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Synthesis of [3α-^{3}H] 17α-Hydroxy pregnenolone and [3α-^{3}H] Pregnenolone

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For the first time, [3α-^{3}H] 17α-hydroxy pregnenolone (1) was synthesized through a multiple step sequence. The presence of [3β-^{3}H] isomer in RP-HPLC purified product was identified by tritium NMR. The [3β-^{3}H] isomer was then separated from [3α-^{3}H] 17α-hydroxy pregnenolone with chiralPAK AD-H column. [3α-^{3}H] pregnenolone (2) was synthesized from commercial available 5-pregnen-3,20-dione in one step with an improved procedure.

Keywords: tritium; 17α-hydroxy pregnenolone; pregnenolone; isomer; tritium-NMR (T-NMR); chiralPAK AD-H column

Introduction

Tritium-labeled 17α-hydroxy pregnenolone and pregnenolone were required to support drug research and discovery at BMS. Although [1,2-^{3}H] or [21-^{3}H] 17α-hydroxy pregnenolone and [7-^{3}H(N)] pregnenolone were commercially available, the cost of the commercial products, in particular 17α-hydroxy pregnenolone, was prohibitive for routine program use.

Several publications report syntheses of different versions of hydrogen isotope-labeled 17α-hydroxy pregnenolone and pregnenolone. A.D.Tait[1] reported a two-step synthesis of [3α-^{3}H] pregnenolone starting from unlabeled pregnenolone. In this instance, a product with a specific radioactivity of 80 mCi/mmol was isolated and purified by thin layer chromatography to 93% radiochemical purity (RCP). Marcel Gut[2] reported the synthesis of [7α-^{3}H] 17α-hydroxy pregnenolone by debromination of a bromo analog of the target compound. Martin I. Shapiro[3] revealed evidence of a major impurity existing in commercially available [7-^{3}H] 17α-hydroxypregnenolone. In addition, Weinman[4] reported the synthesis of [16-^{3}H] 17α-hydroxypregnenolone. Deuterium-labeled 17α-hydroxypregnenolone, such as [21-^{2}H3] and [2,2,4,4,21,21,21-^{2}H7] 17α-hydroxypregnenolone, was prepared, respectively, by Muhammad Akhtar[5] and C. Solleder.[6,7] No publication was found reporting the synthesis of [3α-^{3}H] 17α-hydroxy pregnenolone. However, George E. Joannou[8] reported a multiple step procedure to prepare unlabeled 17α-hydroxy pregnenolone.

In this paper, we report the synthesis of [3α-^{3}H] 17α-hydroxy pregnenolone (17.7 Ci/mmol) and [3α-^{3}H] pregnenolone (10.9 Ci/mmol) in high RCP and yield. The stereo chemical purity of the compounds was further characterized by analyses of RP/NP/ChiralPAK AD-H HPLC and T-NMR.

[3α-^{3}H] 17α-hydroxy pregnenolone (**1**) (Figure 1) and [3α-^{3}H] pregnenolone (**2**) (Figure 2).

Results and discussion

We initially attempted to synthesize [3α-^{3}H] 17α-hydroxy pregnenolone (**1**) by utilizing 3,5-pregnadien-3,17-diol-20-one-3-methyl ether (**3**), a commercially available precursor via reduction of the 3-keto group (Scheme 1). Instead of the desired [3-^{3}H] product (**1**), a mixture of [20α-^{3}H] 4-pregnen-17α,20beta-diol-3-one (**6**) and [20α-^{3}H] 5-pregnen-17α,20 beta-diol-3-one (**7**) were isolated (Scheme 1). After RP-HPLC purification, the (**6**)/(**7**) mixture showed a single peak with 99.7% RCP by RP-HPLC. However, when the product was checked by T-NMR, it showed two peaks (ratio 60:40, Figures 3 and 4). Later, ChiralPAK AD-H HPLC analysis also revealed two peaks (ratio 26:74, Figure 5). After storage in 90% ethanol for 23 days, the product was reanalyzed by ChiralPAK AD-H HPLC and showed a single peak (98.5% RCP), which was identified as (**6**) by comparing it to 4-pregnene-17alpha,20 beta-diol-3-one authentic standard.

During the RP-HPLC purification, an early impurity was collected and identified as [20β-^{3}H] isomer by RP-HPLC and T-NMR. The ratio of [20α-^{3}H] isomer to [20β-^{3}H] isomer in crude product was 5.8:1.

Although the synthesis was unsuccessful, the results we obtained provided interesting information on the conversion from (**7**) to (**6**). The analytical methods we developed were useful later for separation and characterization of the desired products.

Because we failed to obtain the desired product through the initial route, we prepared an intermediate according to the method reported by Geoge E. Joannou.[8] Using the same strategy, we successfully prepared (Scheme 2) [3α-^{3}H] 17α-hydroxy pregnenolone (**1**). The product was initially purified by RP-HPLC and shown as a single peak in chromatogram; however, the T-NMR analysis revealed the existence of [3β-^{3}H] isomer of

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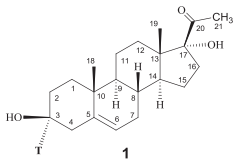


Figure 1. [3α-^{3}H] 17α-hydroxy pregnenolone.

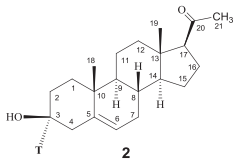


Figure 2. [3α-^{3}H] pregnenolone.

17α-hydroxy pregnenolone (**9**). The [3α-^{3}H] and [3β-^{3}H] isomers of 17α-hydroxy pregnenolone were separated by a chiral HPLC method using ChiralPAK AD column. Both pure isomers were characterized by chiral HPLC and T-NMR analyses (Figures 6–9).

[3α-^{3}H] pregnenolone (**2**) was synthesized by referring to the method reported by A.D.Tait.[1] We utilized a commercially available 5-pregnen-3,20-dione (**10**) as the starting material (Scheme 3). The reaction resulted in a much more complex RP-HPLC chromatogram (Figure 10) compared with the synthesis of [3α-^{3}H] 17α-hydroxy pregnenolone (**1**). This may be because of an impure precursor (**10**), which shows multiple peaks when checked by RP-HPLC (Figure 11). The final [3α-^{3}H] pregnenolone (**2**) product was demonstrated to be ≥99.9% pure when checked by RP/NP/ChiralPAK AD HPLC and T-NMR (Figures 12 and 13). No effort was made to identify and isolate the [3β-^{3}H] pregnenolone isomer during the purification.

Conclusion

We report improved and robust procedures to synthesize [3α-^{3}H] 17α-hydroxy pregnenolone (**1**) and [3α-^{3}H] pregnenolone (**2**). All products were fully characterized by detailed analytical data. With the help of T-NMR and Chiral PAK AD HPLC, the existence of (**7**) and its transformation to (**6**) that had not been reported by previous publications were revealed. The [3β-^{3}H] isomer of 17α-hydroxy pregnenolone, which was not mentioned in previous publications was discovered, then isolated giving a pure product (**1**).

