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A direct stereoselective synthesis of 7β-hydroxytestosterone

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*Although 7β-hydroxytestosterone is a known product of hepatic androgen metabolism, there are no published methods for its chemical synthesis except from the equally difficult to obtain 7β-hydroxy-4-androstene-3,17-dione. We found that several seemingly straightforward routes for its synthesis failed. Consequently, we tried to produce 7β-hydroxytestosterone by enzymatic oxidation of 5-androstene-3β,7β,17β-triol with cholesterol oxidase (*Brevibacterium *sp.), a procedure previously used to synthesize 7β-hydroxy-4-cholesten-3-one from 3β,7β-dihydroxycholesterol (Alexander and Fisher 1995). However, 5-androstene-3β,7β,17β-triol was, at best, a very poor substrate for the enzyme leading to the production of 7β-hydroxytestosterone in only trace amounts. Thus, we explored a strategy for the enzymatic synthesis in which a C_8-ester at C-17 (5-androstene-3β,7β,17β-triol 17-caprylate) would serve to mimic the bulky and hydrophobic side chain of cholesterol and thus allow the C_{19}-steroid to act as an effective substrate. When this ester was incubated with cholesterol oxidase, it was converted efficiently to 7β-hydroxytestosterone-17-caprylate. Attempts to remove the ester group by several mild hydrolytic procedures caused elimination of the 7β-hydroxyl group; we, therefore, obtained 7β-hydroxytestosterone by incubation of the intermediate ester with porcine lipase.* (*Steroids* **62**:482–486, 1997) © *1997 by Elsevier Science Inc.*

Keywords: 7β-hydroxytestosterone; cholesterol oxidase; lipase

Introduction

7β-Hydroxytestosterone is produced as minor constituent of a complex mixture of several metabolites in the microbiological transformation of testosterone by *Botryosphaerica obtusa*[1] and *Rhizopus nigricans*.[2] This compound has been produced synthetically[3] by the reduction of 7β-hydroxy-4-androsten-3,17-dione followed by oxidation of the resulting C-3 allylic hydroxyl group. However, the starting material for this sequence, 7β-hydroxy-4-androsten-3,17-dione, is again produced as a minor component by the microbiological transformation of 4-androstene-3,17-dione by *Xylaria* sp., *Pseudomonas* B20-184, and *Rhizopus stolonifer*.[4–6] 7β-Hydroxytestosterone has been identified as a minor component of a mixture resulting from the metabolism of testosterone by rat liver, produced by the microsomal cytochrome P-450 enzymes RLM_2 and RLM_3.[7] It was noted at that time that a reference standard sample of this compound was not available. We could find no straightforward synthesis of this compound in the literature.

Recently, we required 7β-hydroxytestosterone as an intermediate in our work directed toward the synthesis of ^{125}I-labeled androgens, 7α-iodosteroids, for use as potential imaging agents for androgen-mediated cancers.[8] We initially envisioned 7β-hydroxytestosterone resulting directly from deketalization of 3,3-(ethylenedioxy)-5-androstene-7β,17β-diol. However, this approach failed in a concurrent synthesis of the related steroid 7β-hydroxy-17α-methyltestosterone. Our attempts to remove the 3-ketal group of 3-3-(ethylenedioxy)-17α-methyl-5-androstene-7β,17β-diol by hydrolysis with a variety of mild acid catalysts (acetic acid, adipic acid, oxalic acid, pTsOH) resulted in exclusive production of the corresponding $\Delta^{4,6}$-3-ketone. Oxidative ketal removal with Ph_3CBF_4 in CH_2Cl_2 also failed, producing a complex mixture of products.[9]

We then turned toward enzymatic methods to generate the labile 7β-hydroxy-Δ^4-3-ketone functionality. In a recent paper, Alexander and Fisher[10] described the preparation of 7β-hydroxycholest-4-en-3-one by the enzymatic oxidation of 5-cholesten-3β,7β -diol by cholesterol oxidase, *Brevibacterium* sp. However, we found that, under the conditions described,[10] 5-androstene-3β,7β,17β-triol (Figure 1) was converted to 7β-hydroxytestosterone in only a trace amount, even after 4 days incubation. We thought that if the C_{19} steroid was derivatized in a manner designed to mimic the bulky hydrophobic side chain of cholesterol, the resulting compound might be a better substrate in the enzymatic

