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Synthesis of *ent*-25-hydroxycholesterol

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ABCA1, ATP-binding cassette, subfamily A, member 1; *ent*-25-HC, enantiomer of 25-hydroxycholesterol; 25-HC, 25-hydroxycholesterol; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LXR, liver X receptor; SREBP, sterol regulatory element binding protein

ABSTRACT

25-Hydroxycholesterol (25-HC) appears to play a role in several important biological processes, including regulating cellular cholesterol levels and promoting apoptosis. However, in most cases the mechanisms by which 25-HC elicits its biological effects are not known. Insights into mechanisms of 25-HC action can be gained by studying the activity of its enantiomer (*ent*-25-HC). *ent*-25-HC is physically and chemically identical to 25-HC; however, 25-HC and *ent*-25-HC can be distinguished in chiral environments, like a protein binding site. In order to probe the mechanisms of 25-HC action, we have synthesized the enantiomer of 25-HC (*ent*-25-HC).

1. Introduction

25-Hydroxycholesterol (25-HC) has been implicated in a number of important biological processes, including cellular cholesterol homeostasis, atherosclerosis, and apoptosis. Active research is underway to better characterize the in vivo activities of 25-HC.

In the 1970's in vitro studies showed that 25-HC was much more potent than cholesterol at triggering cells to respond to high sterol levels by decreasing 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity and increasing cholesterol esterification, thereby decreasing synthesis and increasing storage of cholesterol [1,2]. Since that time, 25-HC has been used in numerous studies of cellular cholesterol regulation despite some debate about the physiological relevance of 25-HC. It is now known that 25-HC can be formed from cholesterol by autooxidation or enzymatically by a non-heme, iron-containing protein [3,4]. And some studies report that significant levels of 25-HC are produced in cells [3].

Experiments in cell culture have shown that 25-HC affects cholesterol homeostasis at both the transcriptional and post-translational levels. At the level of transcriptional control, 25-HC decreases activation of the transcription factor sterol regu-

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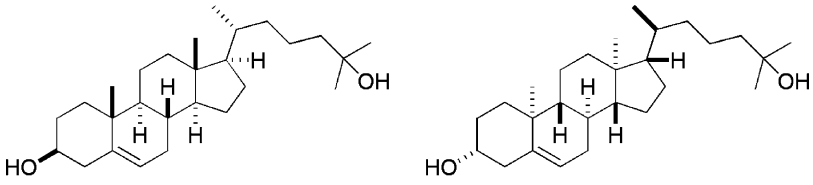

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Fig. 1 – Structures of 25-hydroxycholesterol (1) and *ent*-25-hydroxycholesterol (2).

latory element binding protein (SREBP) by promoting its retention in the ER. This, in turn, leads to decreased production of SREBP-responsive genes, like those encoding LDL receptors and HMG CoA reductase. In addition, 25-HC is an agonist for the liver X receptor (LXR) transcription factor, which increases gene products encoding ATP binding cassette protein, subfamily A, member 1 (ABCA1), a cholesterol efflux pump, and cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile salt synthesis. At the post-translational level, 25-HC promotes the ubiquitination and subsequent degradation of HMG CoA reductase. The fact that 25-HC affects all of these systems has led some to hypothesize that 25-HC, or related oxysterols, may serve as an indicator for high cholesterol in cells [5], leading cells to increase storage and metabolism while decreasing uptake and synthesis of cholesterol.

While 25-HC is more water-soluble than cholesterol ($\log p_{25\text{-HC}} = 7.6$, $\log p_{\text{cholesterol}} = 9.9$),[1] 25-HC is quite hydrophobic and will partition into membranes. To date, only a few biophysical studies have characterized the membrane behavior of 25-HC. In monolayers, 25-HC does not condense other lipids as cholesterol does [6,7]. Liposomes containing 25-HC are more permeable to calcium, sodium and glucose [7,8]. In red blood cell membranes, 25-HC also increases the susceptibility of cholesterol to cholesterol oxidase [9]. These studies clearly indicate that 25-HC may have important effects on cellular membranes.