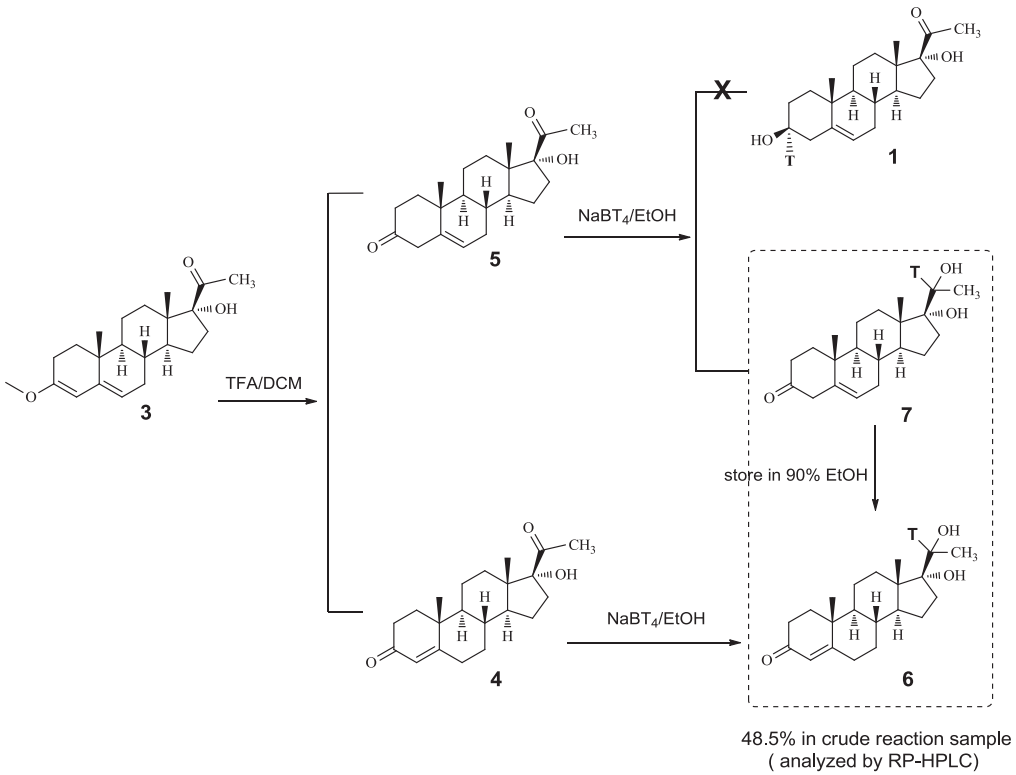
In our experience, the application of ChiralPAK AD-H column was shown to be useful for separating the steroid isomers that are otherwise difficult to separate by either RP or NP HPLC. The separation is likely because of the structural feature of the steroid. As shown in our results, chiral columns may be useful tools in isolation and identification of other steroid isomers.

[3α-^{3}H] 17α-hydroxy pregnenolone and [3α-^{3}H] pregnenolone were used as substrate to develop assays to monitor the activity of the CYP P450 enzyme, CYP17 hydroxylase, and CYP17 lyase. Our in-house generated products show better signal/background ratio when compared with the commercial materials.

Experimental

Materials

(**1**) Cold standard 17α-hydroxy pregnenolone ([387-79-1], Q4710-000), (**2**) cold standard pregnenolone ([145-13-1], Q5500-000), 3,5-pregnadien-



Scheme 1. Attempted synthetic route of [3α-^{3}H] 17α-hydroxy pregnenolone (**1**).

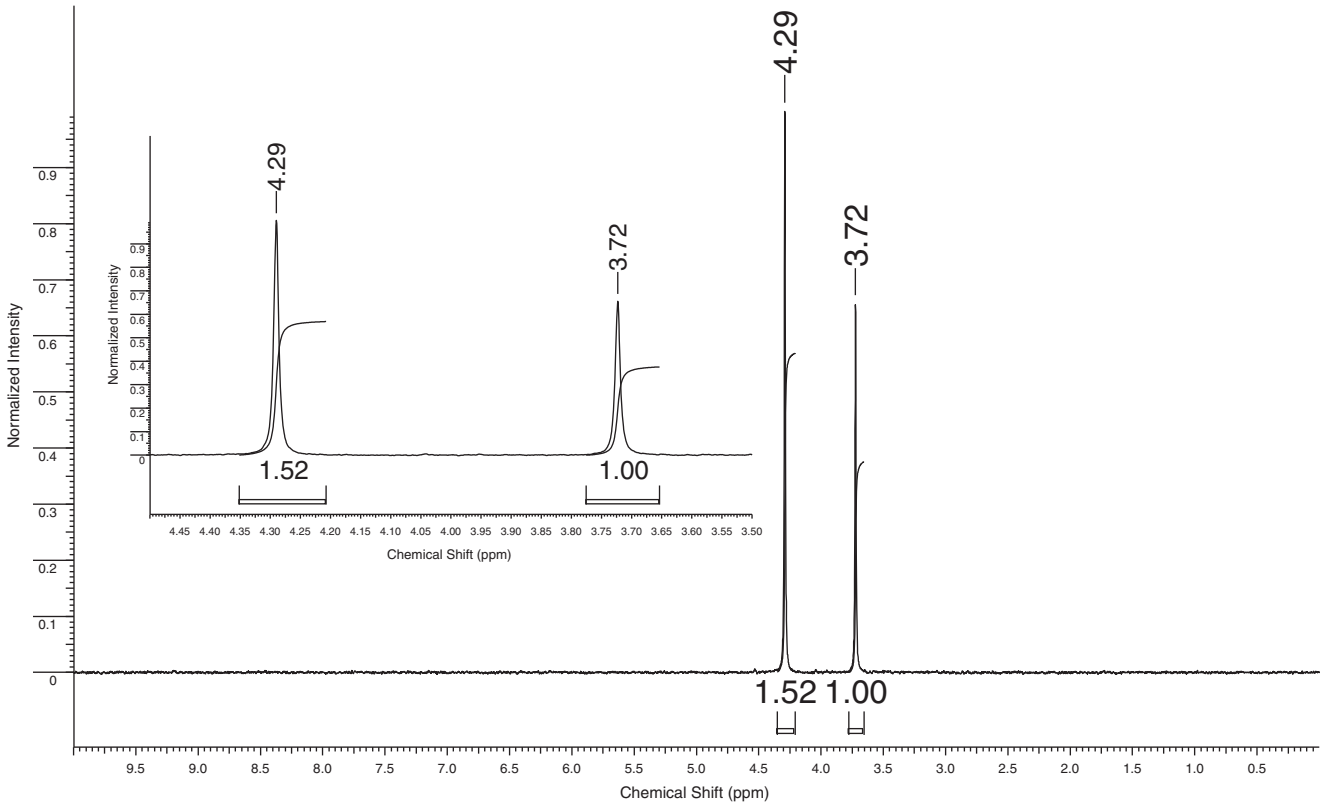


Figure 3. Tritium NMR (T–H decoupled) of [^{3}H] product from Scheme 1 synthesis.

