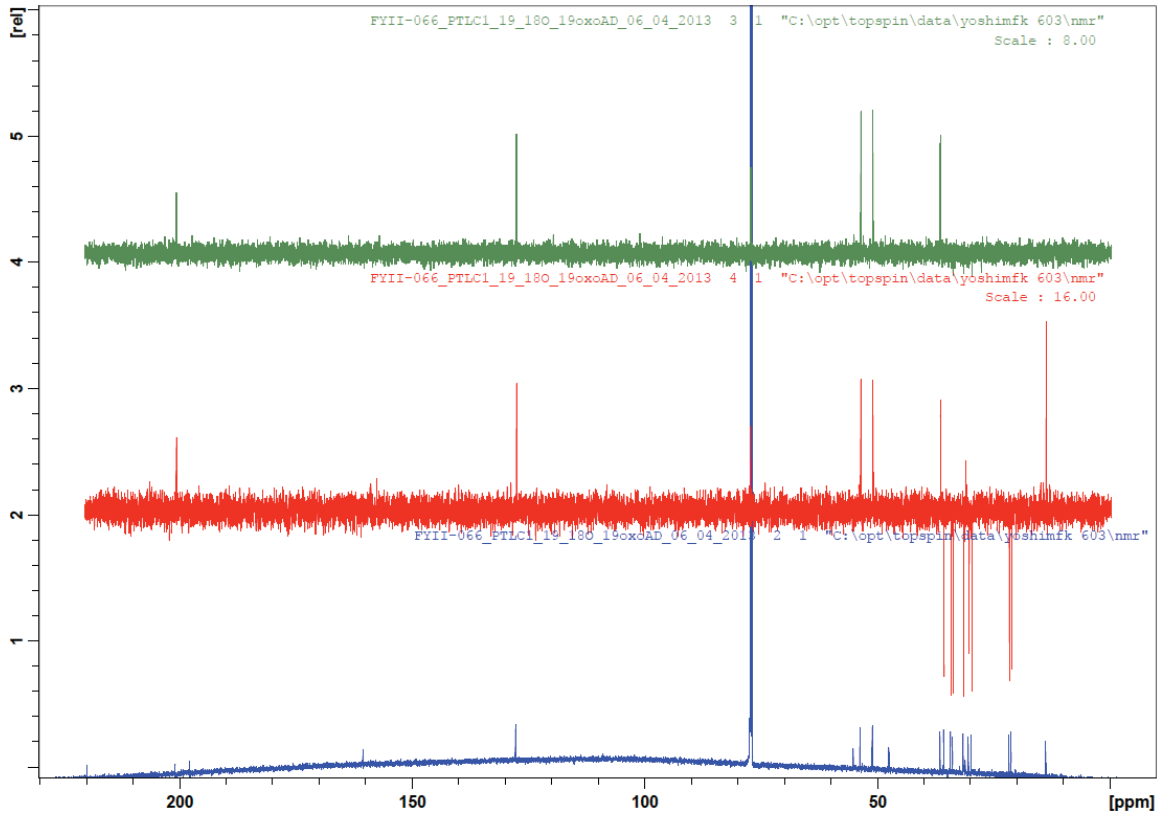
10.46
7.19
4.05

FYII-066_PTLC1_19_18O_19oxoAD_06_04_2013



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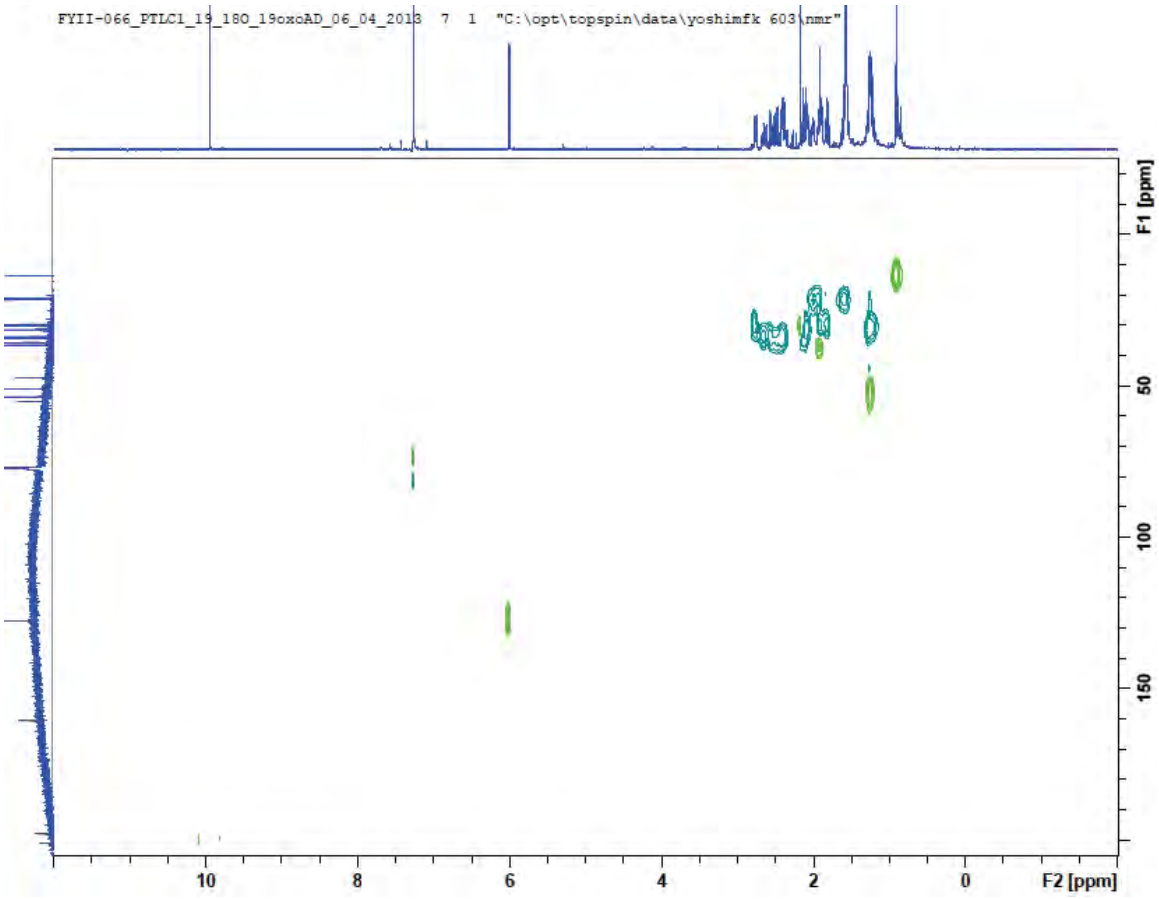
[ppm]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2
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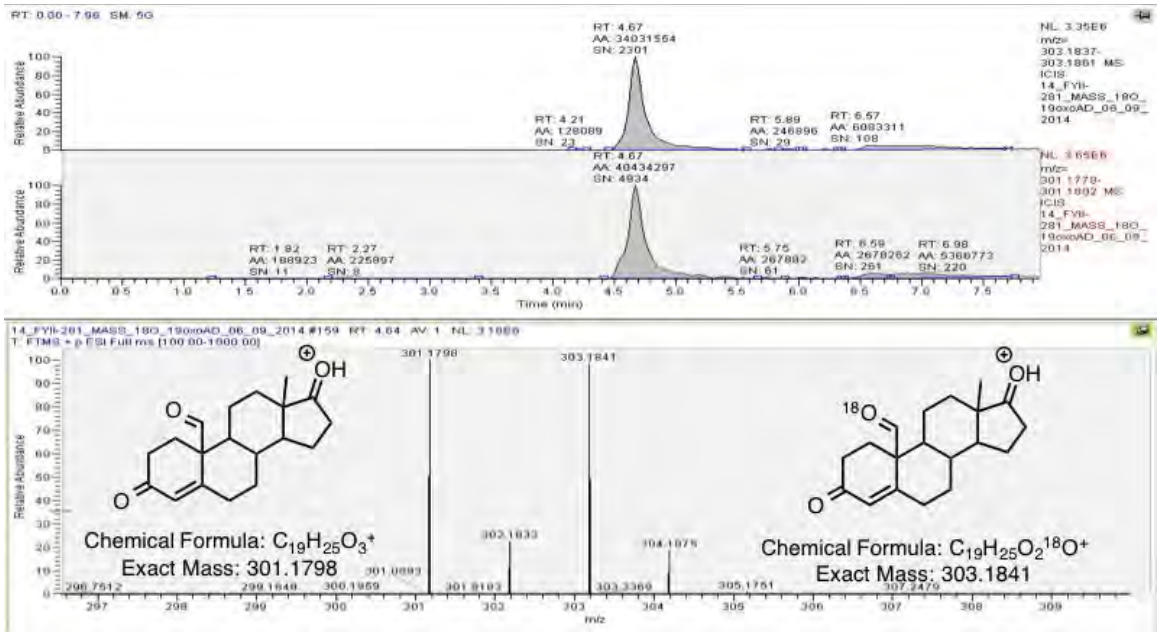
50
100
150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SN: 108
RT: 4.67
AA: 40434297
SN: 4934
RT: 1.92
AA: 188923
SN: 11
RT: 2.27
AA: 225897
SN: 8
RT: 5.75
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Relative Abundance
Time (min)
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302.1833
304.1875
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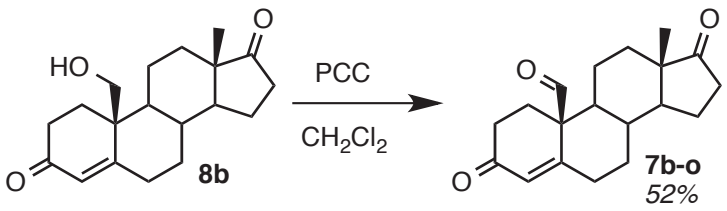
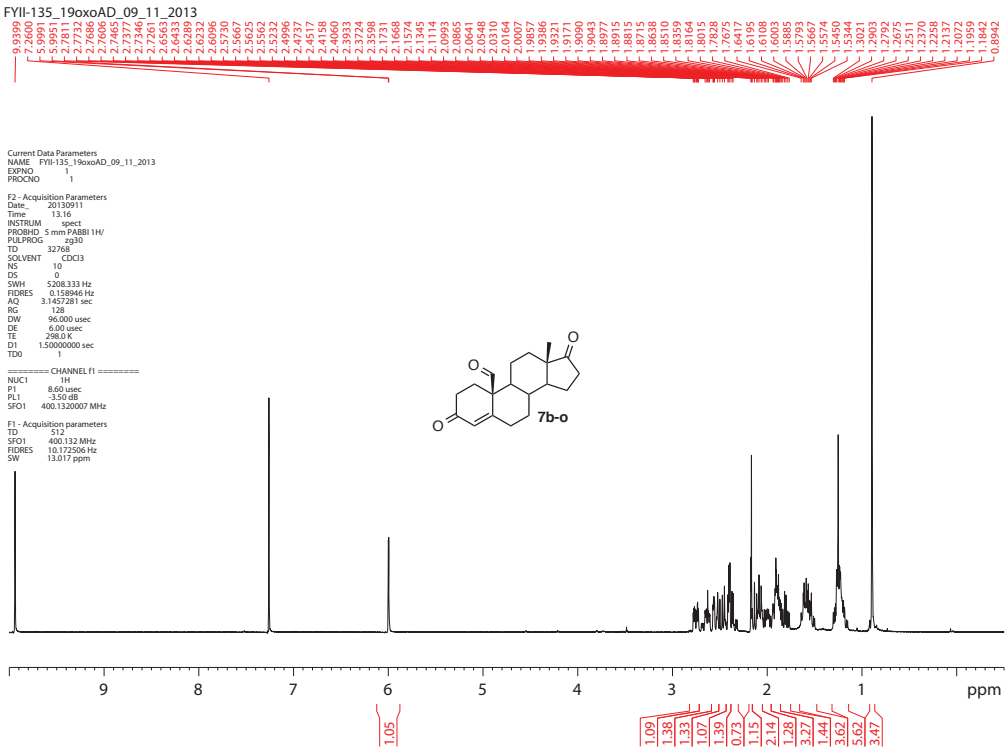
m/z



4.11. 19-Oxoandrostenedione (7b-o). PCC (1.56 g, 7.3 mmol) was added to a solution of 19-hydroxyandrostenedione (2.3 g, 7.3 mmol) in CH_2Cl_2 (300 mL). The reaction was stirred for 5 h. The solvent was evaporated under a stream of air and purified via flash column chromatography (100% hexanes to hexanes:EtOAc, 7:3, v-v) to afford 19-oxoandrostenedione (**7b-o**) (1.20 g, 3.8 mmol, 52%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.91 (s, 1H), 5.97-5.96 (m, 1H), 2.73 (ddd, J_1 = 13.8, J_2 = 5.0, J_3 = 3.1 Hz, 1H), 2.62 (ddd, J_1 = 15.2, J_2 = 5.2, J_3 = 1.9 Hz, 1H), 2.52 (ddd, J_1 = 15.1, J_2 = 4.2, J_3 = 2.5 Hz, 1H), 2.46 (dd, J_1 = 19.2, J_2 = 8.9, 1H), 2.39-2.36 (m, 1H), 2.34-2.29 (m, 1H), 2.13-2.01 (m, 2H), 1.99-1.94 (m, 1H), 1.92-1.83 (m, 4H), 1.78 (dd, J_1 = 13.5, J_2 = 6.2 Hz, 1H), 1.62-1.48 (m, 2H), 1.27-1.16 (m, 3H), 0.87 (s, 3H); ^{13}C NMR ($CDCl_3$: 77.1656) (100 MHz, $CDCl_3$) δ 219.8, 200.9438 (CH, C19), 197.8, 160.6, 127.6, 55.1, 53.6, 51.0, 47.4, 36.5, 35.7, 34.2, 33.8, 31.5, 30.3, 29.7, 21.7, 21.2, 13.7; HRMS (*m*/*z*): calcd for $C_{19}H_{25}O_3$, $[M+H]^+$, 301.1798; found, 301.1795 (Δ 1.0 ppm).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8
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6
5
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3
2
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ppm
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1.33
1.07
1.39
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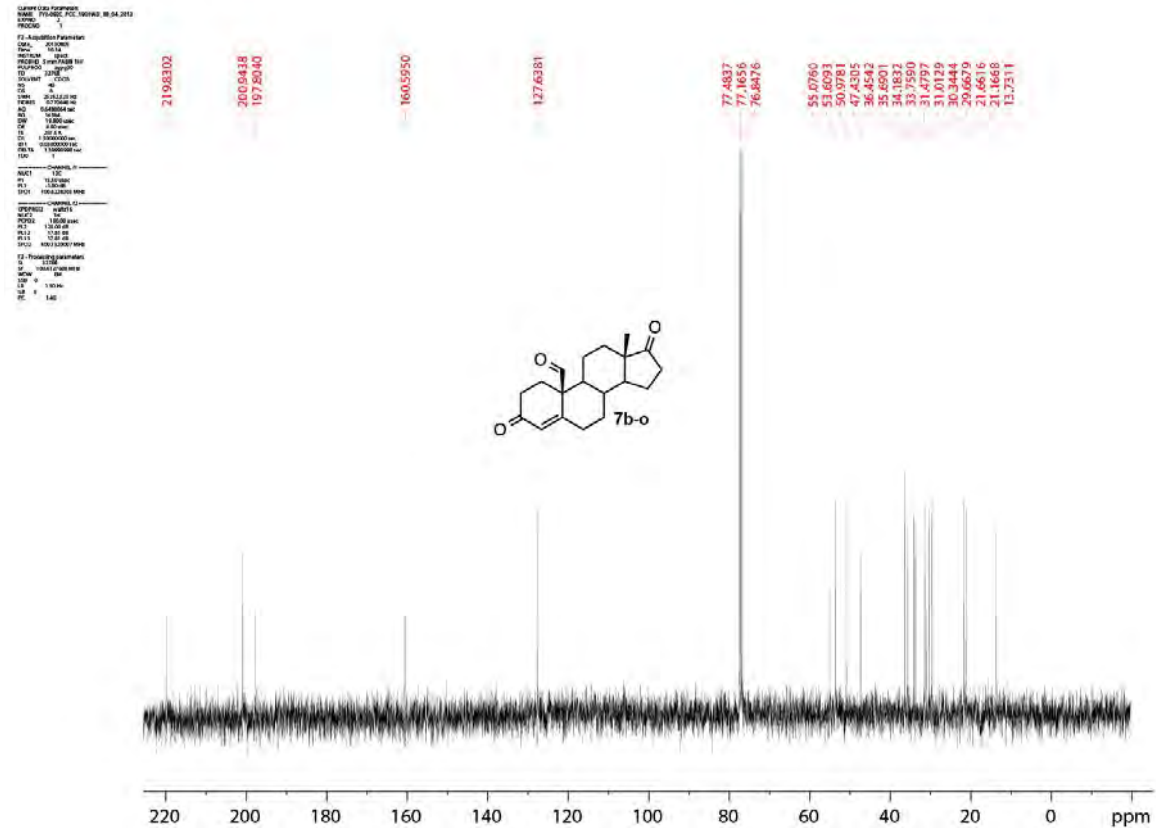
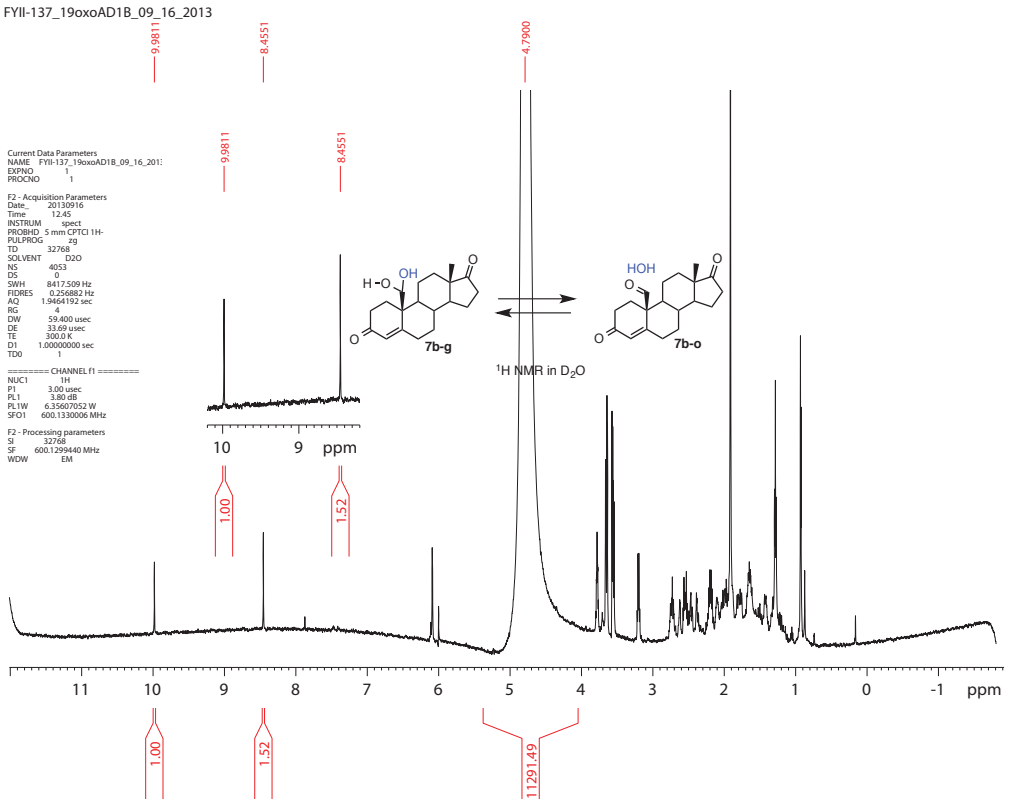
5.62
3.47

FYII-092C_PCC_19OHAD_08_04_2013

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AQ 1.9464192 sec
RG 4
DW 59.400 usec
DE 33.69 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
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ppm
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O
7b-g
HOH
7b-o
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7
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0
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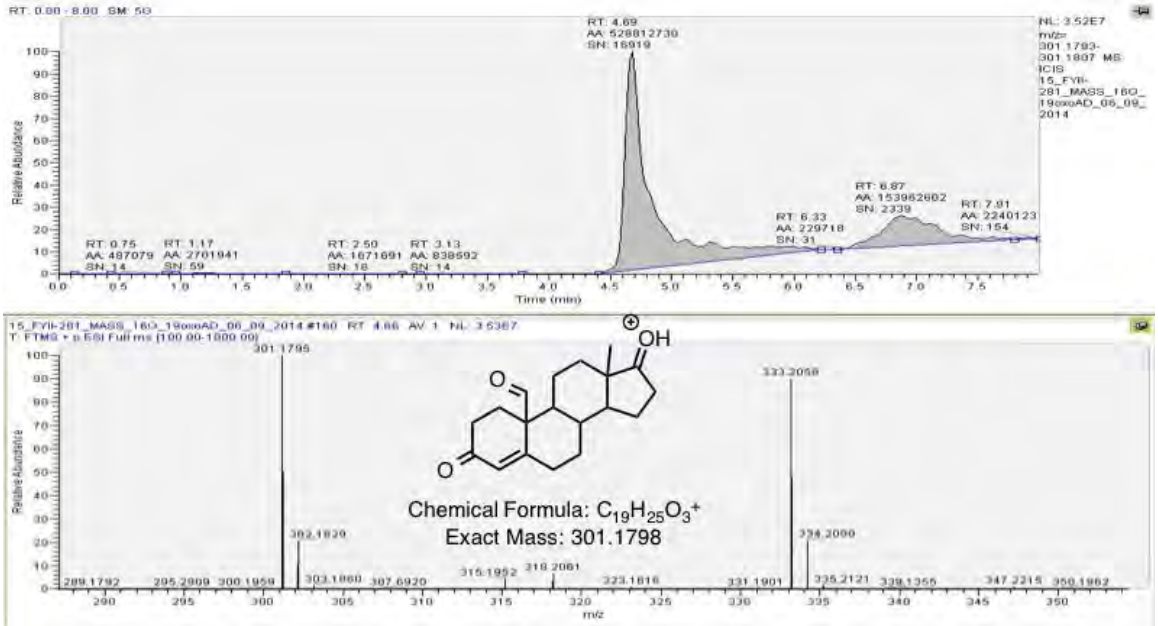
1.52
11291.49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RT: 1.17
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Relative Abundance
Time (min)
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302.1829
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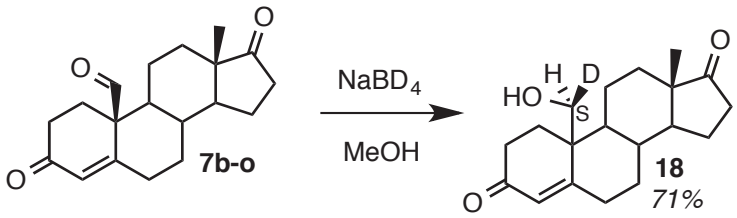
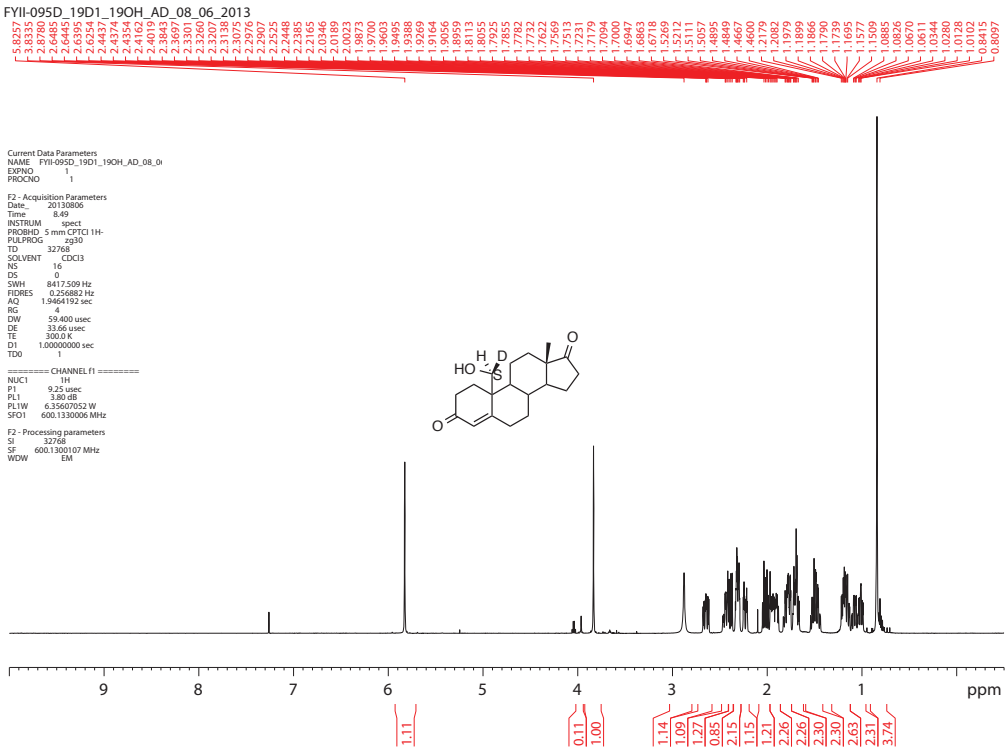
Exact Mass: 301.1798
m/z



4.12. [19-$^{2}H_{1}$]-19-Hydroxyandrostenedione (18). $NaBD_4$ (37 mg, 0.87 mmol) was added to a solution of 19-oxoandrostenedione (**7b-o**) (1.10 g, 3.48 mmol) in CH_3OH (200 mL). The reaction was stirred for 1 h and concentrated in vacuo. The crude material was purified via flash column chromatography (100% hexanes to hexanes:EtOAc, 1:1, v-v and then 10% MeOH/CH_2Cl_2, v-v) to afford 19-deutero-19-hydroxyandrostenedione (**18**) (0.79 g, 2.48 mmol, 71%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.83 (broad s, 1H), 3.96 (broad s, 0.1H), 3.83 (broad s, 1H), 2.88 (broad s, 1H), 2.65 (ddd, J_1 = 17.0, J_2 = 14.0, J_3 = 5.5 Hz, 1H), 2.44 (ddd, J_1 = 14.3, J_2 = 5.2, J_3 = 1.5 Hz, 1H), 2.39 (dd, J_1 = 19.3, J_2 = 8.8 Hz, 1H), 2.33-2.28 (m, 2H), 2.23 (dt, J_1 = 12.4, J_2 = 4.6 Hz, 1H), 2.05-1.99 (m, 1H), 1.97-1.88 (m, 2H), 1.83-1.75 (m, 2H), 1.73-1.66 (m, 2H), 1.54-1.44 (m, 2H), 1.22-1.15 (m, 2H), 1.11-0.99 (m, 2H), 0.84 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 220.7, 200.5, 167.7, 126.5, 65.1 (t, J = 21.4 Hz), 54.0, 51.2, 47.6, 43.8, 35.7, 34.8, 33.4, 33.2, 31.6, 30.9, 21.6, 20.8, 13.8; HRMS (*m*/*z*): calcd for $C_{19}H_{26}DO_3$, $[M+H]^+$, 304.2017; found, 304.2012 (Δ 1.6 ppm). The stereochemistry assignment of C-19(*S*) is based on the Arigoni *et al*. reference.[10]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PL1 3.80 dB
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4
3
2
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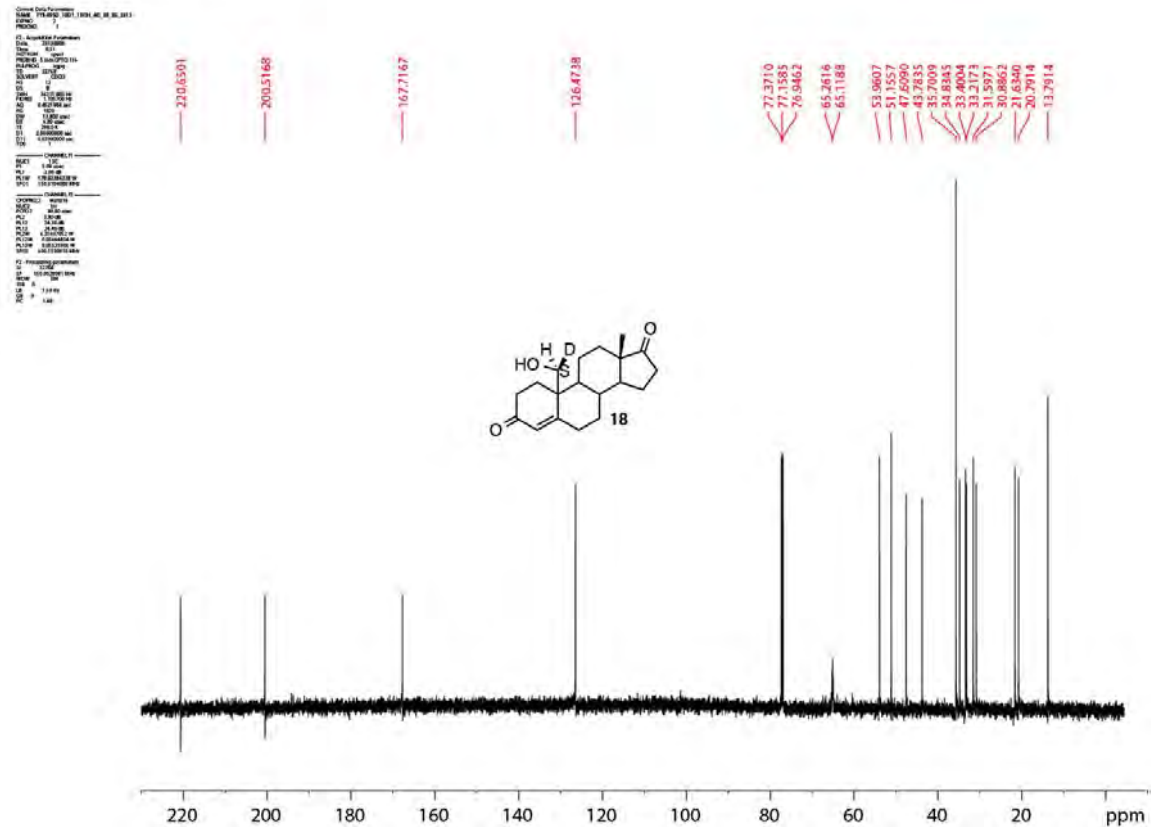
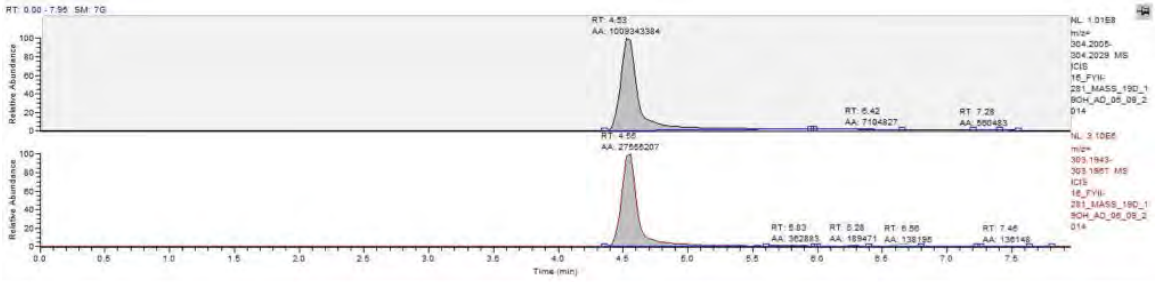
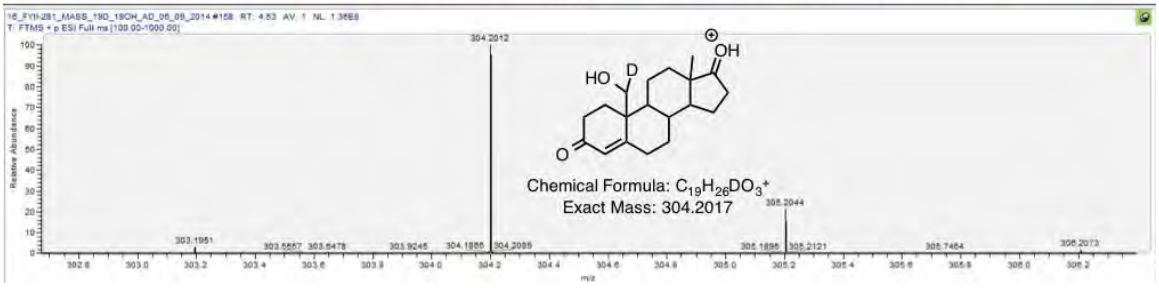
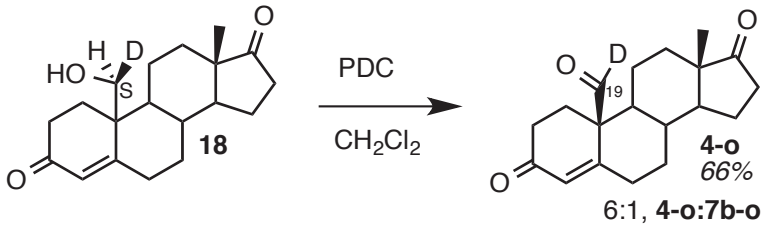
1.15
1.21
2.26
2.26
2.30
2.30
2.63
2.31
3.74

