Isotope-Labeling Studies Support an Electrophilic Compound I Iron Active Species (FeO^{3+}) for the Carbon-Carbon Bond Cleavage Reaction of the Cholesterol Side Chain Cleavage Enzyme, Cytochrome P450 11A1 ($P450_{SCC}$)

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1. Codon optimization and construction of expression vector for human P450 11A1. The website from Integrated DNA Technologies (Coralville, IA) (http://www.idtdna.com/CodonOpt) was used to generate an *Escherichia coli* codon optimization sequence for human P450 11A1 (*CYP11A1*).

A cDNA the sequence for human P450 11A1 (*CYP11A1*) and a C-terminal $(His)_6$ tag was synthesized and ligated into a pCW expression vector (using *Nde*I and *Hind*III restriction sites) by Genewiz (South Plainfield, NJ, USA).

The sequence used for synthesis of *CYP11A1* cDNA:

```
ATGGCAAGCACGCGCAGCCCGCGTCCGTTTAACGAAATTCCGTCTCCAGGCGACAACGGA
 M  A  S  T  R  S  P  R  P  F  N  E  I  P  S  P  G  D  N  G
TGGCTGAATCTCTATCACTTTTGGCGTGAAACGGGCACTCATAAAGTTCACCTGCATCAC
 W  L  N  L  Y  H  F  W  R  E  T  G  T  H  K  V  H  L  H  H
GTCCAGAACTTCCAGAAGTACGGCCCTATCTACCGCGAAAAGCTGGGGAATGTTGAAAGT
 V  Q  N  F  Q  K  Y  G  P  I  Y  R  E  K  L  G  N  V  E  S
GTCTATGTCATCGATCCTGAAGATGTCGCATTGCTGTTTAAAAGTGAGGGCCCGAATCCC
 V  Y  V  I  D  P  E  D  V  A  L  L  F  K  S  E  G  P  N  P
GAACGTTTCCTGATCCCGCCGTGGGTGGCTTACCACCAATATTATCAGCGTCCAATTGGC
 E  R  F  L  I  P  P  W  V  A  Y  H  Q  Y  Y  Q  R  P  I  G
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 V  L  L  K  K  S  A  A  W  K  K  D  R  V  A  L  N  Q  E  V
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 M  A  P  E  A  T  K  N  F  L  P  L  L  D  A  V  S  R  D  F
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 S  D  D  L  F  R  F  A  F  E  S  I  T  N  V  I  F  G  E  R
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 N  V  T  E  M  L  A  G  G  V  D  T  T  S  M  T  L  Q  W  H
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 L  Y  E  M  A  R  N  L  K  V  Q  D  M  L  R  A  E  V  L  A
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 A  R  H  Q  A  Q  G  D  M  A  T  M  L  Q  L  V  P  L  L  K
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 V  N  D  L  V  L  R  D  Y  M  I  P  A  K  T  L  V  Q  V  A
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 I  Y  A  L  G  R  E  P  T  F  F  F  D  P  E  N  F  D  P  T
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 R  W  L  S  K  D  K  N  I  T  Y  F  R  N  L  G  F  G  W  G
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 V  R  Q  C  L  G  R  R  I  A  E  L  E  M  T  I  F  L  I  N
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 L  I  L  M  P  E  K  P  I  S  F  T  F  W  P  F  N  Q  E  A
ACGCAACAACACCATCACCATCACCATTAA
 T  Q  Q  H  H  H  H  H  H  STOP
```
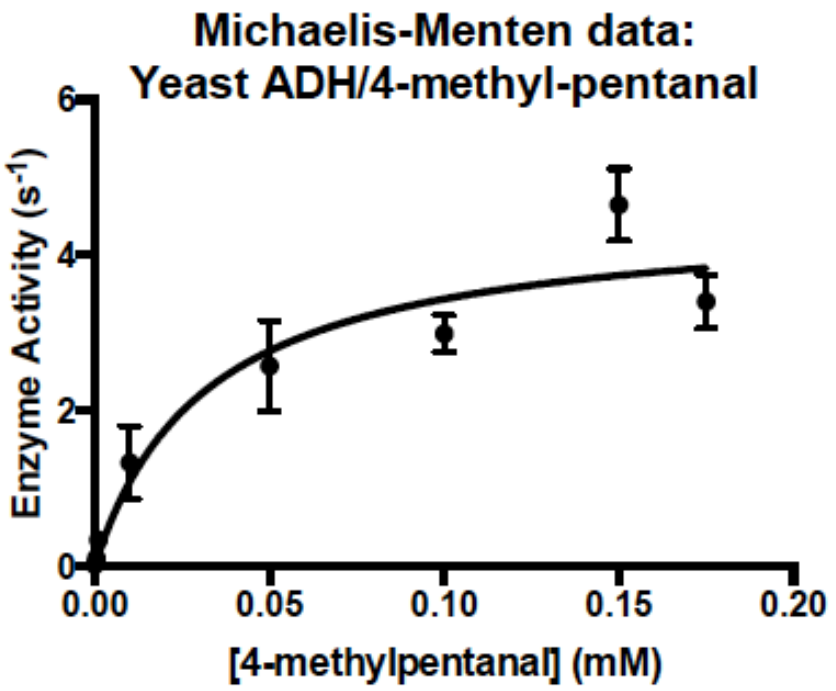

2. Enzyme-Catalyzed Conversion of Isocaproaldehyde to 4-Methylpentan-1-ol.

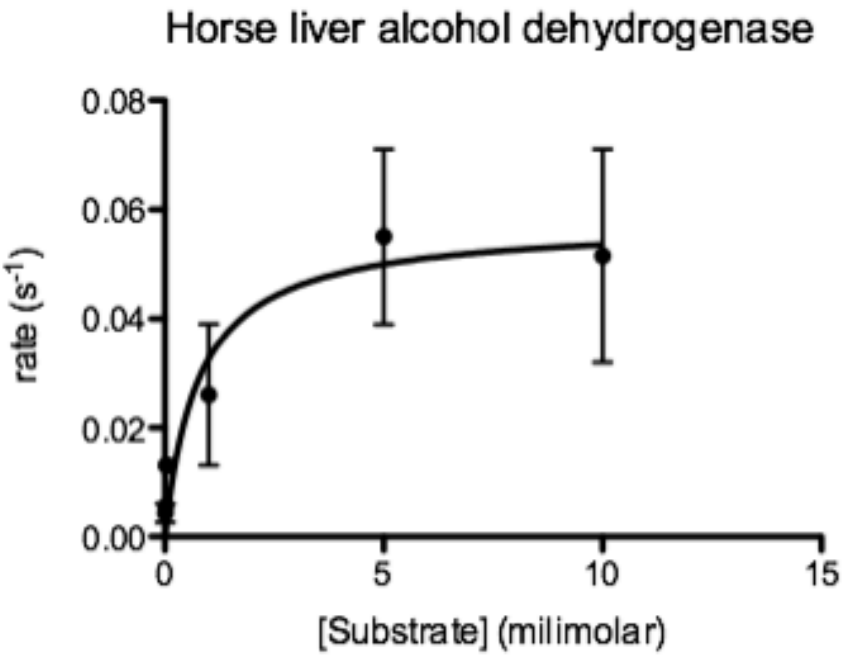
The rate of the enzyme-catalyzed reduction of the isocaproaldehyde (4-methylpentanal) to 4-methylpentan-1-ol (NADH-dependent) was measured by using an OLIS 14 UV/Vis/NIR spectrophotometer (On-Line Instrument Systems, Bogart, GA) with a visible lamp (beam splitter on). The machine was run using OLIS Spectral Works software (Version 4.7.76) and set to 200 increments and 2 reads per datum. The reactions were run in a glass cuvette (1 cm path length) at room temperature. Two enzymes were tested: yeast and horse liver alcohol dehydrogenase (Sigma-Aldrich). Reactions were performed at room temperature.

A master mix was made containing potassium phosphate buffer (100 mM, pH 7.4) and NADH (150 μM), which would be used for each concentration point (970 μL of master mix was used for each concentration point). Isocaproaldehyde stock solutions were made in CH_3CN/H_2O, 1:1, v/v (100 mM, 500 mM, 1 mM, 10 mM, 50 mM, 100 mM, 150 mM, 175 mM, and 200 mM). For each concentration point, 20 μL of substrate was added. The reactions were initiated by the addition of 10 μL of enzyme solution (1 mg/mL) using a plumping device (cuvette mixer, to mix the solution). After the enzyme was added, the decrease in absorbance at 340 nm (i.e. the absorbance maximum of NADH) was monitored for 60-120 seconds. The molar extinction coefficient of NADH at 340 nm is 6,220 $M^{-1}cm^{-1}$, and the reduction rate was calculated accordingly.

Data were plotted using Prism software (Graphpad, La Jolla, CA, version 5.0d) fitting the points using the non-linear regression curve fitting option for the Michaelis-Menten equation. The k_{cat} and K_M values for yeast alcohol dehydrogenase were 4.5 s^{-1} and 32 μM. The k_{cat} and K_M values for horse liver alcohol dehydrogenase were 0.06 s^{-1} and 772 μM.

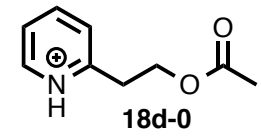


	Enzyme Activity
Michaelis-Menten	
Best-fit values	
Vmax	4.535
Km	0.03201
Std. Error	
Vmax	0.5243
Km	0.01421
95% Confidence Intervals	
Vmax	3.411 to 5.660
Km	0.001521 to 0.06249
Goodness of Fit	
Degrees of Freedom	14
R square	0.9147
Absolute Sum of Squares	3.674
Sy.x	0.5123
Constraints	
Km	Km > 0.0
Number of points	
Analyzed	16



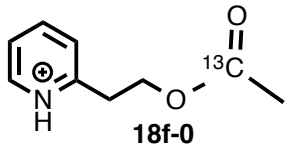
	Enzyme Activity
Michaelis-Menten	
Best-fit values	
Vmax	0.05767
Km	0.7715
Std. Error	
Vmax	0.01059
Km	0.6787
95% Confidence Intervals	
Vmax	0.03458 to 0.08075
Km	0.0 to 2.250
Goodness of Fit	
Degrees of Freedom	12
R square	0.6848
Absolute Sum of Squares	0.002208
Sy.x	0.01356
Constraints	
Km	Km > 0.0
Number of points	
Analyzed	14

3. Detection of naturally abundant mono-^{18}O-isotope incorporated ethylpyridine acetate (18d-1, *m/z* 168.0905, ~0.003%) from synthetic standard.



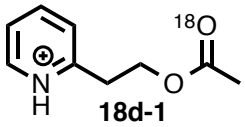
18d-0

Exact Mass: 166.0863



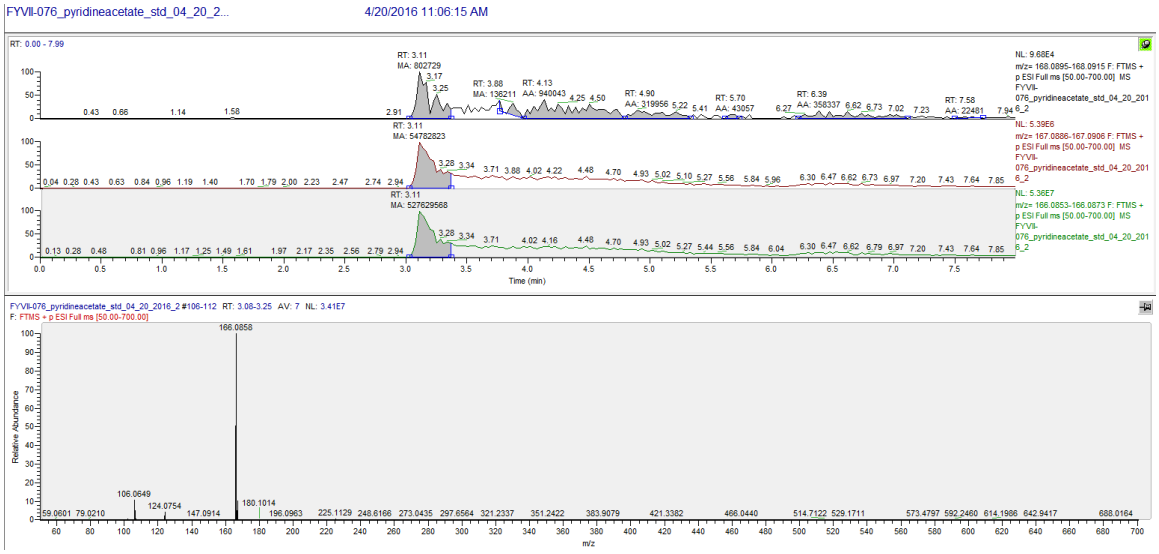
18f-0

Exact Mass: 167.0896



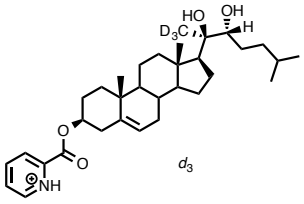
18d-1

Exact Mass: 168.0905



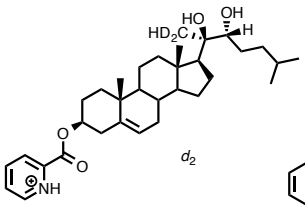
4. LC-MS analysis of picolinoylated 21,21,21-d_3-20,22-dihydroxycholesterol 3a (d_3:d_2:d_1:d_0 ratio 27:64:50:11).

The synthesis of mono-picolinoylated 21,21,21-d_3-20,22-dihydroxycholesterol (picolinoylated **3a**) is presented in the main text. The following *m/z* values were scanned (the location of the picolinoyl group was not verified to be the 3-position): *m/z* 527.3923, 526.3860, 525.3797, 524.3734, corresponding to d_3-, d_2-, d_1-, and d_0-mono-picolinoylated 20,22-dihydroxycholesterol. The peak of interest is at t_R 5.06 min.