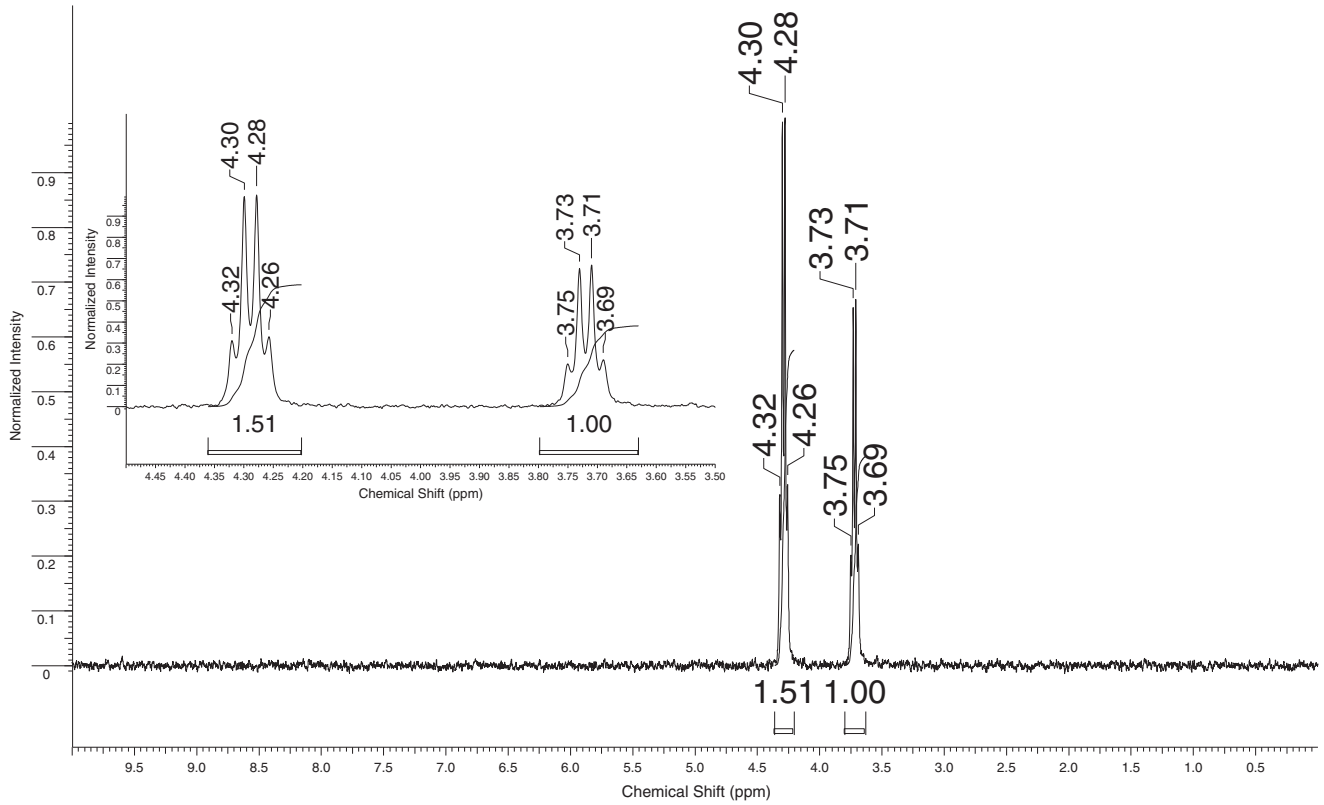


Figure 4. Tritium NMR (T–H coupled) of [^{3}H] product from Scheme 1 synthesis.

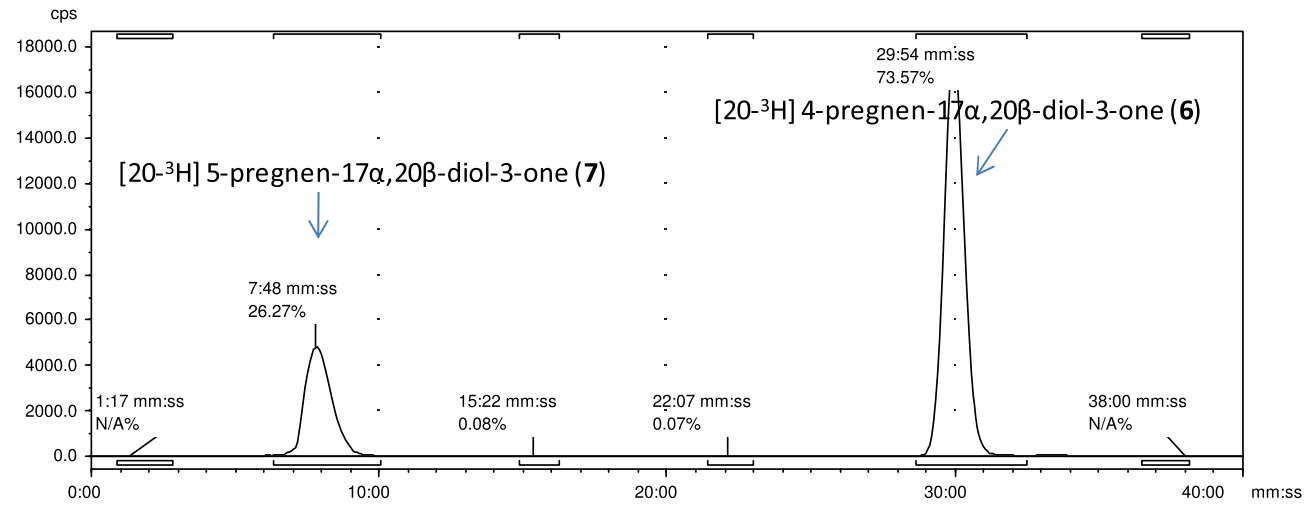
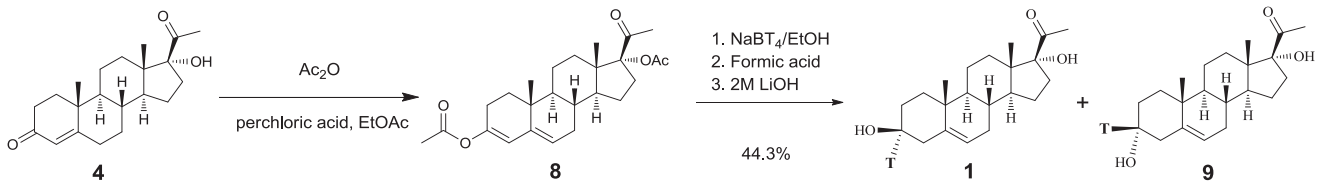


Figure 5. ChiralPAK AD-H HPLC analysis of [^{3}H] product from Scheme 1 synthesis: (4.6 × 250 mm) column; i-PrOH/Hexanes = 10/90; 1.0 mL/min; 210 nm sample: Hot +17α-hydroxy pregnenolone (**1**) cold standard (RT = 15.6 min) co-injection.