FYII-095D_19D1_19OH_AD_08_06_2013

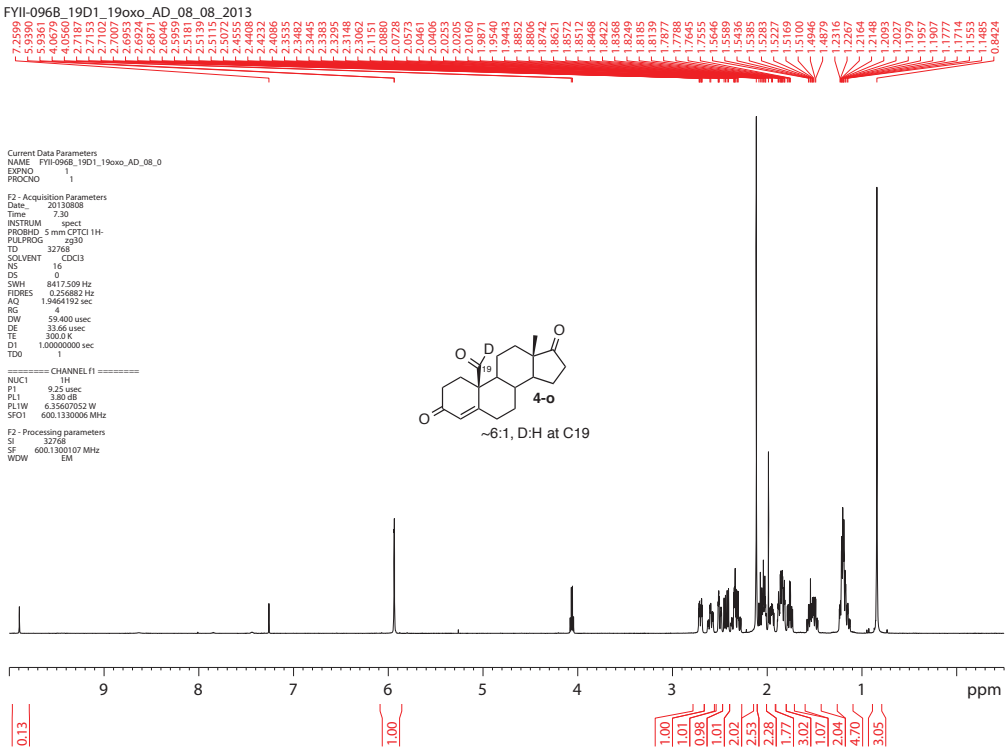








4.13. [19-$^2H_1$]-19-Oxoandrostenedione (4-o). Pyridinium dichromate (936 mg, 2.49 mmol) was added to a solution of 19-deutero-19-hydroxyandrostenedione (**18**) (789 mg, 2.49 mmol) in CH_2Cl_2 (100 mL). The reaction mixture was stirred for 1 h and concentrated. The crude material was purified by flash column chromatography (100% hexanes to hexanes:EtOAc, 1:1, v-v) to afford 19-deutero-19-oxoandrostenedione (**4-o**) (515 mg, 1.63 mmol, 66%). From the analysis of the ^1H NMR spectrum, the aldehyde peak relative to the C4-proton indicated a 6:1 ratio between ^2H:^1H at the C19-position of 19-oxoandrostenedione (6:1, **4-o** to **7b-o**), consistent with HRMS. HRMS (*m*/*z*): calcd for $C_{19}H_{24}DO_3$, $[M+H]^+$, 302.1861; found, 302.1860 (Δ 0.3 ppm).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SWH 8417.509 Hz
FIDRES 0.256882 Hz
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RG 4
DW 59.400 usec
DE 33.66 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
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PL1 3.80 dB
PL1W 6.35607052 W
SFO1 600.1330006 MHz
F2 - Processing parameters
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SF 600.1300107 MHz
WDW EM
4-o
~6:1, D:H at C19
9
8
7
6
5
4
3
2
1
ppm
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1.00
1.00
1.01
0.98
1.01
2.02
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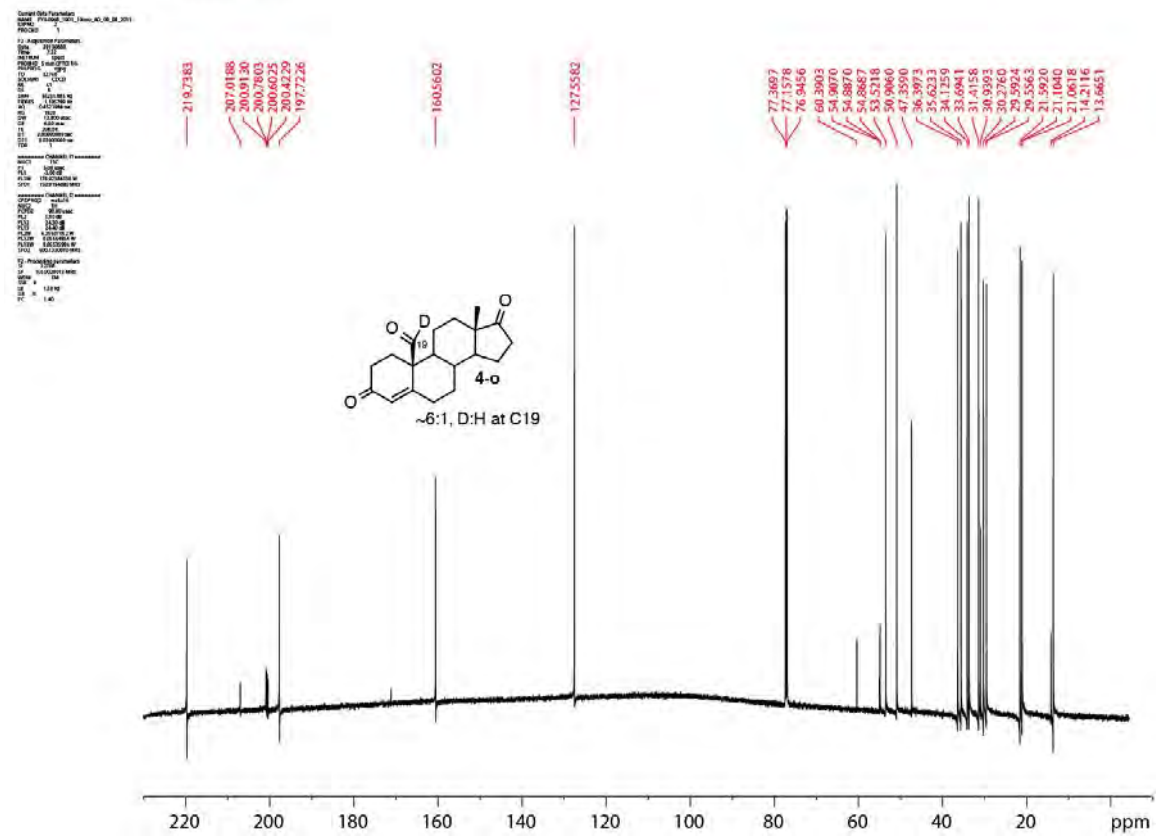
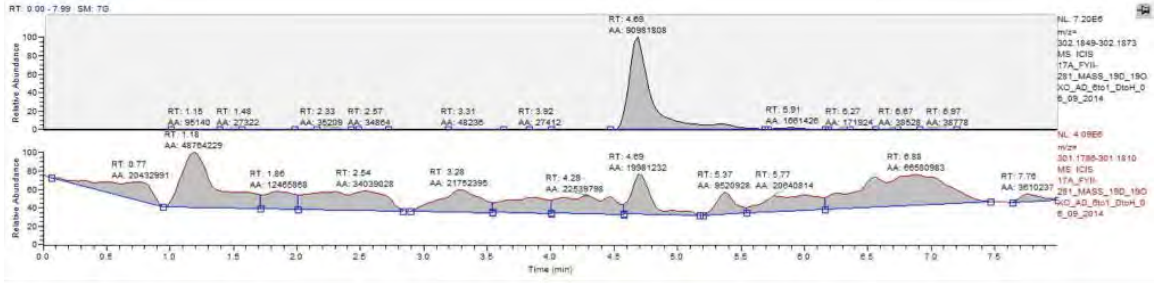
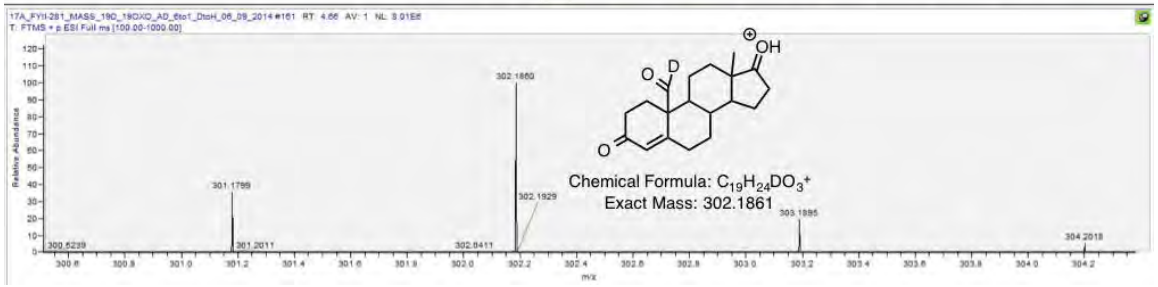
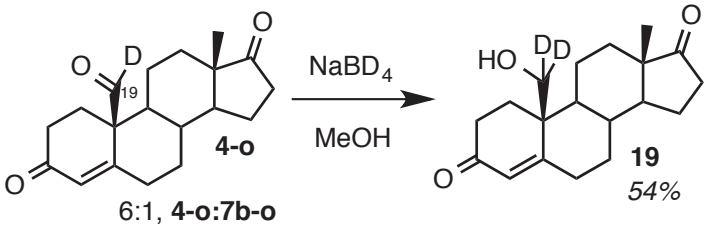
3.02
1.07
2.04
4.70
3.05

FYII-096B_19D1_19oxo_AD_08_08_2013

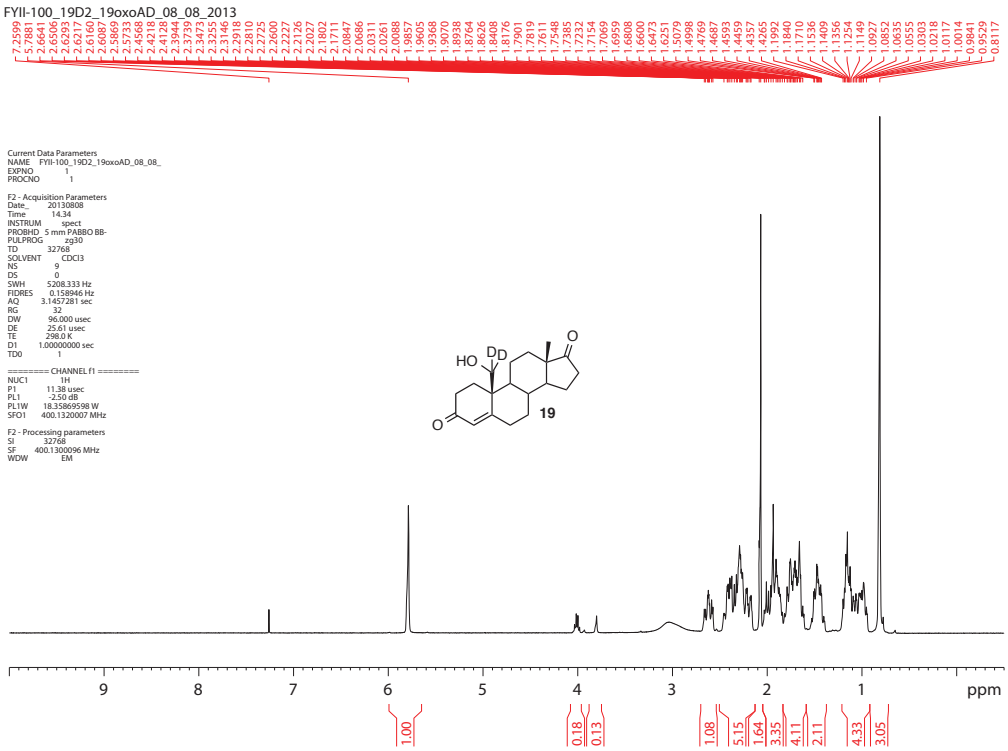








4.14. [19,19-$^2H_2$]-19-Hydroxyandrostenedione (19). $NaBD_4$ (17 mg, 0.41 mmol, 0.25 mol eq) was added to 19-deutero-19-oxoandrostenedione (**4-o**) (~6:1 D:H at C-19, 515 mg, 1.63 mmol) to access 19,19-dideutero-19-hydroxyandrostenedione (**19**) (280 mg, 0.88 mmol, 54%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.79 (m, 1H), 3.93-3.91 (m, 0.03H), 3.80 (m, 0.13H), 2.62 (ddd, J_1 = 19.4, J_2 = 14.2, J_3 = 5.4 Hz, 1H), 2.56-2.17 (m, 5H), 2.03-1.84 (m, 3H), 1.82-1.61 (m, 4H), 1.53-1.39 (m, 2H), 1.20-0.94 (m, 4H), 0.81 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 220.6, 200.5, 167.8, 126.3, 64.7, 53.9, 51.1, 47.5, 43.6, 35.6, 34.8, 33.3, 33.1, 31.5, 30.8, 21.6, 20.7, 13.7.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FIDRES 0.158946 Hz
AQ 3.1457281 sec
RG 32
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DE 25.61 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 11.38 usec
PL1 -2.50 dB
PL1W 18.35869598 W
SFO1 400.1320007 MHz
F2 - Processing parameters
SI 32768
SF 400.1300096 MHz
WDW EM
HO
D
D
O
O
19
9
8
7
6
5
4
3
2
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ppm
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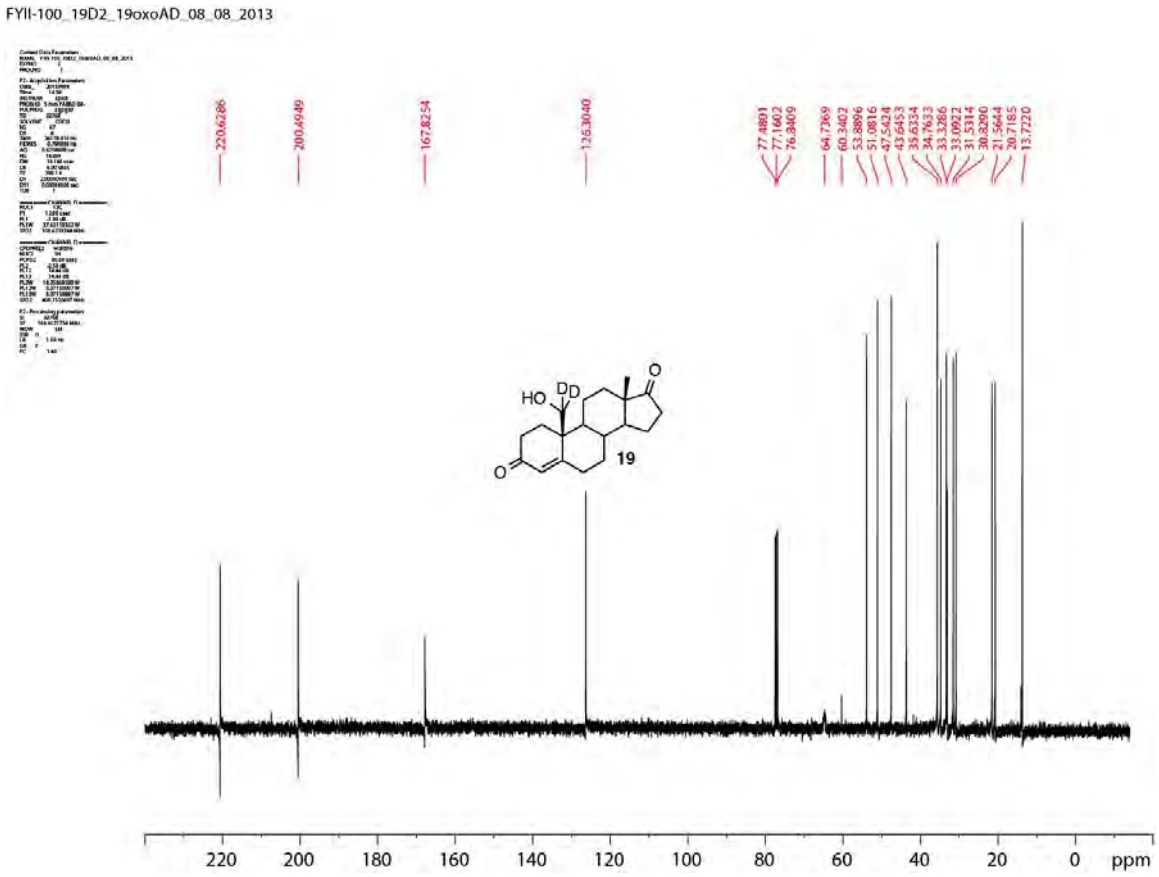
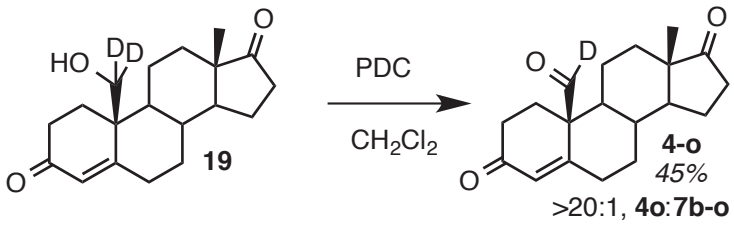
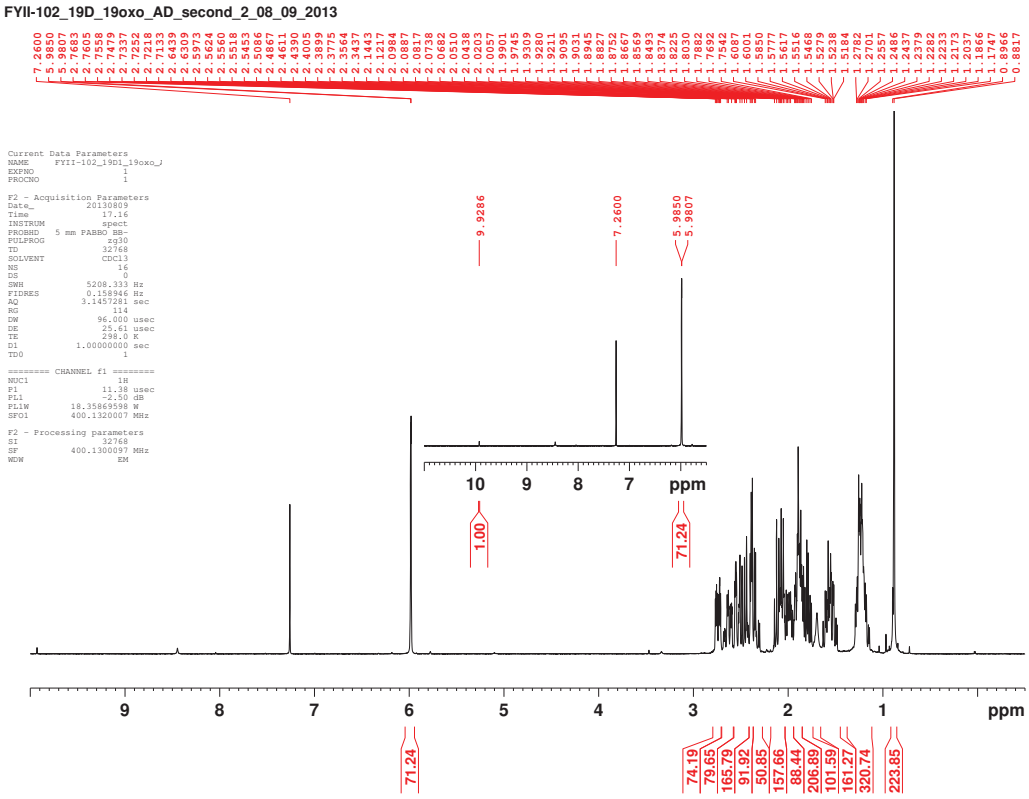
1.08
5.15
1.64
3.35
4.11
2.11
4.33
3.05





4.15. [19-$^2H_1$]-19-Oxoandrostenedione (4-o). Pyridinium dichromate (70 mg, 0.19 mmol, 1 mol eq) was added to 19,19-dideutero-19-hydroxyandrostenedione (**19**) (59 mg, 0.19 mmol) in CH_2Cl_2 (50 mL) to yield 19-deutero-19-oxoandrostenedione (**4-o**) (25 mg, 0.08 mmol, 45%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.99-5.98 (m, 1H), 2.74 (ddd, J_1 = 13.4, J_2 = 5.0, J_3 = 3.1 Hz, 1H), 2.68-2.59 (m, 1H), 2.56-2.30 (m, 5H), 2.14-1.75 (m, 7H), 1.63-1.48 (m, 2H), 1.29-1.14 (m, 4H), 0.88 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 219.7, 200.6 (t,

J = 27.0 Hz), 197.7, 160.6, 127.6, 53.5, 50.9, 47.4, 36.4, 35.6, 34.1, 33.7, 31.4, 30.9, 30.3, 29.56, 21.6, 21.1, 13.7; HRMS (*m*/*z*): calcd for $C_{19}H_{24}DO_3$, $[M+H]^+$, 302.1861; found, 302.1857 (Δ 1.3 ppm).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PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 16
DS 0
SWH 5208.333 Hz
FIDRES 0.158946 Hz
AQ 3.1457281 sec
RG 114
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DE 25.61 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
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PL1 -2.50 dB
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SFO1 400.1320007 MHz
F2 - Processing parameters
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SF 400.1300097 MHz
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7.2600
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10
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8
7
ppm
1.00
71.24
9
8
7
6
5
4
3
2
1
ppm
71.24
74.19
79.65
165.79
91.92
50.85
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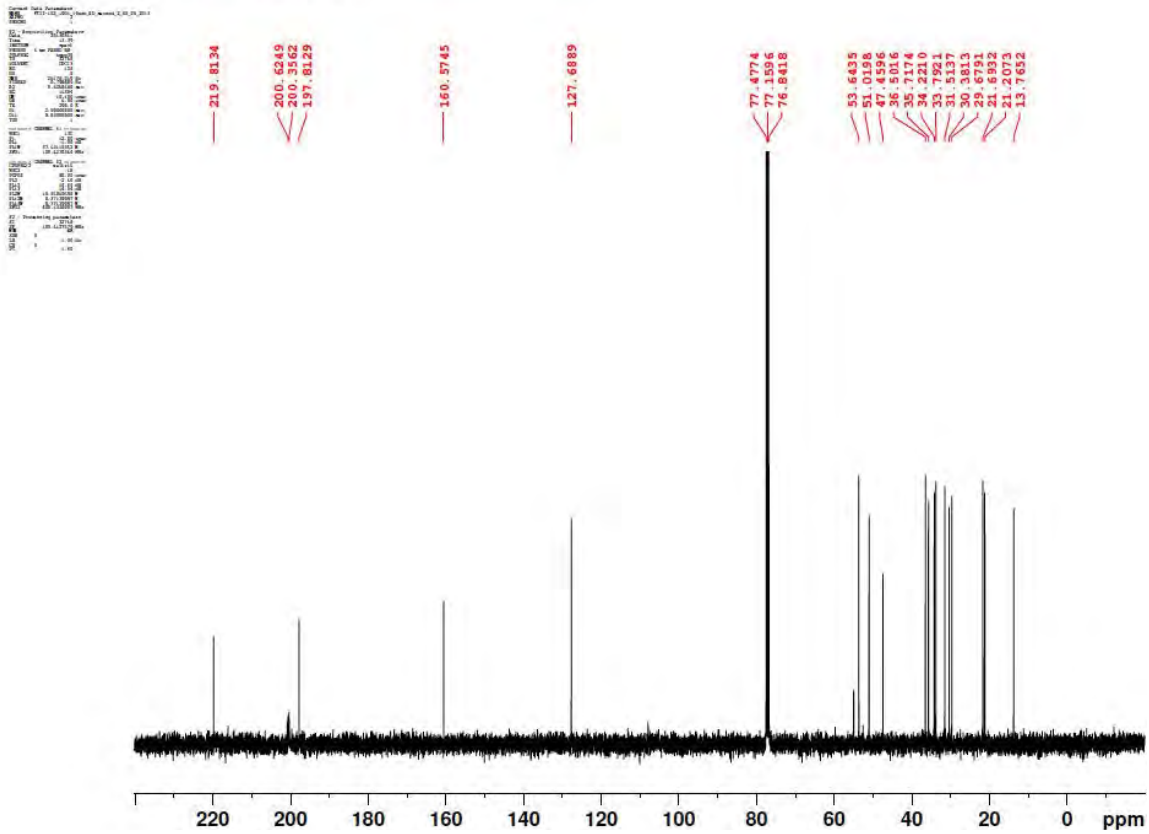
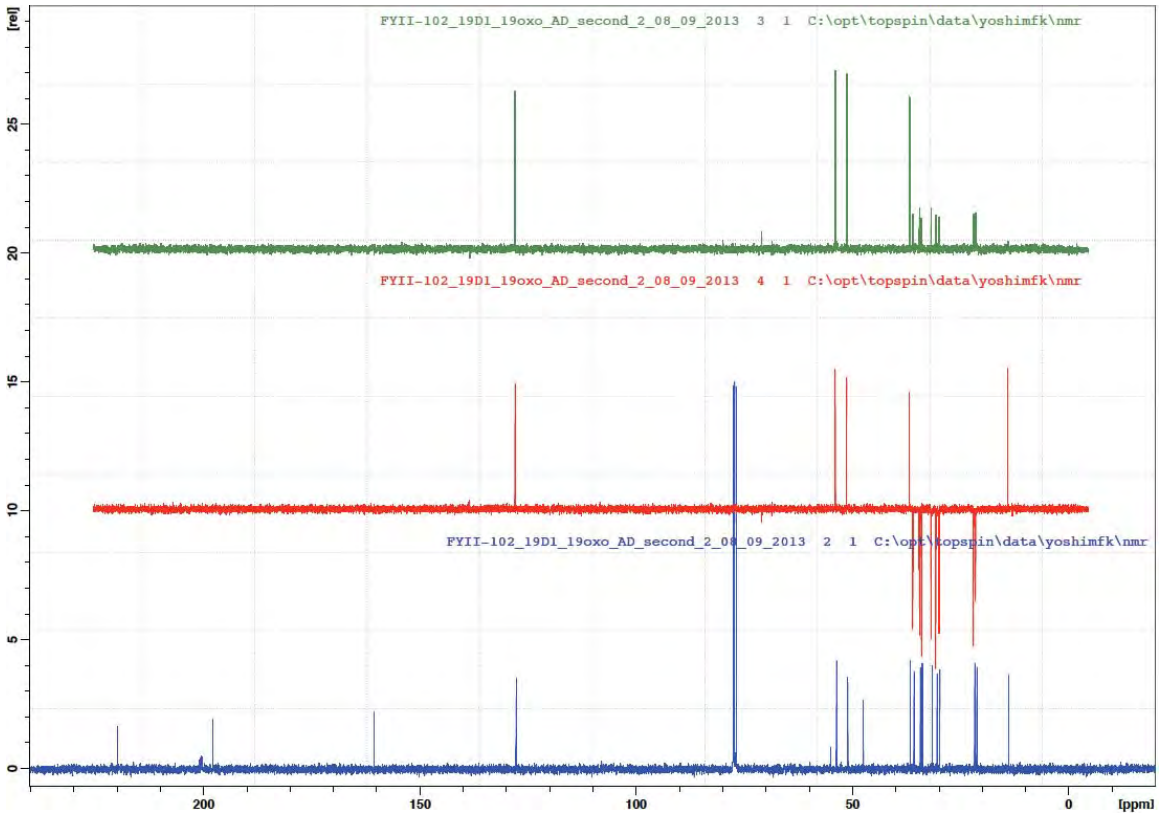
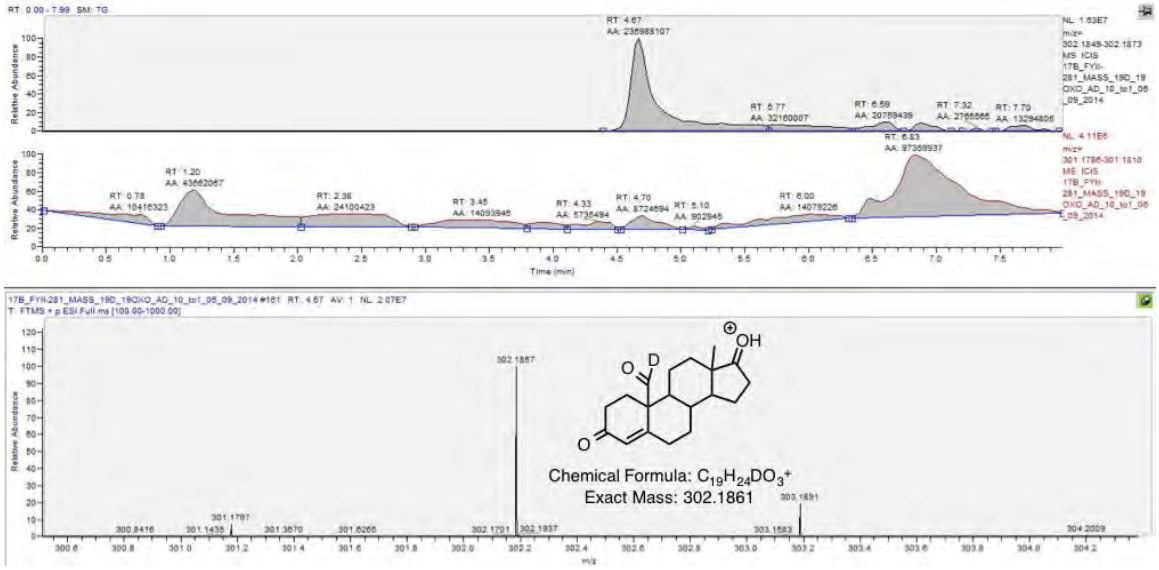
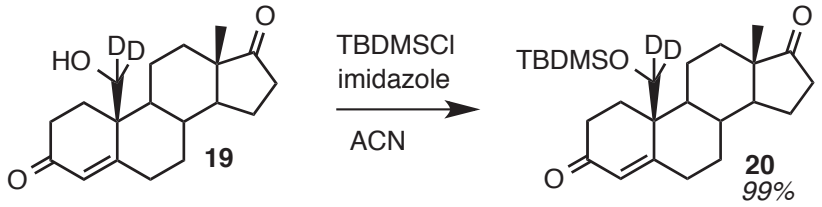
206.89
101.59
161.27
320.74
223.85