Except for LXR binding, the mechanisms by which 25-HC mediates its many biological effects are not known. Understanding the biological activities of 25-HC requires considering its effects on both membranes and proteins. This can be accomplished by using the enantiomer of 25-HC (*ent*-25-HC). Fig. 1 shows 25-HC **1** and *ent*-25-HC **2**. Enantiomers have the same relative configuration and hence physical properties. However, enantiomers have the opposite absolute configuration and thus may be distinguished in chiral environments, such as a protein binding site composed of all L-amino acids (reviewed in [10]). Thus, *ent*-25-HC will affect membranes identically to 25-HC, but may interact differently with proteins. Substituting *ent*-25-HC for 25-HC may provide important insights into the mechanisms of 25-HC action.

Enantiomeric steroids including *ent*-25-HC are not naturally available and must be chemically synthesized. We here report the first synthesis of *ent*-25-HC.

[1] Calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris.

2. Experimental methods

2.1. General methods

Solvents were either used as purchased or dried and purified by standard methodology. All air- and/or moisture-sensitive reactions were carried out under N_2 or Ar using oven-dried glassware and cooled under vacuum or N_2. All extraction solvents were dried with anhydrous Na_2SO_4. Flash chromatography was performed using silica gel (32–63 μm) purchased from Scientific Adsorbents (Atlanta, GA). Optical rotations were determined on a Perkin-Elmer Model 341 polarimeter. Melting points were determined on a Kofler micro hot stage and are uncorrected. IR spectra were recorded as films on an AgCl plate with a Perkin-Elmer 1710 FT-IR spectrophotometer. NMR spectra were recorded at ambient temperature in $CDCl_3$ with a 5 mm probe on a Varian Gemini 2000 operating at 300 MHz (^{1}H) or 75 MHz (^{13}C). ^{1}H and ^{13}C NMR spectra were referenced to $CDCl_3$ (δ 7.27) and (δ 77.00), respectively. Elemental analyses were performed by M-H-W Laboratories (Phoenix, AZ).

2.1.1. *(3α, 8α, 9β, 10α, 13α, 14β, 17α, 20S)-Chol-5-en-24-oic acid, methyl ester* (5)

The methyl ester of (3α, 8α, 9β, 10α, 13α, 14β, 17α, 20S)-3-[[(1,1-dimethylethyl)dimethylsilyl]oxy]chol-5-en-24-oic acid **3a** was prepared as described previously [11]. This synthesis produces a small amount (<10%) of the corresponding methyl ester of *ent*-cholan-24-oic acid **3b**. This mixture of steroids, **3a** and **3b**, (270 mg, 0.54 mmol) was dissolved in Et_2O (15 mL). A mixture of HOAc (1.5 mL), NaOAc (16 mg) and Br_2 (0.06 mL) was then added to the steroid solution. After stirring the mixture for 3 h, water (10 mL) was added. The mixture was extracted with EtOAc (3 × 20 mL) and washed with $NaHCO_3$ (2 × 20 mL) and brine (2 × 20 mL). The organic extracts were dried, filtered and concentrated in vacuo to give an orange oil. The oil was immediately applied to a column packed with silica (15% EtOAc in hexanes). The dibromosteroid **4a** was easily separated from the saturated **4b**. The dibromosteroid **4a** product was recovered as a clear, colorless oil (160 mg, 55%) which was not stable and was used immediately after purification. ^{1}H NMR δ 4.84 (1H, m), 4.44 (1H, m), 3.67 (3H, s, OMe-H), 2.69–1.47 (17H, m), 1.45 (3H, s, 19-H), 1.41–1.07 (8H, m), 0.93 (3H, d, J = 6.32 Hz, 21-H), 0.71 (3H, s, 18-H); ^{13}C NMR δ 174.89, 89.85, 69.31, 56.27, 55.91, 55.30, 51.66, 47.53, 45.89, 42.88, 42.06, 39.73, 37.34, 36.87, 35.48, 31.20, 31.11, 30.97, 30.33, 28.19, 24.15, 21.45, 20.50, 18.40, 12.34.