Scheme 2. Redesigned synthetic route of [3α-^{3}H] 17a-hydroxy pregnenolone (**1**).

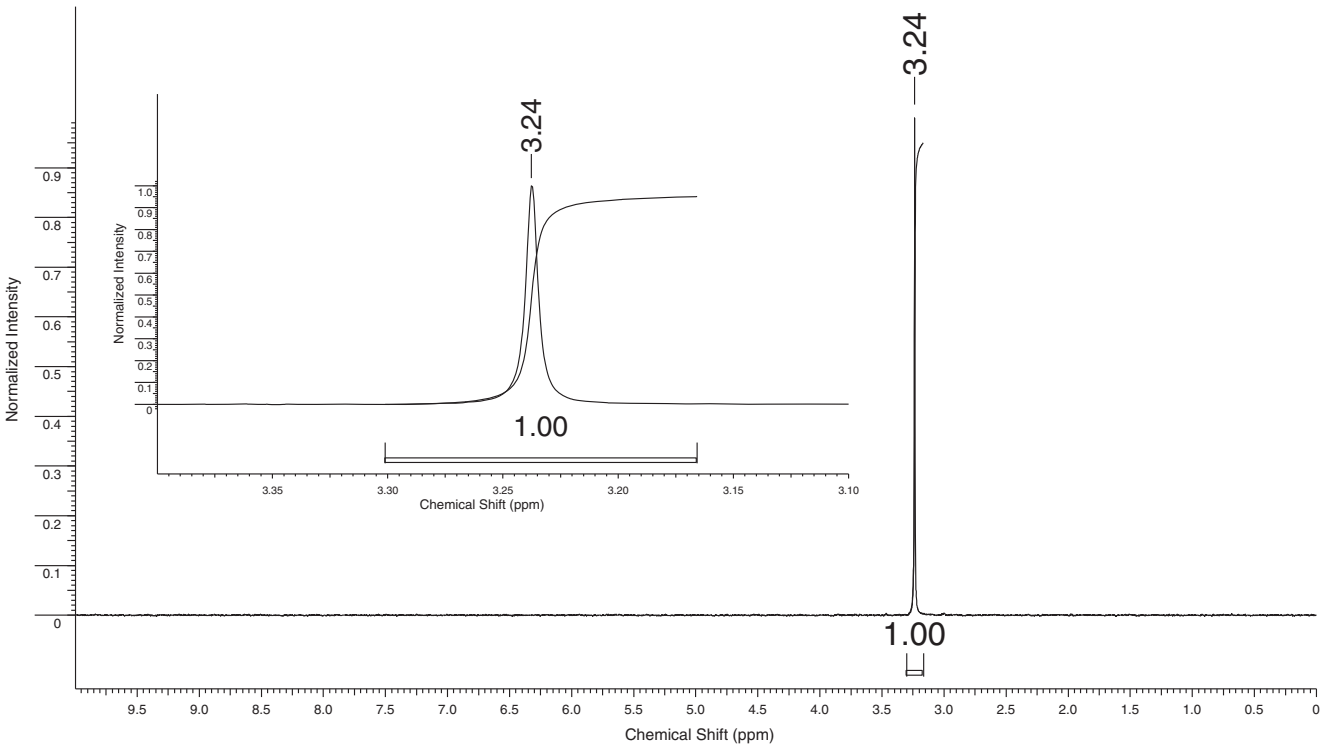


Figure 6. Tritium NMR (T–H decoupled) of [3α-^{3}H] 17α-hydroxy pregnenolone.

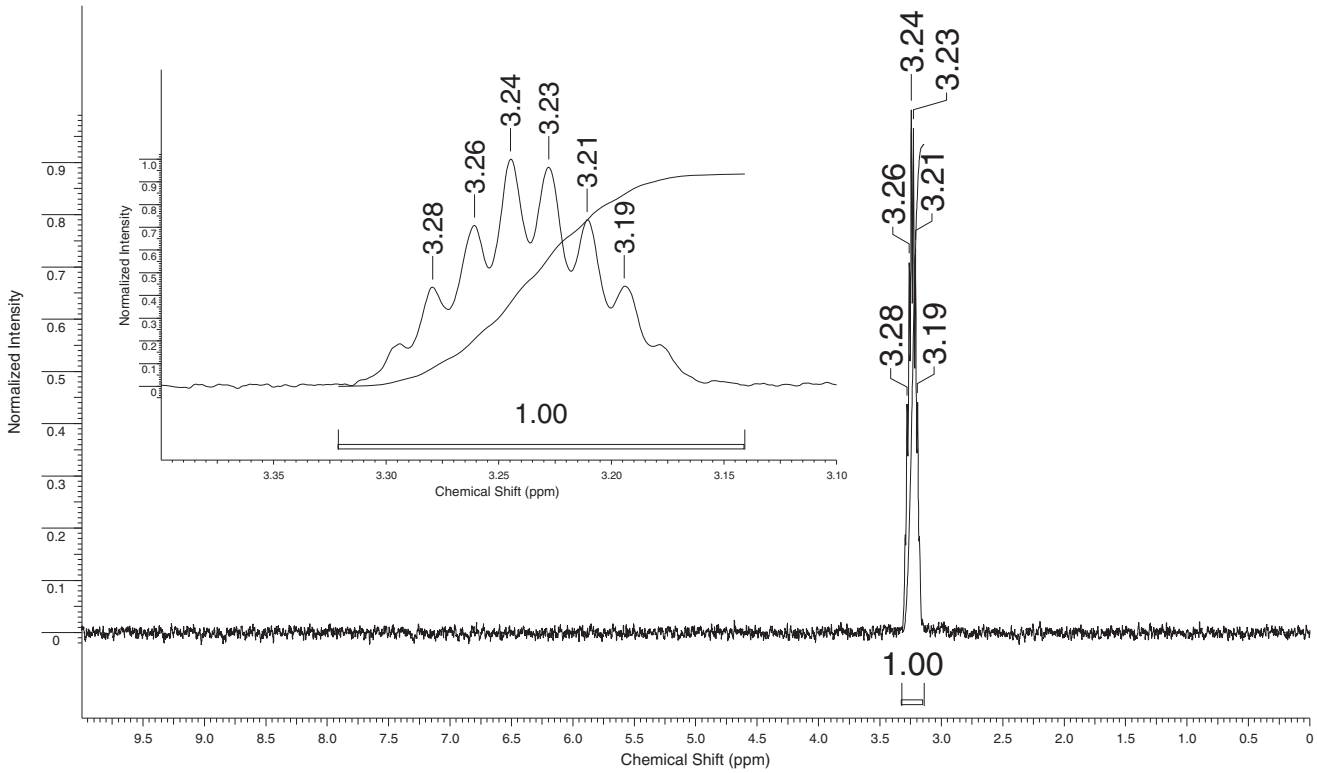


Figure 7. Tritium NMR (T–H coupled) of [3α-^{3}H] 17α-hydroxy pregnenolone.