FYII-102_19D_19oxo_AD_second_2_08_09_2013



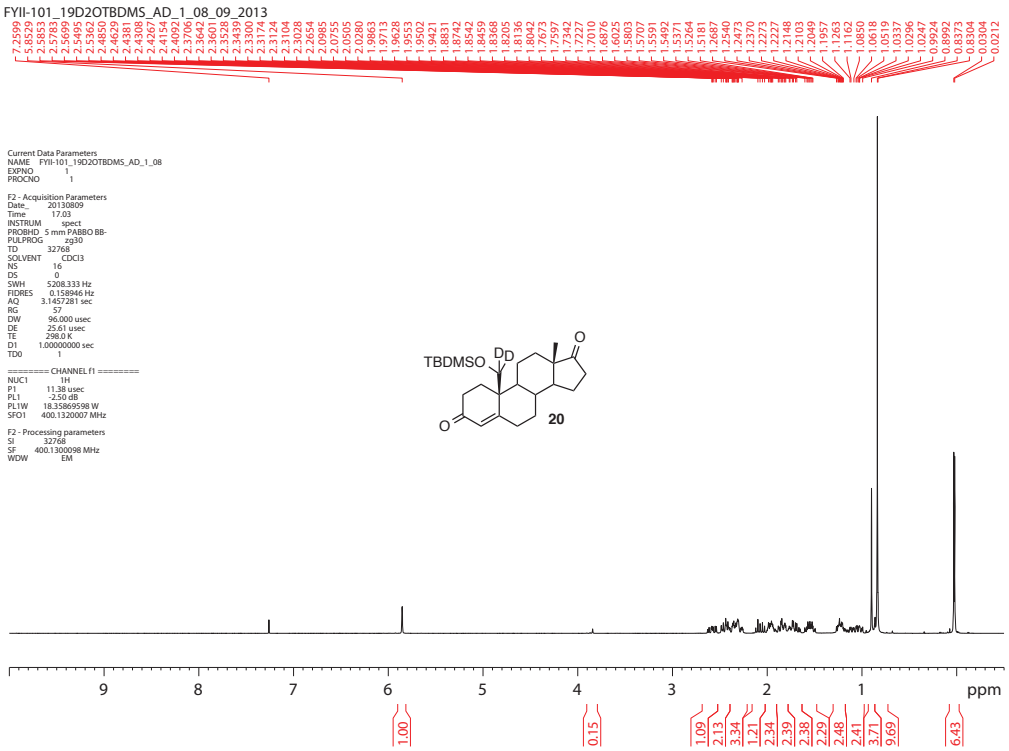






4.16. [19,19-$^2H_2$]-19-Hydroxy-TBDMS androstenedione (20). TBDMS chloride (2.0 g) and imidazole (0.2 g) were added to a solution of 19,19-dideutero-19-hydroxyandrostenedione (**19**) (220 mg, 0.72 mmol). The reaction mixture was stirred for 3 h and concentrated via reduced pressure. The crude material was purified via flash column chromatography (100% hexanes to hexanes:EtOAc, 1:1, v-v) to afford the TBDMS ether (**20**) (300 mg, 0.72 mmol, 99%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.92 (m, 1H), 3.90-3.84 (m, 0.15H), 2.58 (ddd, J_1 = 17.6, J_2 = 15.2, J_3 = 6.2 Hz, 1H), 2.49-2.40 (m, 2H), 2.37-2.26 (m, 3H), 2.12-2.03 (m, 1H), 2.00-1.90 (m, 2H), 1.92-1.80 (m, 2H), 1.78-1.65 (m, 2H), 1.60-1.49 (m, 2H), 1.27-1.20 (m, 2H), 1.11 (qd, J_1 = 12.3, J_2 = 4.0 Hz, 1H),

1.03 (ddd, J_1 = 12.9, J_2 = 11.1, J_3 = 4.1 Hz, 1H), 0.90 (s, 3H), 0.84 (s, 9H), 0.03 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 220.4, 199.0, 167.5, 126.1, 54.1, 51.4, 47.7, 43.5, 36.0, 35.8, 34.8, 33.7, 33.3, 31.8, 30.9, 25.9, 21.8, 21.0, 18.1, 14.0, -5.55, -5.62. HRMS (*m*/*z*): calcd for $C_{25}H_{39}D_2O_3Si$, $[M+H]^+$, 419.2945; found, 419.2939 (Δ 2.2 ppm).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SWH 5208.333 Hz
FIDRES 0.158946 Hz
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TE 298.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
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P1 11.38 usec
PL1 -2.50 dB
PL1W 18.35869598 W
SFO1 400.1320007 MHz
F2 - Processing parameters
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SF 400.1300098 MHz
WDW EM
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DD
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O
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9
8
7
6
5
4
3
2
1
ppm
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0.15
1.09
2.13
3.34
1.21
2.34
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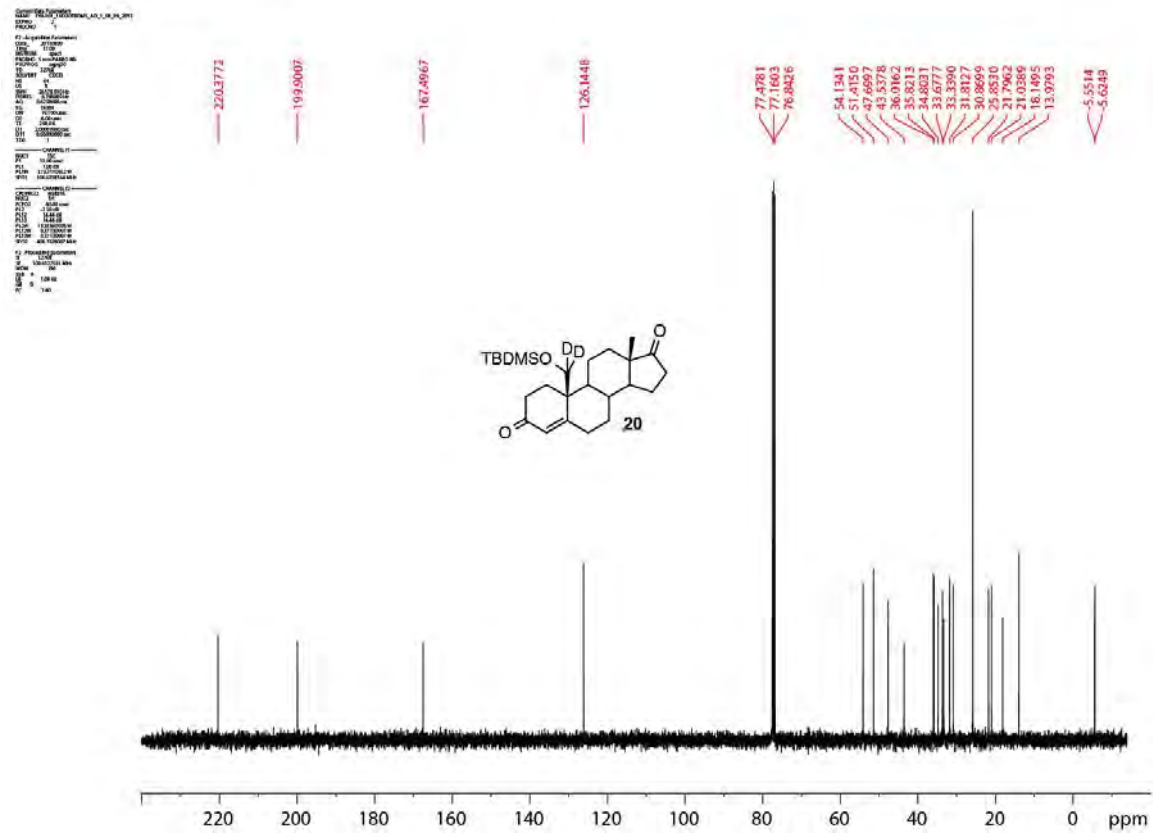
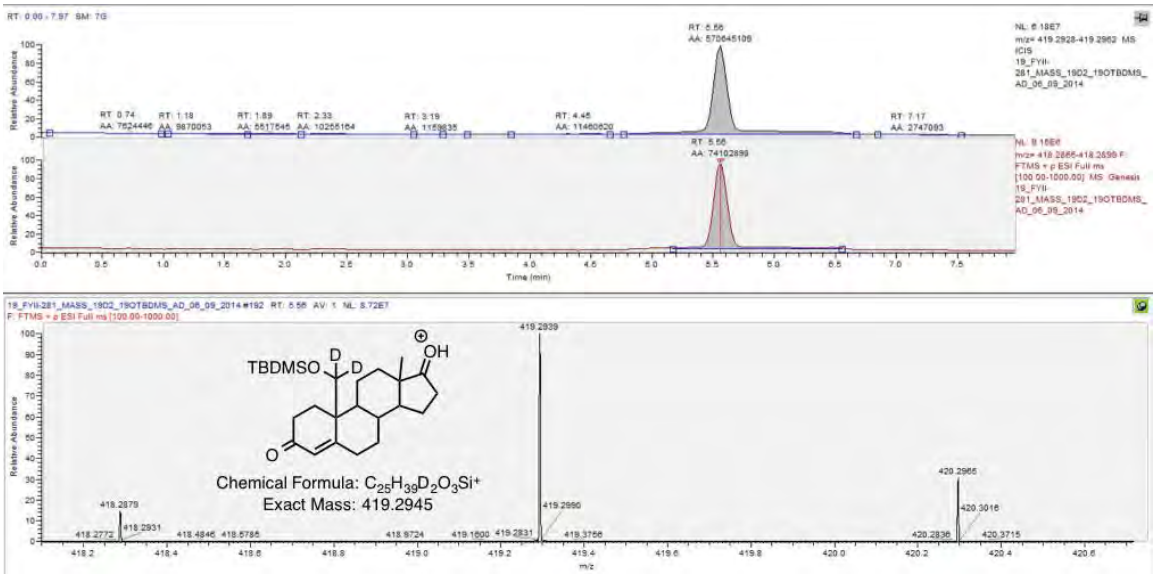
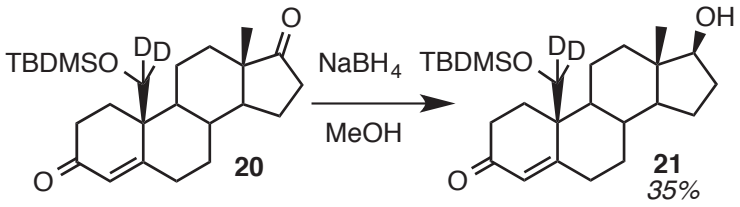
2.48
2.41
3.71
9.69
6.43

FYII-101_19D2OTBDMS_AD_1_08_09_2013

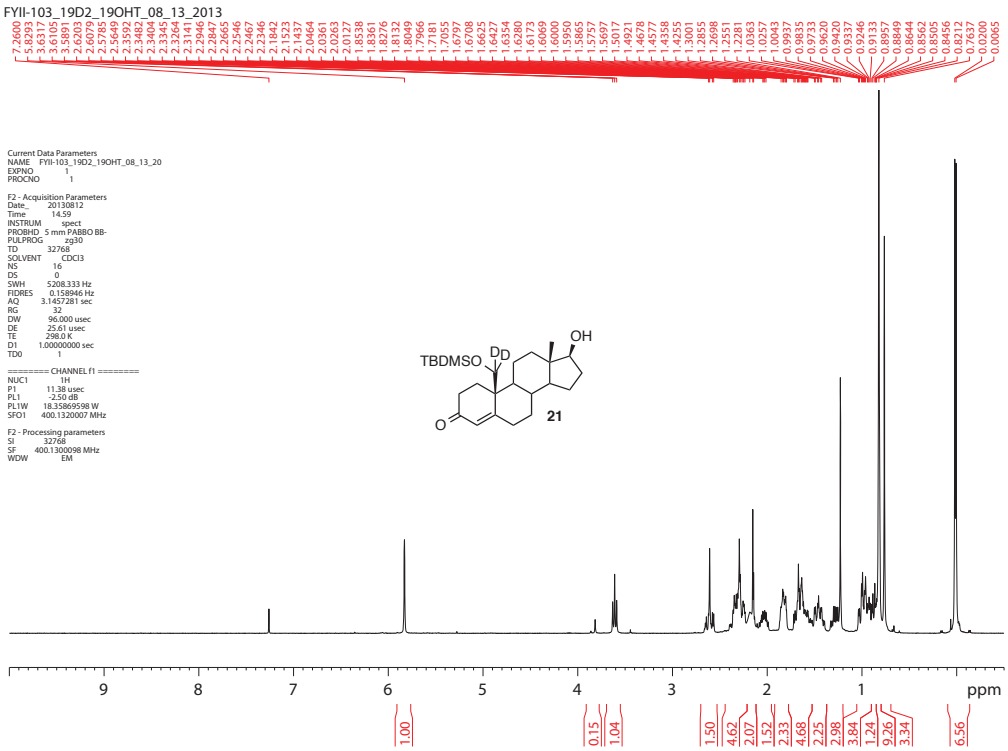






4.17. [19,19-$^2H_2$]-19-Hydroxy-TBDMS testosterone (21). $NaBH_4$ (6.5 mg, 0.70 mmol, 0.25 mol eq) was added to the above TBDMS ether (**20**) (285 mg, 0.68 mmol, 1.00 mol eq) in CH_3OH (100 mL). The reaction was stirred for 30 min. After concentration, the mixture was purified via flash column chromatography (100% hexanes to hexanes:EtOAc, 1:1, v-v) to afford the alcohol (**21**) (100 mg, 0.24 mmol, 35%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.83 (m, 1H), 3.86-3.82 (m, 0.15H), 3.61 (apparent t, J = 8.4 Hz, 1H), 2.65-2.56 (m, 1H), 2.39-2.23 (m, 4H), 2.08-1.99 (m, 1H), 1.85-1.80 (m, 2H), 1.72-1.52 (m, 4H), 1.51-1.39 (m, 2H), 1.33-1.22 (m, 2H), 1.04-0.91 (m, 4H), 0.90-0.85 (m, 1H), 0.82 (s, 9H), 0.76 (s, 3H), 0.02 (s, 3H), 0.01 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.4, 168.3,

126.0, 81.6, 54.2, 51.0, 43.6, 43.0, 37.0, 36.5, 34.9, 33.7, 33.4, 31.7, 30.4, 29.3, 25.8, 23.4, 21.4, 18.1, 11.3, -5.57, -5.66.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DE 25.61 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1
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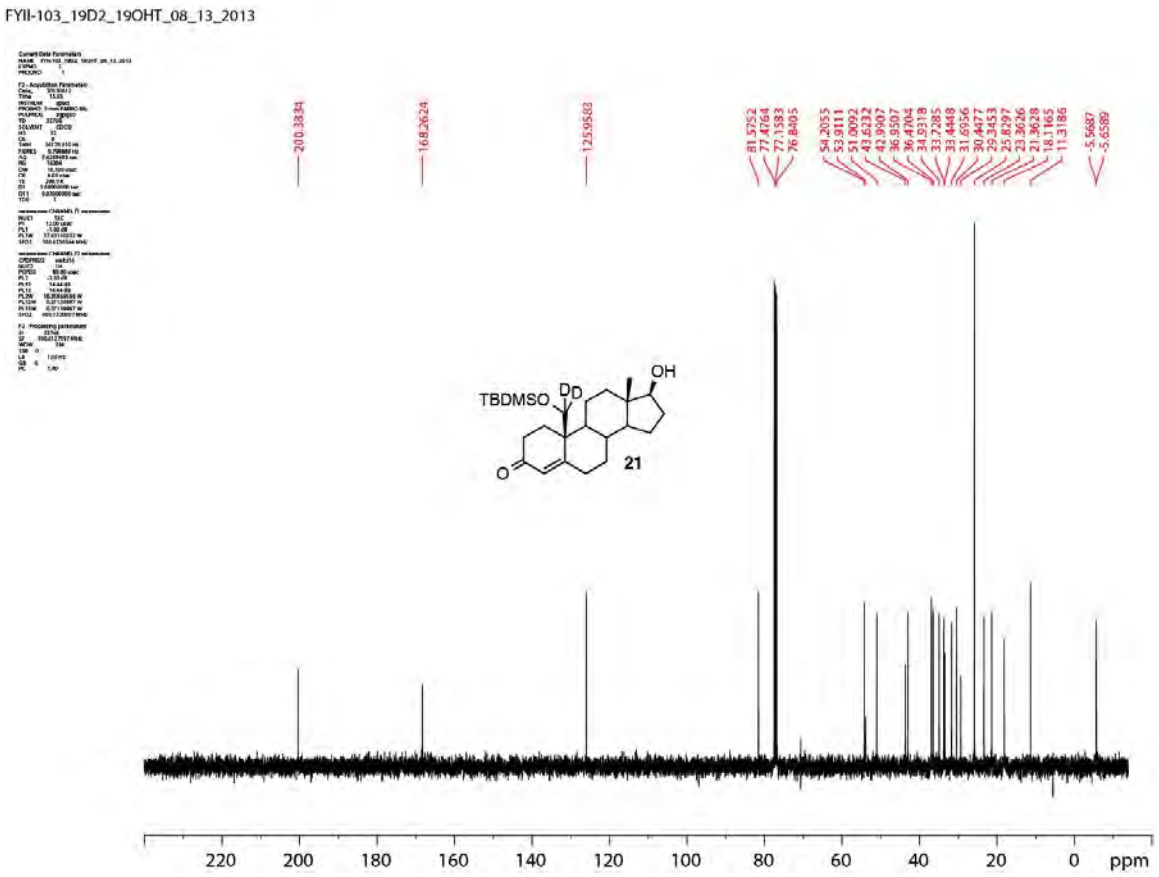
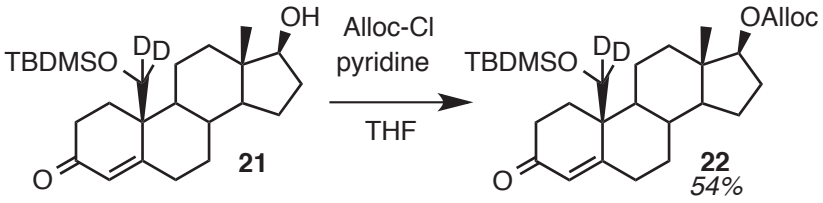
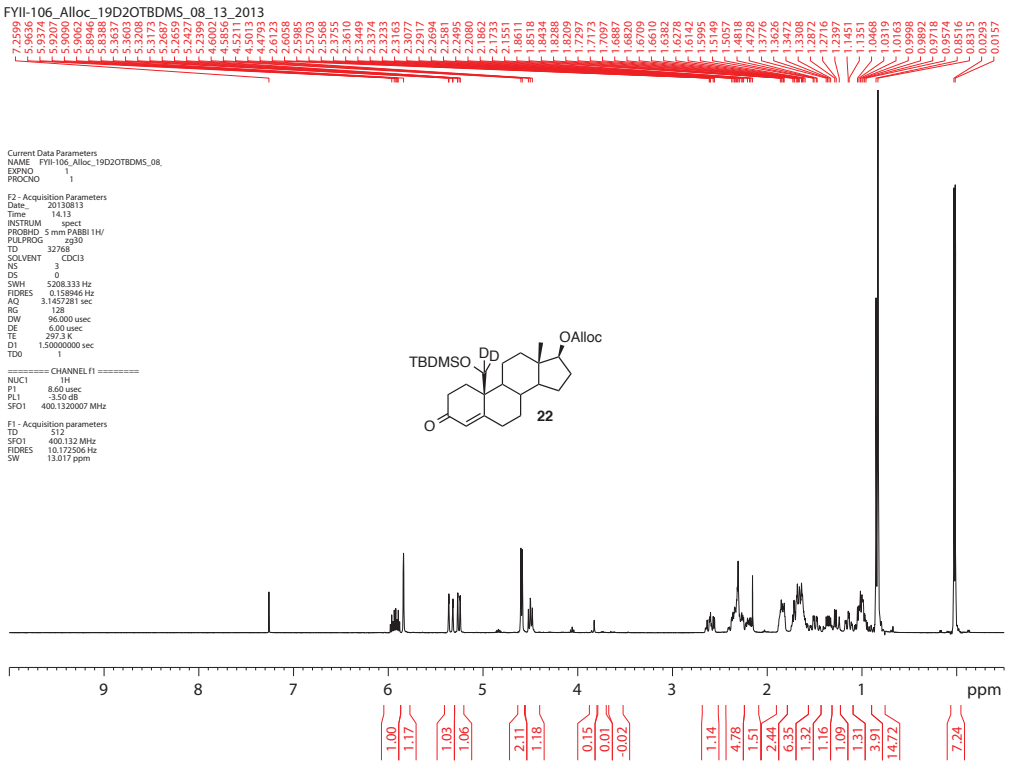




4.18. [19,19-$^2H_2$]-19-Hydroxy-TBDMS testosterone 17β-ol-Alloc (22). Allyl chloroformate (3 mL) was added to a solution of pyridine (5 mL) and the above alcohol (**21**) (100 mg, 0.24 mmol) in THF (100 mL) at 0 °C. The reaction was stirred for 2 h. The reaction mixture was diluted with EtOAc (100 mL) and washed with $NaHCO_3$ (saturated aq. solution, 2 × 50 mL). The organic extract was dried with $MgSO_4$ and concentrated *in*

vacuo. The crude material was purified by preparative TLC (hexanes:EtOAc, 1:1, v-v) to afford the allylic carbonate (**22**) (65 mg, 0.13 mmol, 54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.98-5.88 (m, 1H), 5.84 (m, 1H), 5.34 (dd, J_1 = 17.2, J_2 = 1.2 Hz, 1H); 5.25 (dd, J_1 = 17.2, J_2 = 1.1 Hz, 1H), 4.59 (d, J = 5.8 Hz, 1H), 4.50 (apparent t, J = 8.8 Hz), 2.65-2.56 (m, 1H), 2.41-2.25 (m, 4H), 2.21-2.16 (m, 1H), 1.89-1.81 (m, 2H), 1.75-1.57 (m, 6H), 1.49 (apparent qd, J_1 = 13.4, J_2 = 3.8 Hz, 1H), 1.08-0.92 (m, 3H), 0.85 (s, 3H), 0.83 (s, 9H), 0.03 (s, 3H), 0.02 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 200.1 (C-3), 167.8 (C-5), 131.8, 126.1 (C-4, CH), 119.0, 86.2, 68.4, 54.0, 50.6, 43.6, 42.7, 37.1, 36.2, 34.9, 33.7, 33.4, 31.6, 27.4, 25.8, 23.4, 21.3, 18.1, 12.2, -5.5, -5.6.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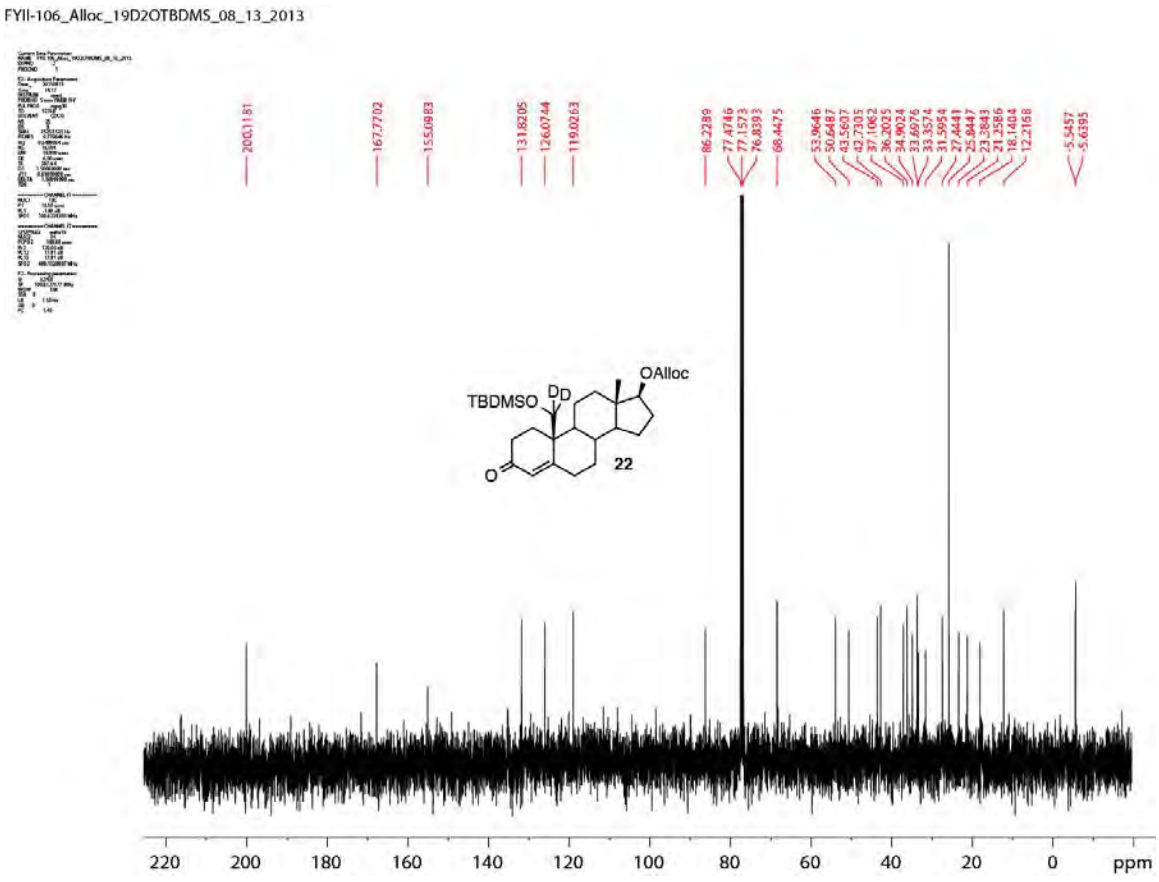
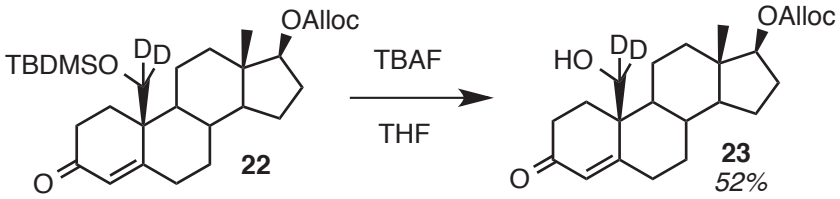
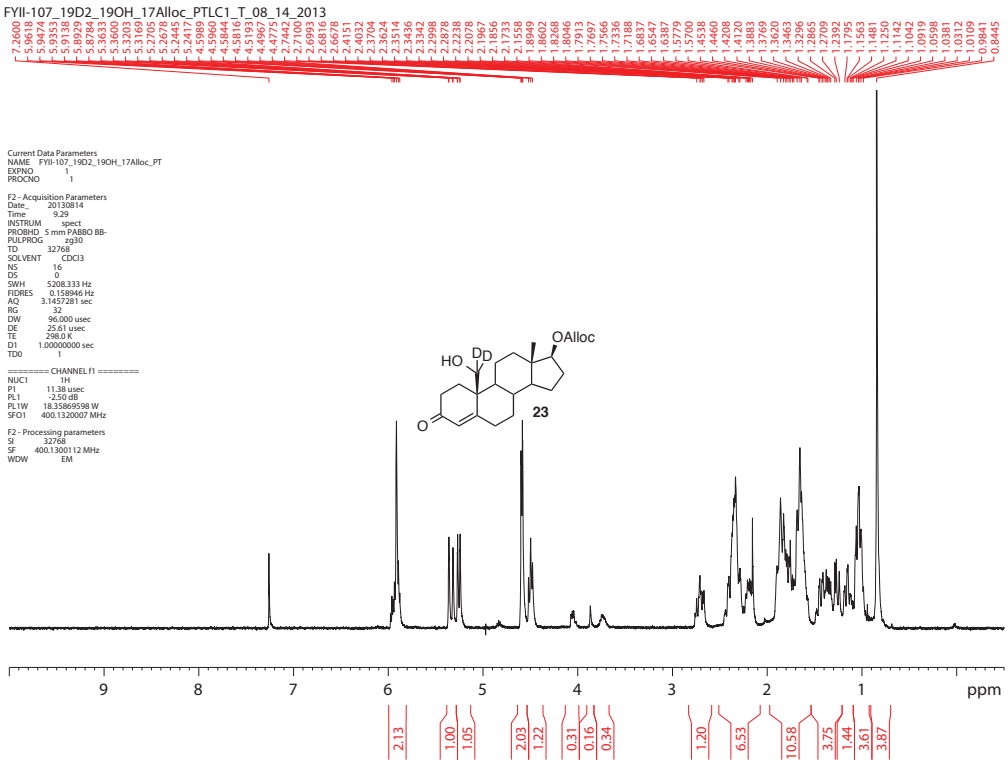




4.19. [19,19-$^2H_2$]-19-Hydroxy-TBDMS testosterone 17β-ol-Alloc (23). TBAF (1 M solution in THF, 0.15 mL, 0.15 mmol) was added to a solution of the above TBDMS ether (**22**) (65 mg, 0.13 mmol) in THF. The reaction was stirred for 10 h and the mixture was purified by preparative TLC to afford the alcohol (**23**) (26 mg, 0.067 mmol, 52%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.96-5.88 (m, 2H), 5.34 (dd, J_1 = 17.2, J_2 = 1.2 Hz, 1H), 5.25 (dd, J_1 = 10.4, J_2 = 1.2 Hz, 1H), 4.59 (dd, J_1 = 5.8, J_2 = 1.2 Hz, 1H), 4.50 (apparent t, J = 7.7 Hz, 1H), 2.74-2.67 (m, 1H), 2.44-2.16 (m, 6H), 1.94-1,55 (m, 10H), 1.48-1.24 (m,