The dibromosteroid **4a** (130 mg, 0.24 mmol) was dissolved in HOAc (10 mL). Zinc dust (0.5 g) was added and the mixture

was stirred at rt for 1.5 h. Ether (10 mL) was added and the zinc was removed by filtration through Celite. The organic phase was washed with water (3 × 20 mL), $NaHCO_3$ (2 × 20 mL), and brine (2 × 20 mL). The organic layer was dried, filtered and concentrated to yield a white solid **5** (80 mg, 90%); mp 141–142.5 °C; $[\alpha]_D^{24} = +39.0$ ($c = 1.0$, $CHCl_3$); IR 2936, 1740, 1438, 1375, 1194, 1173, 1058, 734 cm^{-1}; ^1H NMR δ 5.32 (1H, m, 6-H), 3.64 (3H, s, OMe-H) 3.49 (1H, m, 3-H), 2.37–1.00 (26H, m), 0.98 (3H, s, 19-H), 0.90 (3H, d, $J = 6.32$ Hz, 21-H), 0.65 (3H, s, 18-H); ^{13}C NMR δ 174.90, 140.86, 121.62, 71.63, 56.62, 55.66, 51.38, 49.97, 42.23, 42.14, 39.61, 37.13, 36.34, 35.22, 31.73 (2 × C), 31.47, 30.91, 30.86, 27.95, 24.10, 20.91, 19.22, 18.15, 11.68.

2.1.2. *(3α, 8α, 9β, 10α, 13α, 14β, 17α, 20S)-3-[[(1,1-Dimethylethyl)dimethylsilyl]oxy]chol-5-en-24-ol* (6)

4-(Dimethylamino)pyridine (DMAP) (70 mg, 0.52 mmol), *t*-butyldimethylsilyl chloride (80 mg, 0.52 mmol), CH_2Cl_2 (3 mL), THF (1 mL) and distilled triethylamine (0.75 mL) were added to alcohol **5** (100 mg, 0.26 mmol). The mixture was stirred overnight at rt. Aqueous NH_4Cl (7 mL) was added. After stirring for 10 min, the mixture was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic extracts were dried and concentrated. The residue was passed through a silica plug using CH_2Cl_2 as the eluent and concentrated to yield the protected steroid as a white solid (130 mg, 99%); mp 135.5–137.5 °C; $[\alpha]_D^{24} = +25.2$ ($c = 0.88$, $CHCl_3$); IR 2929, 2895, 2853, 1744, 1636, 1253, 1100, 837, 777 cm^{-1}; ^1H NMR δ 5.31 (1H, m, 6-H), 3.65 (3H, s, OMe-H), 3.47 (1H, m, 3-H), 2.35–1.02 (25H, m), 0.99 (3H, s, 19-H), 0.92 (3H, d, $J = 6.04$ Hz, 21-H), 0.88 (9H, s, $C(CH_3)_3$), 0.66 (3H, s, 18-H), 0.04 (6H, s, $Si(CH_3)_2$); ^{13}C NMR δ 174.84, 141.56, 121.15, 72.55, 56.66, 56.39, 55.66, 51.37, 50.05, 42.70, 42.25, 39.64, 37.25, 36.43, 35.24, 31.94, 31.74, 30.88, 27.98, 25.80 (4 × C), 24.11, 20.89, 19.27, 18.15, 18.10, 11.68, −4.78 (2 × C).