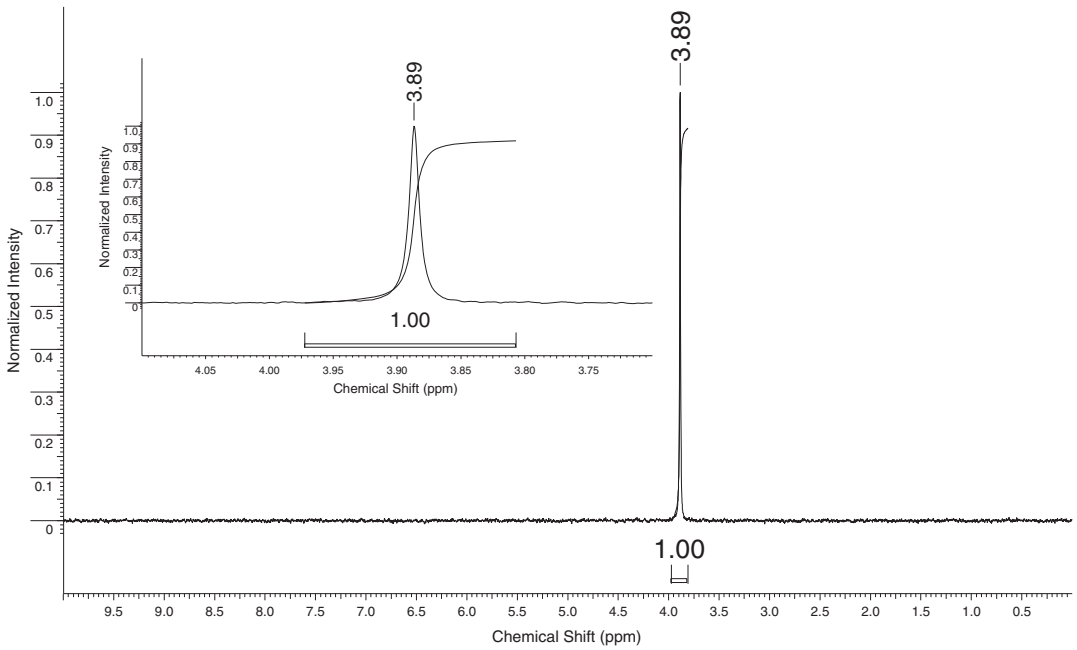


Figure 8. Tritium NMR (T–H decoupled) of [3β-^{3}H] 17α-hydroxy pregnenolone isomer.

3,17-diol-20-one-3-methyl ether (**3**)(P0815-000), 5-pregnen-3,20-dione (**10**)([1236-09-5], Q4850-000), and 4-pregnene-17alpha,20alpha-diol-3-one ([652-69-7], Q1820-000) were purchased from Steraloids Inc., (Newport, RI, USA); (**6**) cold standard 4-pregnene-17alpha,20beta-diol-3-one ([1662-06-2], P6285) and 17α-hydroxyprogesterone ([68-96-2], H5752) (**4**) were purchased from Sigma-Aldrich (St. Louis, MO, USA). $NaBT_4$ (ART-0121, 60–80 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc., (St. Louis, MO, USA)

High-performance liquid chromatography was performed using an Agilent (Santa clara, CA, USA). ChemStation HPLC system equipped with a diode array detector (DAD) and a LabLogic (Brandon, FL, USA). β-RAM radio detector. Luna 5u C18 (2) (4.6 × 150 mm) column for RP-HPLC and Luna Silica (2) 5u (4.6 × 150 mm) column for NP-HPLC were purchased from Phenomenex (Torrance, CA, USA). ChiralPAK AD-H 5u (4.6 × 250 mm) column was purchased from Chiral technologies Inc., (West Chester, PA, USA) General HPLC method is run isocratically until

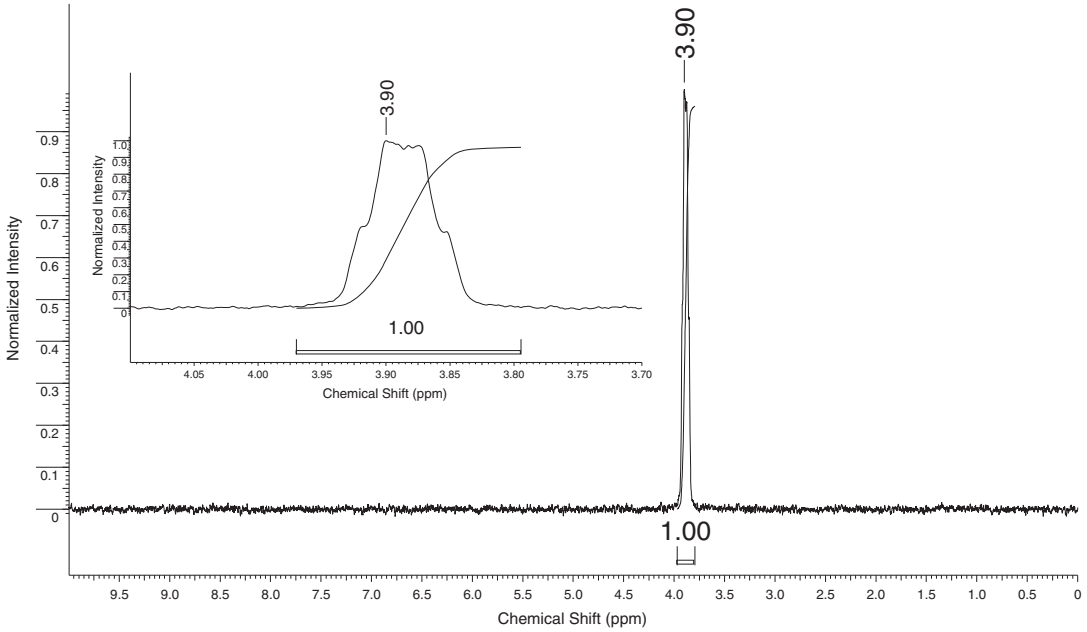
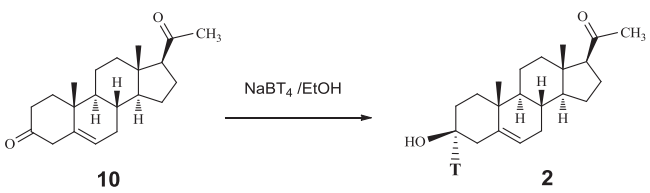


Figure 9. Tritium NMR (T–H coupled) of [3β-^{3}H] 17α-hydroxy pregnenolone isomer.