3H), 1.18-1.15 (m, 1H), 1.04-0.94 (m, 3H), 0.84 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.9, 167.0, 131.8 (CH), 127.1 (CH), 119.1 (CH_2), 86.2 (CH), 68.5 (CH_2), 53.9 (CH), 50.6 (CH), 43.8, 42.7, 37.0 (CH_2), 36.2 (CH), 35.1 (CH_2), 33.6 (CH_2), 33.5 (CH_2), 31.7 (CH_2), 27.4 (CH_2), 23.4 (CH_2), 21.1, 12.2 (CH_3).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5
4
3
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ppm
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1.00
1.05
2.03
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0.34
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3.61
3.87

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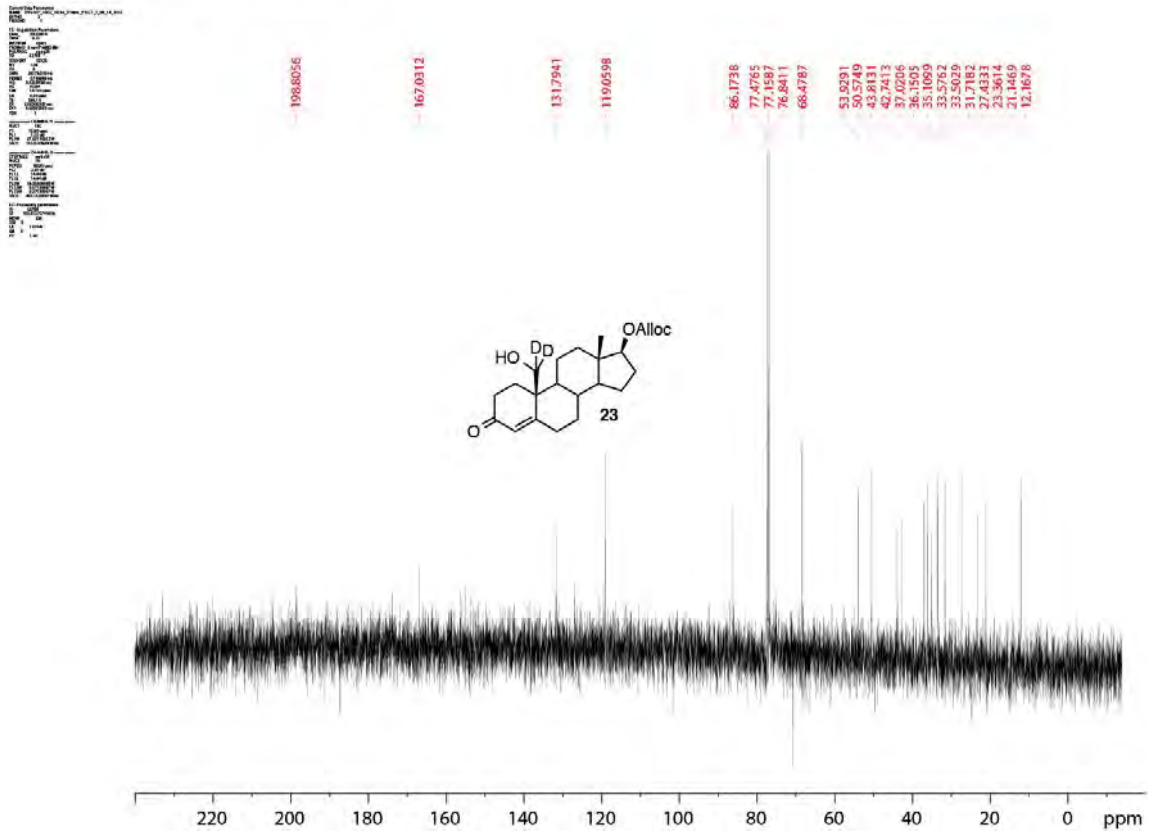
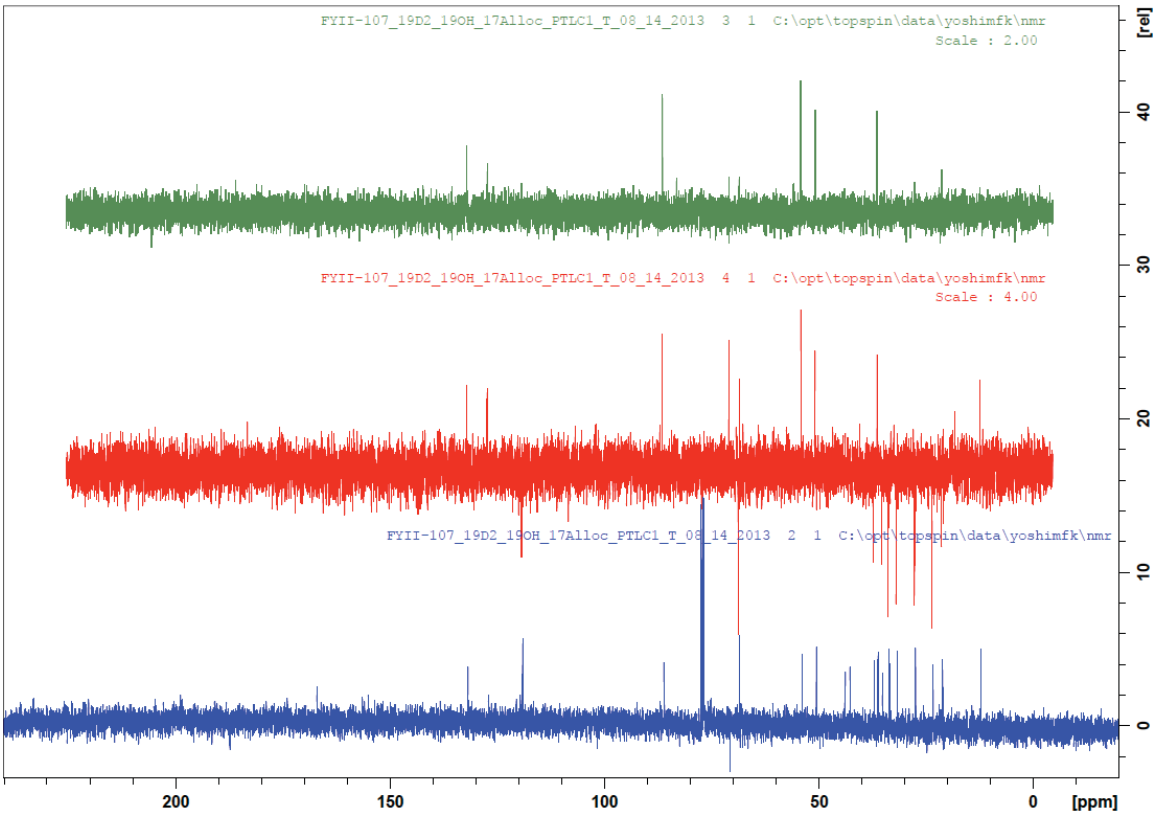
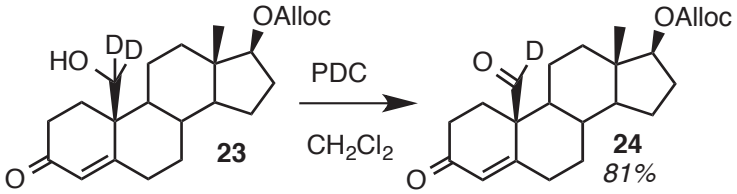
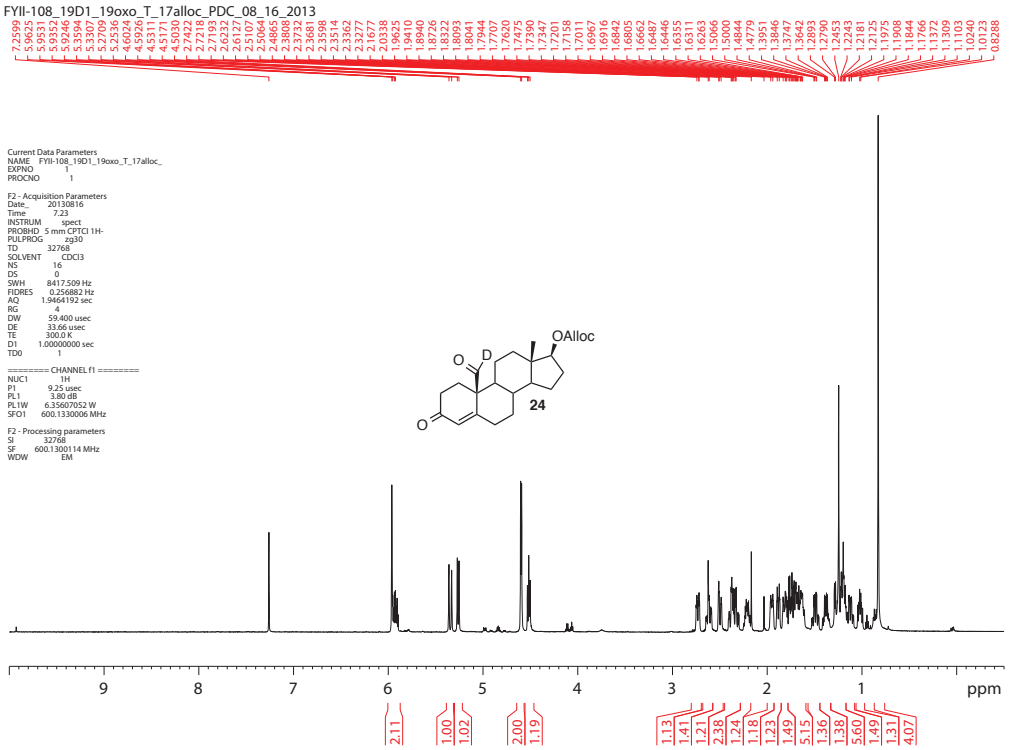






4.20. [19-$^2H_1$]-19-Oxotestosterone 17β-ol-Alloc (24). Pyridinium dichromate (25 mg, 0.067 mmol) was added to a solution of the preceding alcohol (**23**) (26 mg, 0.067 mmol) in CH_2Cl_2 (5 mL). The reaction mixture was stirred for 24 h and purified by preparative TLC to afford 19-deutero-3,19-dioxo-androst-4-ene 17β-allylic carbonate (**24**) (21 mg, 0.054 mmol, 81%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.96 (m, 1H), 5.95-5.90 (m, 1H), 5.35 (d, J = 17.2 Hz, 1H), 5.26 (d, J = 10.4 Hz, 1H), 4.60 (d, J = 5.9 Hz, 2H), 4.52 (apparent t, J = 8.5 Hz, 1H), 2.73 (ddd, J_1 = 13.8, J_2 = 4.6, J_3 = 3.1 Hz, 1H), 2.65-2.59 (m, 1H), 2.51-2.49 (m, 1H), 2.41-2.37 (m, 1H), 2.36-2.30 (m, 1H), 2.24-2.18 (m, 1H), 1.96-1.93 (m,

1H), 1.90-1.87 (m, 1H), 1.83-1.79 (m, 1H), 1.79-1.61 (m, 1H), 1.49 (apparent qd, J_1 = 13.3, J_2 = 4.0 Hz, 1H), 1.42-1.35 (m, 1H), 1.29-1.15 (m, 5H), 1.12 (qd, J_1 = 12.4, J_2 = 4.0 Hz, 1H), 1.04-0.99 (m, 1H), 0.83 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 200.85 (t, J = 26.4 Hz), 198.0, 161.0, 155.1, 131.8 (CH), 127.5, 119.1 (CH_2), 85.9 (CH), 68.5 (CH_2), 53.6 (CH), 50.4 (CH), 42.6, 36.8 (CH), 36.7 (CH_2), 34.3 (CH_2), 33.9 (CH_2), 31.1 (CH_2), 29.6 (CH_2), 27.3 (CH_2), 23.3 (CH_2), 21.4 (CH_2), 12.0 (CH_3).



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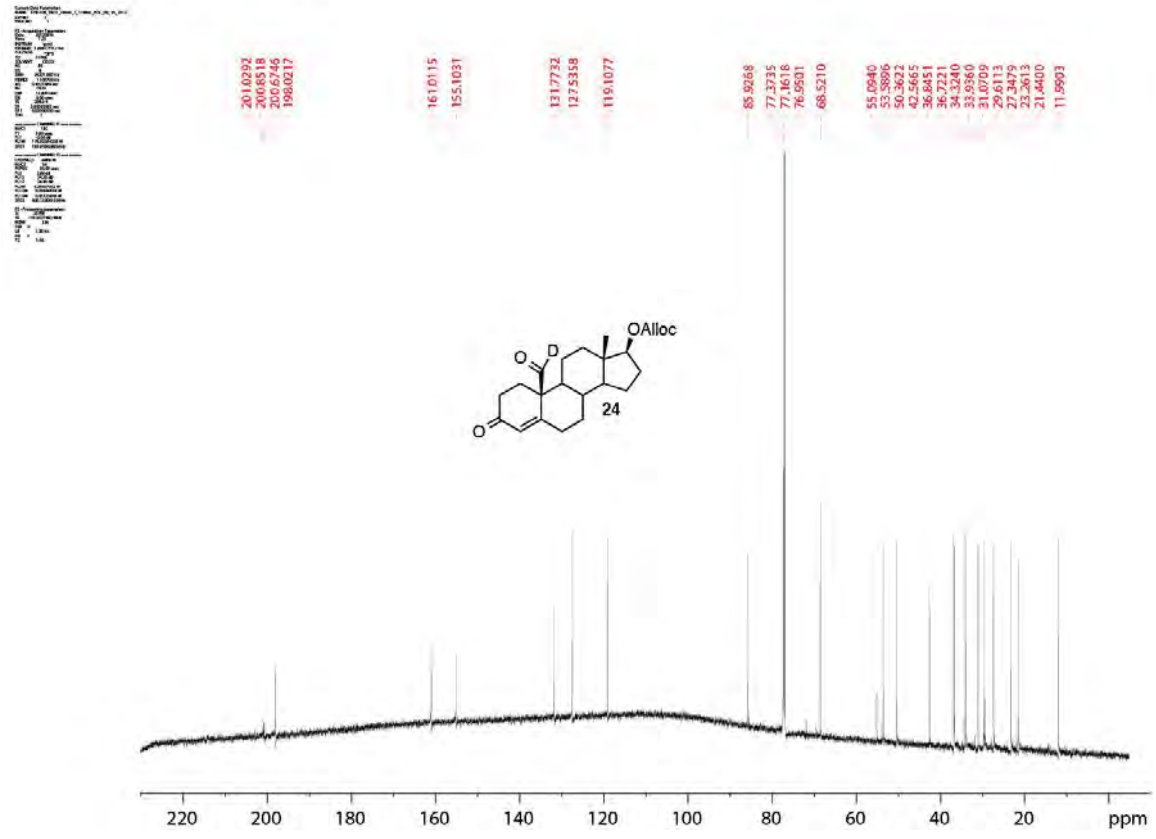
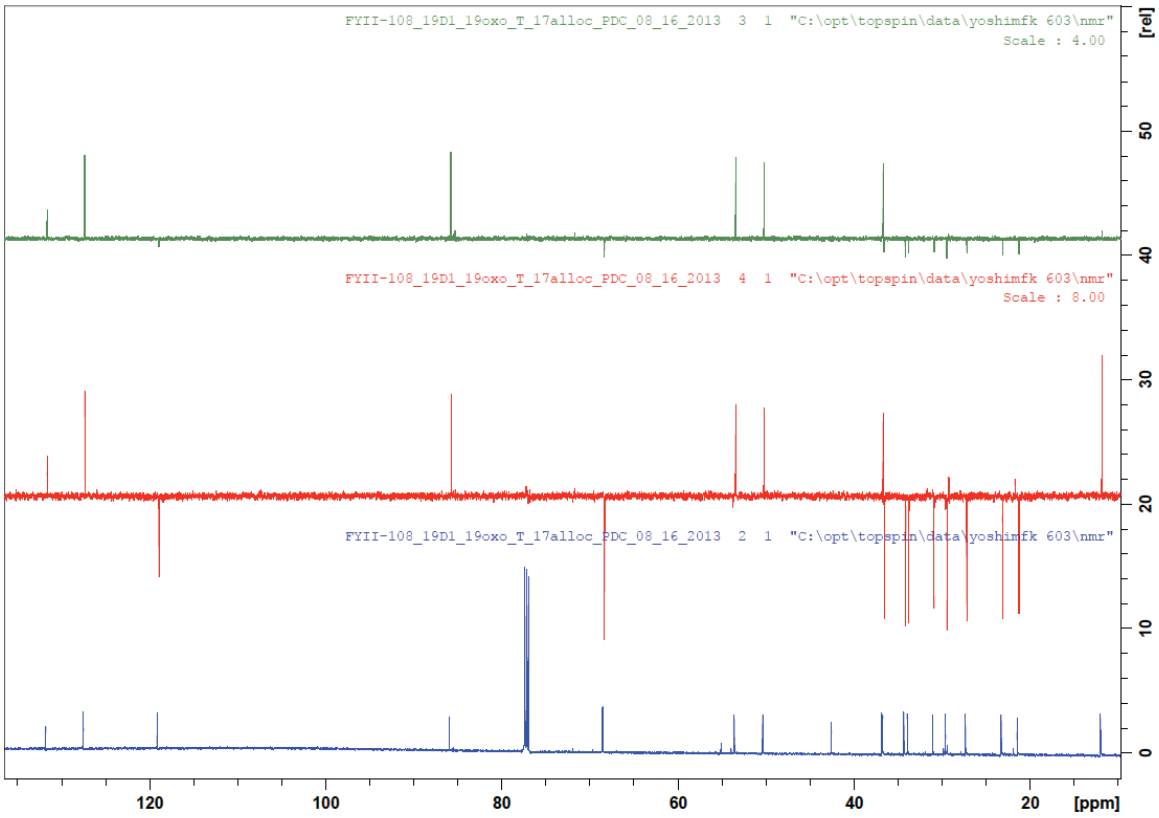
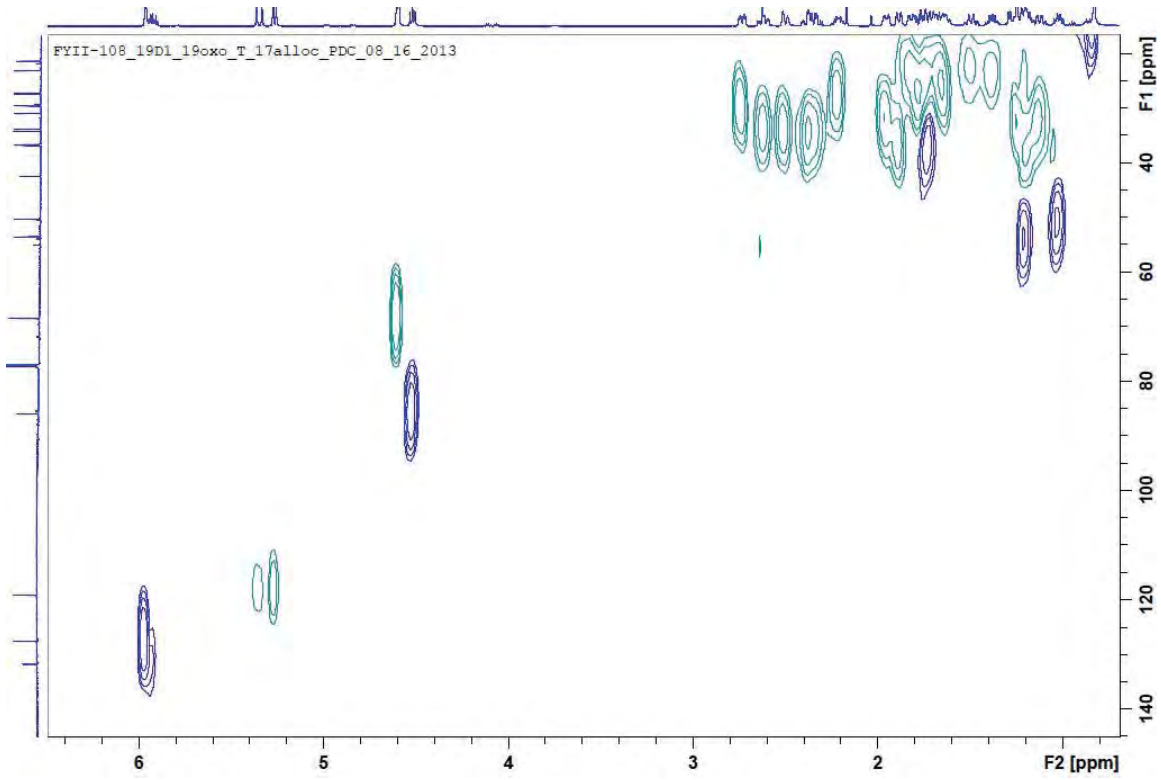
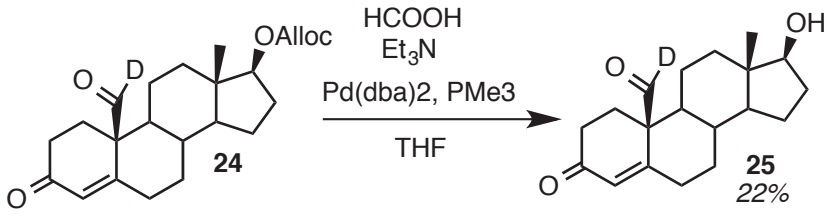


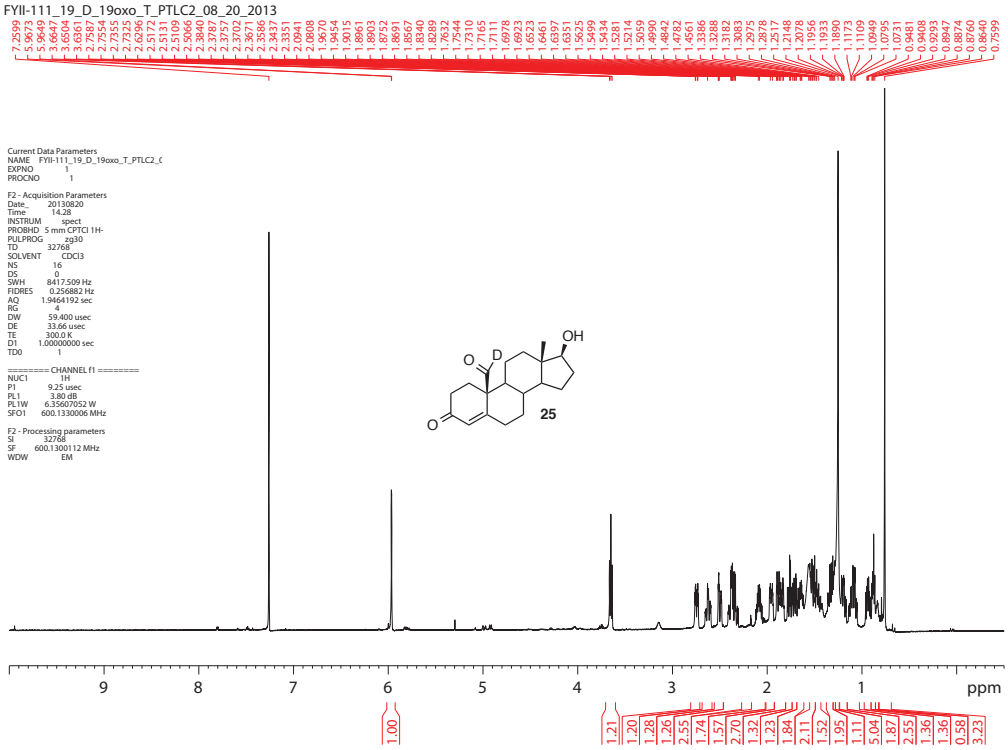






4.21. [19-$^{2}H_{1}$]-19-Oxotestosterone (25). Trimethylphosphine, in THF (1 M solution, 0.2 mL, 0.2 mmol, 7.8 mol eq), was added to $Pd(DBA)_2$ (29 mg, 0.05 mmol, 1.9 mol eq). TEA (0.5 mL, 3.6 mmol) was added followed by formic acid (4 mL) at 0 °C. A solution of allylic carbonate (**24**) (10 mg, 0.03 mmol, 1.0 mol eq) in THF (4 mL) was added and the reaction mixture was stirred for 2 h under Ar. The reaction mixture was under a stream of air and purified via preparative TLC (hexanes: EtOAc: 3:1, v-v) to afford the alcohol (**25**) (2 mg, 0.0066 mmol, 22%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.97-5.96 (m, 1H), 3.65 (apparent t, J = 8.6 Hz, 1H), 2.75 (ddd, J_1 = 13.8, J_2 = 4.9, J_3 = 3.1 Hz, 1H),

2.66-2.60 (m, 1H), 2.50 (ddd, J_1 = 15.1, J_2 = 4.0, J_3 = 2.5 Hz, 1H), 2.41-2.31 (m, 2H), 2.12-2.06 (m, 1H), 1.97-1.94 (m, 1H), 1.90-1.82 (m, 2H), 1.77 (dd, J_1 = 14.0, J_2 = 5.3 Hz, 1H), 1.74-1.69 (m, 1H), 1.68-1.62 (m, 1H), 1.48-1.44 (m, 1H), 1.36-1.28 (m, 2H), 1.22-1.17 (m, 1H), 1.14-1.05 (m, 2H), 0.96-0.91 (m, 1H), 0.89-0.86 (m, 1H), 0.76 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 198.2, 161.3, 127.5, 81.5, 53.9, 50.7, 42.8, 37.2, 36.6, 34.4, 34.0, 31.2, 30.5, 29.8, 29.6, 23.3, 21.6, 11.1; HRMS (*m/z*): calcd for $C_{19}H_{26}DO_3$, $[M+H]^+$, 304.2017; found, 304.2023 (Δ 2.0 ppm).



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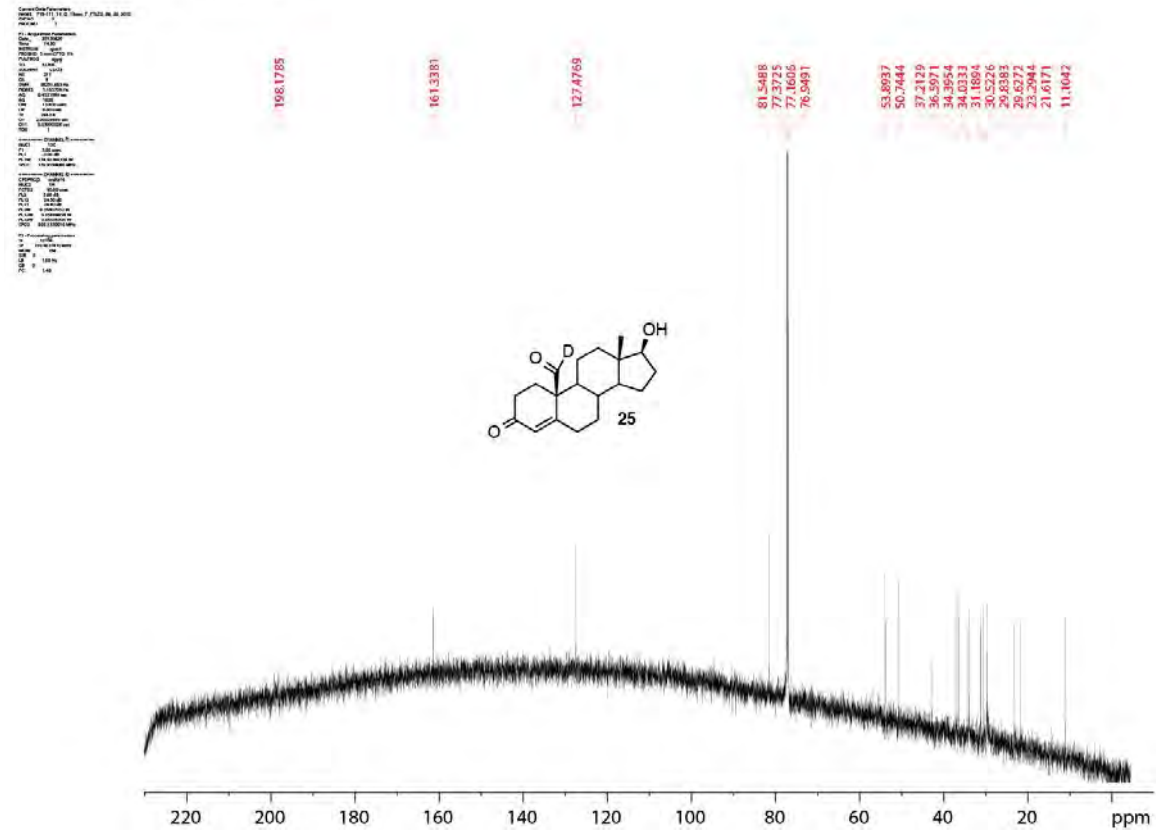
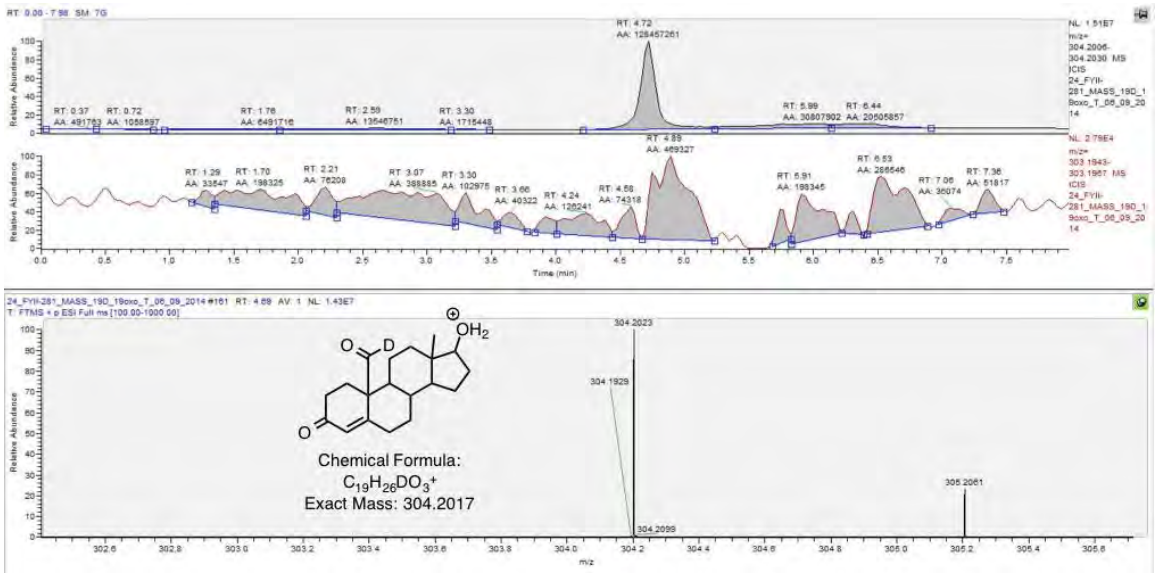
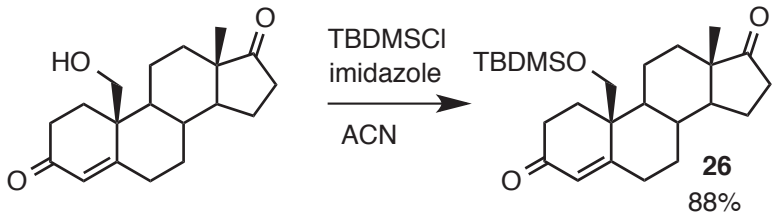
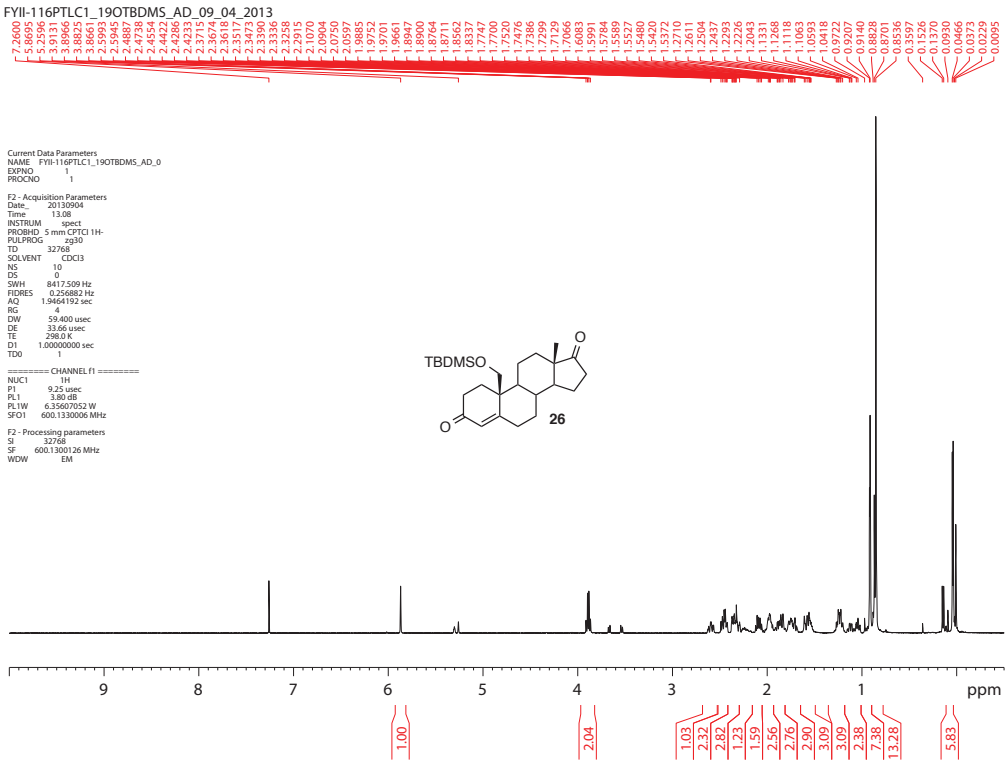