The protected methyl ester (130 mg, 0.26 mmol) was dissolved in Et_2O (8 mL) and added dropwise into a flask containing Et_2O (8 mL) and LAH (60 mg, 1.56 mmol) at 0 °C. After 30 min, reduction of the ester was complete. The reaction was quenched by addition of water (0.06 mL), followed by 15% NaOH (0.06 mL). After stirring for 30 min, additional water (0.18 mL) was added. The solvent was then decanted from the white aluminum salts, which were washed several times with Et_2O. Removal of solvent in vacuo produced a white solid, the alcohol **6** (120 mg, 98%); mp 164–165 °C; $[\alpha]_D^{24} = +28.4$ ($c = 1.0$, $CHCl_3$); IR 3370, 2937, 2857, 1253, 1082, 839, 774 cm^{-1}; ^1H NMR δ 5.32 (1H, m, 6-H), 3.61 (1H, OH), 3.48 (1H, m, 3-H), 2.28–1.03 (27H, m), 1.00 (3H, m, 19-H), 0.94 (3H, d, $J = 6.31$ Hz, 21-H), 0.89 (9H, s, $C(CH_3)_3$), 0.68 (3H, s, 18-H), 0.06 (6H, s, $Si(CH_3)_2$); ^{13}C NMR δ 141.54, 121.13, 72.63, 63.58, 56.77, 55.95, 50.16, 42.79, 42.32, 39.76, 37.35, 36.55, 35.55, 32.06, 31.87, 31.81 (2 × C), 29.37, 28.22, 25.92 (3 × C), 24.25, 21.04, 19.41, 18.67, 18.24, 11.84, −4.62 (2 × C).

2.1.3. *(3α, 8α, 9β, 10α, 13α, 14β, 17α, 20S)-3-[[(1,1-Dimethylethyl)dimethylsilyl]oxy]chol-5-en-24-carbonitrile* (7)

To the alcohol **6** (120 mg, 0.25 mmol), *p*-toluenesulfonylchloride (100 mg, 0.5 mmol) and pyridine (5 mL) were added. The mixture was kept at 4 °C for 2 days. The reaction was then poured into ice/water (10 mL). The white solid was filtered, solubilized with EtOAc, and washed with both 0.5 N HCl (1 × 10 mL) and water (2 × 5 mL). The organic layer was dried, filtered and concentrated in vacuo to give a white solid (130 mg, 80%) which was used without further purification. ^1H NMR δ 7.79 (2H, d, $J = 9.2$ Hz), 7.35 (2H, d, $J = 8.0$ Hz), 5.32 (1H, m, 6-H), 4.00 (2H, dt, $J = 6.59$, 2.47 Hz, 24-H), 3.48 (1H, m, 3-H), 2.45 (3H, s, Ph-CH_3), 2.38–1.00 (28H, m), 0.99 (3H, s, 19-H), 0.89 (9H, s, $C(CH_3)_3$), 0.64 (3H, s, 18H), 0.06 (6H, s, $Si(CH_3)_2$); ^{13}C NMR δ 144.58, 141.51, 133.25, 129.76, 127.85, 121.06, 72.58, 71.20, 56.69, 55.75, 50.10, 42.77, 42.27, 39.71, 37.34, 36.52, 35.15, 32.03, 31.84 (×2), 31.39, 28.09, 25.91 (5 × C), 25.48, 24.19, 21.61, 20.99, 19.38, 18.41, 18.23, 11.78, −4.63 (2 × C).