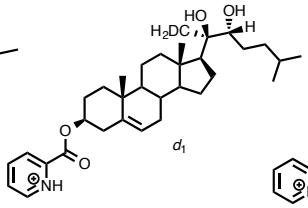
d_3

Exact Mass: 527.3923



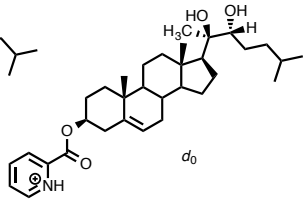
d_2

Exact Mass: 526.3860



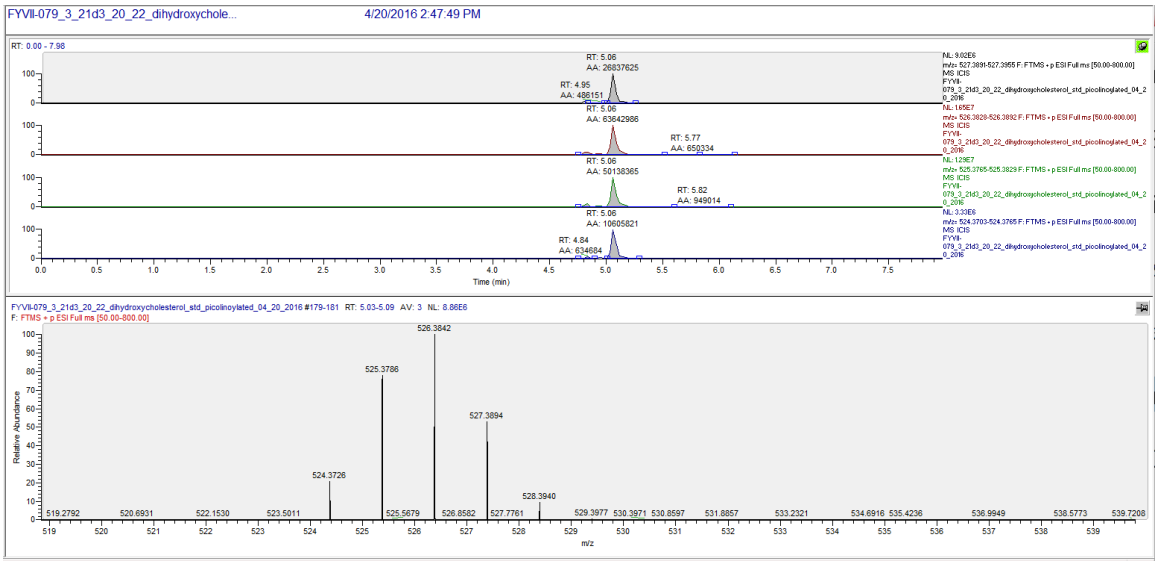
d_1

Exact Mass: 525.3797



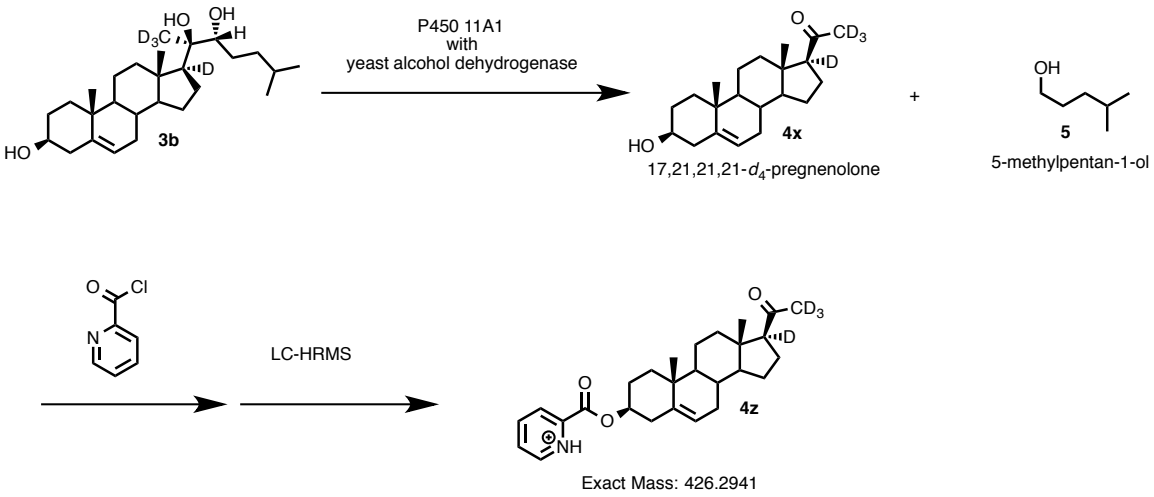
d_0

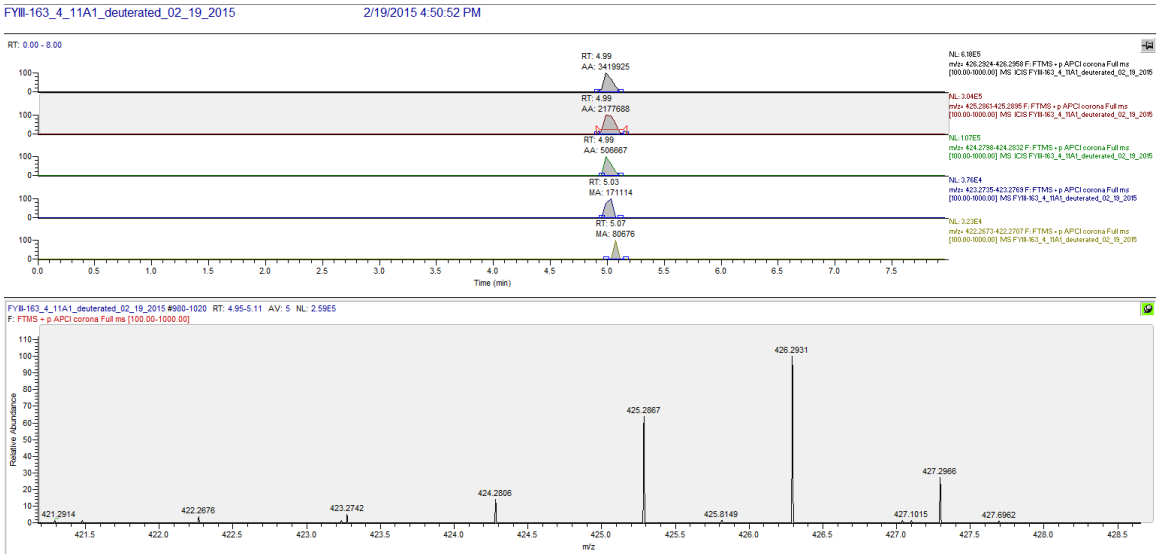
Exact Mass: 524.3734



5. LC-MS analysis of picolinoylated-pregnenolone product using 17,21,21,21-d_4-20,22-dihydroxycholesterol (3b) as the starting material.

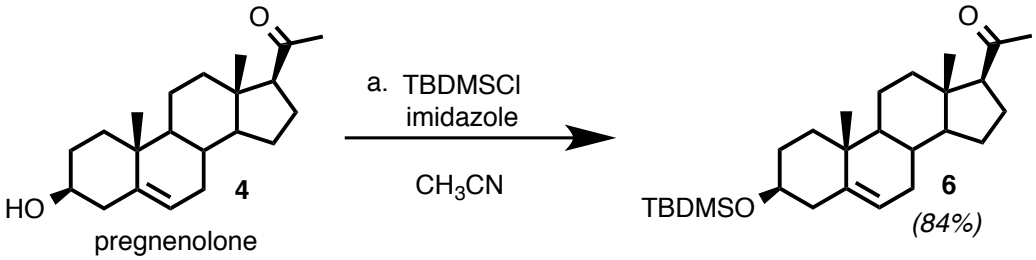
The tune conditions for detecting picolinoylated 4-methyl-pentan-1-ol were used to detect picolinoylated pregnenolone (APCI positive mode). The incubation conditions are described in the manuscript, except that a Thunberg tube, gas train, and $^{18}O_2$ were not used. The incubation was performed in a 16 × 150 mm glass test tube open to air, and the same workup procedure (including derivatization with picolinoyl chloride) described in the main text for the P450 11A1-yeast alcohol dehydrogenase assay was followed. Picolinoylated-tetradeuteropregnenolone (**4z**, calculated: *m/z* 426.2941, found: *m/z* 426.2931, Δ = 2.3 ppm) was detected by LC-MS.



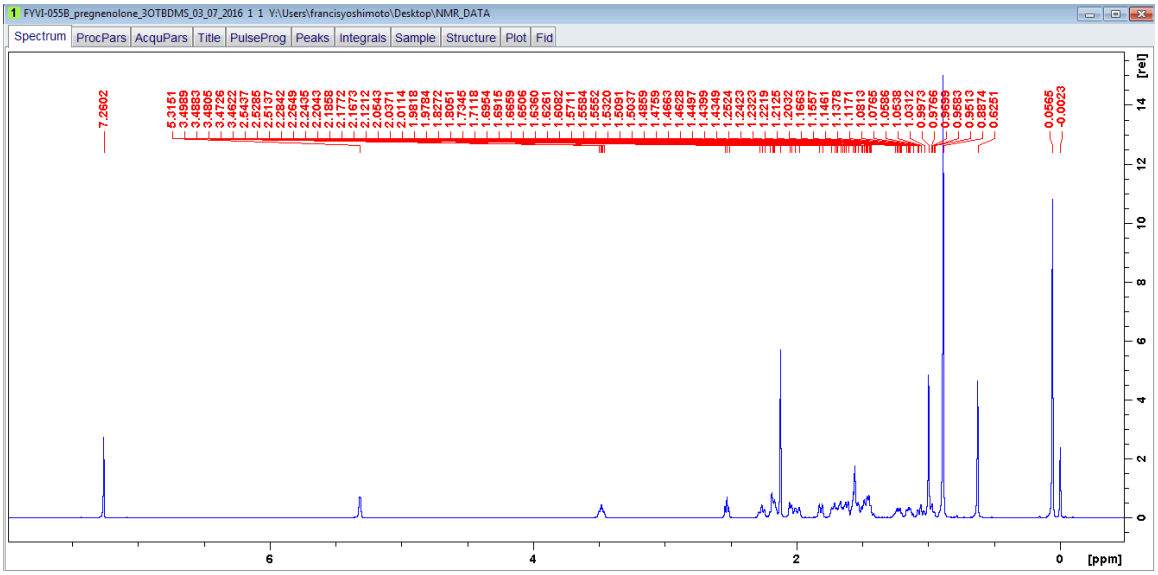


6. Experimental data of synthesized chemicals

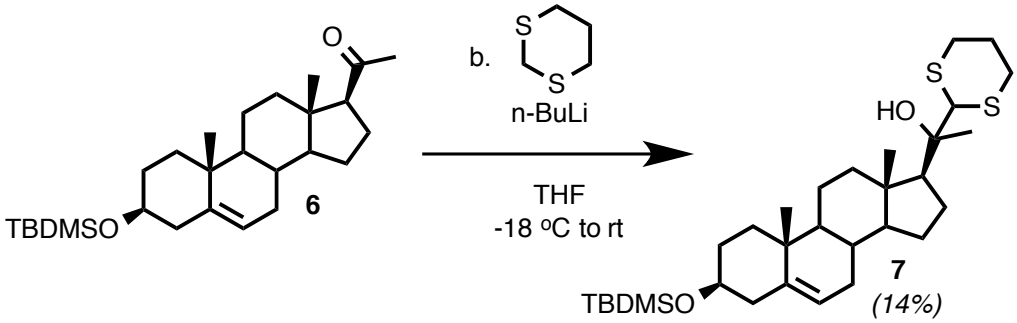
6.1. Pregnenolone-3-OTBDMS (6).



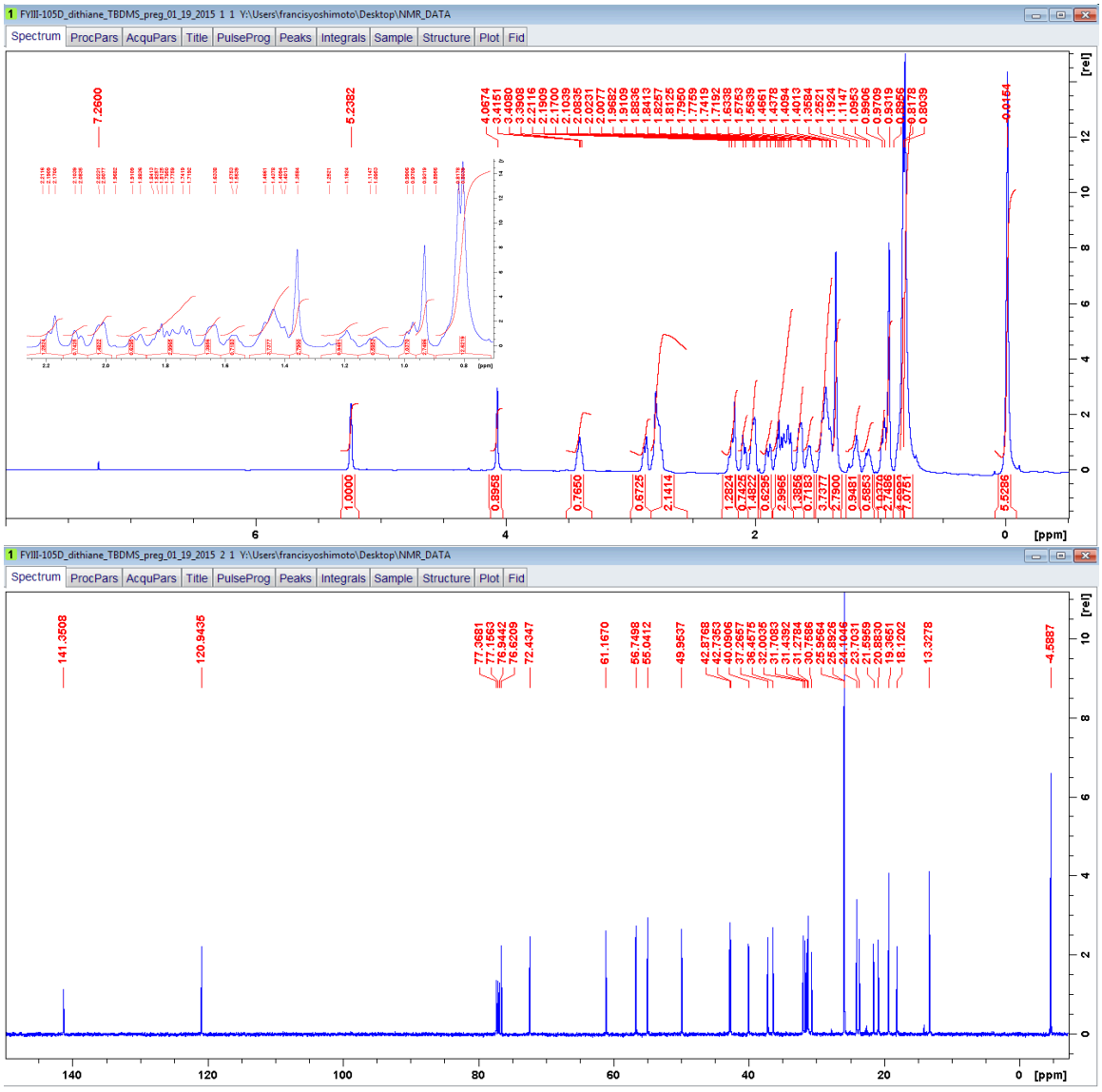
Experimental procedure for the synthesis of TBDMS ether **6**; ^{1}H NMR chemical shifts are reported in the main text of the manuscript. Below is the ^{1}H NMR spectrum (**6**, $CDCl_3$, 600 MHz).



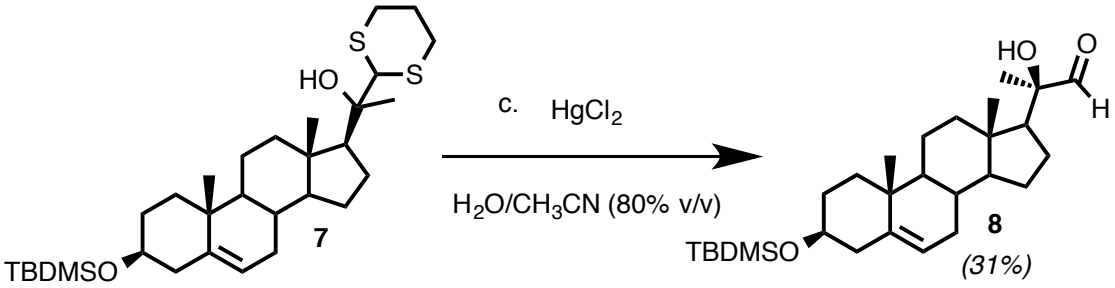
6.2. Dithiane (compound 7).