4.22. 19-[(*tert*-Butyl)dimethylsilyloxy]-androstenedione (26). TBDMS-chloride (2.6 g, 17 mmol, 10 mol eq) was added to a solution of 19-hydroxyandrostenedione (550 mg, 1.7 mmol, 1.0 mol eq) and imidazole (500 mg, 7.3 mmol, 4.3 mol eq) in CH_3CN (50 mL). The solution was stirred for 20 h. The reaction mixture was washed with H_2O (100 mL) and extracted with EtOAc (150 mL). The organic layer was dried *in vacuo* and purified by flash column chromatography (100% hexanes to 50% hexanes/EtOAc, v/v) to afford the TBDMS ether (**26**) (623 mg, 1.5 mmol, 88%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.87 (m, 1H), 3.90 (d, *J* = 9.9 Hz, 1H), 3.87 (d, *J* = 8.5 Hz, 1H), 2.62-2.57 (m, 1H), 2.49-2.42 (m, 2H), 2.37-2.32 (m, 2H), 2.12-2.06 (m 1H), 2.01-1.94 (m, 2H), 1.91-1.82 (m, 2H), 1.77-1.66 (m, 2H), 1.61-1.50 (m, 2H), 1.29-1.18 (m, 3H), 1.15-1.02 (m, 2H), 0.91 (s, 3H), 0.85 (s, 9H), 0.05 (s, 3H), 0.04 (s, 3H); ^{13}C NMR ($CDCl_3$: 77.1585) (150 MHz, $CDCl_3$) δ 220.4, 199.9, 167.5, 126.2, 66.0031 (C19), 54.2, 51.5, 47.7, 43.7, 36.1, 35.9, 34.8, 33.7, 33.4, 31.9, 30.9, 25.8, 21.1, 14.0, -5.60.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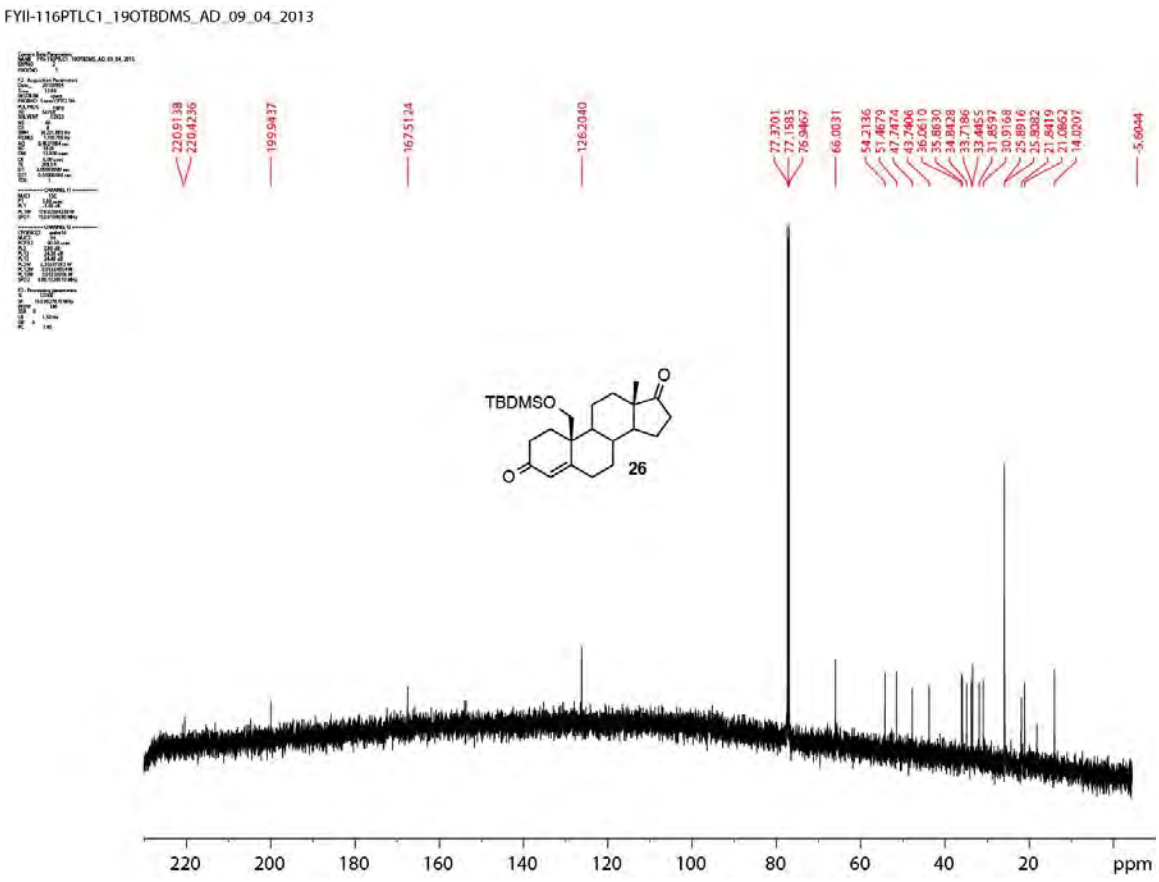
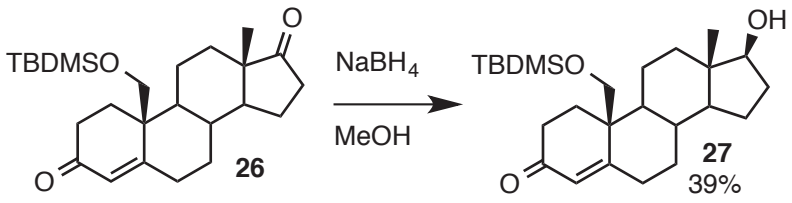
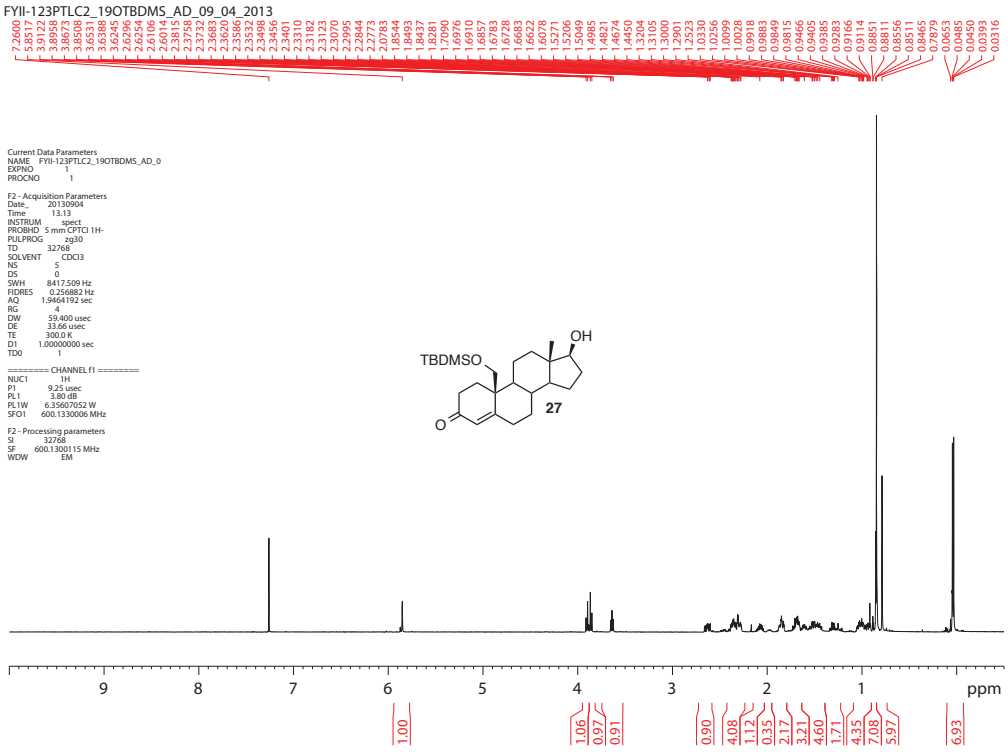
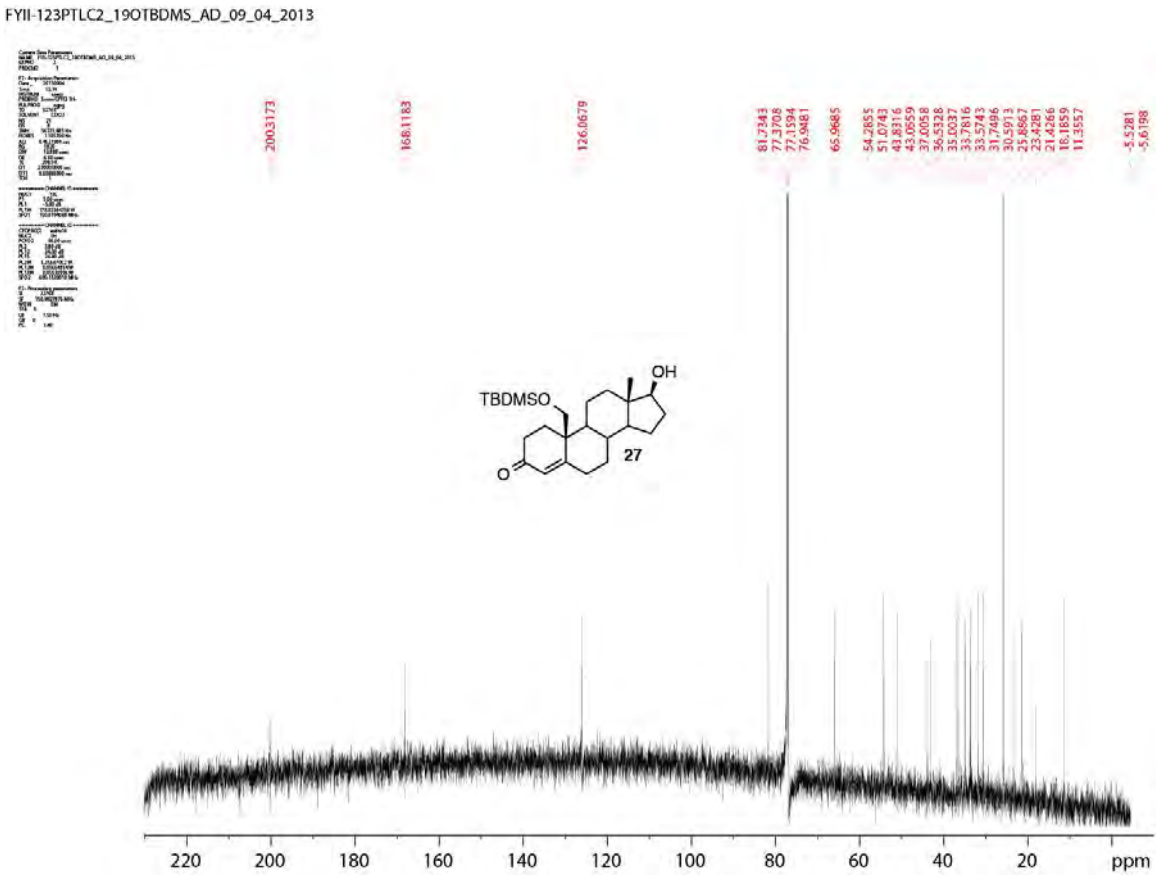


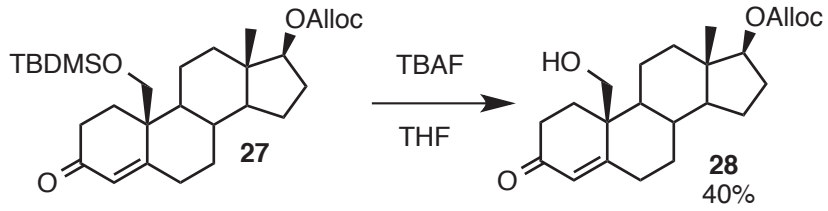


4.23. 19-[(*tert*-Butyl)dimethylsilyloxy]-testosterone (27). To a solution of diketone (**26**) (3.1 g, 7.4 mmol) in CH_3OH (200 mL) was added $NaBH_4$ (0.07 g, 1.9 mmol) and after 10 min, EtOAc (100 mL) was added and the reaction mixture was washed with water (100 mL). The organic layer was dried with $MgSO_4$ and concentrated in vacuo. The crude material was purified via flash column chromatography (100% hexanes to 30% hexanes:EtOAc, v/v) to afford 19-*tert*-butyldimethylsiloxytestosterone (**27**) (1.2 g, 2.9 mmol, 39%). R_f 0.20 (hexanes:EtOAc, 4:1, v-v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.85 (m,

1H), 3.90 (d, J = 10.1 Hz, 1H), 3.86 (d, J = 9.9 Hz, 1H), 3.64 (apparent t, J = 8.6 Hz, 1H), 2.63 (ddd, J_1 = 16.8, J_2 = 14.1, J_3 = 5.4 Hz, 1H), 2.41-2.27 (m, 4H), 2.11-2.04 (m, 1H), 1.88-1.82 (m, 2H), 1.73-1.66 (m, 3H), 1.64-1.43 (m, 4H), 1.34-1.28 (m, 1H), 1.05-0.91 (m, 4H), 0.85 (s, 9H), 0.79 (s, 3H), 0.05 (s, 3H), 0.03 (s, 3H); ^{13}C NMR ($CDCl_3$: 77.1594) (150 MHz, $CDCl_3$) δ 200.3, 168.1, 126.1, 81.7, 65.9685 (C19), 54.3, 51.1, 43.8, 43.1, 37.0, 36.5, 35.0, 33.8, 33.6, 31.7, 30.6, 25.9, 23.4, 21.4, 18.2, 11.4, -5.53, -5.62.

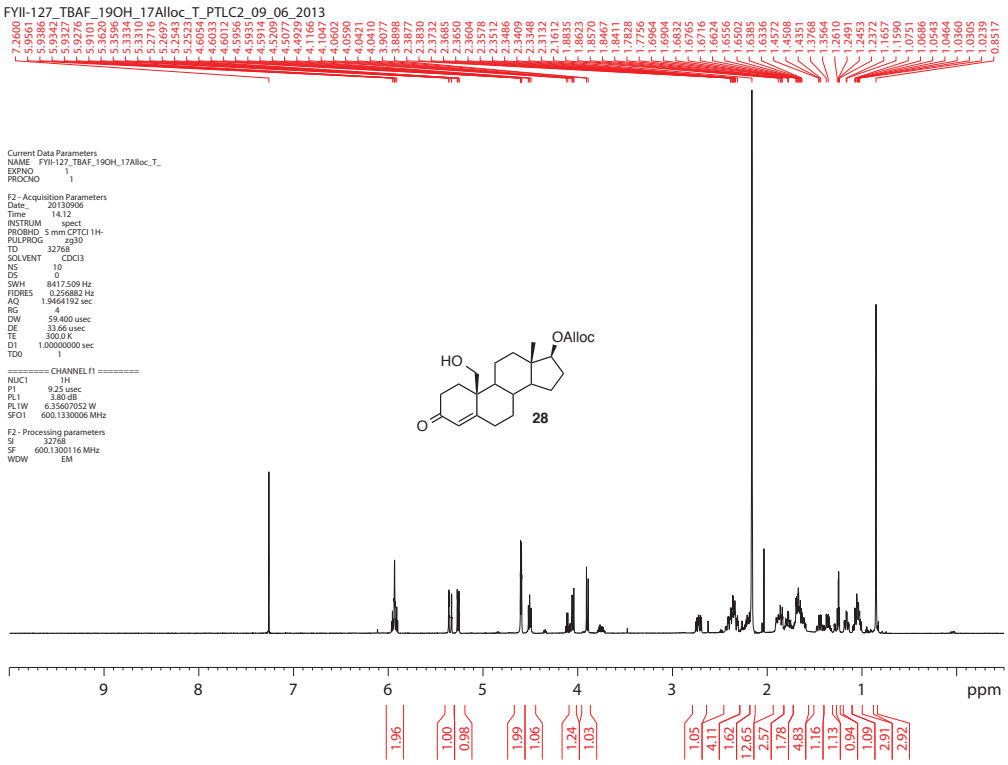






4.24. 19-[(*tert*-Butyl)dimethylsilyloxy]-testosterone 17β-(2-propenyl) carbonate (28). Pyridine (5 mL) was added to a solution of 19-*tert*-butyldimethylsiloxytestosterone (**27**) (1.2 g, 2.9 mmol) in THF at 0 °C followed by allyl chloroformate (3 mL). The reaction was stirred for 3 h and warmed to ambient temperature. The mixture was diluted with EtOAc (200 mL) and washed with $NaHCO_3$ (sat. aqueous, 2 × 40 mL). The organic

extract was washed with brine (2 × 50 mL). The recovered organic layer was dried with $MgSO_4$ and concentrated *in vacuo* and purified by flash column chromatography (100% hexanes to 50% hexanes:EtOAc, v/v) to afford the allylic carbonate (**28**) (0.45 g, 0.90 mmol, 31%). R_f 0.40 (hexanes:EtOAc, 4:1, v-v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.96-5.90 (m, 1H), 5.85 (m, 1H), 5.35 (ddd, J_1 = 14.4, J_2 = 2.82, J_3 = 1.38 Hz, 1H), 5.26 (dd, J_1 = 10.4, J_2 = 1.20 Hz, 1H), 4.60 (dt, J_1 = 5.8, J_2 = 1.3 Hz, 1H), 4.51 (apparent t, J = 7.9 Hz, 1H), 3.88, 3.85 (ABq, J = 10.0 Hz, 2H), 2.61 (ddd, J_1 = 16.9, J_2 = 14.5, J_3 = 5.5 Hz, 1H), 2.41-2.25 (m, 4H), 2.22-2.18 (m, 1H), 1.88-1.82 (m, 2H), 1.73-1.59 (m, 6H), 1.50 (qd, J_1 = 13.1, J_2 = 3.9 Hz, 1H), 1.40-1.32 (m, 1H), 1.15 (qd, J_1 = 13.0, J_2 = 3.8 Hz, 1H), 1.07-0.96 (m, 3H), 0.86 (s, 3H), 0.84 (s, 6H), 0.42 (s, 3H), 0.36 (s, 3H); ^{13}C NMR ($CDCl_3$: 77.1604) (100 MHz, $CDCl_3$) δ 199.8, 176.4, 167.5, 154.9, 131.7, 125.9, 118.8, 86.0538 (C19), 68.3, 65.7, 53.9, 50.5, 43.6, 42.6, 37.0, 36.1, 34.8, 33.6, 33.3, 31.5, 27.3, 25.7, 23.3, 21.1, 18.0, 12.1, -5.68, -5.78.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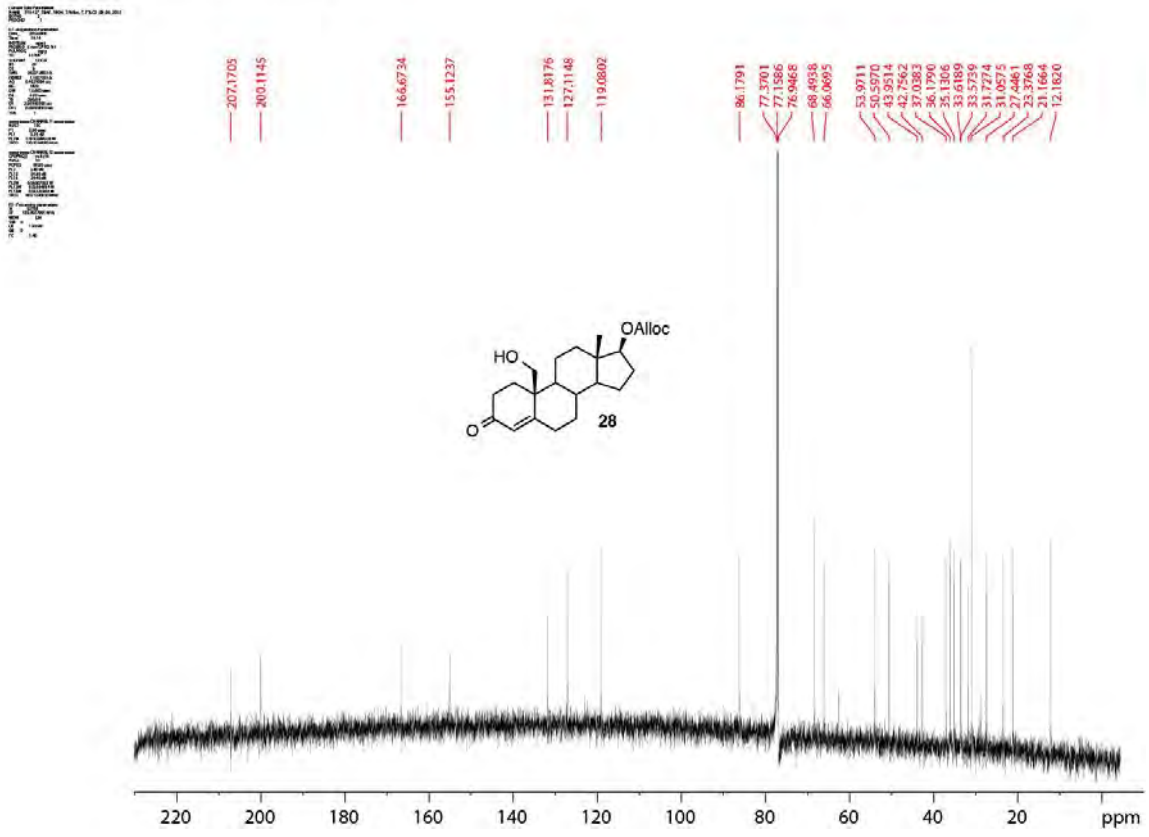
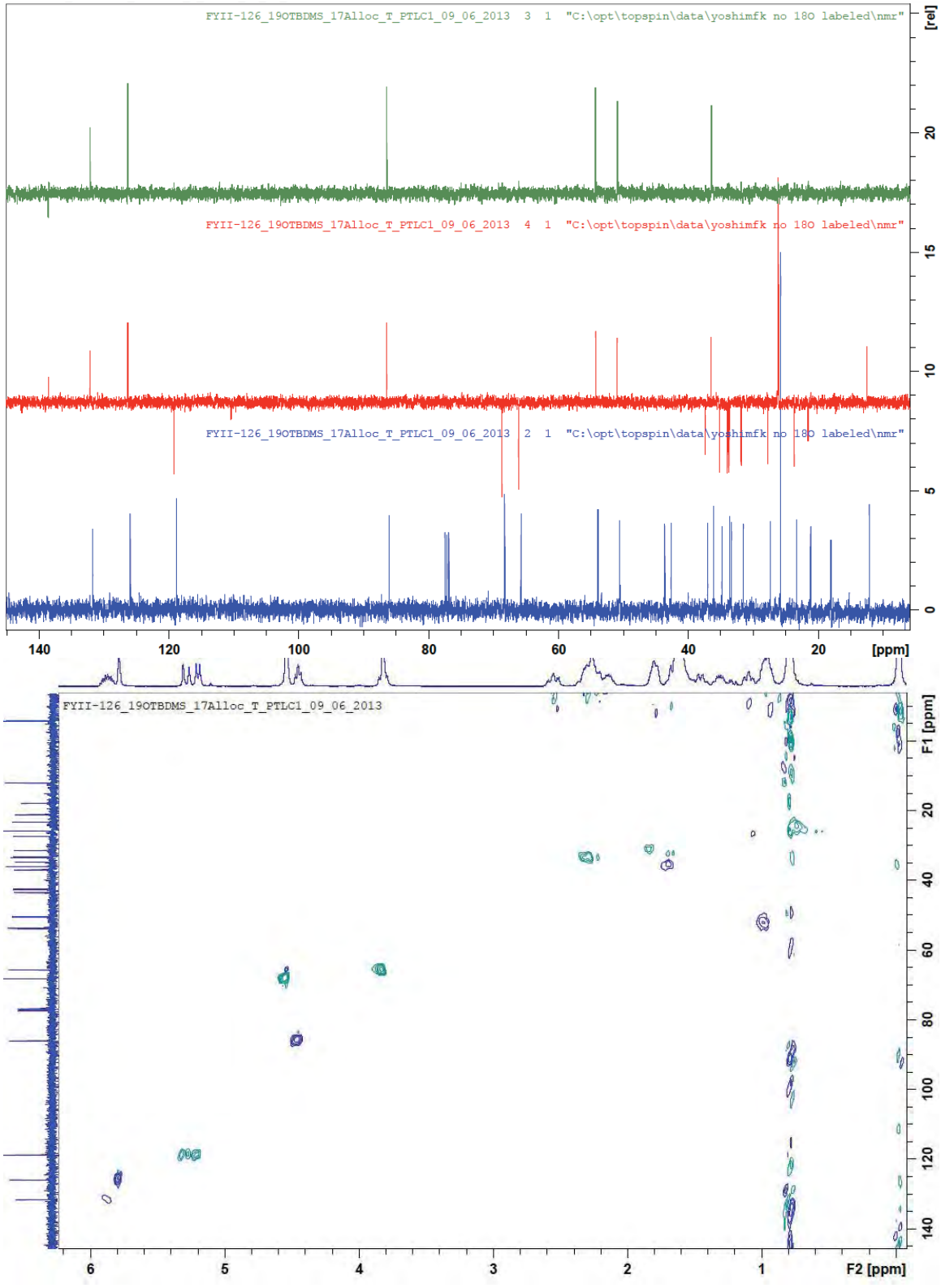
4
3
2
1
ppm
1.96
1.00
0.98
1.99
1.06
1.24
1.03
1.05
4.11
1.62
12.65
2.57
1.78
4.83
1.16
1.13
0.94
1.09
2.91
2.92

FYII-127_TBAF_19OH_17Alloc_T_PTLC2_09_06_2013

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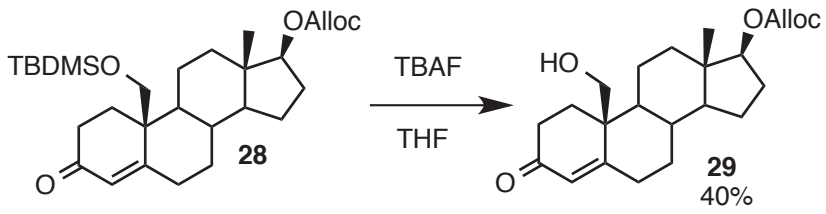
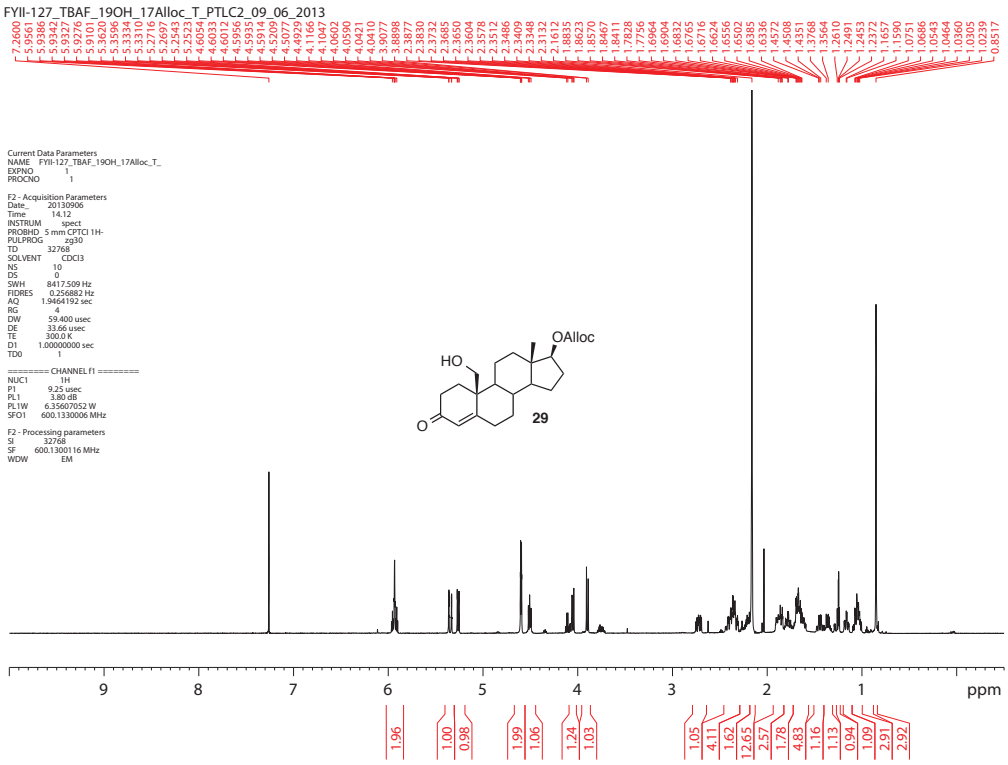