To the dry steroidal tosylate (130 mg, 0.21 mmol) was added NaCN (15 mg, 0.31 mmol). After evacuating and filling the flask with N_2, anhydrous DMSO (5 mL) was added and the mixture was heated to 90 °C for 3.5 h. The reaction was then cooled and added to a mixture of ice and saturated NaCl (20 mL). The organic layer was extracted with EtOAc (3 × 20 mL), washed with brine (2 × 20 mL), and dried with sodium sulfate. After filtration and concentration, the crude material was applied to a column packed with silica gel (15% EtOAc in hexanes). The desired product was recovered as a white solid and recrystallized from Et_2O/hexanes to obtain clear, colorless crystals of the carbonitrile **7** (80 mg, 80%); mp 196–198 °C; $[\alpha]_D^{24} = +32.3$ ($c = 1.0$, $CHCl_3$); IR 2929, 2253, 1256, 1094, 837, 775; ^1H NMR δ 5.32 (1H, s, 6-H), 3.48 (1H, m, 3-H), 2.34–1.03 (27H, m), 1.00 (3H, s, 19-H), 0.94 (3H, d, $J = 6.59$ Hz, 21-H), 0.89 (9H, s, $C(CH_3)_3$), 0.68 (3H, s, 18-H), 0.06 (6H, s, $Si(CH_3)_2$); ^{13}C NMR δ 141.50, 121.06, 119.82, 72.57, 56.71, 55.72, 50.10, 42.77, 42.33, 39.74, 37.34, 36.52, 35.20, 35.00, 32.04, 31.84 (2 × C), 28.17, 25.91 (3 × C), 24.19, 22.16, 21.01, 19.38, 18.49, 18.21, 17.51, 11.81, −4.62 (2 × C). Anal. Calcd. for $C_{31}H_{53}NOSi$: C, 76.95; H, 11.04; N, 2.89. Obsd. C, 77.12; H, 11.26; N, 2.63. A small amount (<10 mg) of the 24-alcohol **6** was also recovered.

2.1.4. *(3α, 8α, 9β, 10α, 13α, 14β, 17α, 20S)-27-Norcholest-5-en-25-one* (8)

THF (6 mL) and MeLi (1.25 mL of 1.6 M solution in Et_2O, 2 mmol) were introduced to a dry flask. The mixture turned cloudy upon cooling to 0 °C. The steroidal nitrile **7** (80 mg, 0.2 mmol) was then dissolved in THF (4 mL) and slowly added to the MeLi solution. The flask was rinsed with additional THF (3 mL) and added to the reaction. The reaction was stirred at 0 °C for 20 min, then at rt for 2.5 h. The reaction was monitored by TLC (10% EtOAc in hexanes), visualized with 2,4-dinitrophenylhydrazine because the nitrile **7** and methyl ketone **8** have identical R_f values. Next, H_2SO_4 in dioxane (3 mL of 3 M solution in 6 mL dioxane) was added to the reaction and heated to 65 °C for 1.5 h. After cooling, the layers were separated. The aqueous layer was neutralized with 10% NaOH and extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine, dried, filtered, and concentrated. The resultant white solid was dissolved in CH_2Cl_2 and applied to a column of silica gel in 25% EtOAc in hexanes. The desired product **8** was recovered as a white solid (60 mg, 90%) and recrystallized from MeOH as thin white crystals; mp 116–118 °C; $[\alpha]_D^{24} = +44.0$ ($c = 0.6$, $CHCl_3$); IR 3435, 2938, 1712, 1375, 1045; ^1H NMR δ 5.35 (1H, m, 6-H), 3.53 (1H, m, 3-H), 2.42–2.23 (4H, m), 2.14 (3H, s, 26-H), 2.09–1.04 (24H, m), 1.01 (3H, s, 19-H), 0.94 (3H, d, $J = 6.59$ Hz, 21-H), 0.67 (3H, s, 18-H); ^{13}C NMR δ 209.45, 140.75, 121.64, 71.76, 56.71, 55.76, 50.07, 44.30, 42.30, 42.26, 39.71, 37.22, 36.47, 35.58, 35.41, 31.86