Scheme 3. Synthesis of [3α-^{3}H] pregnenolone (**2**).

the elution of the target peak and then ramped up using a gradient to elute all components. Peaks were detected with DAD @210 nm and radio detector. Hot and cold standards were mixed together before injection. Tritium and proton NMR spectra were recorded on a Bruker (Billerica, MA, USA). DMX-300 MHz Spectrometer at 320 MHz with DMSO-d6 as solvent. LC-MS was determined on a Thermo (Waltham, MA, USA). LXQ Mass Spectrometer (acetonitrile/water with 0.1% formic acid as mobile phase).

17α-hydroxyprogesterone (4) / pregn-5-ene-17α-ol-3,20-dione (**5**)

To an 11 mg 3,5-pregnadien-3,17-diol-20-one-3-methyl ether (**3**) (31.9 μmol) in 2 mL DCM was added 0.2 mL TFA. The solution was stirred for 1 h at room temperature. A total of 6 mL DCM was added, and the solution was washed with 4 mL each of (i) saturated $NaHCO_3$; (ii) NaCl; and (iii) de-ionized water. The solution was dried with Na_2SO_4 followed by filtering and evaporation to give a quantitative yield (**4**)/(**5**). The product mixture was immediately used for the next step without further analysis and purification.

[20-^{3}H] 4-pregnen-17α,20β-diol-3-one (**6**)

To a 2 mg (**4**)/(**5**) (6 μmol) was added 250 mCi $NaBT_4$ (60–80 Ci/mmol, ~3.1–4.1 μmol) in 2 mL ethanol. After being stirred at room temperature for 3 h, the reaction was terminated by adding 2 mL 10% TFA in ethanol and stirring for 5 min. Labile tritium was removed by three evaporations with ethanol (2 mL each). The crude product was assayed by scintillation counting to contain ~93 mCi radioactivity and was analyzed with

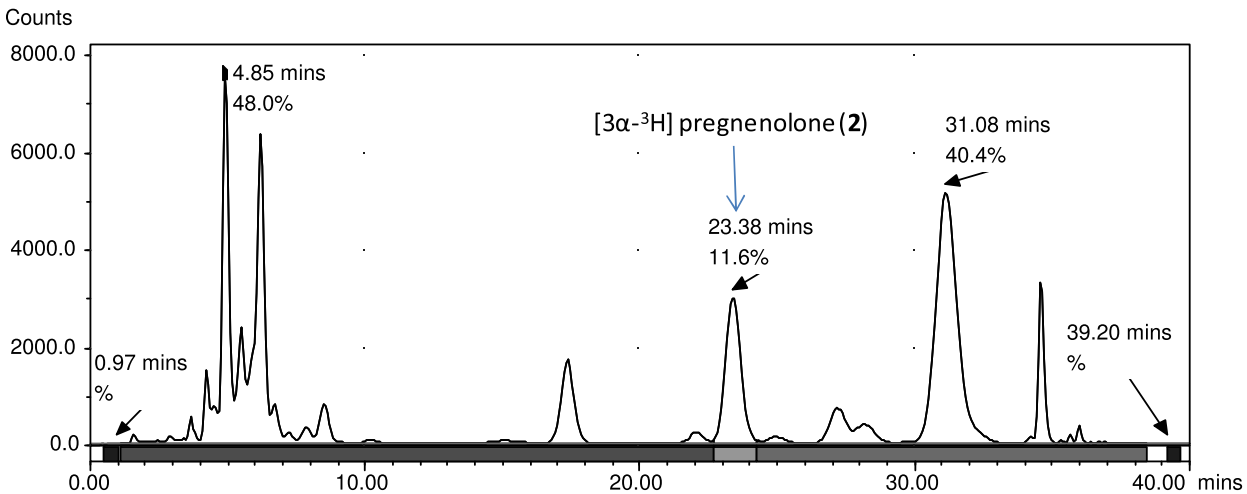


Figure 10. RP-HPLC analysis of Scheme 3 crude product (A: H2O; B: MeOH; 1.2 mL/min; 210nm; 0–30 min 65%B; 30–35 min 65–100%B; 35–40 min 100%B).

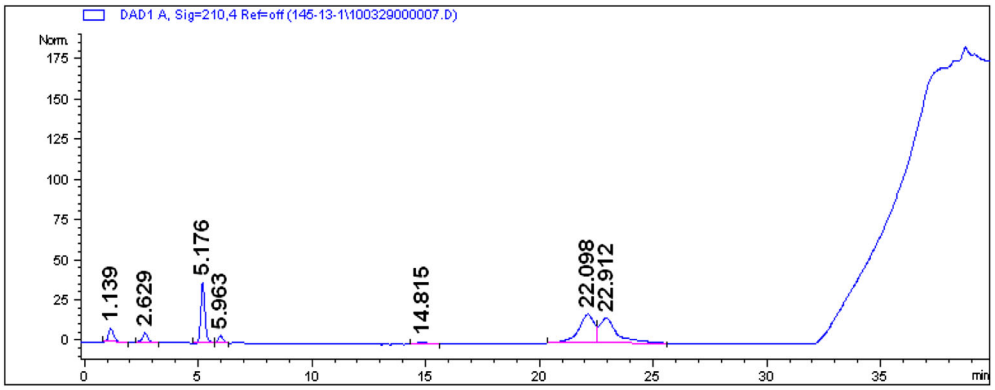


Figure 11. RP-HPLC analysis of 5-pregnen-3,20-dione (**10**) (A: H_2O; B: MeOH; 1.2mL/min; 210nm; 0-30 min 65%B; 30-35min 65-100%B; 35-40 min 100%B).