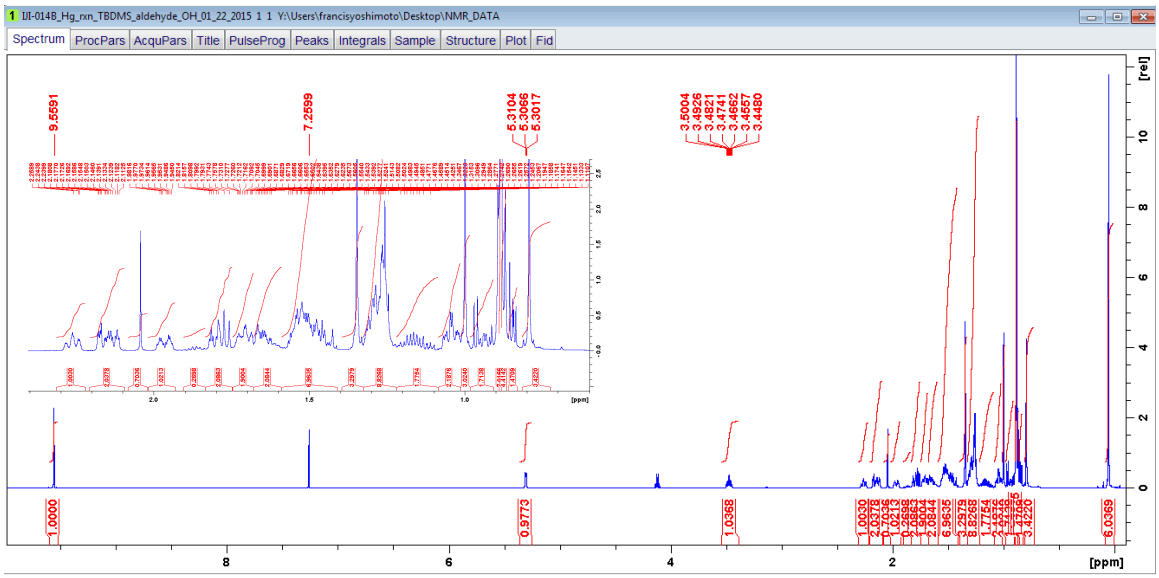
Dithiane **7** was obtained following the procedure in the manuscript. Below are the ^{1}H and ^{13}C NMR spectra (**7**, $CDCl_3$, 600 MHz and 150 MHz).



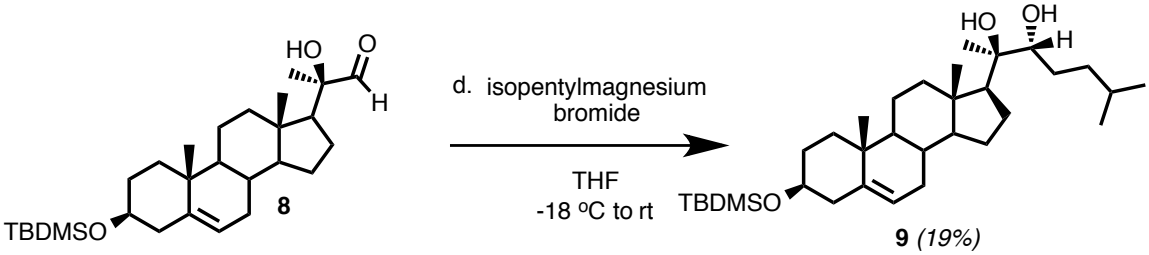
6.3. Hg reaction (8).



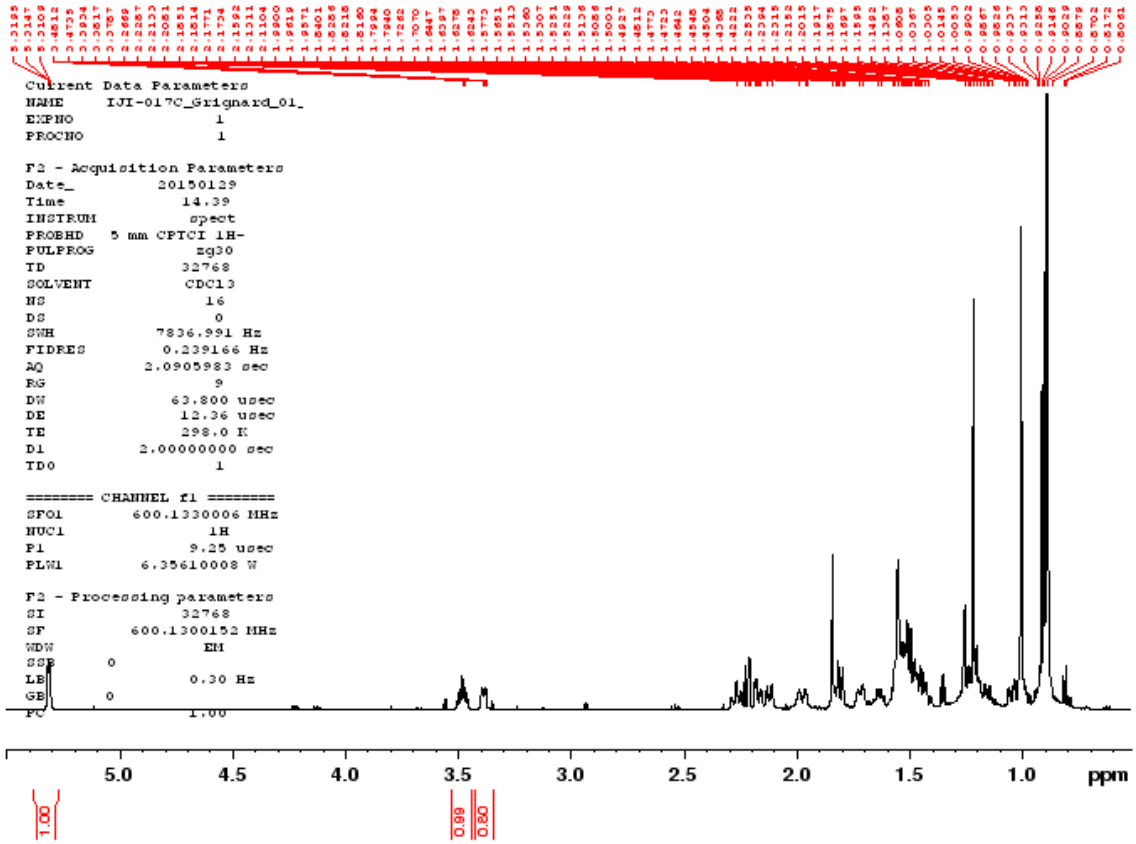
Aldehyde **8** was obtained following the procedure described in the manuscript. Below is the ^{1}H NMR spectrum (**8**, $CDCl_3$, 600 MHz).



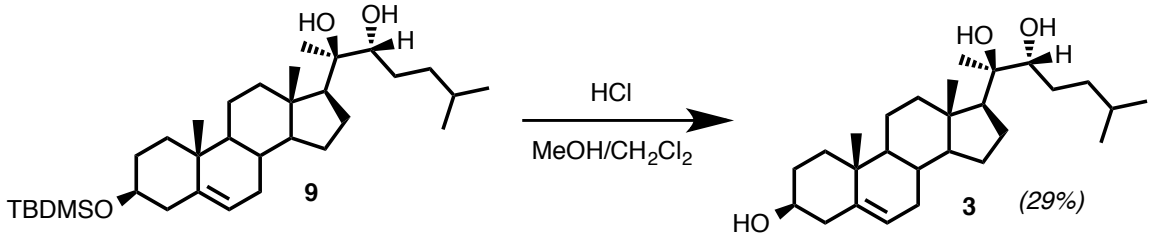
6.4. Grignard adduct (9).



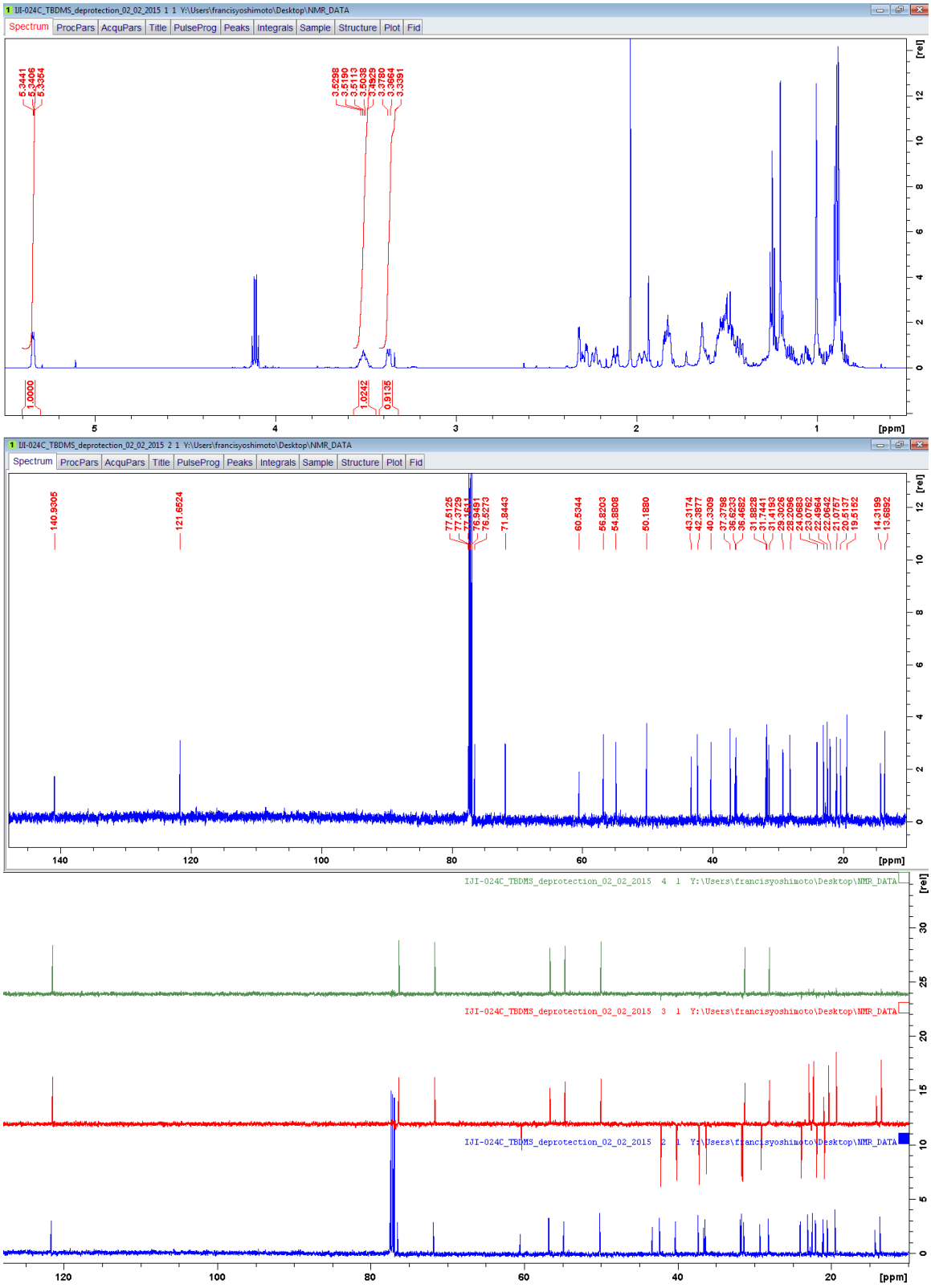
Diol **9** was obtained following the procedure in the manuscript. Below is the ^{1}H NMR spectrum (**9**, $CDCl_3$, 600 MHz).



6.5. TBDMS deprotection (3).



Triol **3** was obtained following the procedure described in the manuscript. Below are the ^{1}H and ^{13}C NMR spectra (**3**, $CDCl_3$, 600 MHz), followed by DEPT90/ DEPT135/^{13}C NMR stacked overlay.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3.3391
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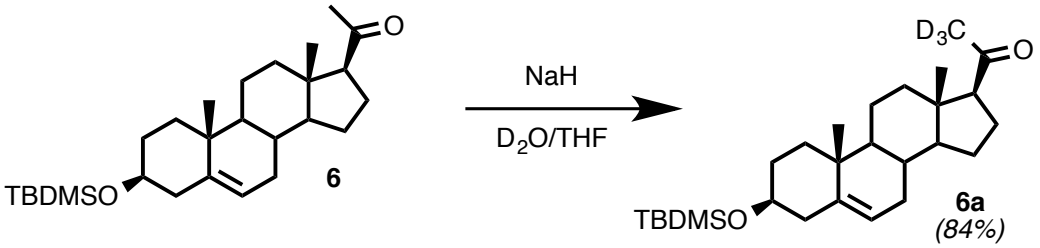
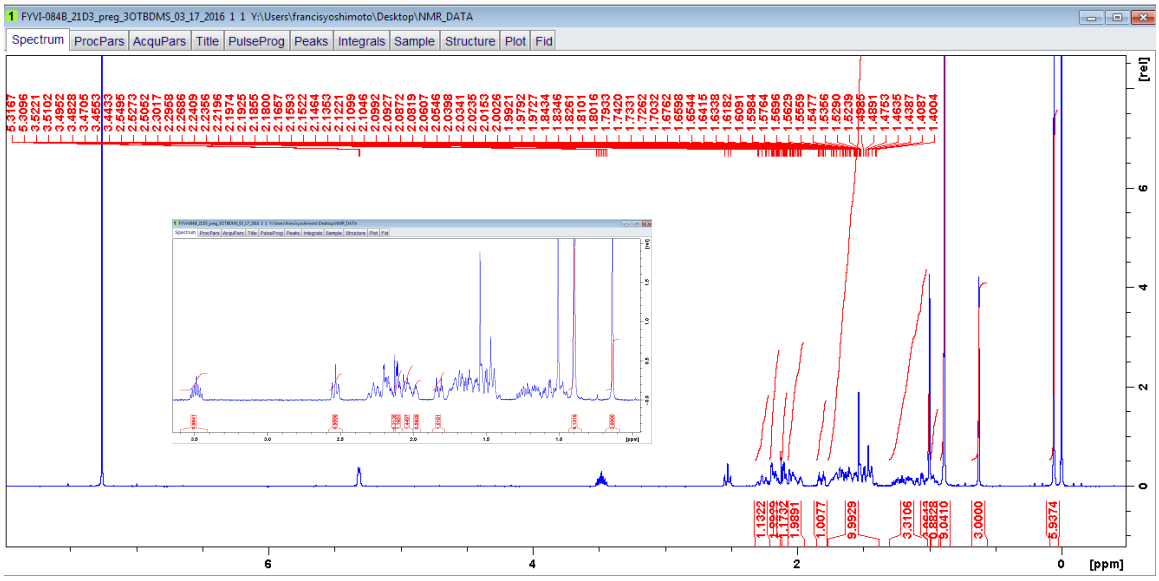
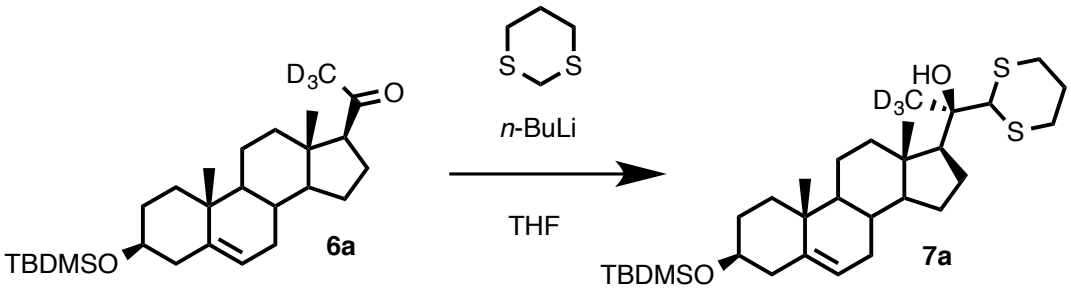
6.6. Synthesis of 21,21,21-d_3-Pregnenolone-3-OTBDMS (6a).



[21,21,21-d_3]-Pregnenolone-3-OTBDMS (**6a**) was obtained following the procedure in the manuscript. Below is the ^{1}H NMR spectrum (**6a**, $CDCl_3$, 600 MHz). The C21-methyl protons (δ 2.12 ppm) integrates to 0.31 while the C17-proton (δ 2.52 ppm) integrates to 0.96, confirming regioselective deuteration at C21. Below is the ^{1}H NMR spectrum (**6a**, $CDCl_3$, 600 MHz).