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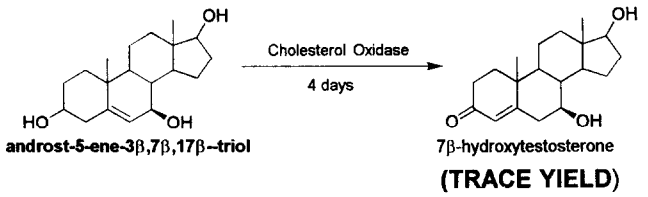

Figure 1 5-Androstene-3β,7β,17β-triol is a poor substrate for cholesterol oxidase (*Brevibacterium* sp.).

reaction with cholesterol oxidase and that the desired transformation would be achieved. To carry out the oxidation to the 7β-hydroxy-Δ^4-3-ketone with the enzyme, we selected to functionalize the substrate with a C_8 ester group at C-17 (Figure 2), and we describe the results of this approach.

Experimental

General methods

Thin-layer chromatography (TLC) was performed using Analtech silica gel plates (GHLF, 0.25 mm) and visualized using phosphomolybdic acid or ultraviolet illumination. Melting points were obtained in a Mel-temp apparatus and are uncorrected. Ultraviolet spectra were recorded on Perkin–Elmer model 552 spectrophotometer. Purification by flash column chromatography was performed according to the procedure of Still[11] using 230–400 mesh silica gel (EM Science, Gibbstown, NJ, USA). Solvents for chromatography and extraction were reagent grade. ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra [broadband decoupled and distortion less enhancement by polarization transfer DEPT)] were recorded at 75 MHz. Low- and high-resolution fast atom bombardment mass spectra (FABMS) were obtained on a VG instrument (ZAB SE) using a matrix of *m*-nitrobenzyl alcohol or Magic Bullet + PEG by Dr. Walter J. McMurray at the Yale University Comprehensive Cancer Center. EI mass spectra were recorded on a Hewlett-Packard 5890 gas chromatograph with a Model 5972 mass selective detector using a 30 m × 0.25 mm HP-5MS column at 200–250°C.

Tetrahydrofuran (THF) was distilled from benzophenone ketyl under N_2 immediately prior to use. Pyridine was distilled from CaH. Caprylyl chloride was purchased from Nu-Chek-Prep (Elysian, Minnesota, USA). Steroids were obtained from Steraloids (Wilton, New Hampshire, USA). Cholesterol oxidase E.C. 1.1.3.6 [9028-76-6] (10 U/mg) from *brevibacterium* sp., catalase E.C. 1.11.1.6 [9001-05-2] from bovine liver and lipase E.C. 3.1.1.3[9001-62-1] type II crude (Steapsin) from porcine pancreas were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) and were used as received without further analysis or purification. Hydroxypropyl-β-cyclodextrin [94035-02-6] and other reagents were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA) and were used without further purification.

3β-tert-Butyldiphenylsiloxy-5-androsten-17-one (2)

A solution of 3β-hydroxy-5-androsten-17-one **1** (3.05 g, 10.5 mmol), imidazole (1.44 g, 21.1 mmol) and *tert*-butyldiphenylsilyl chloride (TBDPSCl) (3.57 mL, 13.7 mmol) in DMF (22 mL) was stirred at rt under N_2 for 5 days. The reaction mixture was poured into H_2O (100 mL) and extracted with CH_2Cl_2 (3×, 50 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 5 × 13 cm column of silica gel using hexanes/EtOAc (3:1) as eluent gave **2** in quantitative yield. Data for **2**: TLC R_f 0.62 (hexanes/EtOAc, 3:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (m, 4H, Ar-H), 7.39 (m, 6H, Ar-H), 5.16 (d, 1H, J = 5.2 Hz, H-6), 3.53 (m, 1H, H-3α), 1.06 (s, 9H, tBu), 1.01 (s, 3H, H-19), 0.86 (s, 3H, H-18).