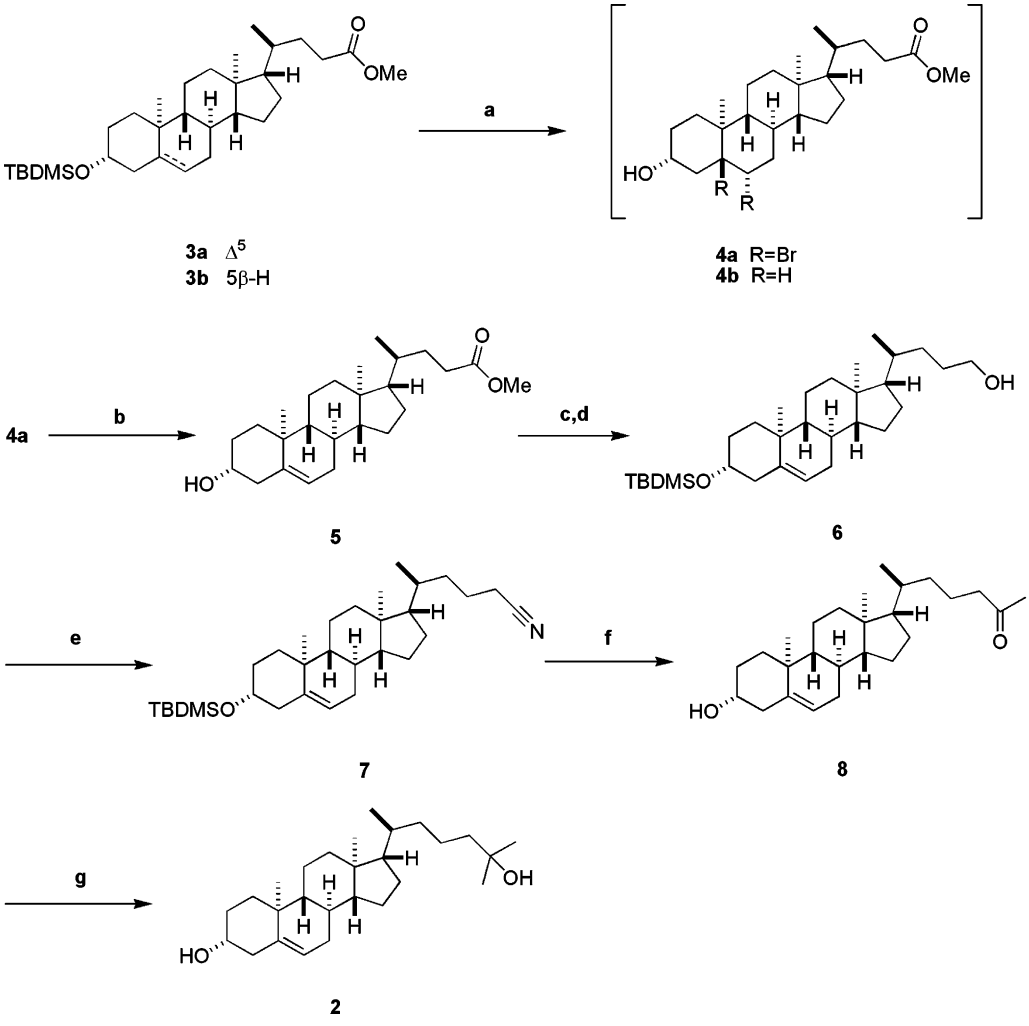
(2 × C), 31.62, 29.84, 28.17, 24.22, 21.04, 20.35, 19.37, 18.58, 11.81. Anal. Calcd. for $C_{26}H_{42}O_2$: C, 80.77; H, 10.95. Obsd. C, 80.45; H, 10.65.