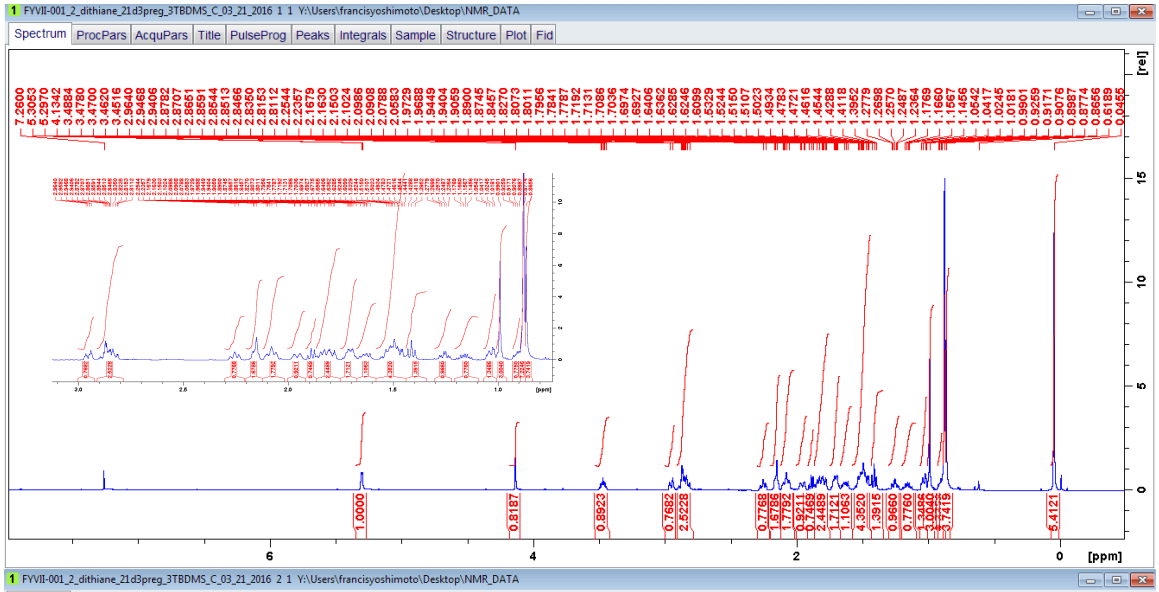


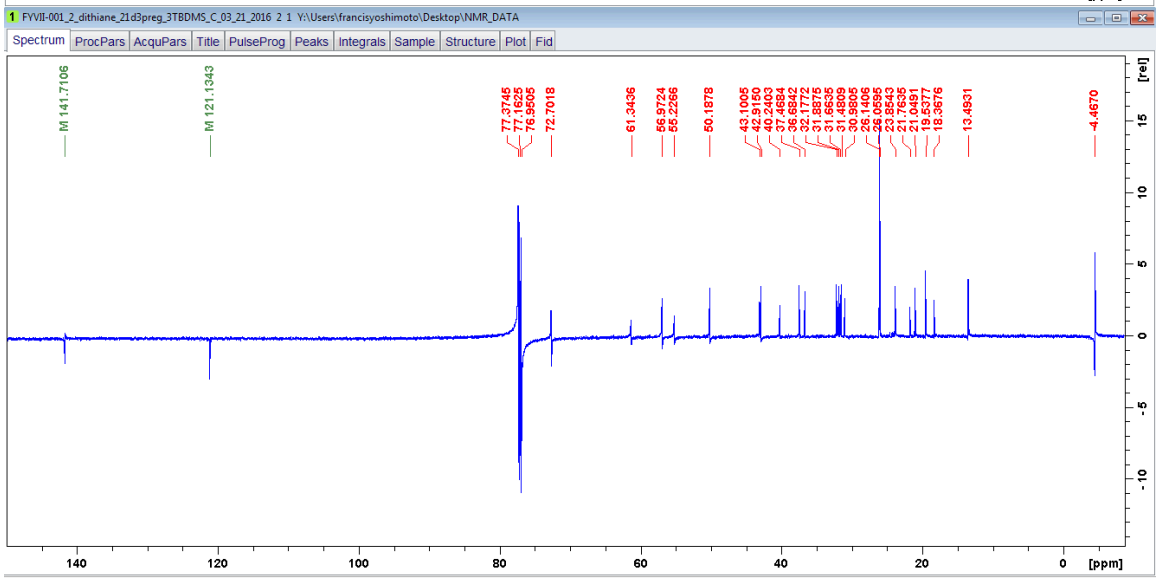
6.7. Deuterated dithiane (7a).

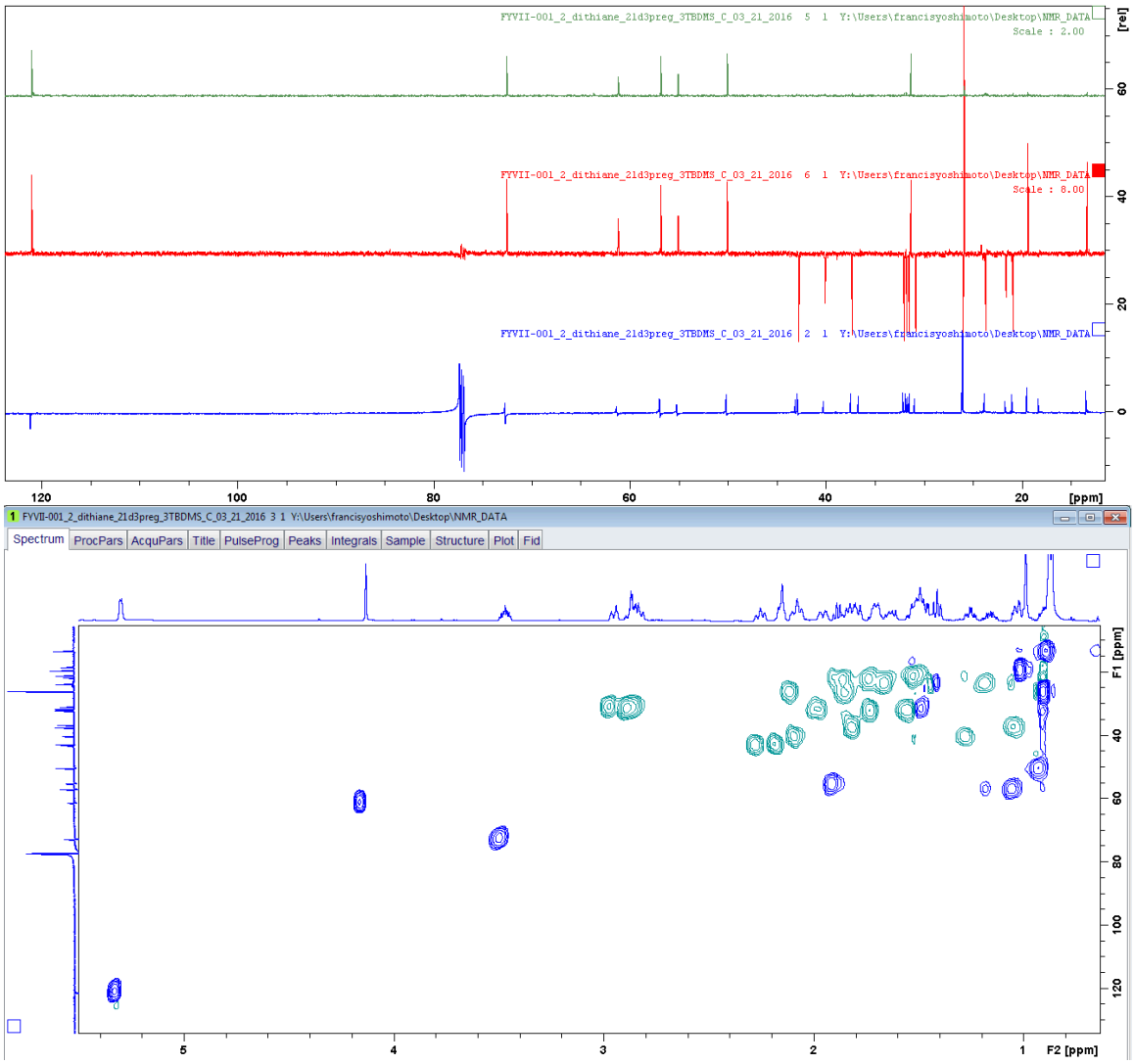


n-BuLi (3.68 mL, 2.5 M in diethyl ether, 9.2 mmol, 2.0 mol eq) was added to dithiane (1.11 g, 9.2 mmol, 2.0 mol eq) in THF (50 mL) at -78 °C. The reaction was gradually warmed to -20 °C over 1.5 hr and cooled back to -78 °C. [21,21,21-d_3]-Pregnenolone-3-OTBDMS (**6a**, 2.0 g, 4.6 mmol) in THF (20 mL) was added. After 2.5 h, the reaction was quenched upon addition of H_2O (100 mL). The reaction mixture was extracted with ethyl acetate (200 mL). The organic extract was concentrated with reduced pressure and

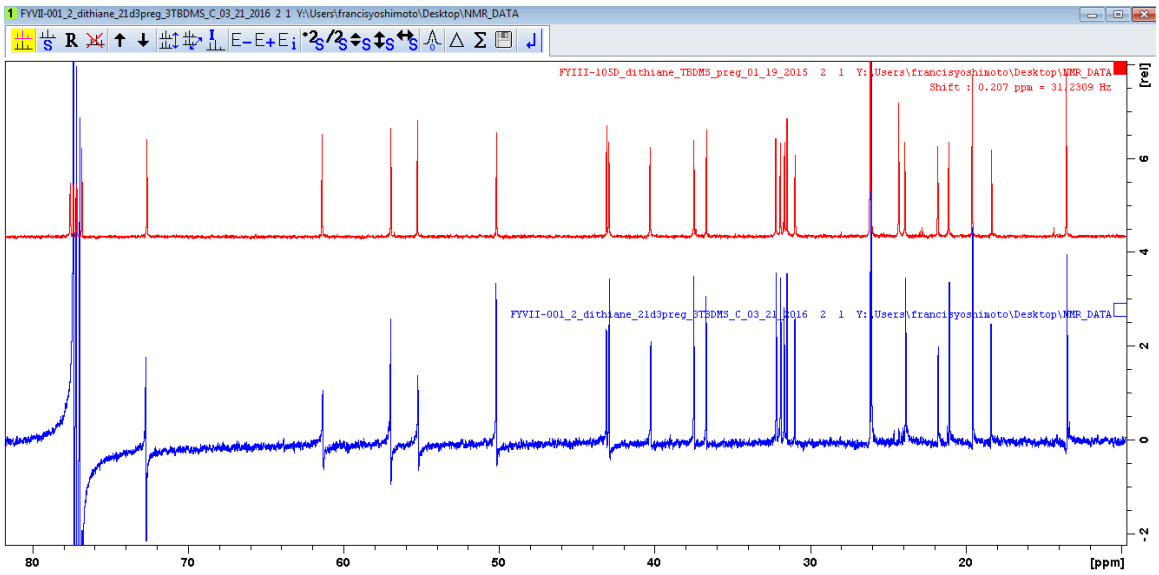
purified by flash column chromatography (100% hexanes to 50% hexanes in ethyl acetate, v/v) to yield dithiane **7a** (0.61 g, 1.1 mmol, 55%). R_f 0.53 of **7a** (hexanes:ethyl acetate, 4:1, v-v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.32-5.29 (m, 1H), 4.13 (broad s, 1H), 3.51-3.43 (m, 1H), 2.97-2.92 (m, 1H), 2.90-2.79 (m, 2H), 2.29-2.22 (m, 1H), 2.19-2.12 (m, 1H), 2.12-2.04 (m, 1H), 1.99-1.93 (m, 1H), 1.92-1.76 (m, 3H), 1.75-1.67 (m, 2H), 1.67-1.60 (m, 1H), 1.57-1.38 (m, 5H), 1.25 (td, J_1 = 12.9 Hz, J_2 = 4.3 Hz, 1H), 1.21-1.12 (m, 1H), 1.06-1.01 (m, 1H), 0.99 (s, 3H), 0.93-0.89 (m, 1H), 0.88 (s, 9H), 0.87 (s, 3H), 0.46 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 141.7, 121.1, 72.7, 61.3, 57.0, 55.2, 50.2, 43.1, 42.9, 40.2, 37.5, 36.7, 32.2, 31.9, 31.7, 31.5, 31.0, 26.14, 26.06, 23.9, 21.8, 21.0, 19.5, 18.4, 13.5, -4.5. Following are the ^{1}H and ^{13}C NMR spectra (compound **7a**, $CDCl_3$, 600 MHz), followed by DEPT90/ DEPT135/^{13}C NMR stacked overlay, HSQC spectra of **7a**.



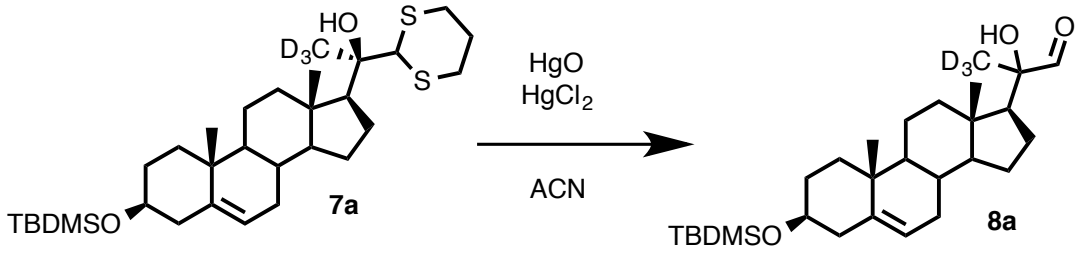




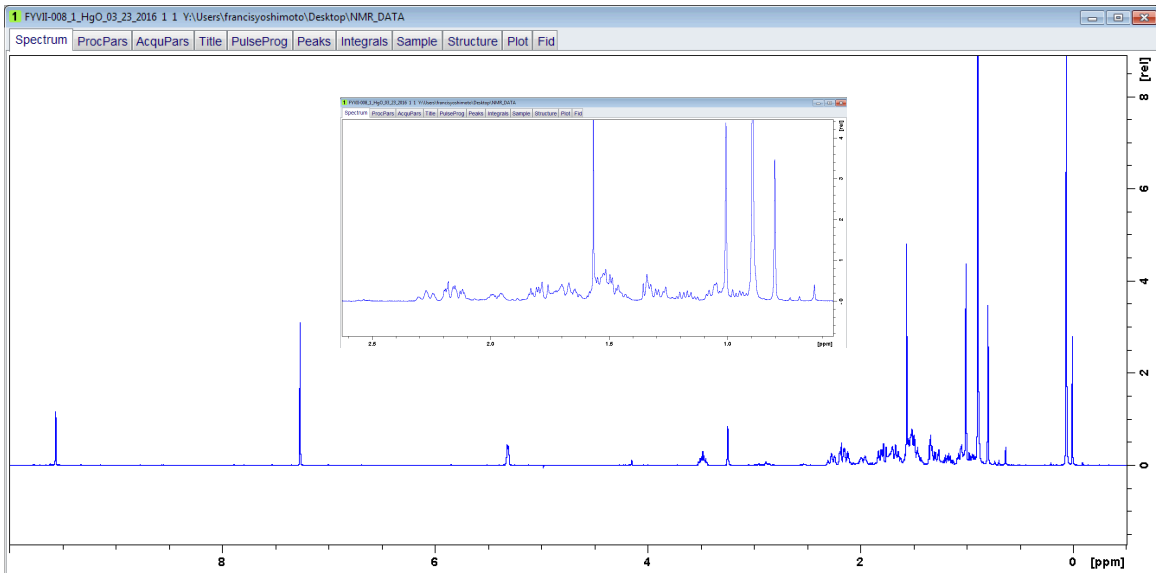
^{13}C NMR spectra overlay of compounds **7** and **7a** (non-deuterated vs. deuterated, δ - 10 ppm—81 ppm) – notice the absence of the peak at δ 24.1 ppm in deuterated compound **7a** corresponding to C21:



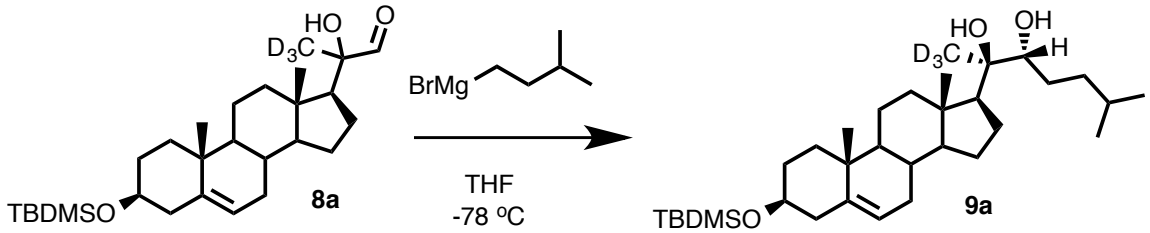
6.8. Hg reaction to form deuterated aldehyde (8a).