4.25. 19-Hydroxytestosterone 17β-(2-propenyl) carbonate (29). TBAF (0.90 mL of a 1 M solution in THF, 0.90 mmol) was added to a solution of TBDMS ether (**28**) (0.45 g, 0.90 mmol) was in THF (100 mL). The reaction was stirred for 1 h and diluted with EtOAc (100 mL). The reaction mixture was washed with water (2 × 50 mL) and the organic extract was concentrated *in vacuo*. The crude material was purified by flash column chromatography (100% hexanes to 30% hexanes:EtOAc, v/v) to afford the alcohol (**29**) (140 mg, 0.36 mmol, 40%) as a white solid. R_f 0.50 (hexanes:EtOAc, 1:1, v-v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.93 (m, 1H), 5.96-5.90 (m, 1H), 5.34 (ddd, J_1 = 17.2, J_2 = 2.8, J_3 = 1.4 Hz, 1H), 5.26 (dt, J_1 = 5.8, J_2 = 1.3 Hz, 2H), 4.51 (apparent t, J = 7.9 Hz, 1H), 4.06 (dd, J_1 = 10.9, J_2 = 0.72 Hz, 1H), 3.90 (d, J = 10.8 Hz, 1H), 2.72 (ddd, J_1 = 16.4, J_2 = 13.3, J_3 = 4.9 Hz, 1H), 2.44-2.31 (m, 4H), 2.28-2.18 (m, 2H), 1.91-1.84 (m, 2H), 1.81-1.74 (m, 1H), 1.72-1.59 (m, 5H), 1.44 (qd, J_1 = 13.1, J_2 = 3.9 Hz, 1H), 1.40-1.32 (m, 1H), 1.25 (dd, J_1 = 9.4, J_2 = 7.1 Hz, 1H), 1.16 (td, J_1 = 13.0, J_2 = 4.08 Hz, 1H), 1.10-1.01 (m, 3H), 0.85 (s, 3H); ^{13}C NMR ($CDCl_3$: 77.1586) (100 MHz, $CDCl_3$) δ 207.2, 200.1, 166.7, 155.1, 131.8, 127.1, 119.0, 86.2, 68.5, 66.0695 (C19), 54.0, 50.6, 44.0, 42.8, 37.0, 36.2, 35.1, 33.6, 35.1, 33.62, 33.57, 31.7, 31.1, 27.4, 23.4, 21.2, 12.2.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PROCNO 1
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TE 300.0 K
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TD0 1
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F2 - Processing parameters
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OAlloc
O
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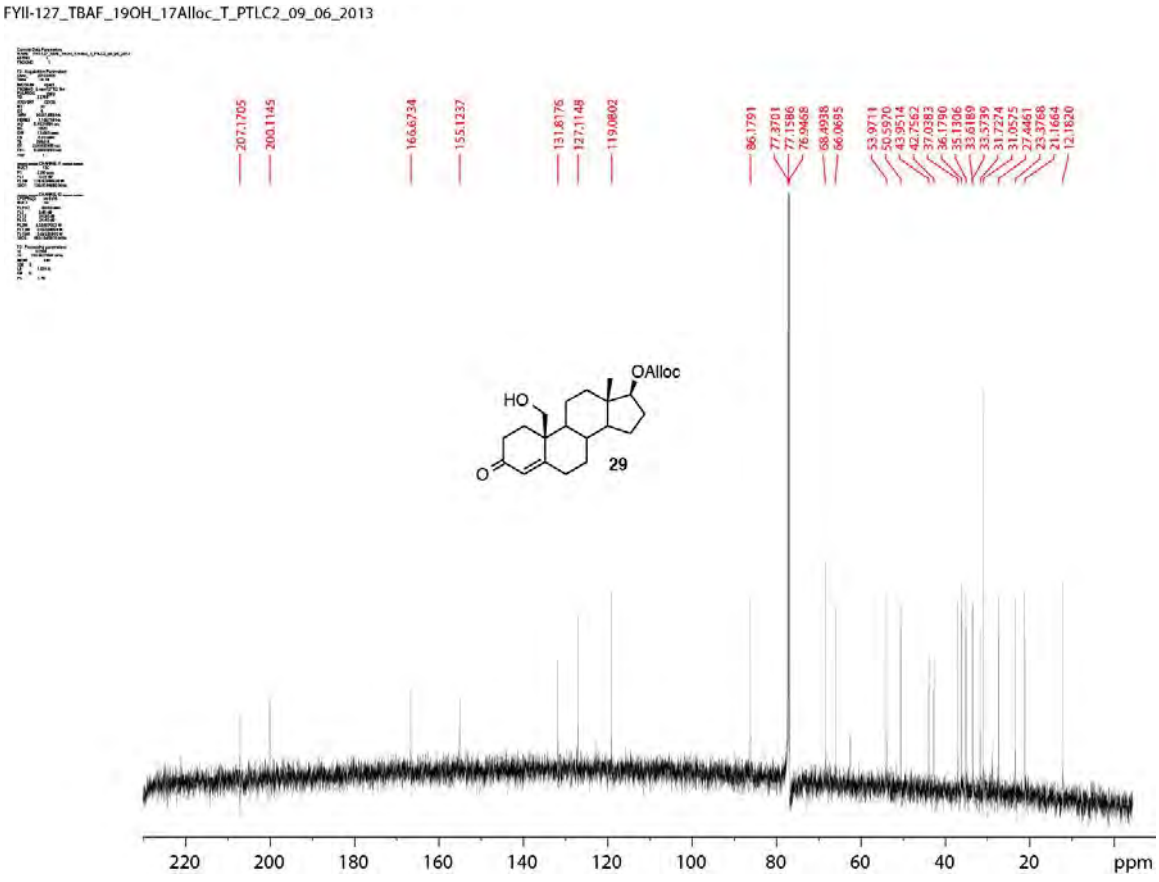
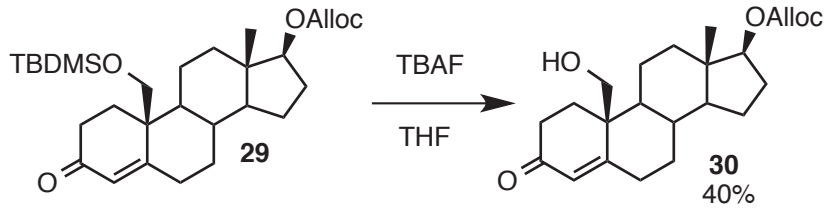
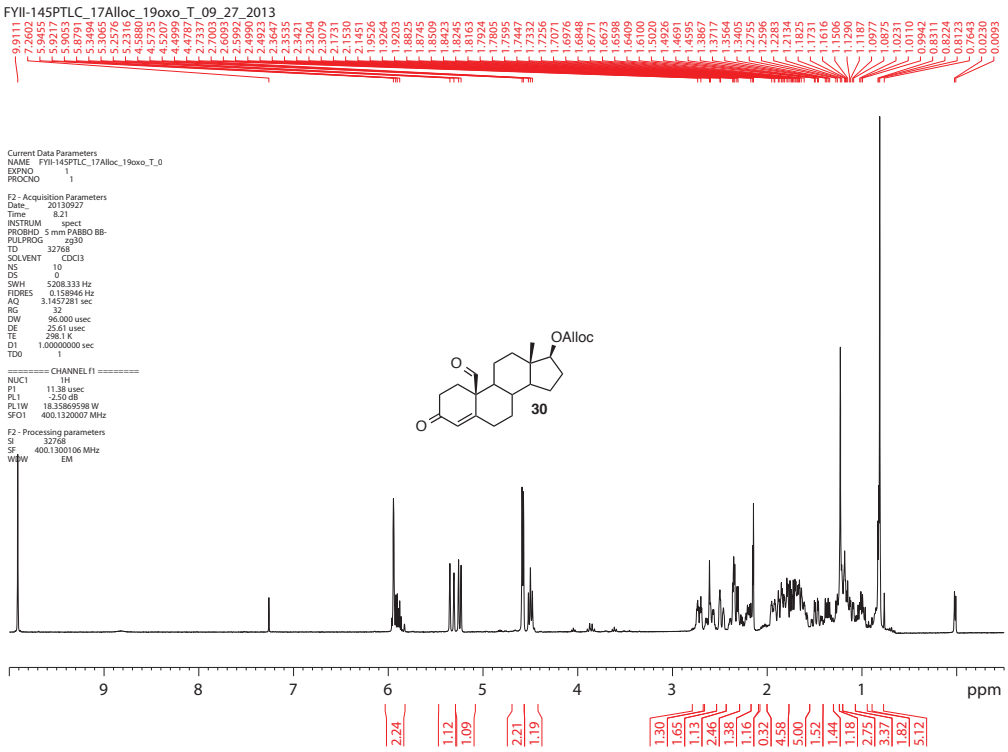
8
7
6
5
4
3
2
1
ppm
1.96
1.00
0.98
1.99
1.06
1.24
1.03
1.05
4.11
1.62
12.65
2.57
1.78
4.83
1.16
1.13
0.94
1.09
2.91
2.92





4.26. 19-Oxotestosterone 17β-(2-propenyl) carbonate (30). PCC (80 mg, 0.37 mmol, 1 mol eq) was added to a solution of alcohol (**29**) (140 mg, 0.36 mmol) in CH_2Cl_2 (20 mL) and the reaction was stirred for 30 min. The reaction mixture was concentrated under a stream of air and purified by preparative TLC (40% hexanes:EtOAc, v/v) to afford aldehyde (**30**) (90 mg, 0.25 mmol, 45%). R_f 0.79 (hexanes:EtOAc, 1:1, v-v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.91 (s, 1H), 5.96-5.83 (m, 1H), 5.95 (m, 1H), 5.33 (d, J =

17.2 Hz, 1H), 5.24 (d, *J* = 10.4 Hz, 1H), 4.58 (d, *J* = 5.8 Hz, 2H), 4.50 (apparent t, *J* = 8.3 Hz, 1H), 2.74-2.69 (m, 1H), 2.61-2.55 (m, 1H), 2.50-2.44 (m, 1H), 2.36-2.31 (m, 2H), 2.22-2.15 (m, 1H), 1.97-1.78 (m, 2H), 1.76-1.55 (m, 3H), 1.53-1.43 (m, 1H), 1.43-1.31 (m, 1H), 1.21-1.07 (m, 2H), 1.07-0.93 (m, 1H), 0.81 (s, 3H); ^{13}C NMR ($CDCl_3$: 77.1582) (100 MHz, $CDCl_3$) δ 201.1203 (C19, CH), 198.0, 161.1, 155.0, 131.7 (CH), 127.4 (CH), 119.0 (CH_2), 85.9 (CH), 68.5 (CH_2), 55.2, 53.5, 50.3, 42.5, 36.8, 34.3, 33.9, 31.0, 29.6, 27.3, 23.2, 21.4, 11.9 (CH_3).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PROCNO 1
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TD 32768
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TE 298.1 K
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PL1W 18.35869598 W
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F2 - Processing parameters
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WDW EM
OAlloc
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O
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8
7
6
5
4
3
2
1
ppm
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1.09
2.21
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1.13
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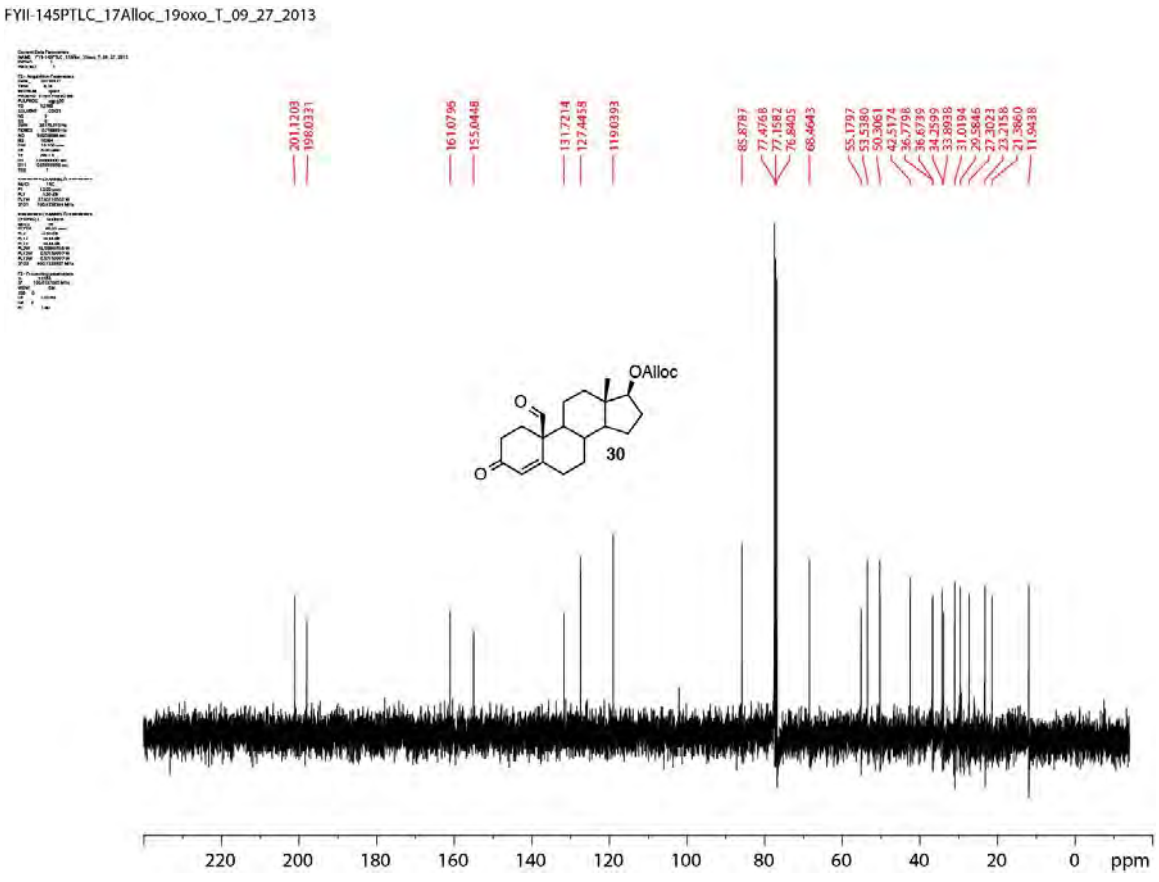
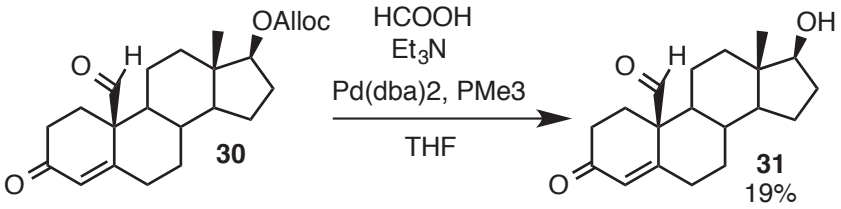
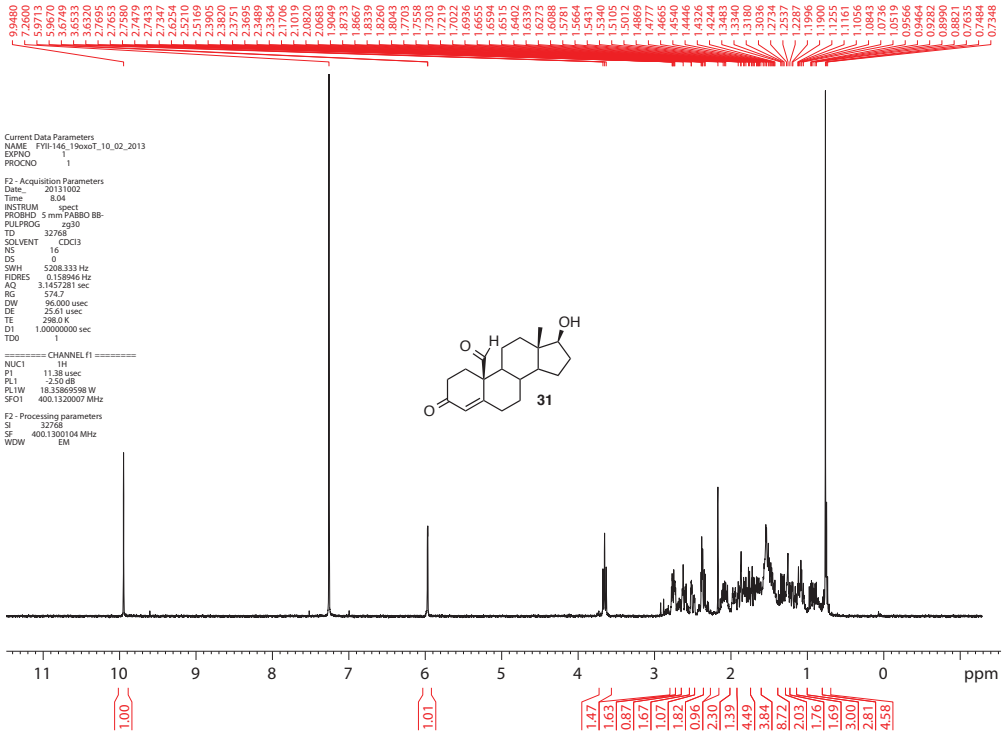
5.12

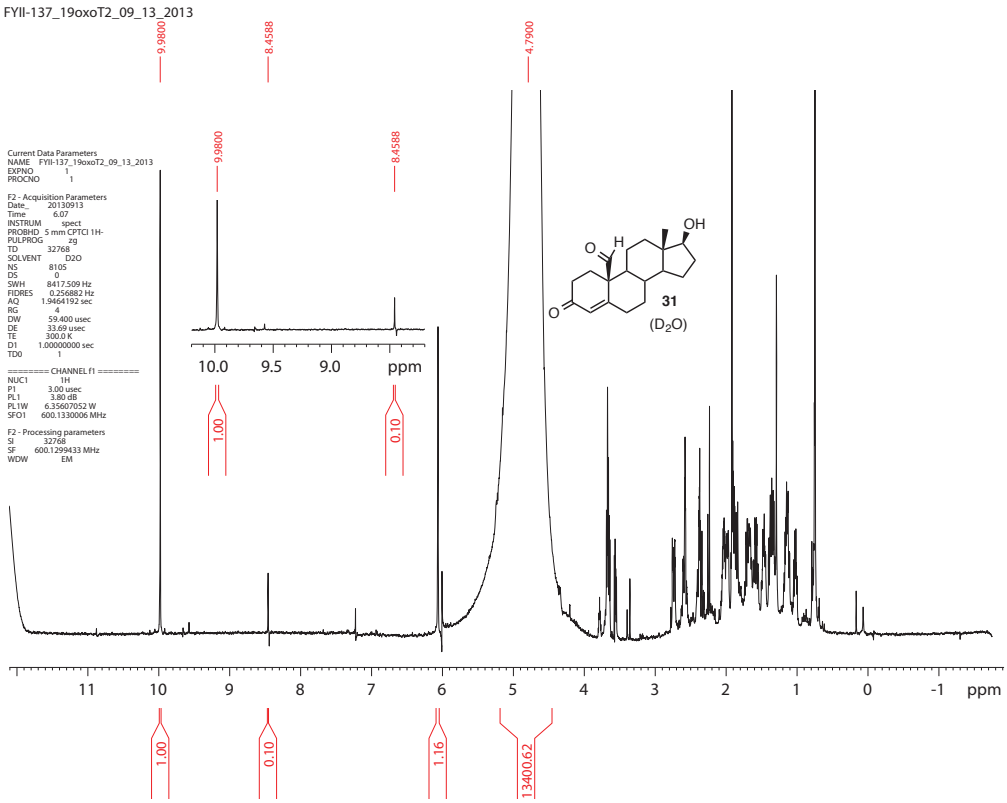




4.27. 19-Oxotestosterone (31). Trimethylphosphine (1 mL of a 1 M solution in THF, 1 mmol) was added to a flask containing $Pd(DBA)_2$ (173 mg, 0.3 mmol) under Ar. TEA (0.5 mL) was added followed by formic acid (2.0 mL). A solution of allylic carbonate (**30**) (90 mg, 0.25 mmol) in THF (5 mL) was added to the reaction mixture and the reaction was stirred for 30 min. The reaction mixture was concentrated under a stream of

air and purified by preparative TLC (30% hexanes:EtOAc, v/v) to afford 19-oxotestosterone (**31**) (15 mg, 0.047 mmol, 19%). R_f 0.15 (hexanes:EtOAc, 1:1, v/v). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.95 (s, 1H), 5.97 (m, 1H), 3.65 (apparent t, J = 8.6, 1H), 2.75 (ddd, J_1 = 14.1, J_2 = 5.3, J_3 = 3.6 Hz, 1H), 2.67-2.56 (m, 1H), 2.50 (ddd, J_1 = 15.2, J_2 = 4.1, J_3 = 2.4 Hz, 1H), 2.43-2.30 (m, 2H), 2.14-2.02 (m, 1H), 1.98-1.93 (m, 1H), 1.91-1.75 (m, 2H), 1.74-1.60 (m, 2H), 1.38-1.30 (m, 2H), 1.25-1.15 (m, 2H), 1.13-1.05 (m, 3H), 0.97-0.82 (m, 2H), 0.76 (s, 3H).

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RG 4
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DE 33.69 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
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PL1 3.80 dB
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SFO1 600.1330006 MHz
F2 - Processing parameters
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9.5
9.0
ppm
1.00
0.10
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H
OH
O
31
(D2O)
11
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6
5
4
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2
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ppm
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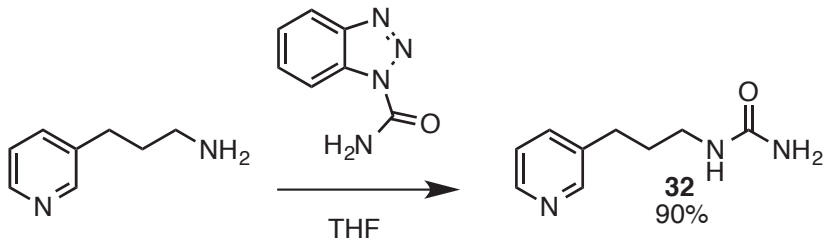
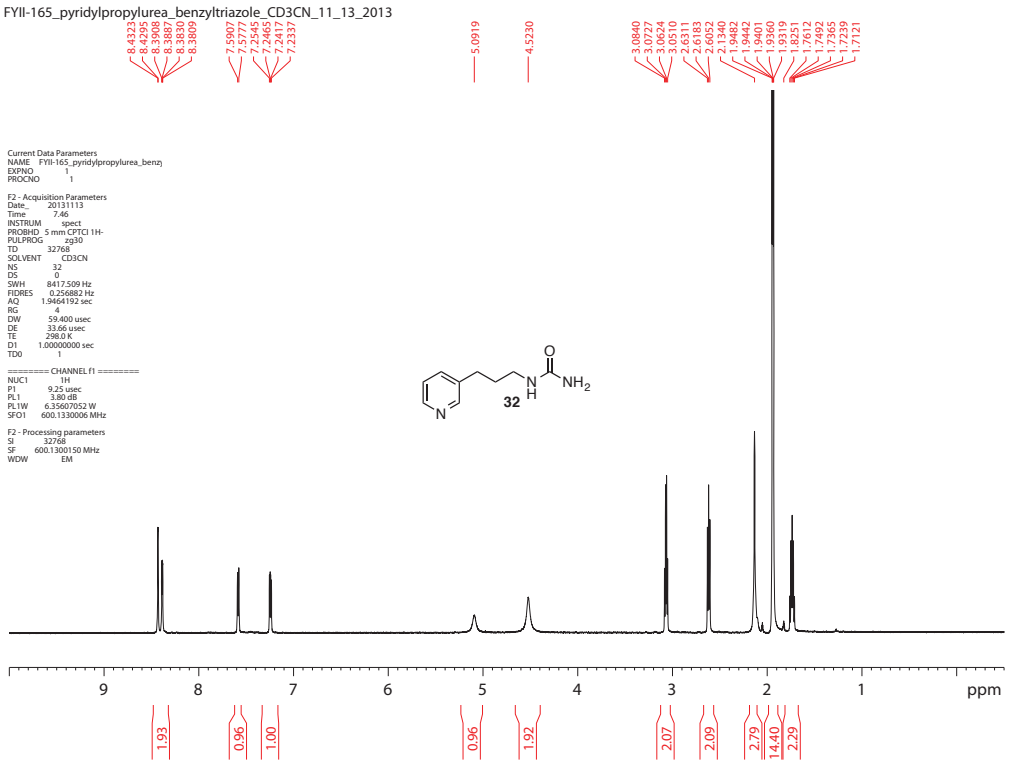


4.28. N^1-(3-(Pyridin-3-yl)-propyl)-urea (32). 3-(Pyridin-3-yl)propan-1-amine (0.84 g, 6.2 mmol) was added to a solution of benzotriazole-1-carboxamide (1.00 g, 6.2 mmol) in THF (25 mL) and the solution was stirred under reflux for 1 h. The resulting solution was concentrated and purified by flash column chromatography (100% hexanes to 50% hexanes:EtOAc, v-v to 10% CH_3OH in CH_2Cl_2, v-v) to afford the urea (**32**) as a white solid (1.0 g, 5.6 mmol, 90%). R_f 0.38 (CH_2Cl_2:CH_3OH, 9:1, v-v). ^{1}H NMR (600 MHz, D_2O) δ 8.49-8.43 (m, 2H), 7.96-7.94 (m, 1H), 7.56-7.54 (m, 1H), 3.06 (apparent t, J = 7.7 Hz, 2H), 2.84-2.81 (m, 2H), 2.05-2.02 (m, 2H); ^{1}H NMR (600 MHz, CD_3CN) δ 8.45 (d, J = 1.6 Hz, 1H), 8.41 (dd, J_1 = 4.7, J_2 = 1.3 Hz, 1H), 7.61-7.60 (m, 1H), 7.26 (dd, J_1 = 7.7, J_2 = 4.8 Hz, 1H), 5.1 (broad s, 1H), 4.54 (broad s, 2H), 3.09 (q, J = 6.8 Hz, 1H), 2.64 (t, J = 7.9 Hz, 1H), 1.78-1.73 (m, 2H); HRMS (*m*/*z*): calcd for $C_9H_{14}N_3O$, $[M+H]^+$, 180.1131; found, 180.1127 (Δ 2.2 ppm).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3.0840
3.0727
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3.0510
3.0311
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2.1340
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PROCNO 1
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FIDRES 0.256882 Hz
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RG 4
DW 59.400 usec
DE 33.66 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1
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PL1 3.80 dB
PL1W 6.35607052 W
SFO1 600.1330006 MHz
F2 - Processing parameters
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SF 600.1300150 MHz
WDW EM
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H
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32
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6
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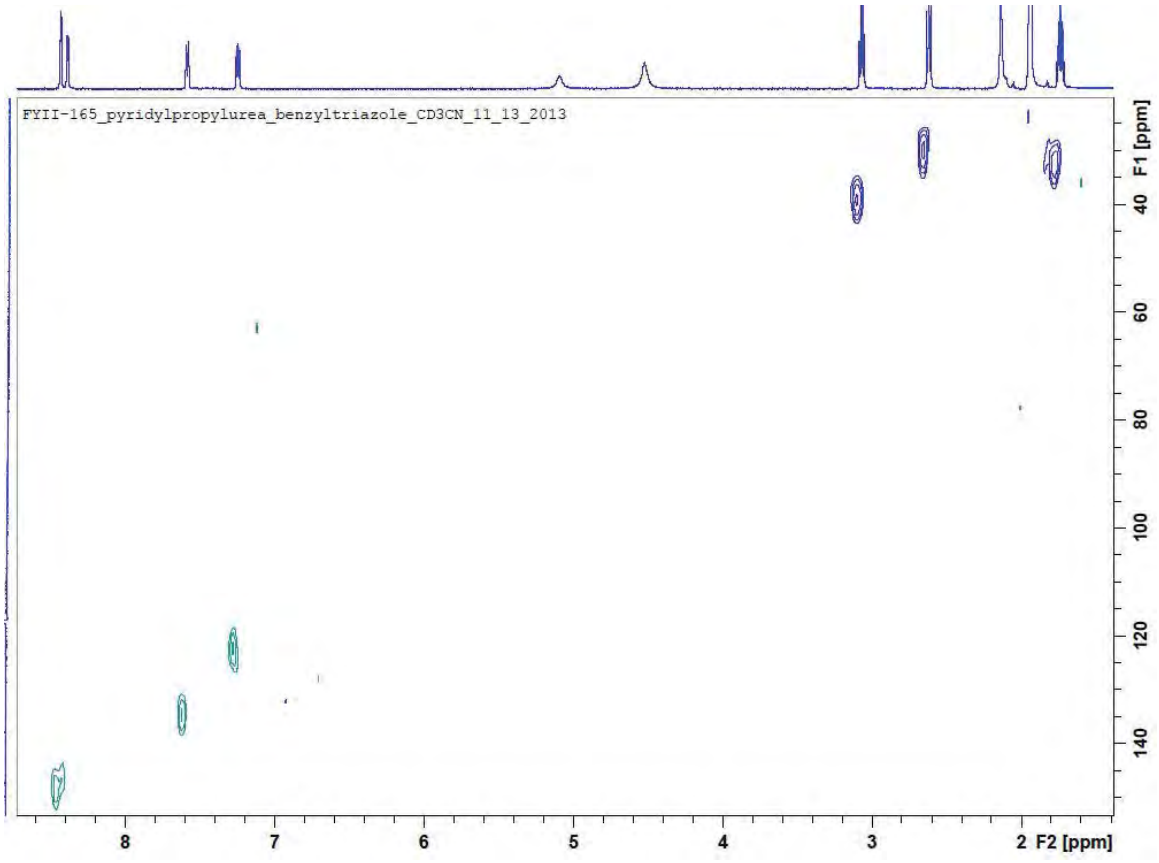
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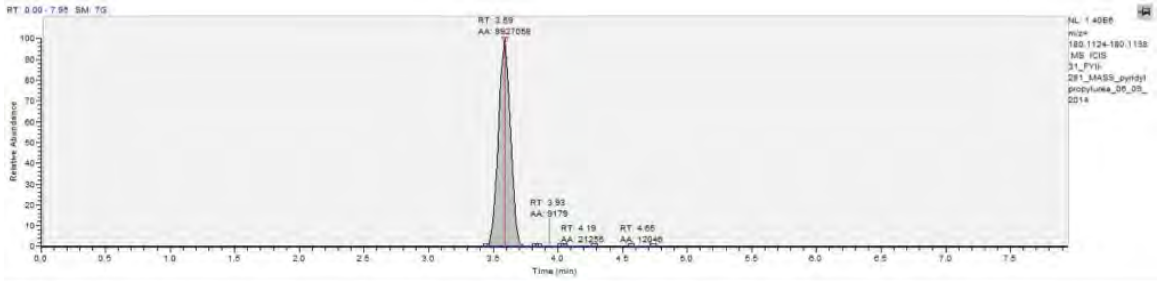
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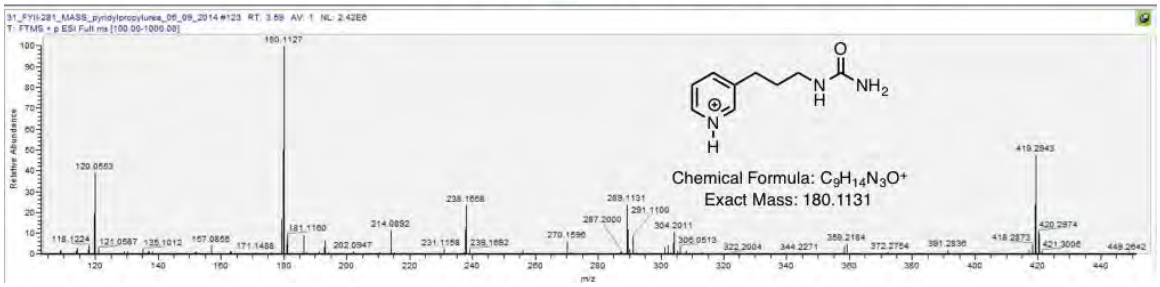
Relative Abundance
Time (min)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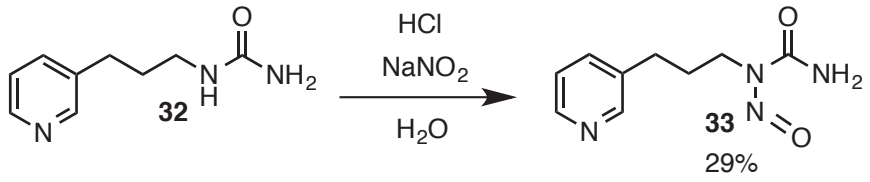
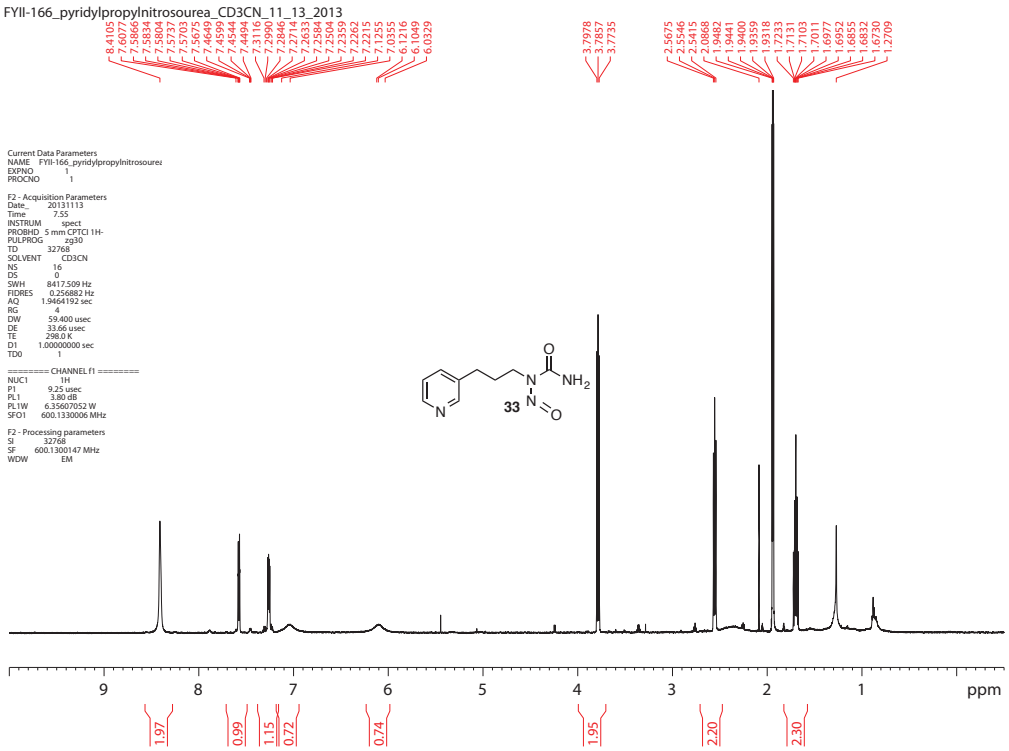
Exact Mass: 180.1131
Relative Abundance
m/z