2.1.5. *(3α, 8α, 9β, 10α, 13α, 14β, 17α, 20S)-25-Hydroxycholesterol (2)*

The steroid methyl ketone **8** (55 mg, 0.15 mmol) and THF (5 mL) were added to a dry flask. The mixture was cooled to 0 °C. Then MeLi was added dropwise (4.7 mL of 1.6 M solution in Et_2O, 7.5 mmol). The mixture was stirred on ice for 30 min and at rt for an additional 90 min. Then water (5 mL) was added cautiously to the reaction mixture. The layers were separated and the aqueous phase was extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (2 × 10 mL), dried, filtered, and concentrated. The resultant white solid was applied to a silica gel column (30% EtOAc in hexanes) to separate the alcohol product **2** from the methyl ketone starting material. Approximately 25% of the methyl ketone **8** was recovered. The desired product **2** was recovered as a white solid (35 mg, 60%); mp 174–175 °C; $[\alpha]_D^{24} = +37.7$ ($c = 0.44$, $CHCl_3$); IR 3306, 2935, 1465, 1377, 1058, 734; ^{1}H NMR δ 5.35 (1H, m, 6-H), 3.53 (1H, m, 3-H), 2.29–1.24 (25H, m), 1.22 (6H, 26 and 27-H), 1.17–1.05 (4H, m), 1.02 (3H, s, 19-H), 0.93 (3H, d, $J = 6.59$ Hz, 21-H), 0.69 (3H, s, 18-H); ^{13}C NMR δ 140.76, 121.70, 71.79, 71.11, 56.75, 56.05, 50.10, 44.41, 42.28 (×2), 39.76, 37.23, 36.49, 36.44, 35.72, 31.88 (×2), 31.64, 29.35, 29.18, 28.23, 24.26, 21.06, 20.74, 19.38, 18.66, 11.85. Anal. Calcd. for $C_{27}H_{46}O_2$: C, 80.54; H, 11.51. Obsd. C, 80.70; H, 11.52.

3. Results and discussion

The strategy we used for preparing *ent*-25-HC was similar to that used to make *ent*-desmosterol [11]; namely, we prepared the *ent*-steroid nucleus and then added the side chain piecewise. This strategy required the production of the steroidal methyl ester **3a** (Scheme 1). However, as described in [11], the method used to make **3a** also produces a small amount (<10%) of the corresponding saturated stanol **3b**. Sterol **3a** and stanol **3b** cannot be separated by chromatography. In the earlier work, the small amount of stanol was removed when the side chain was complete, containing only



Scheme 1 – (a) (i) Br_2, HOAc, NaOAc, (ii) chromatography, 55%; (b) Zn, HOAc, 90%; (c) TBDMSCI, DMAP, $MeCI_2$, THF, TEA, 99%; (d) LiAIH, Et_2O, 0 °C, 98%; (e) (i) *p*-TsCI, pyr, 4 °C, (ii) NaCN, DMSO, 90 °C, 80%; (f) (i) MeLi, THF, 0 °C, (ii) H_2SO_4, dioxane, 65 °C, 90%; (g) MeLi, THF, 60%.

hydrocarbons, e.g. *ent*-TBDMS-desmosterol or *ent*-TBDMS-cholesterol. We predicted that such a separation would not be possible in this work because the side chain would contain some functionality, i.e. the 25-hydroxyl group. Therefore, we sought to remove the saturated material by a different method.

We reacted esters **3a** and **3b** with Br_2 in HOAc. These conditions removed the silyl protecting group from the 3-hydroxyl group in both compounds. Otherwise, ester **4b** did not react, while **3a** was converted to the 5,6-dibromide **4a**. The dibromide **4a** and saturated **4b** were easily separated by chromatography. Prolonged storage (several days) of the dibromide resulted in significant degradation of the product. Thus, after separation, the dibromide **4a** was immediately debrominated by treatment with Zn in HOAc to give the desired methyl ester of *ent*-chol-5-en-3-ol **5**, uncontaminated by the saturated steroid.

The alcohol **5** was easily reprotected and the methyl ester in the side chain was reduced to give the alcohol **6**. This primary alcohol was converted to the tosylate, which was then displaced by NaCN to give the carbonitrile **7**. Methylation followed by acidic workup afforded the methyl ketone **8**. Subsequent methylation provided the tertiary alcohol in the desired product, *ent*-25-HC **2**.

The overall yield of *ent*-25-HC from the methyl ester **3** was 21% and from the steroidal precursor *ent*-testosterone the yield was 6%. Efforts are currently underway to use *ent*-25-hydroxycholesterol as a tool to understand the mechanisms by which 25-hydroxycholesterol affects cellular cholesterol homeostasis and cholesterol transport.

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