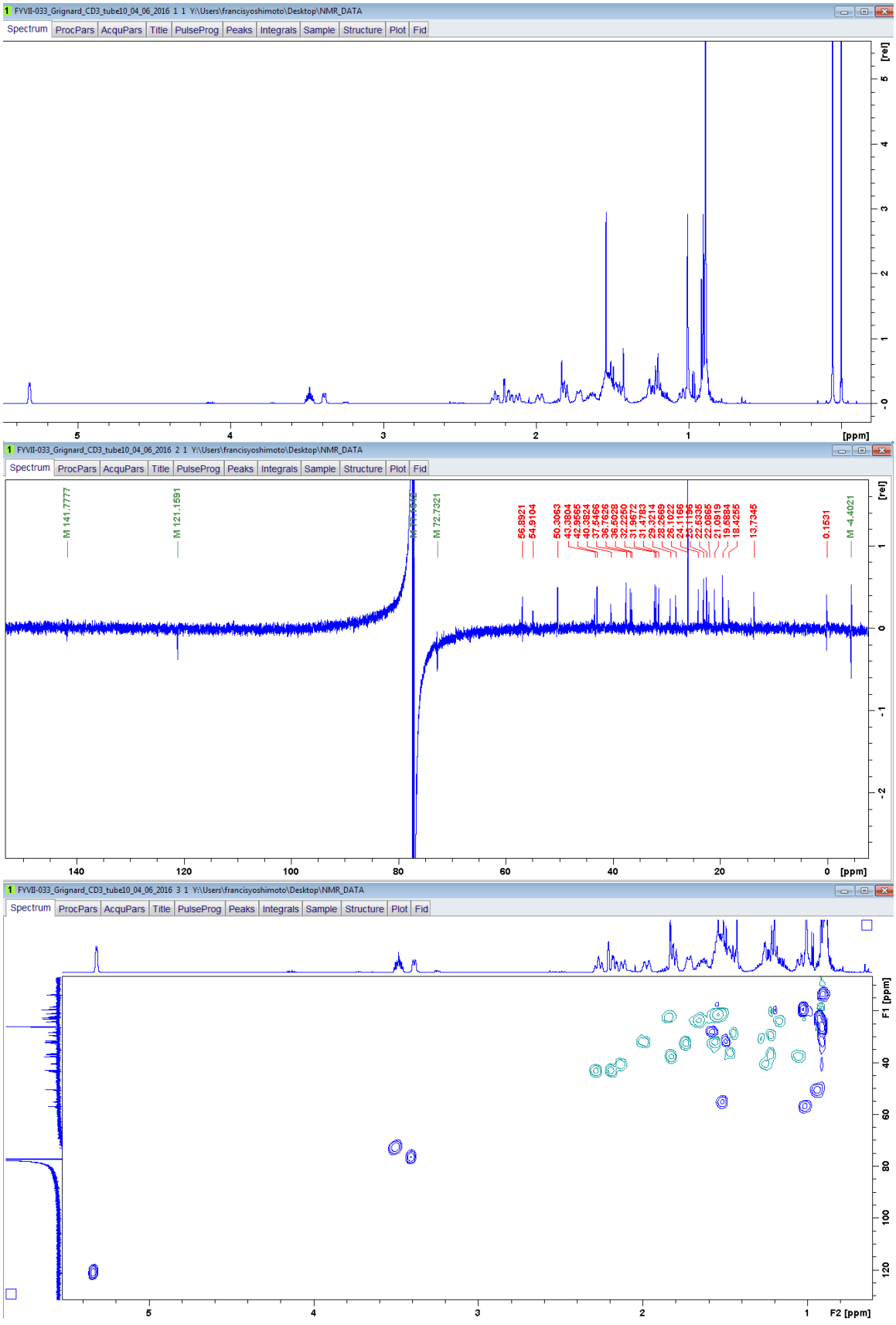
The ratio of acetonitrile to water (100:1 or 10:1, v/v) is important for optimum yield (i.e. excess water lowers the yield of the reaction). Dithiane **7a** (0.61 g, 1.1 mmol), HgO (0.48 g, 2.2 mmol, 2.0 mol eq), $HgCl_2$ (0.60 g, 2.2 mmol, 2.0 mol eq) were dissolved in acetonitrile (100 mL) and water (100 mL). The reaction was stirred at reflux overnight. The mixture was filtered through a short pad of Celite. The concentrated material was purified by flash column chromatography (100% hexanes to 50% hexanes in ethyl acetate, v/v) to afford aldehyde **8a** (**8a**, 0.08 g, 0.17 mmol, 15%) as a white solid. R_f 0.63 of **8a** (hexanes:ethyl acetate, 4:1, v-v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.56 (s, 1H), 5.31-5.30 (m, 1H), 3.51-3.43 (m, 1H), 3.240-3.237 (m, 1H), 2.30-2.24 (m, 1H), 2.19-2.09 (m, 2H), 2.02-1.92 (m, 1H), 1.85-1.76 (m, 1H), 1.76-1.60 (m, 4H), 1.59-1.39 (m, 7H), 1.36-1.24 (m, 3H), 1.22-1.11 (m, 1H), 1.09-1.02 (m, 2H), 1.00 (s, 3H), 0.98-0.90 (m, 1H), 0.89 (s, 12H), 0.79 (s, 3H), 0.05 (s, 6H). Below is the ^{1}H NMR spectrum (**8a**, $CDCl_3$, 600 MHz).



6.9. C21-Deuterated Grignard adduct (9a).



The procedure described in the manuscript to convert aldehyde **8** to diol **9** was followed to convert **8a** to **9a**. *Diol **9a** was reactive with acetone at room temperature to form the acetonide (see synthesis of **3z**). Therefore, the use of acetone to dissolve diol **9a** was avoided. R_f 0.41 of **9a** (hexanes:ethyl acetate, 4:1, v-v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.32-5.31 (m, 1H), 3.51-3.45 (m, 1H), 3.41-3.36 (m, 1H), 2.30-2.23 (m, 1H), 2.21 (d, J = 2.7 Hz, 1H), 2.17 (ddd, J_1 = 13.4 Hz, J_2 = 4.7 Hz 2.2 Hz, 1H), 2.14-2.09 (m, 1H), 2.01-1.94 (m, 1H), 1.85-1.77 (m, 3H), 1.75-1.69 (m, 1H), 1.69-1.59 (m, 2H), 1.59-1.39 (m, 11H), 1.32-1.09 (m, 7H), 1.08-1.02 (m, 1H), 1.00 (s, 3H), 0.91 (d, J = 6.9 Hz, 1H), 0.89 (broad s, 13H), 0.88-0.84 (m, 1H), 0.56 (s, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 141.8, 121.2, 72.7, 56.9, 54.9, 50.3, 43.4, 43.0, 40.4, 37.5, 36.8, 36.5, 32.2, 32.0, 31.5, 29.3, 28.3, 26.1, 24.1, 23.1, 22.5, 22.1, 21.1, 19.6, 18.4, 13.7, 0.15, -4.4. Below are the ^{1}H and ^{13}C NMR spectra (**9a**, $CDCl_3$, 600 and 150 MHz), HSQC, NOESY, and COSY spectra.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[rel]
[ppm]
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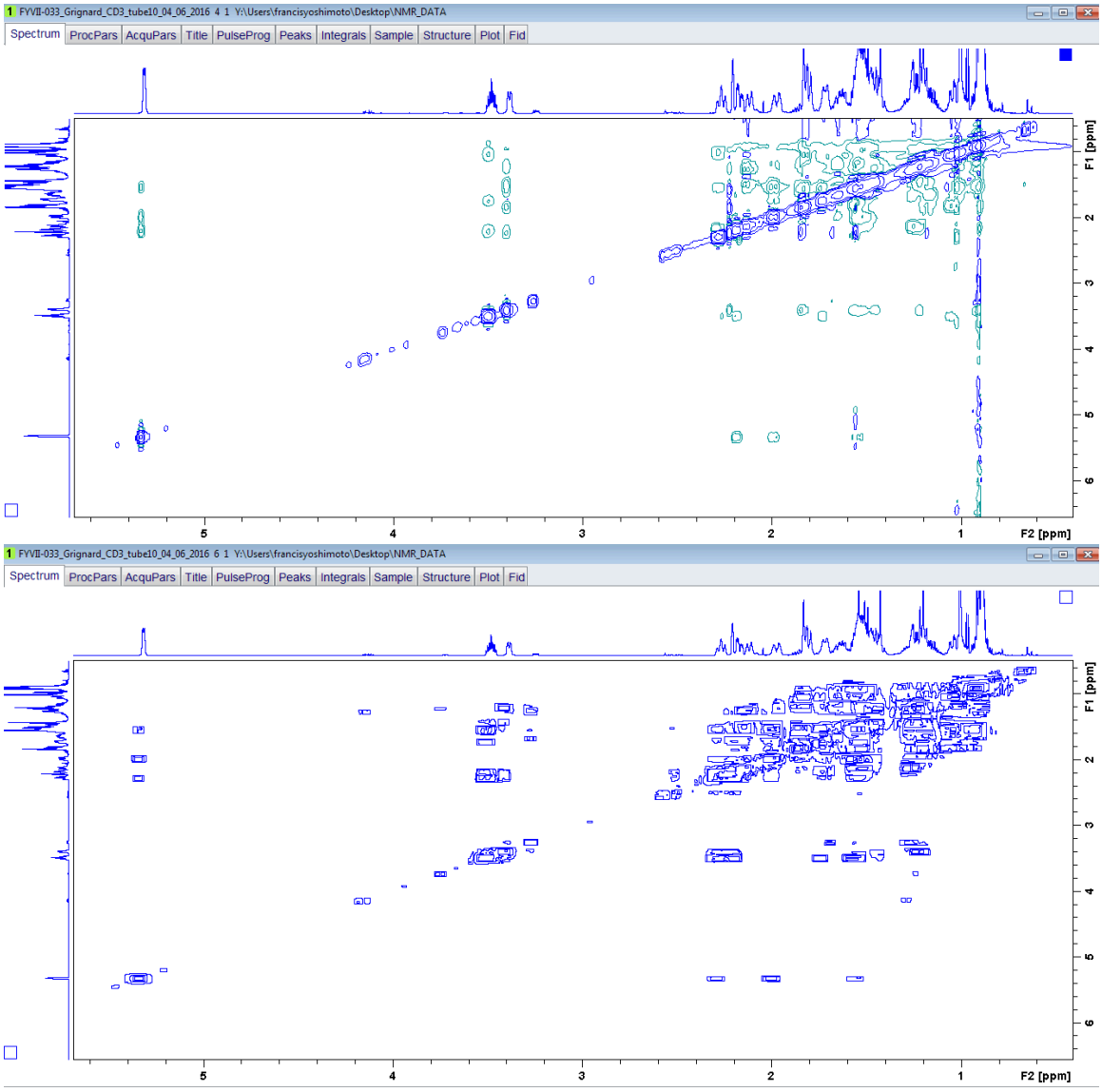
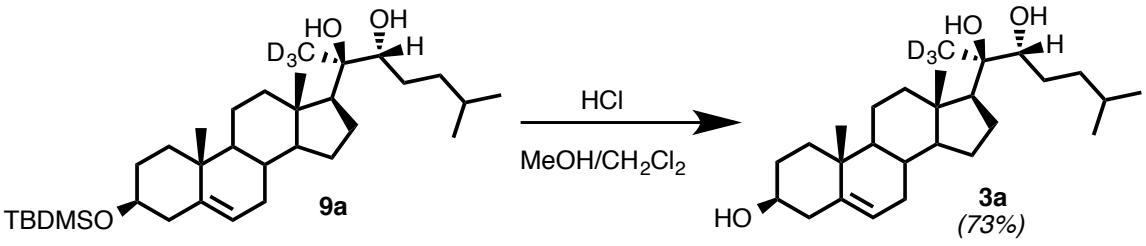
M 77.1068
M 72.7321
56.8921
54.9104
50.3063
43.3804
42.9585
40.9824
37.1466
36.7626
36.6028
32.2250
31.9672
31.4783
29.3214
28.9859
28.1022
24.1166
23.1196
22.5335
22.0865
21.0919
19.6584
18.4255
13.7345
0.1531
M -4.4021
[rel]
[ppm]
1 FYVII-033_Grignard_CD3_tube10_04_06_2016 3 1 Y:\Users\francisyoshimoto\Desktop\NMR_DATA
Spectrum ProcPars AcquPars Title PulseProg Peaks Integrals Sample Structure Plot Fid
F1 [ppm]
F2 [ppm]

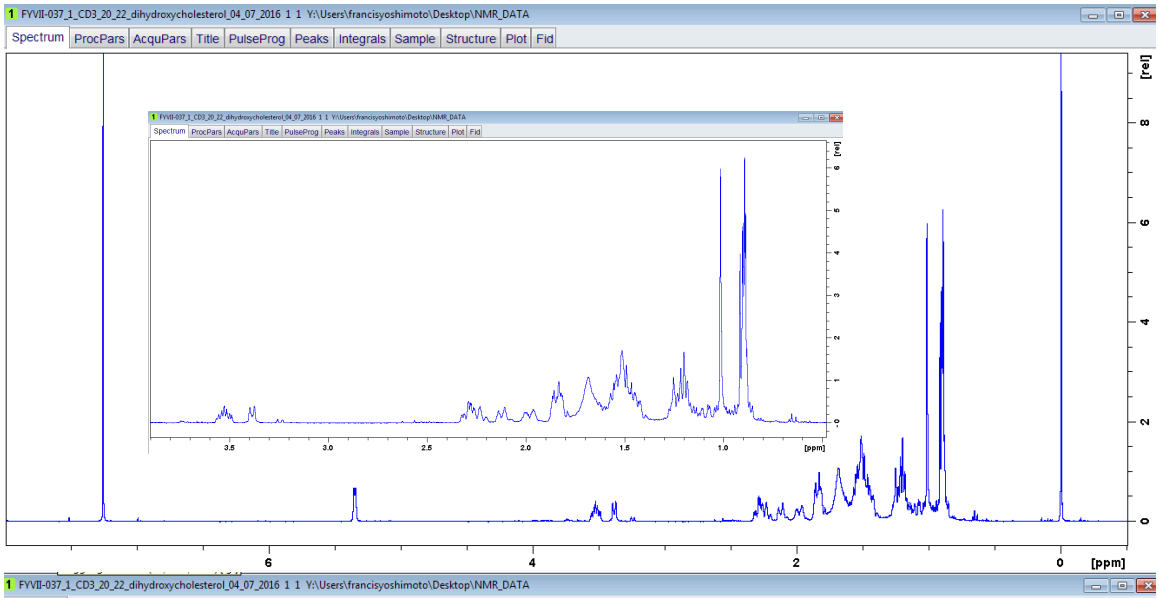


6.10. Synthesis of 21,21,21-d_3-3β,20*R*,22*R*-triol (3a).



Beginning with **9a** (7 mg, 13 μmol) and following the procedure in the main text for **9** to **3**, the triol **3a** was obtained (4 mg, 9.5 μmol, 73%). Similar to diol **9a**, triol **3a** is reactive with acetone to form the 20,22-acetonide, so the use of acetone was avoided to dissolve