4.29. N^1-Nitroso-N^1-(3-(pyridin-3-yl)-propyl)urea (33). Conc HCl (11.6 M, 1 mL) was added to a solution of the above urea (**32**) (450 mg, 2.5 mmol) in H_2O (20 mL), and the solution was cooled to 0 °C. $NaNO_2$ (173 mg, 2.5 mmol) was added and the reaction was allowed to warm to 23 °C and stirred overnight. The reaction mixture was diluted with CH_2Cl_2 (200 mL) and $NaHCO_3$ (50 mL, sat. aqueous solution) was added. The CH_2Cl_2 layer was dried with $MgSO_4$ and the organic layer was dried under a stream of nitrogen to yield the nitrosourea (**33**) (150 mg, 0.72 mmol, 29%). R_f 0.13 (CH_2Cl_2:CH_3OH, 9:1, v-v). ^{1}H NMR (600 MHz, CD_3CN) δ 8.43 (m, 2H), 7.60 (dt, J_1 = 7.7, J_2 = 2.0 Hz, 1H), 7.28 (dd, J_1 = 7.7, J_2 = 4.8 Hz, 1H), 7.06 (broad s, 1H), 6.13 (broad s, 1H), 3.81 (t, J = 7.3 Hz, 2H), 2.57 (t, J = 7.8 Hz, 2H), 1.74-1.69 (m, 2H); HRMS (*m/z*): calcd for $C_9H_{13}N_4O_2$, $[M+H]^+$, 209.1033; found, 209.1033 (Δ <0.1 ppm).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7.3116
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7.2584
7.2504
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DW 59.400 usec
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TE 298.0 K
D1 1.00000000 sec
TD0 1
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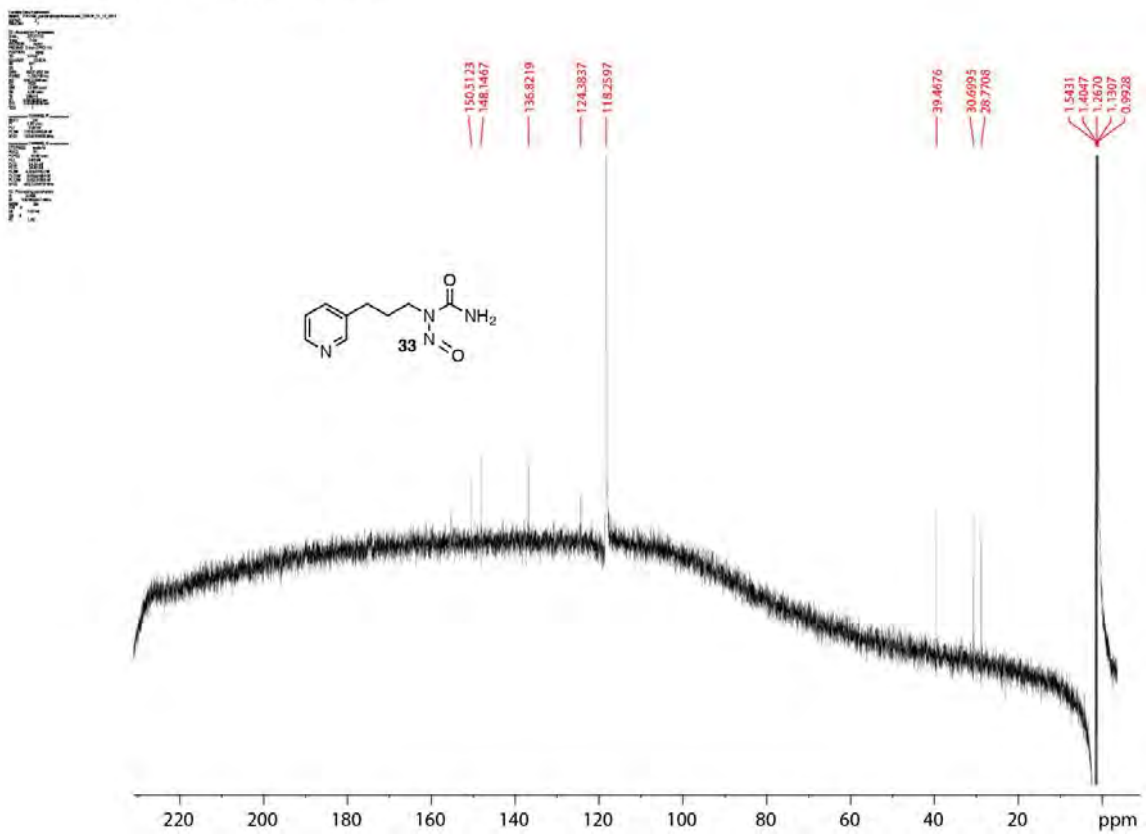
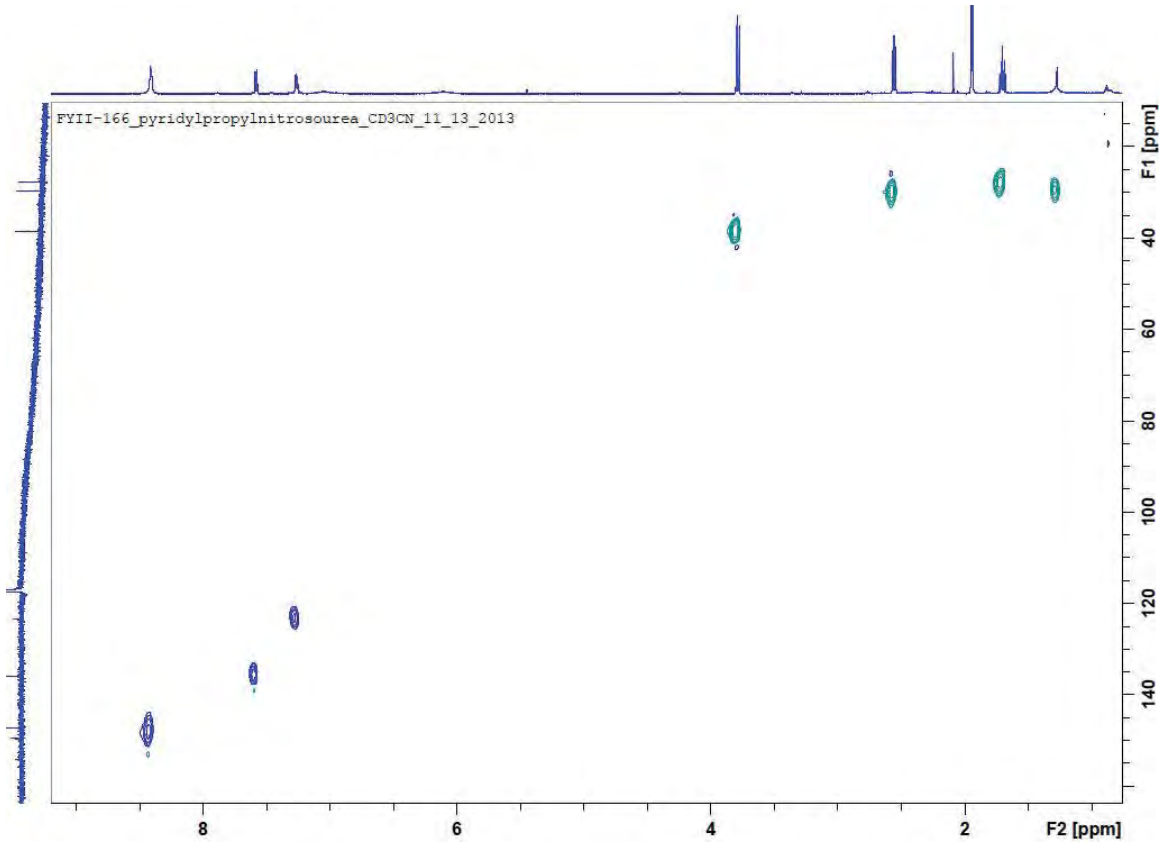
FYII-166_pyridylpropylnitrosourea_CD3CN_11_13_2013




FYII-166_pyridylpropylnitrosourea_CD3CN_11_13_2013
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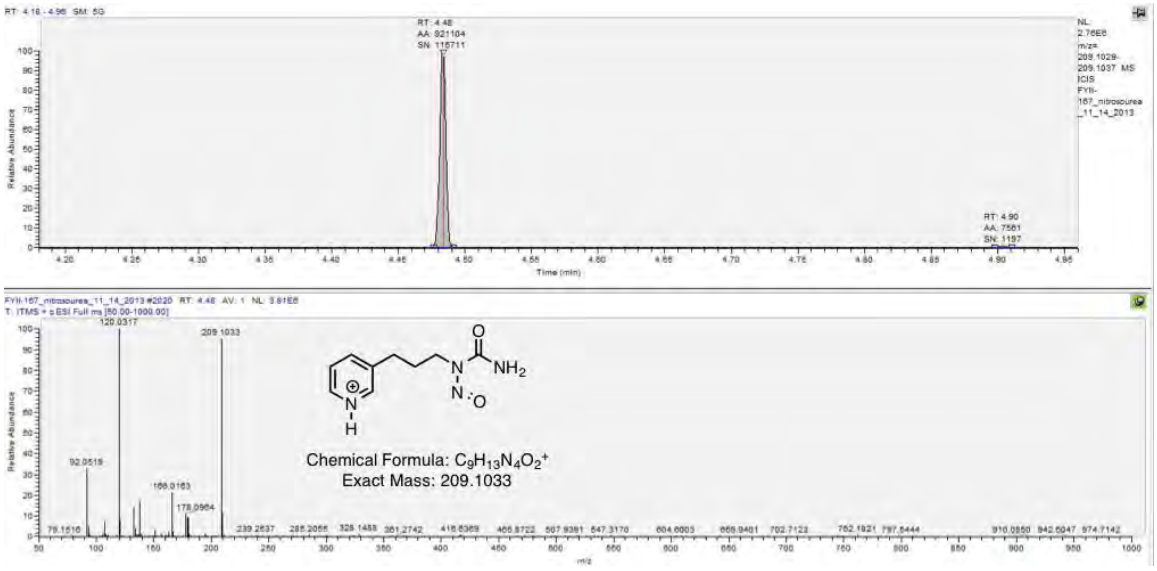
F2 [ppm]
8
6
4
2
40
60
80
100
120
140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AA: 921104
SN: 115711
RT: 4.90
AA: 7561
SN: 1197
Relative Abundance
Time (min)
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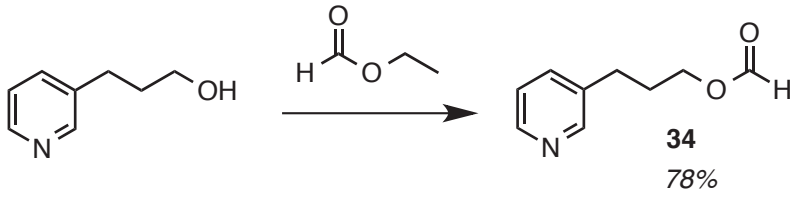
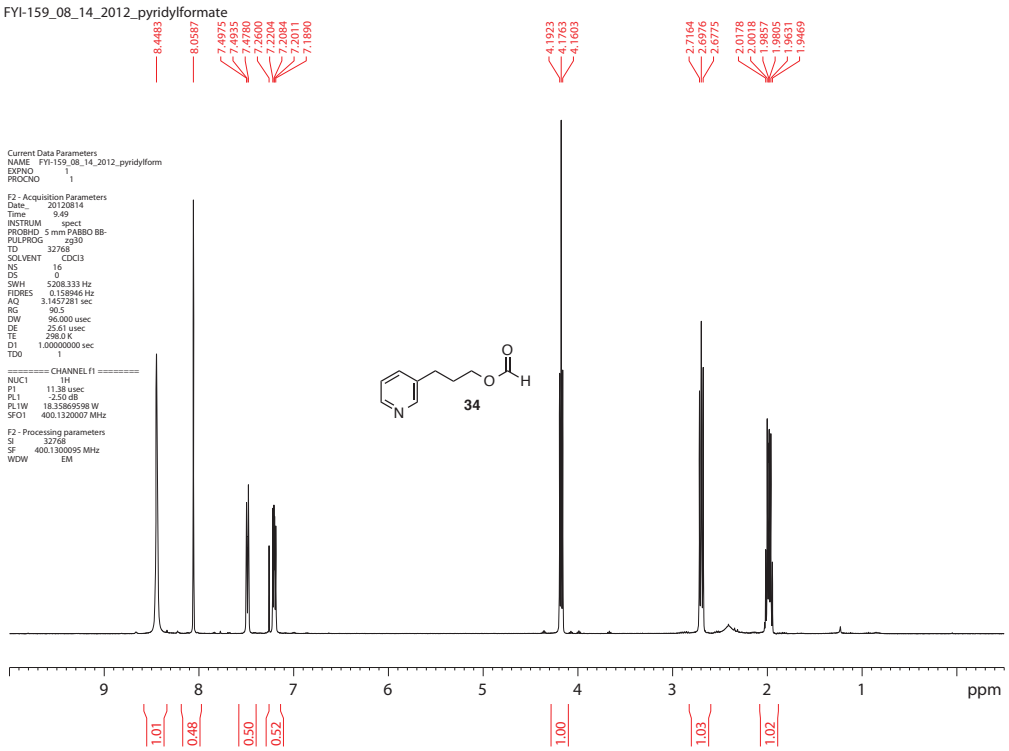
178.0964
Chemical Formula: C9H13N4O2+
Exact Mass: 209.1033



4.30. 3-(Pyridin-3-yl)propyl formate (34). 3-(3-Pyridyl)-1-propanol (1.0 mL, 7.8 mmol) and ethyl formate (50 mL) were stirred in a round bottom flask equipped with a magnetic stirrer, under reflux, for 48 h. The reaction mixture was concentrated and purified by preparative TLC (hexanes:EtOAc, 1:1, v-v) to afford (3-(3-pyridyl)-1-propyl) formate (**34**) (1 g, 6.1 mmol, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.45 (m, 2H), 8.06 (s, 1H), 7.50-7.48 (m, 1H), 7.20 (dd, J_1 = 7.7, J_2 = 4.9 Hz, 1H), 4.18 (t, J = 6.4 Hz, 2H), 2.70 (t, J = 8.1 Hz, 2H), 2.02-1.95 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.0, 150.0, 147.8, 136.3, 135.9, 123.5, 62.9, 29.9, 29.3.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7.4935
7.4780
7.2600
7.2204
7.2084
7.2011
7.1690
4.1923
4.1763
4.1603
2.7164
2.6976
2.6775
2.0178
2.0018
1.9857
1.9805
1.9631
1.9469
Current Data Parameters
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PROCNO 1
F2 - Acquisition Parameters
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Time 9.49
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PULPROG zg30
TD 32768
SOLVENT CDCl3
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DS 0
SWH 5208.333 Hz
FIDRES 0.158946 Hz
AQ 3.1457281 sec
RG 90.5
DW 96.000 usec
DE 25.61 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 11.38 usec
PL1 -2.50 dB
PL1W 18.35869598 W
SFO1 400.1320007 MHz
F2 - Processing parameters
SI 32768
SF 400.1300095 MHz
WDW EM
O
O
H
N
34
9
8
7
6
5
4
3
2
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ppm
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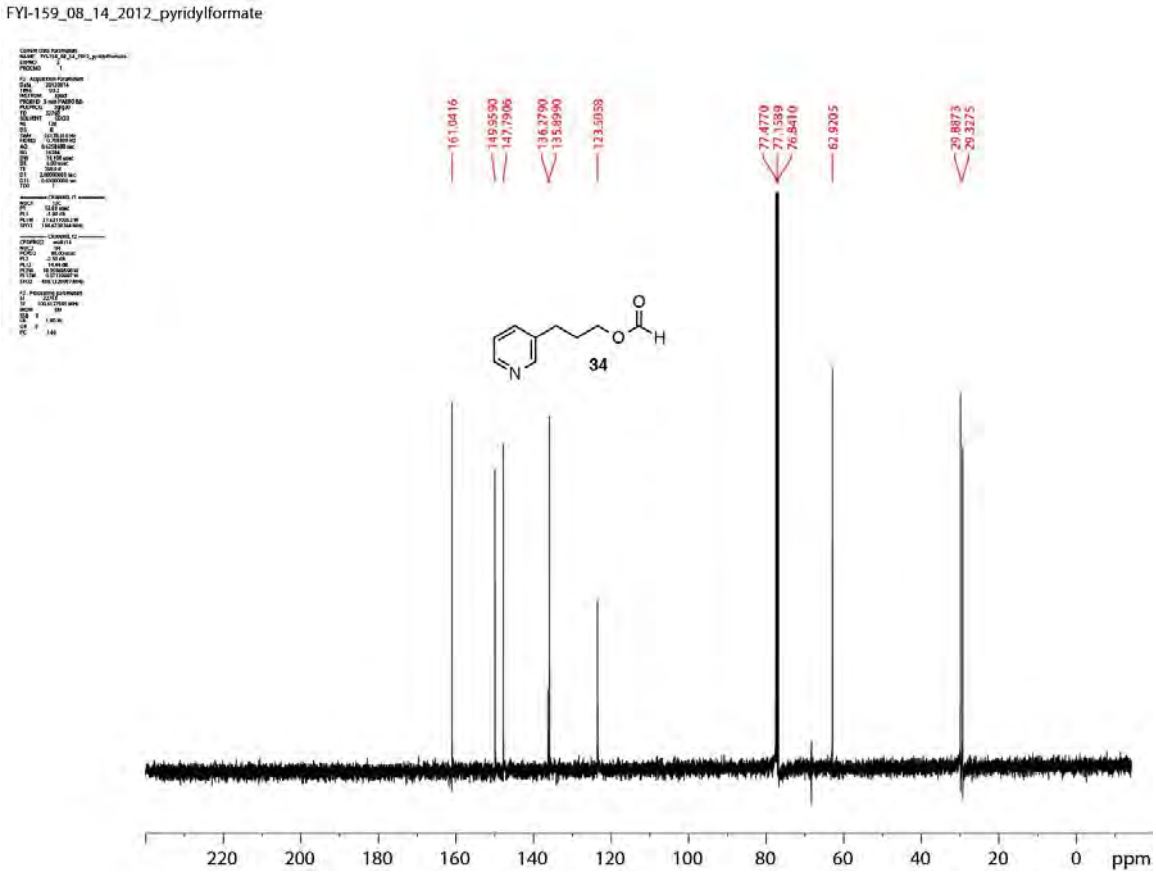
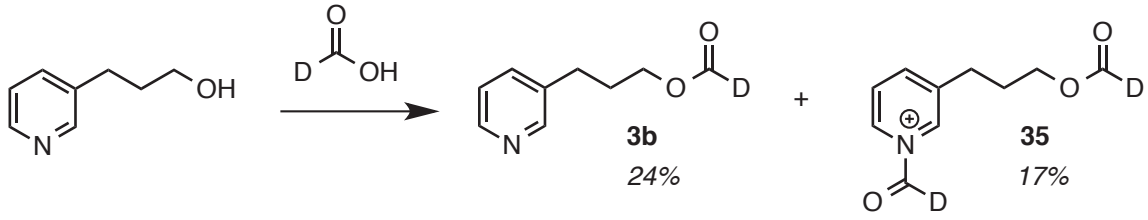
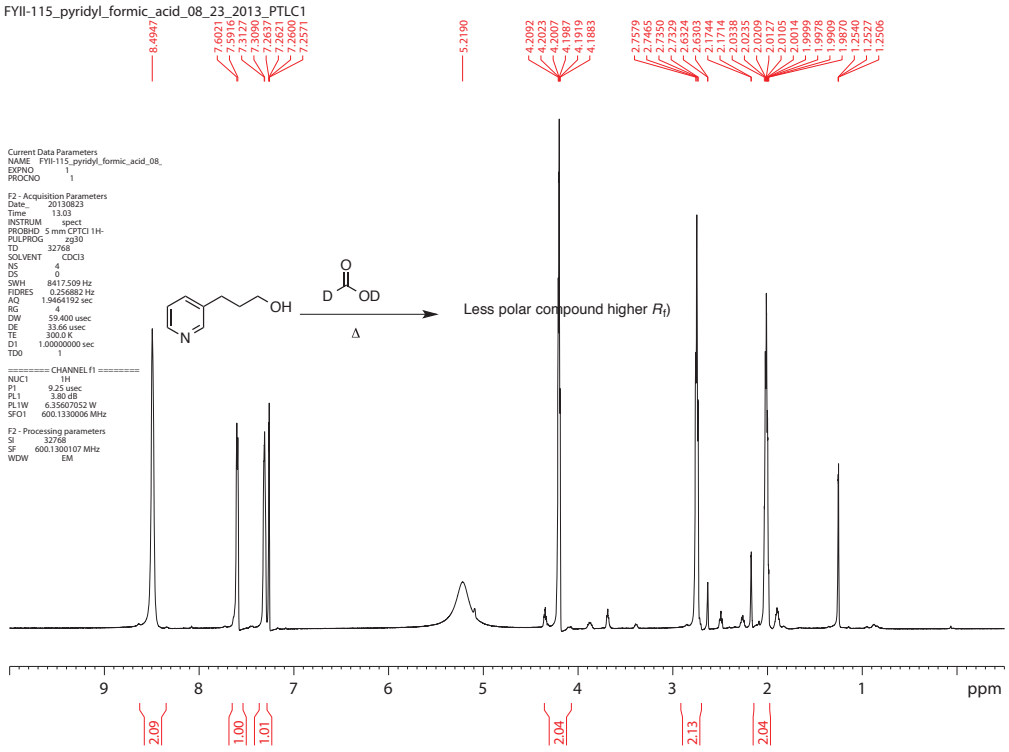
0.48
0.50
0.52
1.00
1.03
1.02

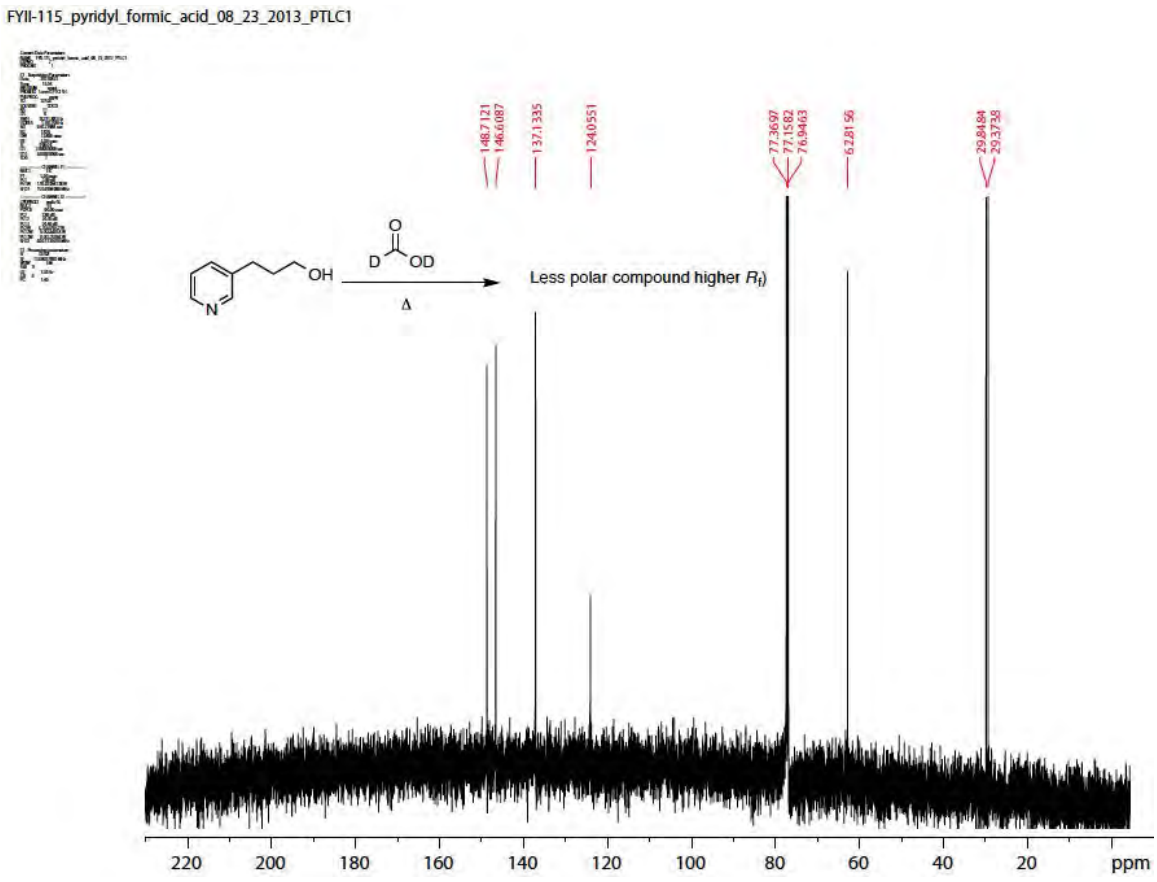




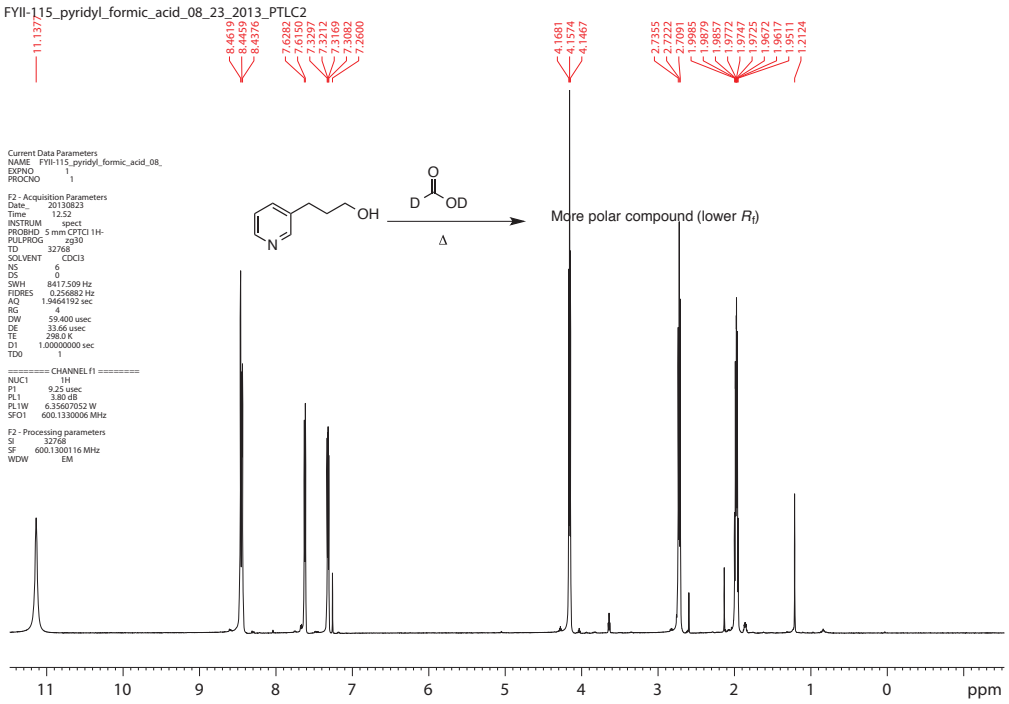
4.31. [Formyl-^{2}H]-3-(pyridin-3-yl)propyl formate (3b). 3-(3-Pyridyl)-1-propanol (0.2 mL, 0.21 g, 1.5 mmol) and d_2-formic acid (0.1 mL) were stirred in THF (2 mL) in a 4 mL screw cap vial equipped with a stir bar. The reaction was heated in a water bath at 50 °C for 24 h and purified directly by preparative TLC (hexanes:EtOAc, 1:1, v-v) to yield (3-

(3-pyridyl)-1-propyl)-deutero-formate (**3b**) (less polar, monoformate, 60 mg, 0.36 mmol, 24%) and [formyl-^{2}H]-3-(pyridin-3-yl)propyl formate (**35**) (more polar, diformate, 50 mg, 0.26 mmol, 17%). Monoformate (**3b**): ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.45 (m, 2H), 7.55-7.54 (m, 1H), 7.21-7.20 (m, 1H), 4.16-4.14 (m, 2H), 2.71-2.58 (m, 2H), 1.98-1.85 (m, 2H).