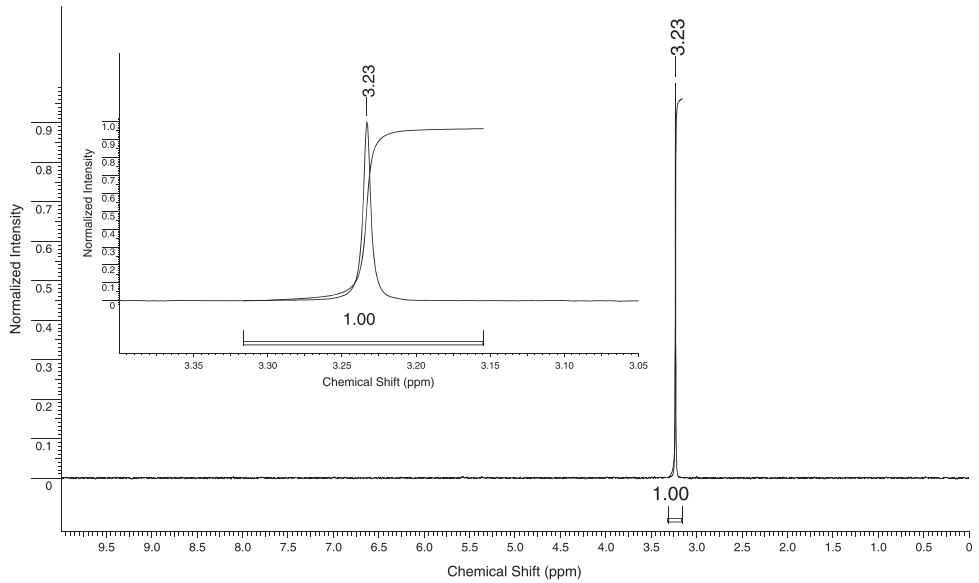


Figure 12. Tritium NMR (T–H decoupled) of [3α-^{3}H] pregnenolone.

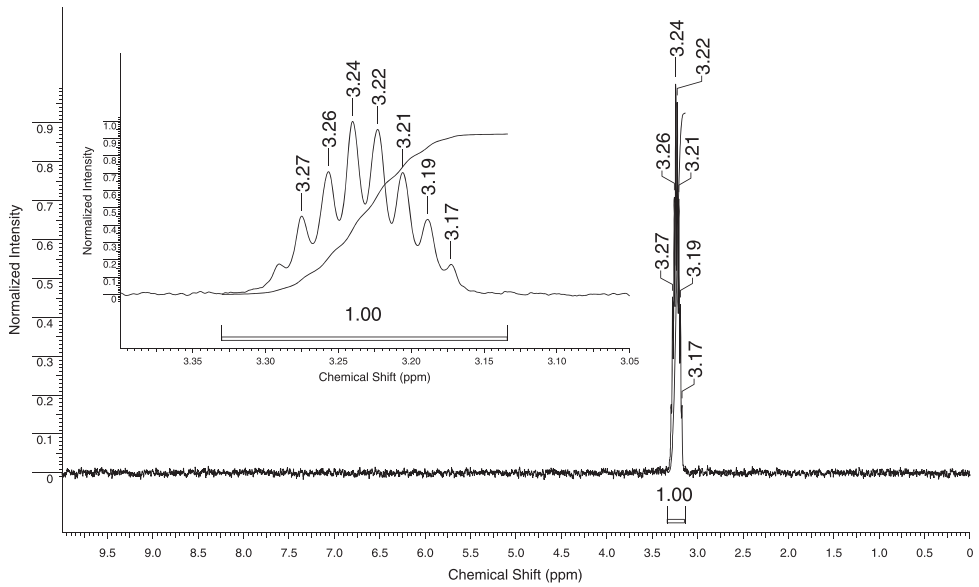


Figure 13. Tritium NMR (T–H coupled) of [3α-^{3}H] pregnenolone.

Table 1. Summary of analytical experiments on product (**6**) from Scheme 1

Storage days	RP-HPLC (MeOH/H2O = 60/40, 1.2 mL/min)	LC-MS ESI$^+$ (100% relative abundance)	T-NMR or H-NMR (δ ppm)	ChiralPAK AD-H (i-PrOH/Hexanes = 10/90, 1.0 mL/min)	NP-HPLC	ChiralCEL OD-H (i-PrOH/Hexanes = 10/90, 1.0 mL/min)
0	Hot (**6**) and (**7**)—13.5 min (99.7%) Cold (**6**)—13.5 min Cold (**1**)—13.2 min Cold [652-69-7]—11.2 min	Hot (**6**) and(**7**)—335.42 *m/z* Cold (**6**)—333.42 *m/z* Cold (**1**)—315.25 *m/z*				
1			Hot (**6**)—3.72 (40%) Hot (**7**)—4.29 (60%) Cold (**6**)—3.75 (H-20) Cold [652-69-7]—3.61 (H-20)			
16				Hot (**6**)—29.9 min (73.6%) Hot (**7**)—7.8 min (26.3%) Cold (**1**)—15.6 min		
19					i-PrOH/Hexanes = 5/95, 1.2 mL/min Hot (**6**) and (**7**)—34.0 min (100%) Cold (**1**)—11.2 min	
22					i-PrOH/Hexanes = 10/90, 1.0 mL/min Hot (**6**) and (**7**)—11.7min (100%) Cold (**6**)—11.4 min	
23				Hot (**6**)—28.3 min (98.5%) Hot (**7**)—10.3 min (1.5%) Cold (**6**)—28.1 min		Hot (**6**)—19.8 min (98.5%) Hot (**7**)—8.1 min (1.3%) Cold (**6**)—19.7 min

Table 2. Summary of analytical experiments on early impurity from Scheme 1

Sample	RP-HPLC (MeOH/H2O = 60/40, 1.2 mL/min)	LC-MS ESI$^+$ (100% relative abundance)	T-NMR or H-NMR (δ ppm)	ChiralPAK AD-H (i-PrOH/Hexanes = 10/90, 1.0 mL/min)	ChiralCEL OD-H (i-PrOH/Hexanes = 10/90, 1.0 mL/min)
Hot impurity	11.3 min (99.0%)	335.42 *m/z*	3.57 (30%) * 4.24 (70%)		
Cold [652-69-7]	11.2 min	333.42 *m/z*	3.61 (H-20)	58.8 min	33.9 min
Cold [1662-06-2]	13.4 min	333.42 *m/z*	3.75 (H-20)	31.3 min	23.4 min

*T–H coupled T-NMR shows the exact same quartets as the major product (**6**).

Table 3. Summary of [3α-^{3}H] 17α-hydroxy pregnenolone (**1**) synthesis from Scheme 2

Compound	Activity (mCi)	S.A. (Ci/mmol)	RP HPLC (MeOH/H2O = 55/45, 1.2 mL/min)	NP HPLC (i-PrOH/Hexanes = 5/95, 1.2 mL/min)	ChiralPAK AD-H (i-PrOH/Hexanes = 10/90, 1.0 mL/min)	TNMR or H-NMR (δ ppm)	LC-MS ESI$^+$ (100% relative abundance)
(**1**)	31	17.7	Hot (**1**)—26.1 min (99.9%) Cold (**1**)—26.1 min	Hot (**1**)—8.7 min (99.9%) Cold (**1**)—8.5 min	Hot (**1**)—14.5 min (100%) Cold (**1**)—14.4 min	Hot (**1**)—-3.24 (100%) Cold (**1**)—3.27 (H-3)	Hot (**1**)—317.2 *m/z*
(**9**)	19	15.8	Hot (**9**)—25.0 min (99.7%) Cold (**1**)—25.2 min	Hot (**9**)—8.9 min (99.7%) Cold (**1**)—8.5 min	Hot (**9**)—19.3 min (99.7%) Cold (**1**)—14.5 min	Hot (**9**)—3.89 (100%)	Hot (**9**)—299.3 *m/z* *
(**1**) and (**9**)	50		Not separated	Not separated	Well Separated	Separated peaks	Same spectrium with Cold (**1**)—315.2 *m/z* **

S.A., specific activity.
*Hot (**9**) MS: 299.3 *m/z* (100% relative abundance) and 317.2 *m/z* (70% relative abundance).
Cold (1**) MS: 297.3 *m/z* (40% relative abundance) and 315.2 *m/z* (100% relative abundance).