triol **3a**. Ethyl acetate, hexanes, methanol, and dichloromethane were solvents used with compounds **9a** and **3a**. The R_f of **3a** 0.59 (hexanes:ethyl acetate, 1:1, v-v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.38-5.33 (m, 1H), 3.58-3.48 (m, 1H), 3.41-3.36 (m, 1H), 2.35-2.18 (m, 1H), 2.16-2.06 (m, 1H), 2.04-1.94 (m, 1H), 1.89-1.78 (m, 3H), 1.76-1.39 (m, 10H), 1.30-1.12 (m, 6H), 1.12-1.03 (m, 1H), 1.02 (s, 3H), group of singlets with 9H integration: (0.916, 0.904, 0.900, 0.893, 0.888); ^{13}C NMR (150 MHz, $CDCl_3$) δ 140.9, 121.7, 76.6, 71.9, 56.8, 50.2, 43.4, 42.4, 43.3, 36.7, 36.5, 31.9, 31.8, 31.4, 29.3, 28.2, 24.1, 23.1, 22.5, 21.1, 19.5, 13.7. Below are the ^{1}H and ^{13}C NMR spectra (**3a**, $CDCl_3$, 600 and 150 MHz).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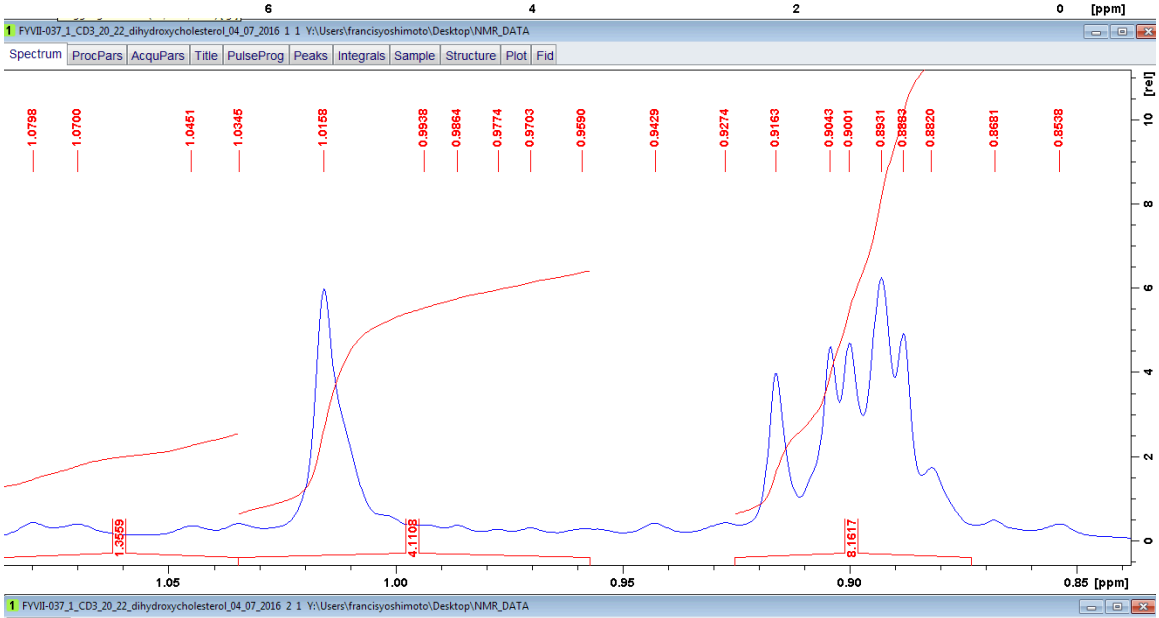
Spectrum ProcPars AcquPars Title PulseProg Peaks Integrals Sample Structure Plot Fid
[rel]
[ppm]


FYVII-037_1_CD3_20_22_dihydroxycholesterol_04_07_2016 1 1 Y:\Users\francisyoshimoto\Desktop\NMR_DATA
Spectrum ProcPars AcquPars Title PulseProg Peaks Integrals Sample Structure Plot Fid
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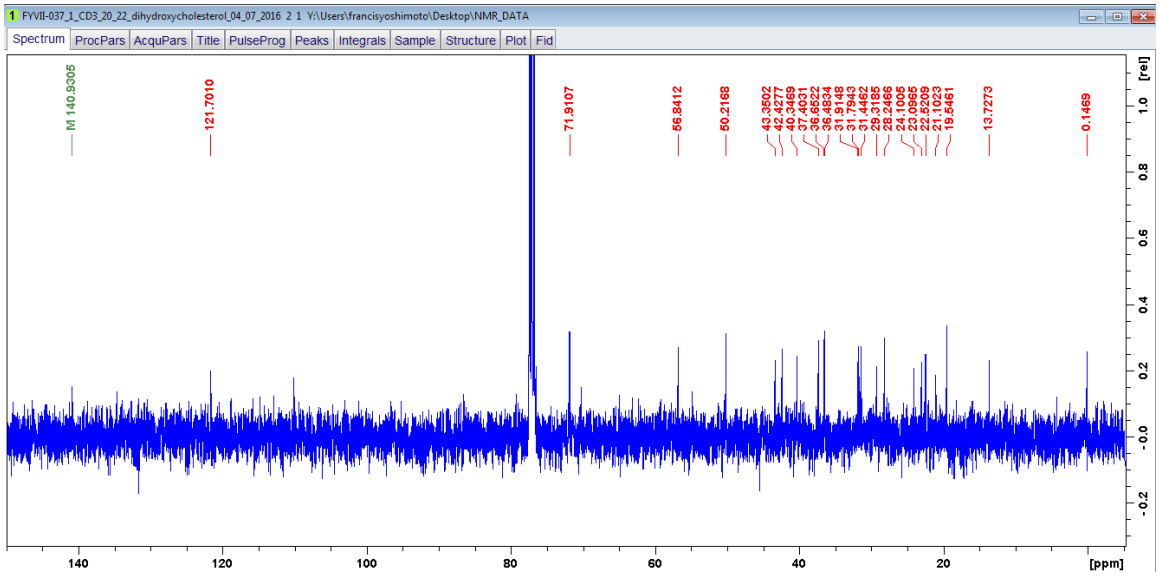
1.0700
1.0451
1.0345
1.0158
0.9938
0.9864
0.9774
0.9703
0.9590
0.9429
0.9274
0.9163
0.9043
0.9001
0.8931
0.8883
0.8820
0.8681
0.8538
1.3559
4.1108
8.1617
[rel]
[ppm]


FYVII-037_1_CD3_20_22_dihydroxycholesterol_04_07_2016 2 1 Y:\Users\francisyoshimoto\Desktop\NMR_DATA
Spectrum ProcPars AcquPars Title PulseProg Peaks Integrals Sample Structure Plot Fid
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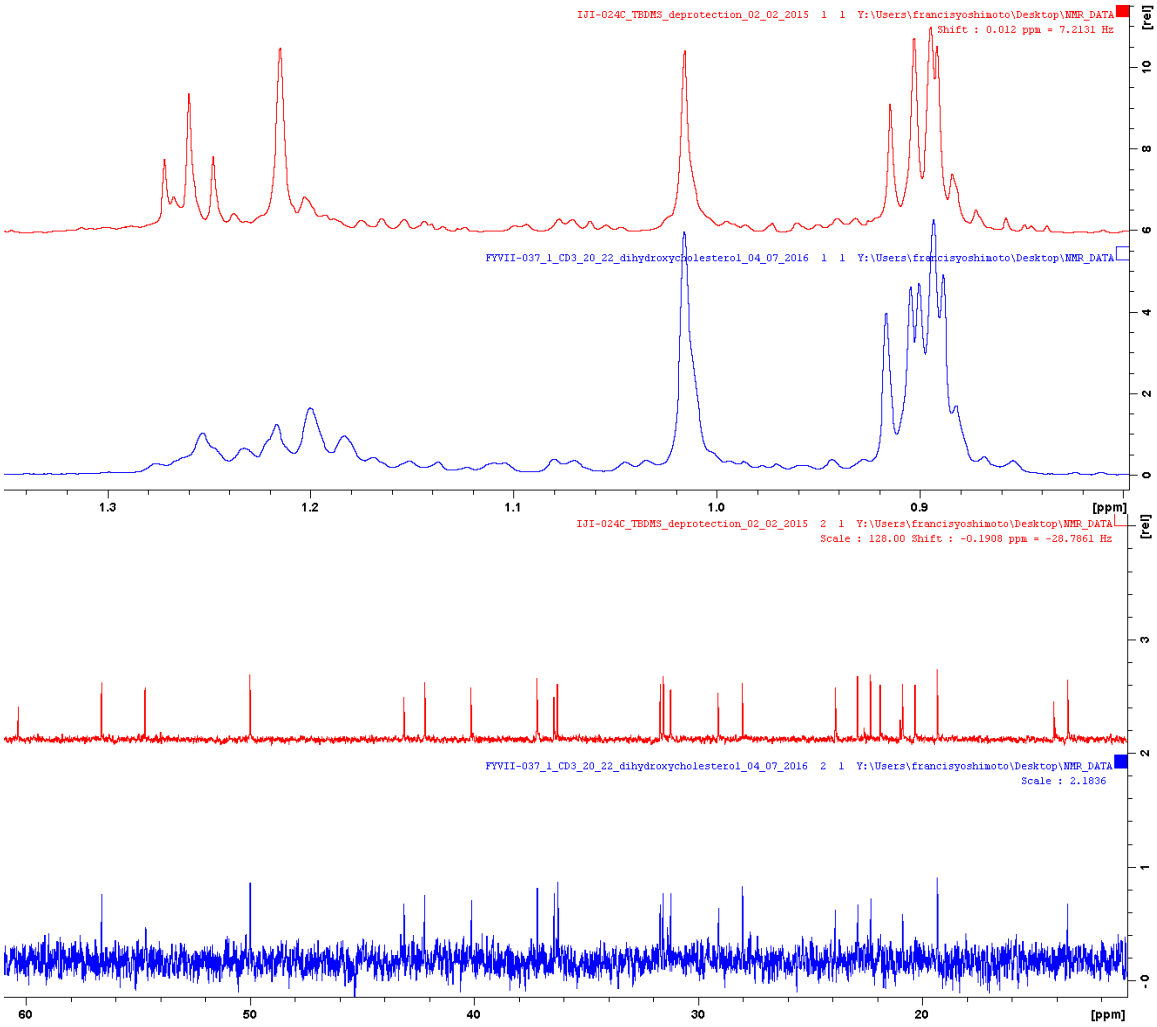
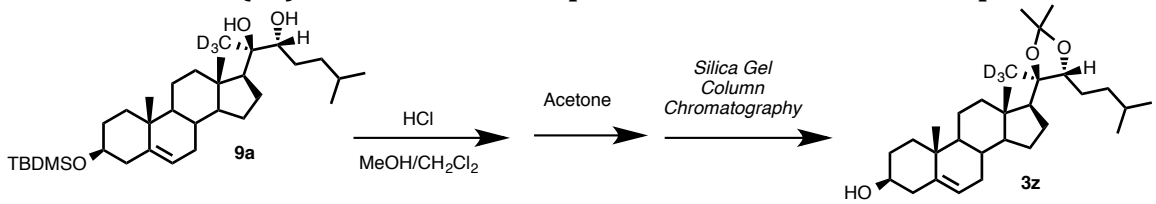
121.7010
71.9107
56.8412
50.2168
43.3502
42.4277
40.3469
37.4031
36.8522
36.4834
31.9148
31.7943
31.4462
29.3185
28.2466
24.1005
23.8565
23.6529
21.1023
19.5461
13.7273
0.1469
[rel]
[ppm]

Overlay of 20*R*,22*R*-dihydroxycholesterol (**3**) and 21,21,21-d_3-20*R*,22*R*-dihydroxycholesterol (**3a**) in the upfield region:

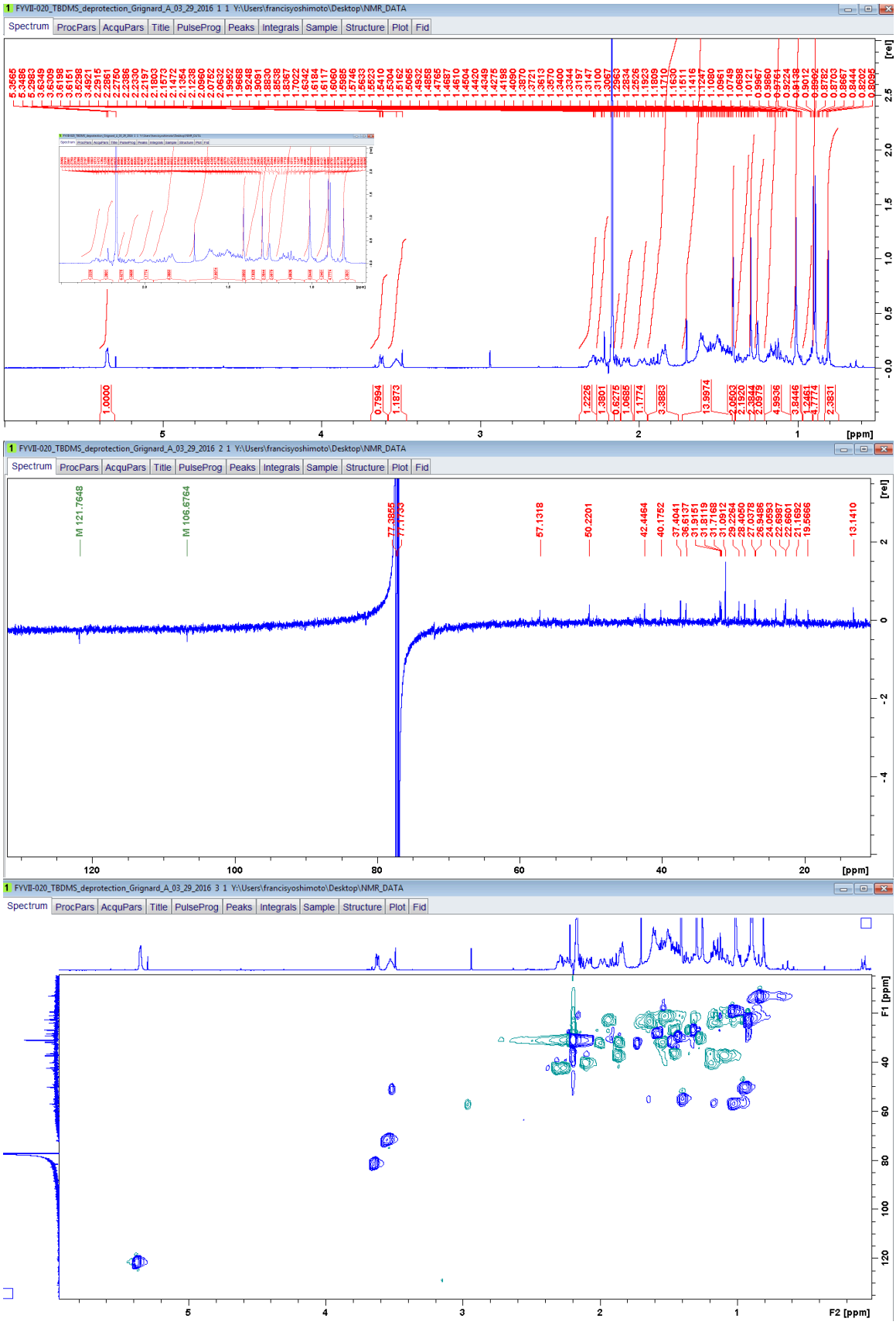


6.11. Acetonide (3z) formation with exposure to acetone from compound 9a.

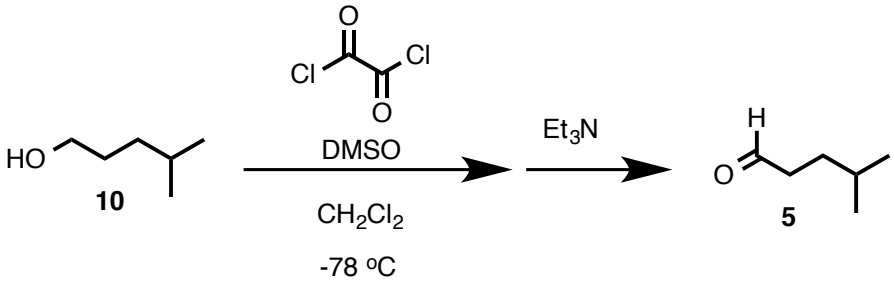


The TBDMS-deprotection procedure to yield triol **3a** was performed, however, before purification by flash column chromatography, acetone was used to dissolve the triol (**3a**) residue to load the material on the silica gel column. The only recovered material was the acetonide (**3z**). ^{1}H NMR of **3z** (600 MHz, $CDCl_3$) δ 5.37-5.33 (m, 1H), 3.63 (dd, J_1 = 9.4 Hz, J_2 = 2.6 Hz, 1H), 3.57-3.48 (m, 1H), 2.33-2.27 (m, 1H), 2.27-2.19 (m, 1H), 2.16-2.11 (m, 1H), 2.12-2.05 (m, 1H), 2.02-1.95 (m, 1H), 1.95-1.79 (m, 3H), 1.69-1.42 (m, 13H), 1.41 (s, 3H), 1.39-1.30 (m, 2H), 1.30 (s, 3H), 1.20-1.03 (m, 5H), 1.01 (s, 3H), 0.95-0.91 (m, 1H), 0.90 (s, 3H), 0.89 (s, 3H), 0.81 (s,