Diformate (35): ^{1}H NMR (600 MHz, $CDCl_3$) δ 11.14 (broad s, 1H), 8.46 (broad s, 1H), 8.44 (d, J = 5.0 Hz, 1H), 7.62 (d, J = 7.9 Hz, 1H), 7.32 (dd, J_1 = 7.7, J_2 = 5.0 Hz, 1H), 4.16 (apparent t, J = 6.5 Hz, 2H), 2.72 (apparent t, J = 8.0 Hz, 2H), 2.0-1.97 (m, 2H). The diformate was assigned because there were two distinct triplets corresponding to formyl carbons at 164.3 (J = 31 Hz) and 160.8 ppm (J = 34 Hz) in the ^{13}C NMR spectrum.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7.6150
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7.3169
7.3082
7.2600
4.1681
4.1574
4.1467
2.7355
2.7222
2.7091
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1.9672
1.9617
1.9511
1.2124
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EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
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Time 12.52
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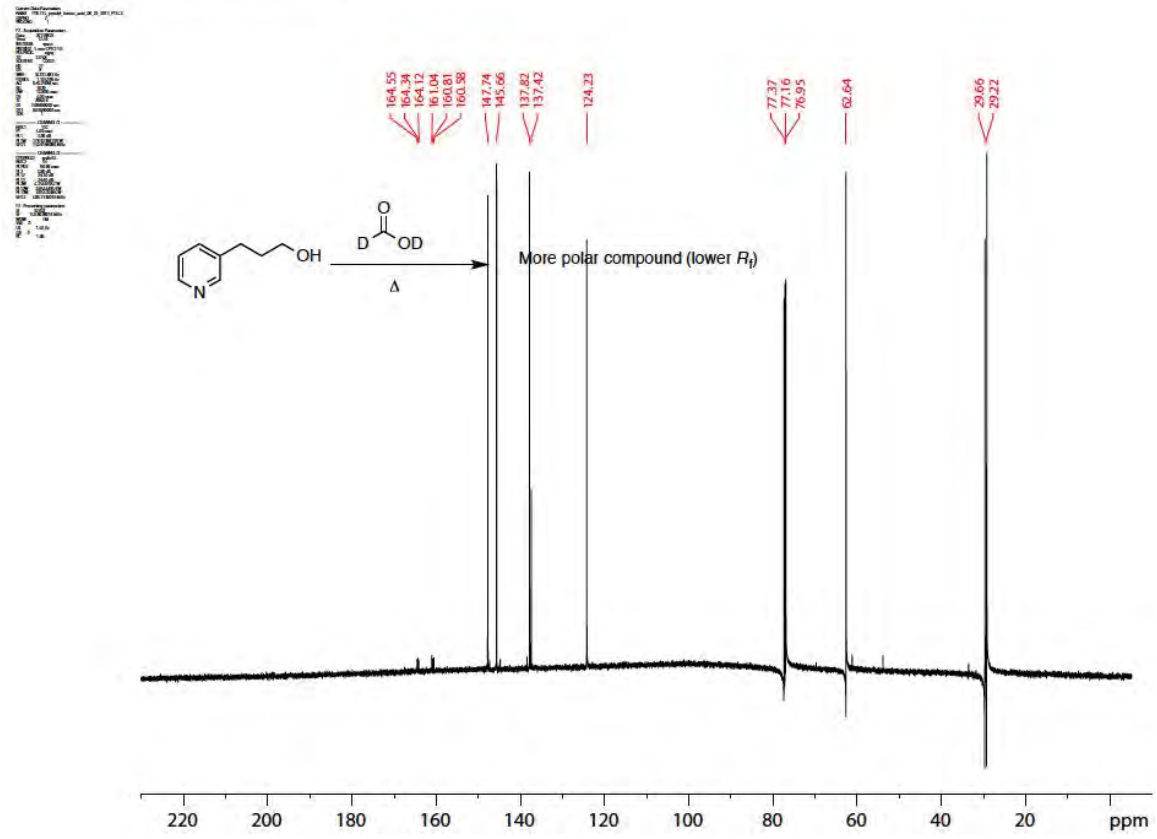
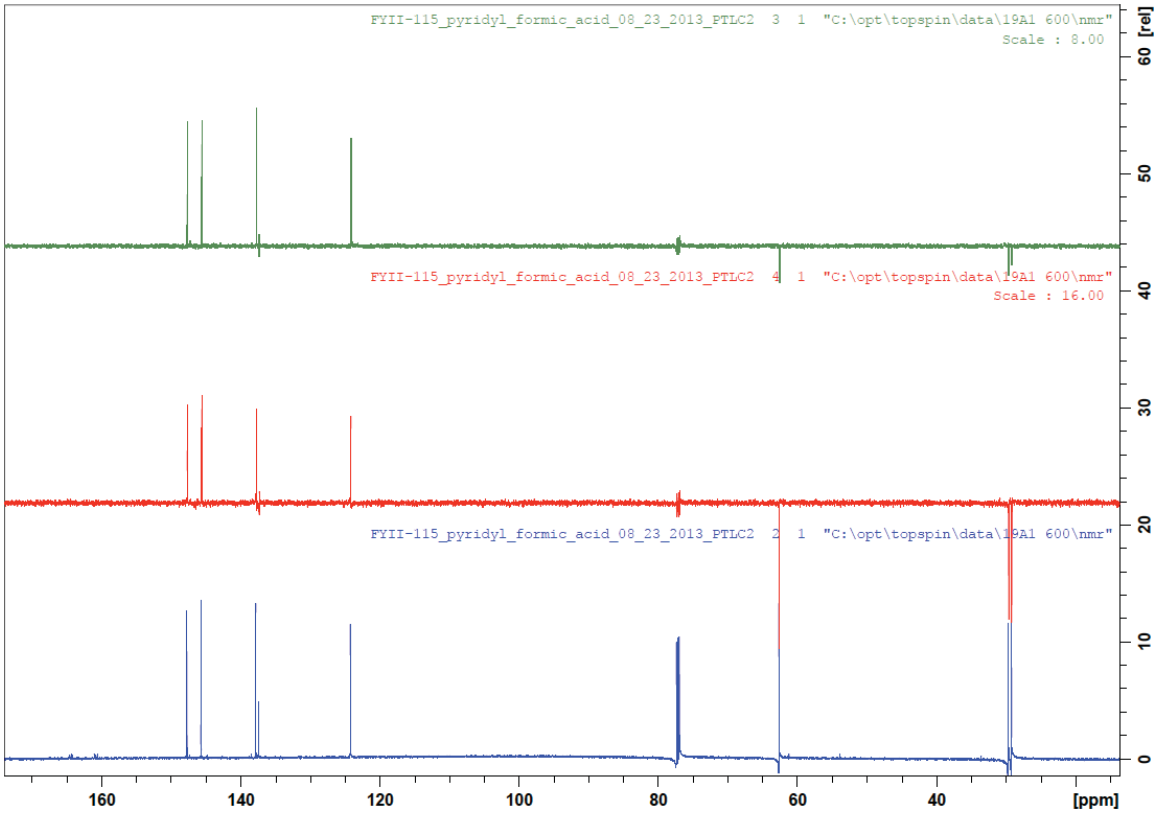
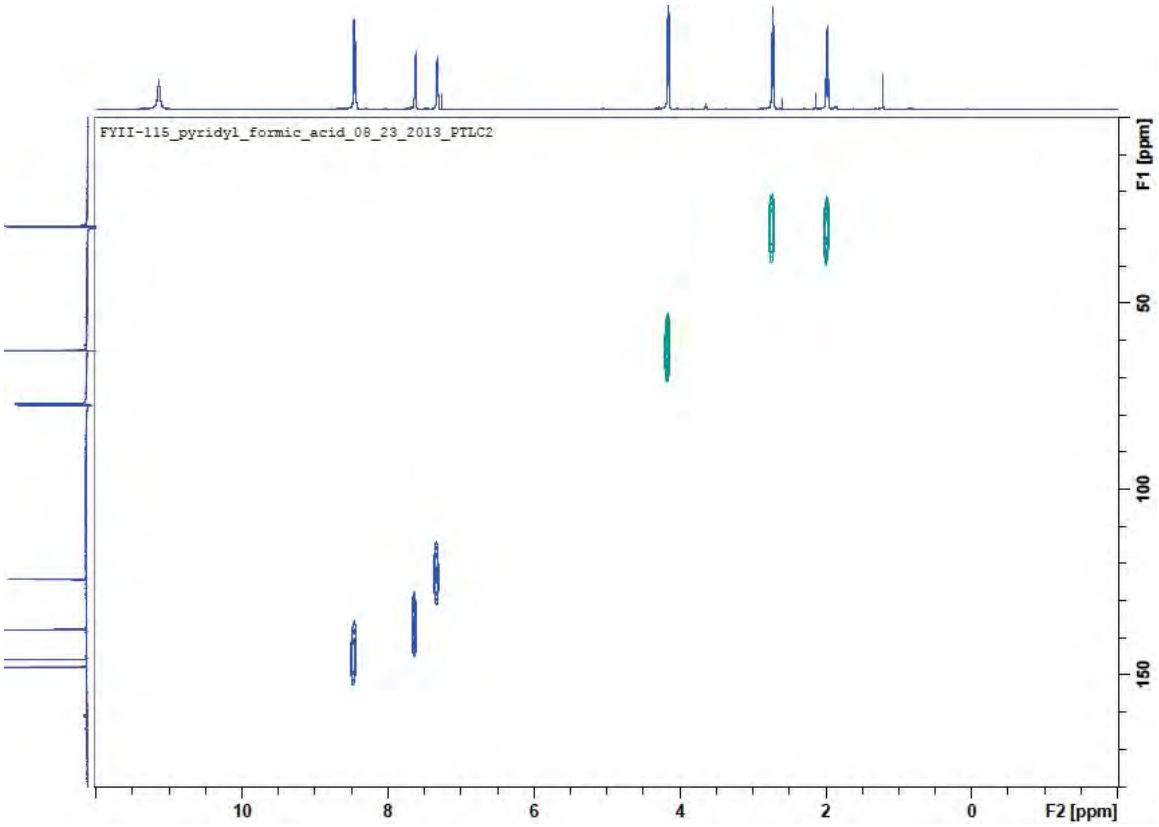
PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 6
DS 0
SWH 8417.509 Hz
FIDRES 0.256882 Hz
AQ 1.9464192 sec
RG 4
DW 59.400 usec
DE 33.66 usec
TE 298.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 9.25 usec
PL1 3.80 dB
PL1W 6.35607052 W
SFO1 600.1330006 MHz
F2 - Processing parameters
SI 32768
SF 600.1300116 MHz
WDW EM
OH
N
O
D
OD
Δ
More polar compound (lower R_f)
11
10
9
8
7
6
5
4
3
2
1
0
ppm

FYII-115_pyridyl_formic_acid_08_23_2013_PTLC2







5. Enzyme incubations

$^{18}O_2$ Incubations

P450 19A1 (20 pmol), *E. coli* recombinant rat NADPH-P450 reductase (240 pmol), and 1,2-α-didodecanoyl-*sn*-glycero-3-phosphocholine (30 nmol), were added to a Thunberg tube containing 800 μL of 50 mM potassium phosphate buffer (pH 7.4) followed by 35 nmol of [19-^{2}H]-19-oxo-androstenedione or -testosterone. An NADPH-regenerating system[11] was added to the Thunberg cap. The capped Thunberg tube, sealed with Dow Corning high vacuum (silicone lubricant) grease, was briefly evacuated (~ 10 mm Hg) and backfilled with argon gas, through at least three cycles, and finally placed

under vacuum and injected (through a serum cap attached to the sidearm of the Thunberg tube) with $^{18}O_2$ gas from the pressurized cylinder (99.9% ^{18}O content). The contents of the tube and cap were mixed and the incubation proceeded at 37 °C for 10 min, with gentle shaking at 100 rpm. The sealed tube was cooled to 0 °C, and CH_2Cl_2 (5 mL) was quickly added, followed by 400 μL of an ice-cold solution of 3 M aqueous HCl. The layers were mixed with a vortex device for 2 seconds, and the organic phase was added to a tube containing anhydrous $MgSO_4$ (~200 mg) and then filtered through a Pasteur pipet containing a cotton plug. The dried diazo reagent **2** (*vide infra*, in diethyl ether) was added, and the contents of the tube were mixed briefly and dried under an N_2 stream. The dried extracts were dissolved in CH_3OH (60 μL) and analyzed using LC-MS.

A similar procedure was used for control reactions with P450 3A4 and testosterone. The components included 160 nM *E. coli* recombinant P450 3A4,[12,13] 280 nM *E. coli* recombinant NADPH-P450 reductase,[3] 160 nM *E. coli* recombinant human cytochrome b_5,[14] 50 mM potassium phosphate buffer (pH 7.4), 1,2-*a*-didodecanoyl-*sn*-glycero-3-phosphocholine (30 μM), and 35 μM testosterone. Following the incubation (10 min), the products were extracted into two volumes of CH_2Cl_2 and the concentrated material was analyzed for ^{18}O content in the product 6β-hydroxytestosterone by LC-MS[15] using HRMS (Orbitrap).

*Diazo reagent (**2**) preparation*

The diethyl ether and dichloromethane solvents were filtered with basic alumina (Brockman Activity I, Fisher) before use in order to remove any endogenous formic acid.

Diethyl ether (2 mL) was added to the nitrosourea **32** (~3 mg) in a screw-cap vial, followed by an equal volume of KOH (30% aqueous, w/v). The capped vial was mixed using a vortex device for 10 seconds, and the diethyl ether layer was collected into a tube and dried with $MgSO_4$. The solution was filtered and the diethyl ether solution of **2** was used for derivatization.

Radioactive assays (time course)

Incubations were run in a total volume of 50 μL. A stock solution was made containing P450 19A1 (100 nM, 500 μL, 50 pmol), *E. coli* recombinant rat P450 reductase[3] (22 μM, 25 μL, 500 pmol), and 30 μM 1,2-α-didodecanoyl-*sn*-glycero-3-phosphocholine. The test tube was gently mixed and incubated room temperature for 5 min. Potassium phosphate (pH 7.4) was added to the solution to 50 mM. In a separate test tube steroid (4-[^{14}C]-androstenedione: 50 μCi/2.5 mL, 48 mCi/mmol, 100 μL, 2 μCi, 42 nmol, or 4-[^{14}C]-testosterone: 50 μCi/1.25 mL, 58.8 mCi/mmol, 61 μL, 2.45 μCi, 42 nmol) was added and the C_2H_5OH was evaporated under a stream of N_2. The master mix containing P450 19A1, NADPH-P450 reductase, phospholipid, and potassium phosphate buffer was added to the tube containing dried steroid, which was mixed and preincubated at 37 °C for 5 s. An NADPH-regenerating system was added.[11] The reaction was shaken at 50 rpm, 37 °C, and at varying times 50-μL aliquots were quenched with 10 μL of CH_3OH and centrifuged at 14,000 *g*. The centrifuged mixtures (50 μL each) were directly injected onto the HPLC-flow counting system.

6. Liquid Chromatography

UPLC-MS

The sequence of the runs began after the delta for pressure change reached ≤ 40, and five injections of the pyridyl formate standard were necessary until retention times stabilized (usually at 3.86 min for the pyridyl formate standard: *m/z* 166.0863). The injection volume for each run was 5 μL. The liquid chromatography was run on an Acuity UPLC with a gradient of 100% A to 100% B from 0 to 4 min and to 100% A to 5.3 min, holding at 100% A until 8 min. The flow rate was 0.3 mL/min. The mobile phases were as follows: solvent A: 95% H_2O/5% CH_3CN, v-v (10 mM $NH_4CH_3CO_2$), solvent B: 100% CH_3OH (10 mM $NH_4CH_3CO_2$).

Radioactivity Assays

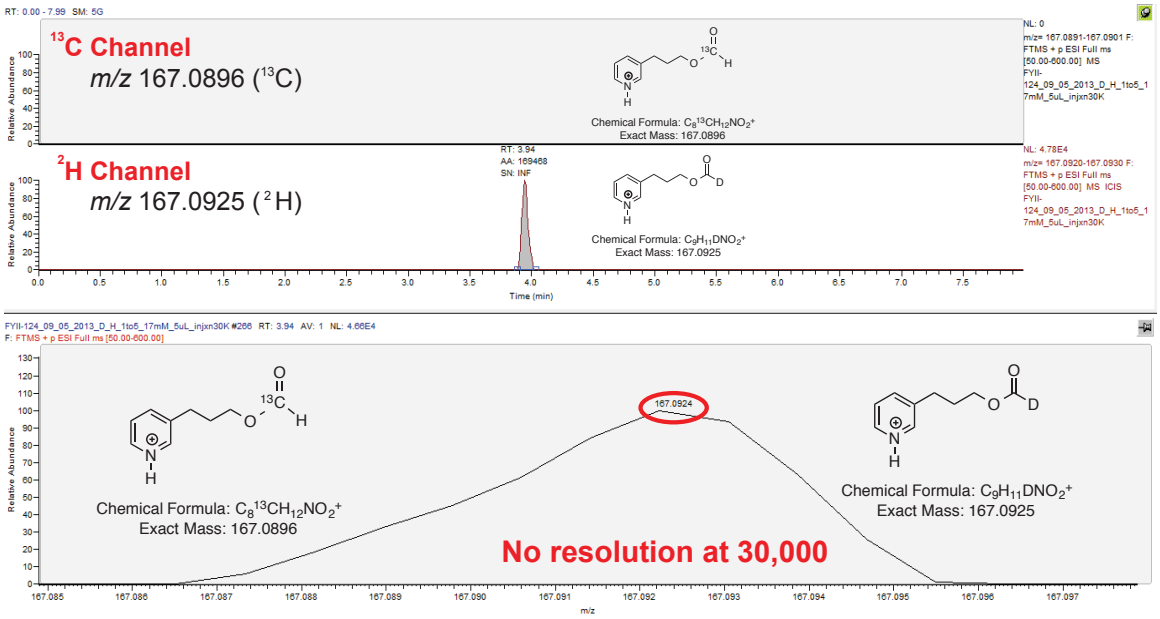
The solvent systems were solvent A: 83% H_2O/17% THF, v-v and solvent B: 83% CH_3OH, 17% THF (v-v). The gradient was from 10% B (v/v) from 0 min to 2 min, then to 100% B from 2 min to 20 min, and then to 10% B (v/v) from 21 min to 30 min. The injection volume for each run was 50 μL. The flow rate was 0.6 mL/min. In the preliminary trials, the THF solvent contained 250 ppb butylated hydroxytoluene as stabilizer (which did not affect the UV absorption). After the conditions were optimized, THF with no stabilizer was used and the solvents were sparged with argon. Amber bottles

were used to store the solvents. The flow rate for the scintillation cocktail (LabLogic, Order No. SG-BXX-05) was 3.0 mL/min.

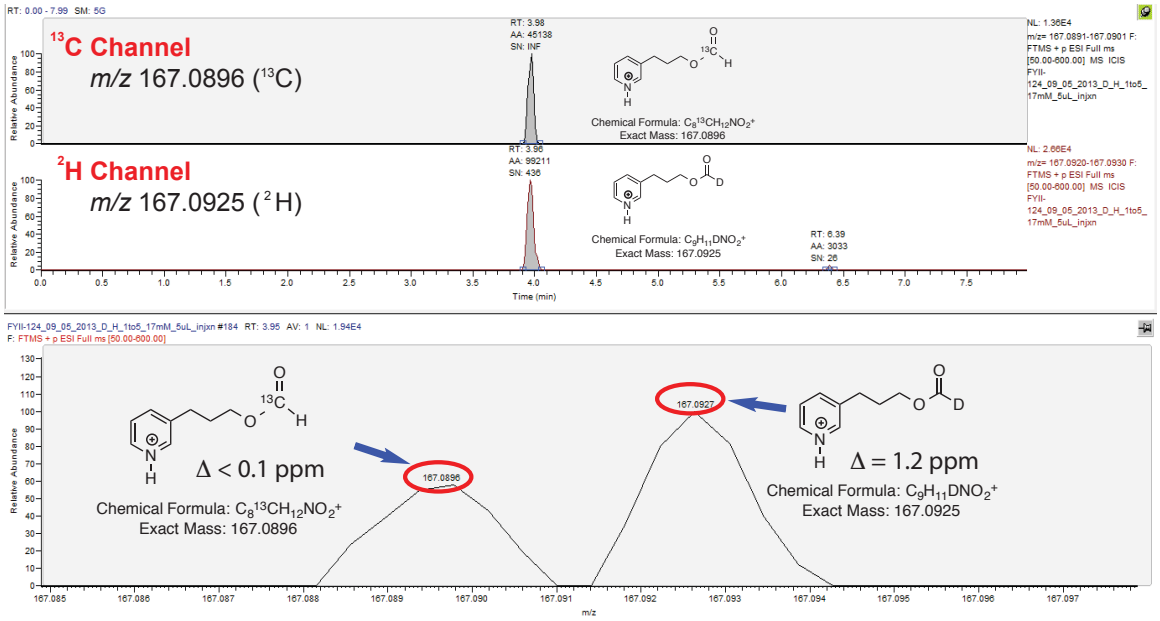
7. Formic Acid Resolution from Background ^{13}C (Natural Abundance) vs. ^{2}H

In order to determine the minimum resolution required to distinguish between the deuterated formate and ^{13}C-formate from the background, a mixture of deuterated (**3b**) and non-deuterated formate (**33**) (1:5, 17 mM deuterated formate **3b** in CH_3OH to 17 mM non-deuterated formate **33** in CH_3OH, v/v) was made. This mixture was injected onto the LTQ Orbitrap with different resolution settings. It was determined that a minimum of 60,000 resolution was necessary to distinguish the ^{13}C-formate from the ^{2}H-formate. Therefore, for all incubation analyses, the Orbitrap resolution setting was set to 100,000 to avoid any overlapping between the two masses.

(A) Formate analysis with resolution set at 30,000 (*m/z* 167.0925 Area = 169468, retention time = 3.94 min)



(B) Formate analysis with resolution set at 60,000 (*m/z* 167.0896 Area = 45138, retention time = 3.98 min and *m/z* 167.0925 Area = 99211, retention time = 3.96 min)

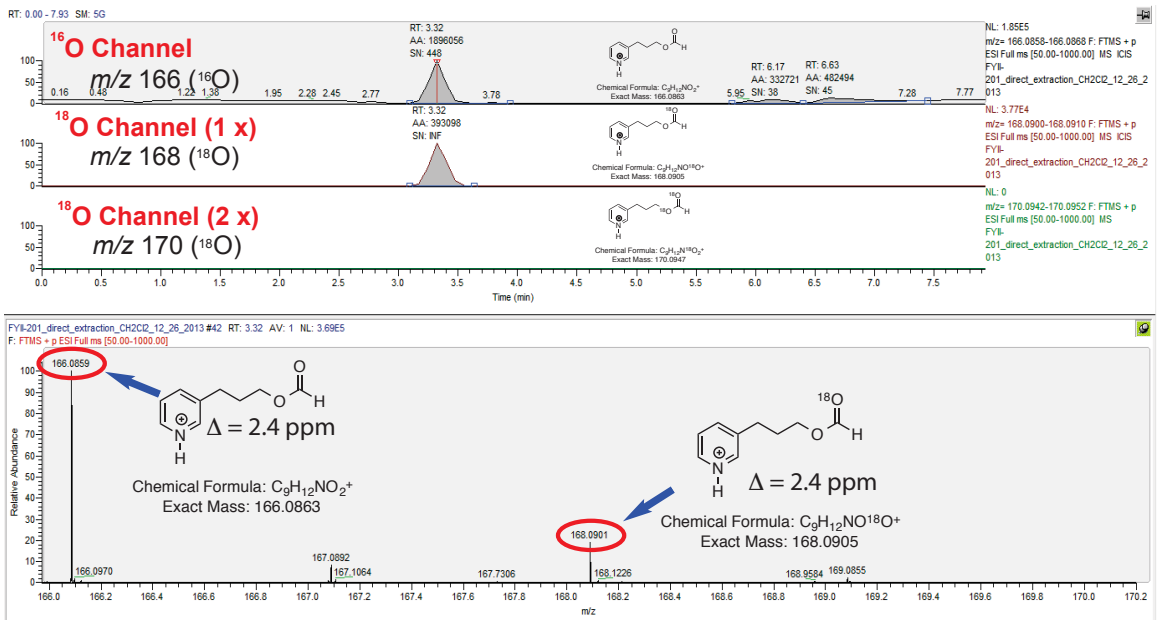


8. Formic Acid Oxygen Exchange Trials: ^{18}O-Atom Exchange with $H_2{}^{18}O$

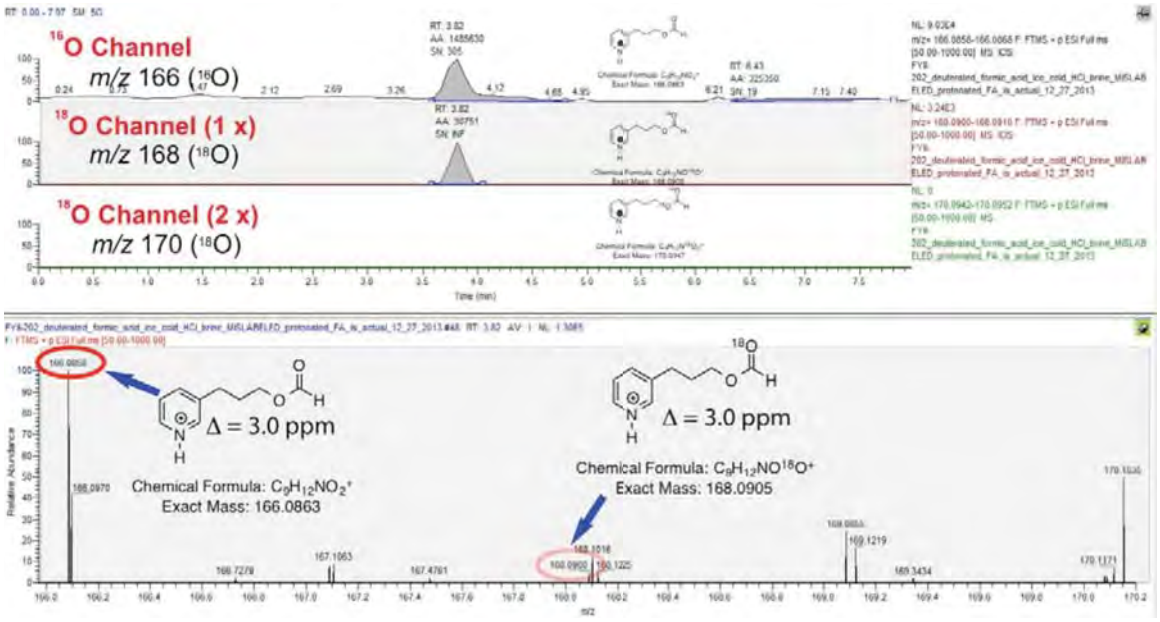
In order to verify minimal exchange of the formic acid oxygen atoms with the medium during the extraction process,[16] the following control experiments were run.

Conc HCl (50 μL) was added to a 10 mL solution of 100 mM potassium phosphate buffer (pH 7.4), to mimic the assay conditions. The pH was determined to be 3.26.

A solution of $H_2{}^{18}O$ (45 μL), potassium phosphate buffer (pH 7.4, 1 M, 5 μL), and formic acid (1 μL) was shaken at 100 rpm in a 37 °C water bath for 10 min. HCl (15 μL of 3 M solution) and then CH_2Cl_2 (1 mL) was added, and organic layer was extracted. The CH_2Cl_2 layer was dried with $MgSO_4$ (~100 mg) and filtered through a Pasteur pipet packed with a cotton plug. Diazo reagent **2** (in diethyl ether, *vide supra*) was added and the mixture was dried (under an N_2 stream) and analyzed by LC-MS. A ratio of ~8:1 of ^{16}O-formate to (mono-) ^{18}O-formate was detected. (*m/z* 166.0863 Area = 1896056, retention time = 3.32 min and *m/z* 168.0905 Area = 393098, retention time = 3.32 min)



The HCl addition was repeated at 0 °C in the process, and the ratio of ^{16}O-formate to (mono-) ^{18}O-formate was ~46:1. (*m/z* 166.0863 Area = 1485630, retention time = 3.82 min and *m/z* 168.0905 Area = 30751, retention time = 3.82 min)



9. Calibration Results of High-Resolution Mass Spectrometer (LTQ Orbitrap).

Pierce ® LTQ ESI Positive Ion Calibration Solution was used to calibrate the instrument. The solution was injected through direct infusion with a 500 μL Hamilton syringe at 10 μL/min. The calibration was repeated until FT analyzer temperature was stable.

```
10:24:09:   Running FT mass calibration for
10:24:09:     universal mass range / positive ions
10:24:09:      Reference masses m/z 195.087652 .. 1721.958701.
10:24:12:      0.100000E6: 0.513548ppm, B=47498244.352850, C=3019634.689456
10:24:14:      0.300000E6: 0.310400ppm, B=47498235.466133, C=2452835.774328
10:24:17:      1.000000E6: 0.216308ppm, B=47498180.009665, C=2022496.422894
10:24:19:      3.000000E6: 0.329624ppm, B=47498077.301204, C=2547059.500599
10:24:20:        [ Info: 0.769479 ppm NLC at m/z 200 ]
10:24:21:   Running FT mass calibration for
10:24:21:     small mass range / positive ions
10:24:21:      Reference masses m/z 138.066190 .. 524.264964.
10:24:24:      0.100000E6: 0.323068ppm, B=47498377.723982, C=-4856711.406524
10:24:26:      0.300000E6: 0.265279ppm, B=47498334.065378, C=-2876656.291302
10:24:29:      1.000000E6: 0.177453ppm, B=47498287.734953, C=675188.370958
10:24:33:      3.000000E6: 0.196351ppm, B=47498205.287604, C=5367901.355345
10:24:34:  FT Mass Calibration (pos) SUCCESSFUL
```

Signature _____

References for Supporting Information

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