Table 4. Summary of [3α-^{3}H] pregnenolone (**2**) synthesis from Scheme 3

Compound	Activity (mCi)	S.A. (Ci/mmol)	RP HPLC (MeOH/H_2O = 65/35, 1.2 mL/min)	NP HPLC (i-PrOH/Hexanes = 5/95, 1.0 mL/min)	ChiralPAK AD-H (i-PrOH/Hexanes = 10/90, 1.0 mL/min)	T-NMR or H-NMR (δ ppm)	LC-MS ESI^+ (100% relative abundance)
Hot (**2**)	18	10.9	24.1 min (99.9%)	8.7 min (99.9%)	8.4 min (99.9%)	3.23 (100%)	299.3 *m/z* ($[M+H]^+ - H_2O$)
Cold (**2**)			24.1 min	8.5 min	8.3 min	3.26 (H-3)	299.3 *m/z* ($[M+H]^+ - H_2O$)

S.A., specific activity.

RP-HPLC (MeOH/H_2O = 60/40) to contain a major peak (48.5%) and an early impurity (8.3%). The major peak lined up with 17α-hydroxy pregnenolone (**1**) cold standard, and the early impurity lined up with 4-pregnene-17alpha,20alpha-diol-3-one cold standard. The crude product was purified by RP-HPLC using the following conditions: Phenomenex Luna 5u C18 (2) (10 × 250 mm); MeOH/H_2O = 60/40; 3 mL/min; UV@210 nm. The two target radioactive fractions were collected and evaporated to dryness. The major product was redissolved in 90% ethanol (1 mCi/mL) and stored in freezer at −70 °C. This product was assayed to contain 45 mCi radioactivity with specific activity of 16.4 Ci/mmol when checked by MS. All analytical experiments on this major product are described and summarized in Table 1. Meanwhile, after being analyzed by RP-HPLC and LC-MS, all the early impurity was dissolved in DMSO-d6 for T-NMR analysis, and analysis results are showed in Table 2.

*Pregna-3,5-dien-20-one, 3,17-dihydroxy-, diacetate (**8**)*

To a suspension of 17α-hydroxyprogesterone (**4**)(1.00 g, 3.03 mmol) in ethyl acetate(80 mL) was added perchloric acid (0.04 mL, 0.46 mmol) in ethyl acetate (10 mL) and acetic anhydride (10 mL, 106 mmol). This resulted in a homogenous, amber-colored solution. After being stirred for 30 min at room temperature, the reaction was judged to be complete by HPLC. The reaction was concentrated in vacuum to a white solid, which was recrystallized from methanol to give diacetate product (**8**) (0.49 g, 1.17 mmol, 39% yield) as fine colorless crystals. LC-MS (ESI^+): $[M+H]^+$ = 415.2 *m/z*. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.71 (1H), 5.41 (1H), 2.14 (3H), 2.12 (3H), 2.05 (3H), 1.00 (3H), 0.67 (3H)

[3α-^{3}H] 17α-hydroxy pregnenolone (**1**)

To a 5.1 mg pregna-3,5-dien-20-one, 3,17-dihydroxy-, diacetate **(8)** (12.3 μmol) was added 500 mCi $NaBT_4$ (60–80 Ci/mmol, ~6.3–8.3 μmol) in 2 mL ethanol. After stirring at room temperature for 18 h, 20 mL formic acid was added dropwise. The solution was stirred for 5 min then evaporated to dryness. The residue was redissolved in 2 mL EtOH, and 2 mL 2 M aqueous LiOH was added. The mixture was stirred at room temperature for 1 h and remained clear. After 2 mL 2 M aqueous NaOH was added, the mixture turned cloudy. The reaction was run at room temperature for another 2 h. EtOH was removed by evaporation, and the aqueous solution was then extracted with 3 × 4 mL EtOAc. The combined organic phase was dried with Na_2SO_4 and evaporated to dryness. Labile tritium was removed by three evaporations with ethanol (2 mL each). The crude product was assayed by scintillation counting to contain ~173 mCi radioactivity and analyzed with RP-HPLC (MeOH/H_2O = 55/45) to contain 44% product. RP-HPLC purification was performed with the following condition: Phenomenex Luna 5u C18 (2) (10 × 250 mm); MeOH/H_2O = 55/45; 3 mL/min; UV@210 nm. 59 mCi product was collected with RCP 95.2% checked by RP-HPLC (MeOH/H_2O = 55/45). This product showed two peaks (57.9% and 36.8%) when analyzed with ChiralPAK AD-H column (i-PrOH/Hexanes = 10/90). A ChiralPAK AD column purification was utilized as follows: 10 μ (10 × 250 mm), i-PrOH/Hexanes = 10/90, 3 mL/min, UV@210 nm. The pure products, (**1**) and (**9**) were isolated then analyzed with the result shown in Table 3.

[3α-^{3}H] pregnenolone (**2**)

To a 2 mg 5-pregnen-3, 20-dione (**10**) (6.4 μmol) was added 250 mCi $NaBT_4$ (60–80 Ci/mmol, ~3.1–4.1 μmol) in 2 mL ethanol. The reaction was stirred at room temperature for 4.5 h. Labile tritium was removed by three evaporations with ethanol (2 mL each). The crude product was assayed by scintillation counting to contain ~108 mCi radioactivity. RP-HPLC (MeOH/H_2O = 65/35) showed that 13.6% radioactivity was co-eluted with (**2**) cold standard. The crude product was purified by RP-HPLC: Phenomenex Luna 5u C18 (2) (10 × 250 mm), MeOH/H_2O = 65/35, 3 mL/min, UV@210 nm. The product fraction was collected and assayed to contain 20 mCi radioactivity. This product shows 95.1% RCP by RP-HPLC (MeOH/H_2O = 65/35) and 93.0% RCP by ChiralPAK AD-H HPLC (i-PrOH/Hexanes = 10/90). 18 mCi pure product (**2**) was obtained

through a ChiralPAK AD column purification: 10 μ (10 × 250 mm), i-PrOH/Hexanes = 10/90, 3 mL/min, UV@210 nm. The summary of the analysis results is shown in Table 4.

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Conflict of Interest

The authors did not report any conflict of interest.

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