3β-tert-Butyldiphenylsiloxy-5-androstene-17β-ol (3)

A solution of ketone **2** (6.24 g, 11.8 mmol) in THF (148 mL) and H_2O) (1.5 mL) was stirred at rt as sodium borohydride (580 mg, 15.4 mmol) was added. The reaction mixture was stirred at rt for 5.5 h, poured into saturated aqueous NH_4Cl, and extracted with CH_2Cl_2 (3×, 100 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo giving 6.53 g of a clear oil, which was used without further purification in the next step. Data for **3**: TLC R_f 0.33 (hexanes/EtOAc, 3:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.68 (m, 4H, Ar-H), 7.38 (m, 6H, Ar-H), 5.12 (d, 1H, J = 4.9 Hz, H-6), 3.61 (t, 1H, J = 8.5 Hz, H-17α), 3.52 (m, 1H, H-3α), 1.06 (s, 9H, tBu), 1.00 (s, 3H, H-19), 0.73 (s, 3H, H-18).

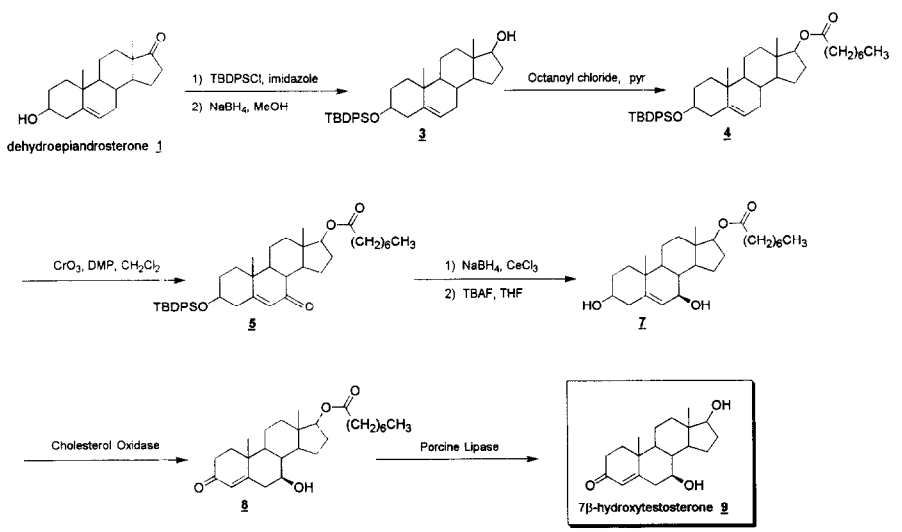

Figure 2 The synthetic scheme for 7β-hydroxytestosterone.

3β-tert-Butyldiphenylsiloxy-5-androsten-17β-ol 17-caprylate (4)

A solution of alcohol **3** (480 mg, 0.91 mmol), octanoyl chloride (200 mg, 1.23 mmol) in pyridine (1 mL) was stirred at rt for 15 h. The reaction mixture was poured into saturated aqueous $CuSO_4$ and extracted with CH_2Cl_2 (3×, 20 mL). Combined organic extracts were washed with brine (30 mL), dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 16 cm column of silica gel using hexanes/EtOAc (10:1) as eluent gave 489 mg (82%) of **4** as a clear colorless oil. Data for **4:** TLC R_f 0.66 (hexanes/EtOAc, 5:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.66 (m, 4H, Ar-H), 7.37 (m, 6H, Ar-H), 5.11 (d, 1H, J = 4.7 Hz, H-6), 4.57 (t, 1H, J = 8.2 Hz, H-17α), 3.52 (m, 1H, H-3α), 1.05 (s, 9H, tBu), 0.98 (s, 3H, H-19), 0.87 (t, 3H, J = 7.2 Hz, CH_3), 0.77 (s, 3H, H-18).