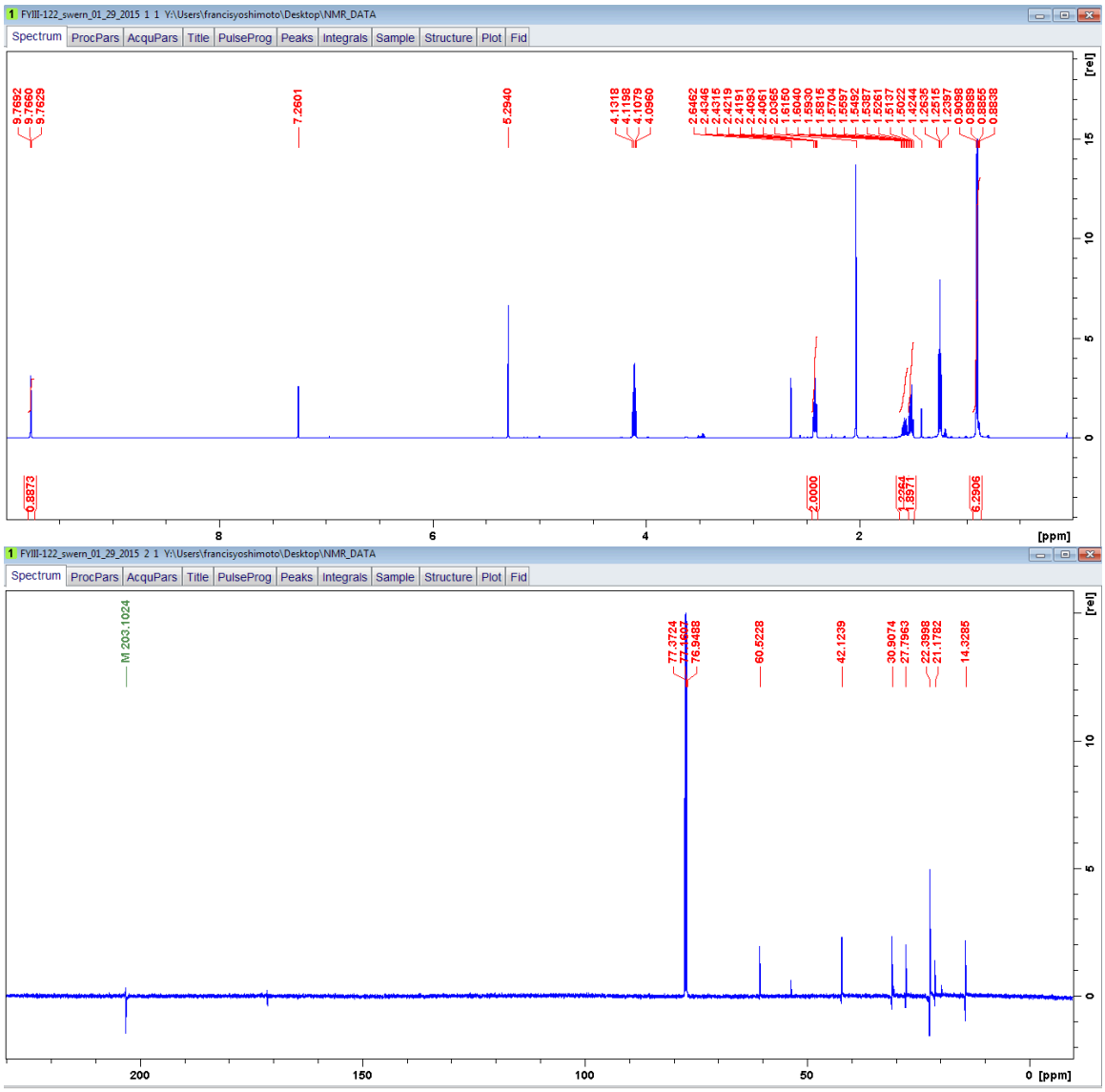
3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 121.8, 106.7, 57.1, 50.2, 42.4, 40.2, 37.4, 36.6, 31.9, 31.8, 31.7, 31.1, 29.2, 28.4, 27.0, 26.9, 24.1, 22.9, 22.70, 22.66, 21.2, 19.6, 13.1, 0.13. Attempts to convert the acetonide back to the triol (compound **3a**) with HCl (0.5 mL, 12 M) in CH_3OH (5 mL) at room temperature for 24 h resulted in major decomposition to an unwanted compound. Following are the ^{1}H, ^{13}C NMR, and HSQC spectra (**3z**, $CDCl_3$, 600 and 150 MHz).

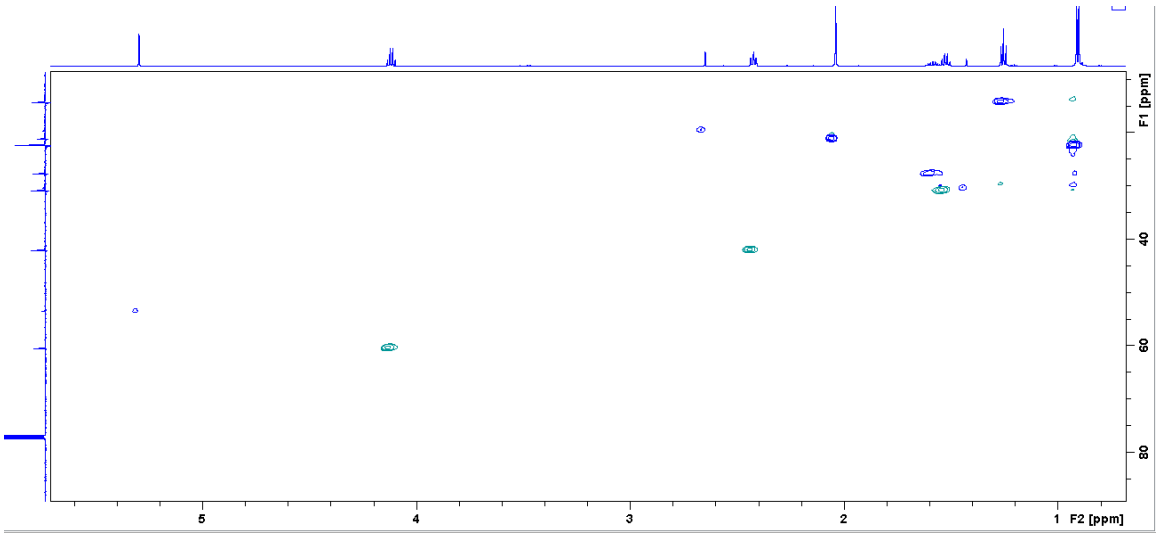

FYVII-020_TBDMS_deprotection_Grignard_A_03_29_2016 1 1 Y:\Users\francisyoshimoto\Desktop\NMR_DATA
Spectrum ProcPars AcquPars Title PulseProg Peaks Integrals Sample Structure Plot Fid
[rel]
[ppm]
FYVII-020_TBDMS_deprotection_Grignard_A_03_29_2016 2 1 Y:\Users\francisyoshimoto\Desktop\NMR_DATA
M 121.7648
M 106.6764
77.3855
77.1730
57.1318
50.2201
42.4464
40.1752
37.4041
36.6137
31.9151
31.8319
31.7168
31.0912
29.2264
28.4050
27.0078
26.9488
24.0959
22.6987
22.6601
21.1692
19.5666
13.1410
FYVII-020_TBDMS_deprotection_Grignard_A_03_29_2016 3 1 Y:\Users\francisyoshimoto\Desktop\NMR_DATA
F1 [ppm]
F2 [ppm]

6.12. Synthesis of isocaproaldehyde (5) (see main text for procedure).

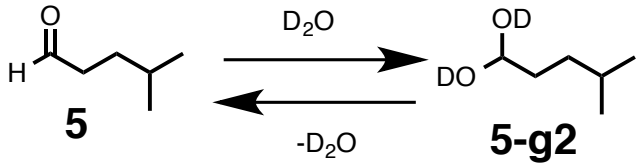


Following are the ^{1}H and ^{13}C NMR spectra (**5**, $CDCl_3$, 600 and 150 MHz).

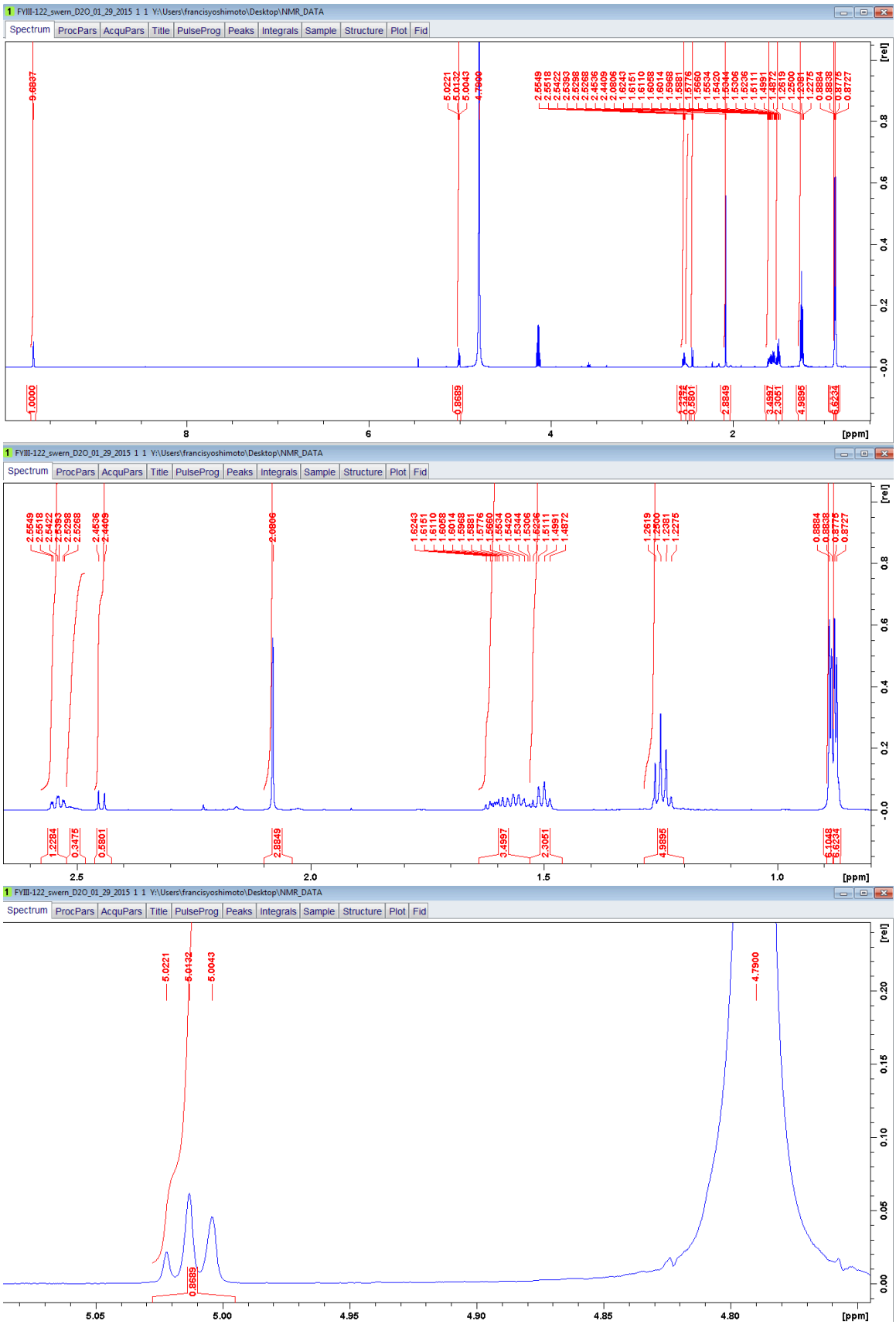




^{1}H NMR spectrum of isocaproaldehyde (5) in D_2O (potassium phosphate buffer, pD = 7.4, as described in the manuscript).



The integrations of the aldehyde and *gem*-dihydroxymethine (**5** and **5-g2**) can be calculated to determine the ratio of the aldehyde and *gem*-diol (1.0:0.9) in D_2O (600 MHz). Similarly, the methyl protons (C5, δ 0.88, 0.89) can also be used to determine the same ratio. Below is the ^{1}H NMR spectrum (**5**, D_2O, 600 MHz) along with the expanded regions (δ 0.80-2.65 ppm and δ 4.75-5.10 ppm.