3β-tert-Butyldiphenylsiloxy-17β-hydroxy-5-androsten-7-one 17-caprylate (5)

This procedure is based on the literature method.[12] A 250 mL three-neck flask containing CrO_3 (7.46 g, 74.7 mmol) in anhydrous CH_2Cl_2 (107 mL) was stirred by a mechanical stirrer at −23°C for 2 min. A portion of 3,5-dimethylpyrazole (7.18 g, 74.7 mmol) was added, and the reaction was stirred at −23°C for 15 min. To this was added a solution of olefin **4** (489 mg, 0.747 mmol) in CH_2Cl_2 (10 mL), and the reaction mixture was stirred at −23°C for 4 h, allowed to warm to rt, and poured onto a 3 × 20 cm column of flash silica gel with a head volume of 100 mL of eluent. The column was eluted with EtOAc/hexanes (3:1), and products containing fractions were combined and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 15 cm column of silica gel using hexanes/EtOAc (10:1) as eluent gave 219 mg (44%) of **5** as a clear, colorless oil. Data for **5:** TLC R_f 0.18 (iso-octane/EtOAc, 10:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.65 (m, 4H, Ar-H), 7.39 (m 6H, Ar-H), 5.46 (s, 1H, H-6), 4.59 (t, 1H, J = 8.1 Hz, H-17α), 3.60 (m, 1H, H-3α), 1.16 (s, 3H, H-19), 1.06 (s, 9H, tBu), 0.87 (t, 3H, J = 7.0 Hz, CH_3), 0.77 (s, 3H, H-18). FAB MS (*m/z* 669 $[MH]^+$.

3β-tert-Butyldiphenylsiloxy-5-androstene-7β, 17β-diol 17-caprylate (6)

This procedure is based on the literature method.[13] Enone **5** (219 mg, 0.327 mmol) and cerium (III) chloride heptahydrate (122 mg, 0.327 mmol) were stirred in a mixture of THF (2 mL) and methanol (2 mL) at room temperature, and sodium borohydride (12.4 mg, 0.327 mmol) was added in five portions over 1 min. The reaction mixture was stirred at rt for 1 h, poured into saturated aqueous NH_4Cl, and extracted with EtOAc (3×, 75 mL). Combined organic extracts were washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was used as in the next step without further purification. Data for **6:** TLC R_f 0.36 (hexanes/EtOAc, 4:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.68 (m, 4H, Ar-H), 7.40 (m, 6H, Ar-H), 5.04 (s, 1H, H-6), 4.58 (t, 1H, J = 8.4 Hz, H-17α), 3.75 (br s, 1H, H-7α), 3.52 (m, 1H, H-3α), 1.06 (s, 9H, tBu), 1.03 (s, 3H, H-19), 0.88 (t, 3H, J = 6.3 Hz, CH_3), 0.79 (s, 3H, H-18).

5-androstene-3β7β,17β-triol-17 caprylate (7)

A solution of crude alcohol **6** and nBu_4NF (980 μL, 1M solution in THF) in THF (20 mL) was stirred at rt for 24 h. The reaction mixture was poured into 20 mL of 10% sodium metabisulfite (20 mL) and extracted with CH_2Cl_2 (3×, 75 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo. Purification of the residue by flash chromatography on a 2 × 17 cm column of silica gel using EtOAc/hexanes (2:1) as eluent gave 99 mg of **7** (70%, two steps from **5**) as a clear colorless oil. Data for **7:** TLC R_f 0.17 (hexanes/EtOAc, 1:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.30 (s, 1H, H-6), 4.62 (t, 1H, J = 8.4 Hz, H-17α), 3.87 (d, 1H, J = 8.2 Hz, H-7α), 3.56 (m, 1H, H-3α), 1.07 (s, 3H, H-19), 0.89 (t, 3H, J = 7.5 Hz, CH_3), 0.83 (s, 3H, H-18).