FVIII-122_swern_D2O_01_29_2015 1 1 Y:\Users\francisyoshimoto\Desktop\NMR_DATA
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5.0221
5.0132
5.0043
4.7900
2.5549
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2.5268
2.4536
2.4409
2.0806
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1.6058
1.6014
1.5968
1.5881
1.5776
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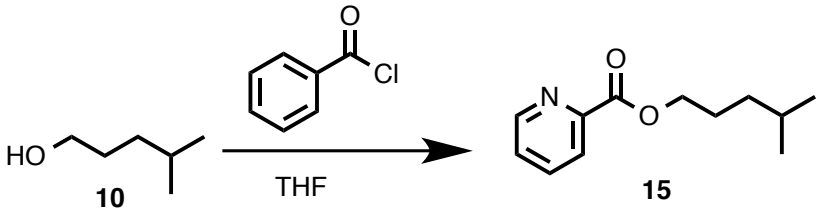
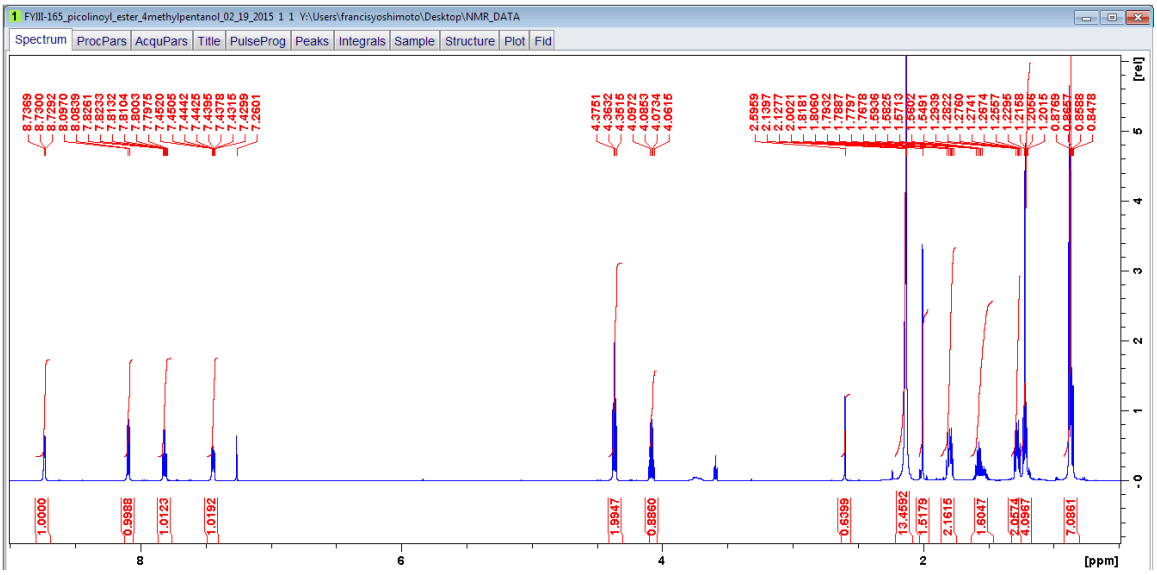
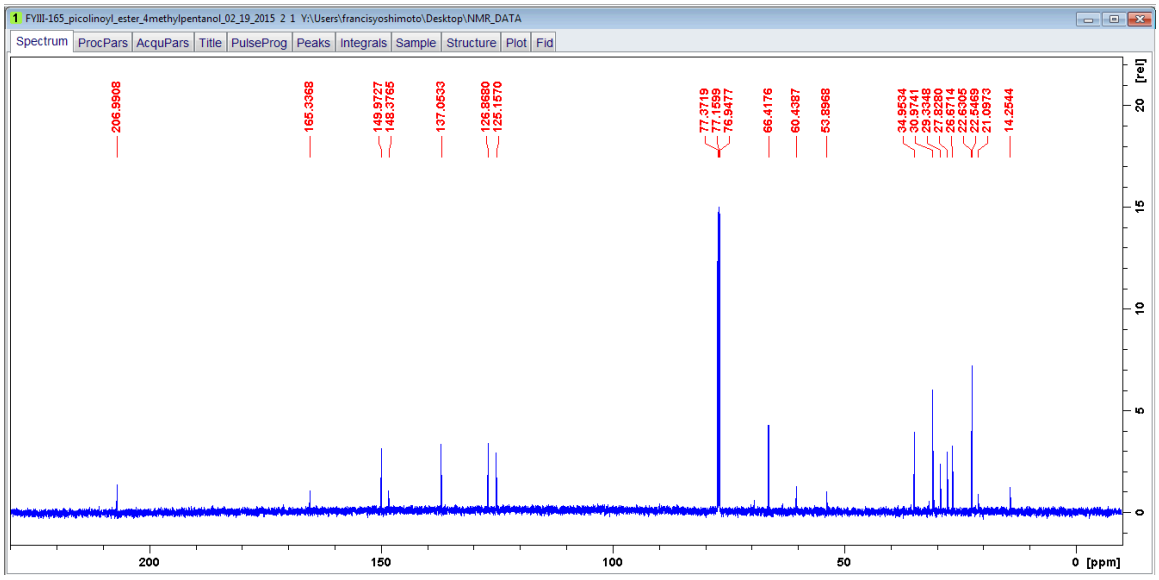
1.5236
1.5111
1.4991
1.4872
1.2619
1.2500
1.2381
1.2275
0.8884
0.8838
0.8775
0.8727
1.0000
0.8689
1.2284
0.3476
0.5801
2.8849
3.4997
2.3051
4.9895
6.1045
6.6234
[rel]
[ppm]

6.13. Synthesis of 4-methyl-pentan-1-ol picolinoyl ester (15).

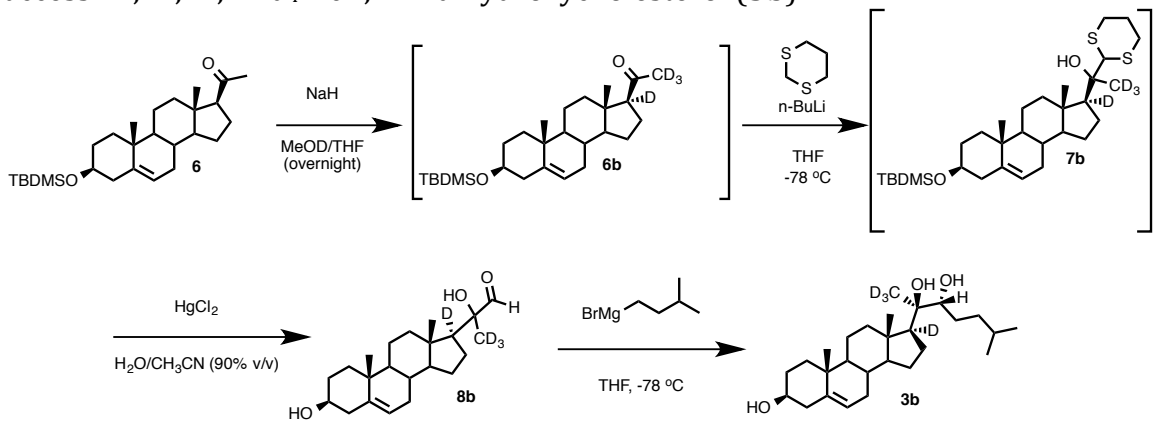


Picolinoyl chloride (200 mg, 1.4 mmol) was added to a solution of 4-methyl-pentan-1-ol (100 mg, 0.98 mmol) in THF (10 mL). The reaction was stirred at room temperature for 2 hr and concentrated by reduced pressure. The crude material was purified by preparative TLC (2,000 μM thickness of silica, 1:1 ethyl acetate:hexanes, v/v) to afford picolinoyl ester **15** as an oil (20 mg, 0.10 mmol, 10%). R_f 0.5 of compound **15** (hexanes:ethyl acetate, 1:1, v-v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.73 (d, J = 4.9 Hz, 1H), 8.09 (d, J = 7.7 Hz, 1H), 7.81 (td, J_1 = 7.7 Hz, J_2 = 1.6 Hz, 1H), 7.44 (ddd J_1 = 7.7 Hz, J_2 = 4.6 Hz, J_2 = 0.9 Hz, 1H), 4.36 (t, J = 7.1 Hz, 1H), 1.82-1.77 (m, 2H), 1.61-1.49 (m, 2H), 1.31-1.25 (m, 2H), 0.87 (d, J = 6.5 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 165.3, 150.0, 148.4, 137.1, 126.9, 125.2, 66.4, 35.0, 29.3, 27.8, 26.7, 22.5. Following are the ^{1}H and ^{13}C NMR spectra (**15**, $CDCl_3$, 600 and 150 MHz).



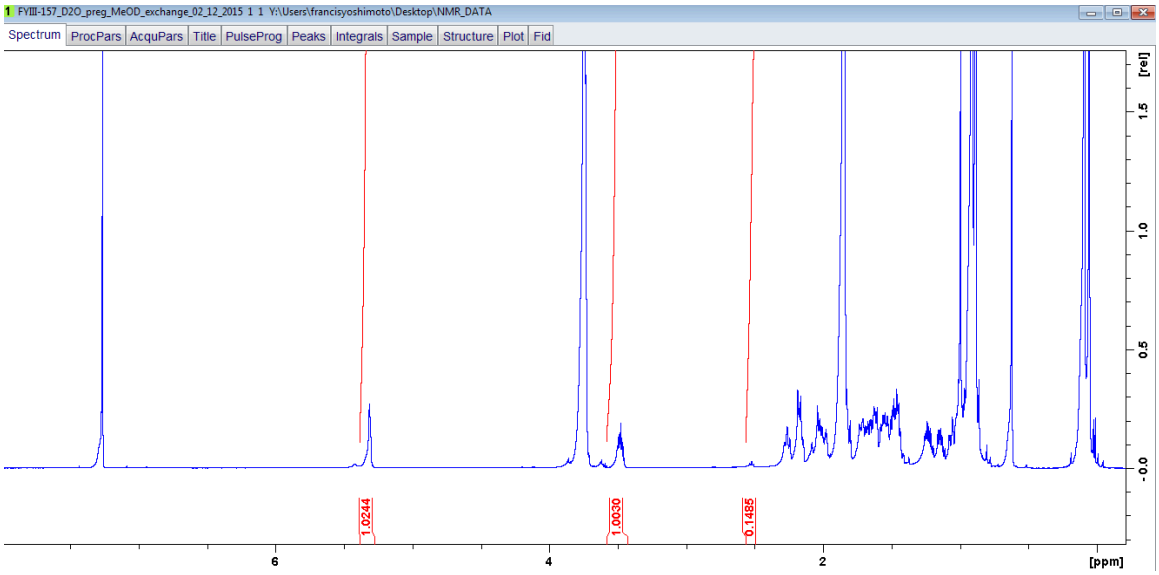


6.14. 4-Step Synthesis of 3b. The following reaction sequence was followed to access 17,21,21,21-d$_4$-20*R*,22*R*-dihydroxycholesterol (**3b**).



6.14.1. Synthesis of 17,21,21,21-*d*$_4$-pregnenolone-3-*O*-*tert*-butyldimethylsilyl ether (6b).

NaH (3.0 g, 125 mmol) was added to a solution of pregnenolone-3-O-*tert*-butyldimethylsilyl ether **6** (5.5 g, 12.8 mmol) in THF (100 mL) and D_2O (20 mL) at 0 °C. The flask was evacuated and backfilled with argon. MeOD (5 mL, 99%) was added and the reaction was stirred overnight. The reaction mixture was concentrated, and the crude material (17,21,21,21-*d*$_4$-pregnenolone-3-*O*-*tert*-butyldimethylsilyl ether, **6b**) was directly used for the next step (compound **6b** to compound **7b**). Following is the ^{1}H NMR spectrum of the crude material (**6b**, $CDCl_3$, 600 MHz).



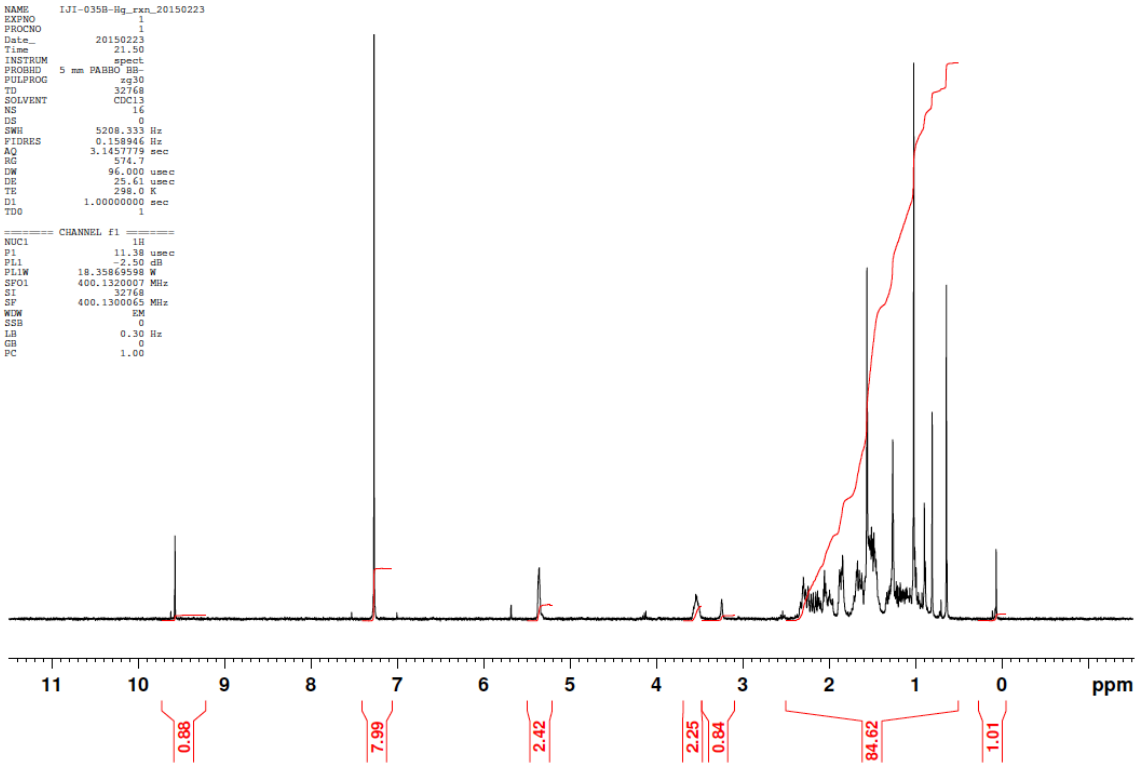
6.14.2. Synthesis of 17,21,21,21-d_4-dithiane (7b).

n-BuLi (14.7 mL, 2.5 M, 37 mmol) was added to a solution of 1,3-dithiane (4.8 g, 40 mmol) in THF (100 mL) at -78 °C. After 1 h, **6b** (crude material from the previous step) was added in THF (50 mL) and was stirred for 1.5 h during which the reaction was warmed gradually to -20 °C. The reaction was quenched with H_2O (100 mL). The reaction mixture was extracted with ethyl acetate (250 mL) and concentrated under reduced pressure. The crude material (~2.3 g) was used directly for the next reaction.

6.14.3. Synthesis of 3-hydroxy-aldehyde (8b).

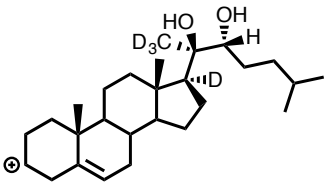
A mixture of HgO (1.3 g, 6.2 mmol, 1.5 mol eq), $HgCl_2$ (2.2 g, 8.2 mmol, 2.0 mol eq), and dithiane (2.3 g, 4.1 mmol) in acetonitrile:H_2O (150 mL:10 mL) was refluxed for 2.5 hr. The reaction mixture was filtered through a short pad of silica gel with ethyl acetate (100 mL) to remove the mercury salts. The eluent was concentrated under reduced pressure and purified by flash column chromatography (100% hexanes to 50% hexanes/ethyl acetate, v/v) to afford 3-hydroxy-aldehyde (**8b**, 1.9 g, 5.4 mmol, 42% for three steps from **6b**) as a white solid. Below is the ^{1}H NMR spectrum of

compound **8b** ($CDCl_3$, 400 MHz).

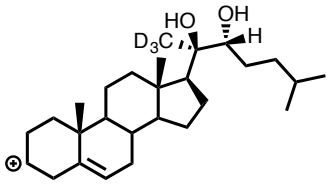


6.14.4. Synthesis of 17,21,21,21-d_4-20*R*,22*R*-dihydroxycholesterol (3b).

Isopentylmagnesium bromide (32 mL of a 2.5 M solution in diethyl ether, 82 mmol) was added to 3-hydroxyaldehyde (1.9 g, 4.09 mmol) in THF (150 mL) at -78 °C. After 2 hr, the reaction was quenched with H_2O (100 mL) at -78 °C and warmed to room temperature. The reaction mixture was extracted with ethyl acetate (2 × 150 mL). The combined organic extracts were concentrated with reduced pressure and purified by flash column chromatography (100% hexanes to 50% hexanes and ethyl acetate v/v) to afford title compound (500 mg, 1.18 mmol, 29%) as an impure mixture but contained the desired product (**3b**) from an LC-MS analysis (calculated $[M\text{-}H_2O]^+$: *m/z* 405.3665, found: *m/z* 405.3657, Δ = 1.9 ppm, APCI-positive). This mixture was directly used for the incubation with P450 11A1.



Exact Mass: 405.3665



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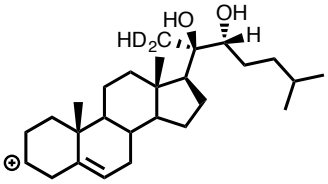

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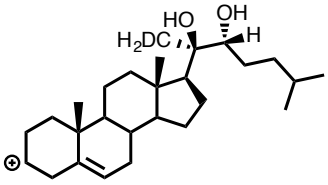

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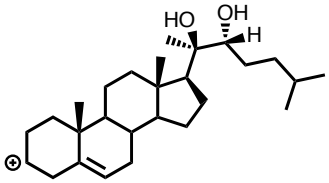

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