7β,17β-dihydroxy-4-androsten-3-one 17-caprylate (8)

This procedure is based on the published method.[10] In a 100-mL flask was placed 82.1 mg (0.189 mmol) of diol 7, 370 mg (0.247 mmol) of hydroxypropyl-β-cyclodextrin in 5 mL of MeOH. The solvent was evaporated in vacuo, and the residue was dissolved in 0.06M pH 7 potassium phosphate buffer (68 mL). Catalase (23.4 μL of a 5.7 × 10^6 U/mL solution) and cholesterol oxidase (107 U) were added, and the milky white mixture was stirred at rt open to the air for 4.25 days. The reaction mixture was poured into brine (50 mL) and extracted with CH_2Cl_2 (3×, 75 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo, giving a clear colorless oil. Purification of the residue by flash chromatography on a 2 × 16 cm column of silica gel eluting with hexane/EtOAc (1:1) gave 72.9 mg (89%) of **8** as a clear colorless oil. Data for **8:** TLC R_f 0.60 (EtOAc/hexanes, 2:1). Ultraviolet (MeOH) λ_{max} (ε), 242 nm (10,425). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.77 (d, 1H, 1.4 Hz, H-4), 4.60 (dd, 1H, J = 8.9, 8.1 Hz, H-17α), 3.48 (ddd, 1H, J = 10.7, 9.6, 5.2 Hz, H-7α), 1.22 (s, 3H, H-19), 0.90 (t, 3H, J = 6.6 Hz, CH_3), 0.86 (s, 3H, H-18). FAB MS (*m/z*) 431 $[MH]^+$, FAB(+) HRMS (MB+PEG) calcd. for $C_{27}H_{43}O_4$, (*m/z*) 431.3161. Found: 431.3149.

7β-Hydroxytestosterone (9)

To a 25-mL flask was added 17.3 mg (0.04 mmol) of enone **8**, 86.5 mg (0.0577 mmol) of hydroxypropyl-β-cyclodextrin and MeOH (3 mL). The solids were dissolved with gentle warming, the solvent was evaporated under vacuum, and the residue was suspended in 0.5M pH 7.1 phosphate buffer (17.3 mL). Porcine lipase (173 mg of a 35–70 U/mg protein) was added, and the mixture was stirred for 24 h at rt under argon. The reaction mixture was extracted with EtOAc (3×, 30 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated in vacuo, giving a clear oil. Purification of the residue by flash chromatography on a 2 × 15 cm column of silica gel using EtOAc/hexanes (5:1) as eluent gave 20.5 mg of a clear, colorless oil. Further purification of the oily residue on HPLC using a I-60 protein column (1 cm × 30 cm, Waters Associates Milford, MA) eluting with 3% iPrOH/CH_2Cl_2 at 1.5 mL/min (retention time 12.5 min) with ultraviolet detection at 280 nm, then flash column chromatography on 0.5 × 4 cm column of silica gel eluting with EtOAc/hexanes (3:1) gave 9.2 mg (75%) of **9** as a white solid. Data for **9:** mp 197.5–199.5°C (acetone); lit.[3] mp 196–197°C; TLC R_f 0.18 (EtOAc/hexanes, 2:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.76 (s, 1H, H-4), 3.64 (t, 1H, J = 8.5 Hz, H-17α), 3.46 (ddd, 1H, J = 11.1, 9.4, 5.2 Hz, H-7α), 1.23 (s, 3H, H-19), 0.82 (s, 3H, H-18). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.36 (C-3), 167.47 (C-5); 124.71 (C-4), 81.08 (C-17), 74.90 (C-7), 50.67 (C-14), 49.86 (C-9), 43.49 (C-13), 43.03 (C-8), 42.24 (C-6), 38.01 (C-10), 36.26 (C-12), 35.64 (C-1), 33.90 (C-2), 30.70 (C-16), 26.33 (C-15), 20.54 (C-11), 17.31 (C-19), 11.10 (C-18). EI MS (*m/e*) (relative intensity) 304 (41, M+), 286 (9), 244 (7), 227 (7), 159 (13), 125 (17), 124 (100). FAB(+)HRMS (MB+PEG) calcd. for $C_{19}H_{28}O_3$ (*m/z*) 304.2038. Found: 304.2050.

Results and discussion

Several straightforward routes that we attempted for the synthesis of 7β-hydroxytestosterone **9** failed because of the tendency of the 7β-hydroxy-Δ^4-3-ketone to eliminate and form the $\Delta^{4,6}$-3-ketone. Consequently, we tried to synthesize **9** by adapting an enzymatic method previously used to synthesize 7β-hydroxy-4-cholesten-3-one.[10] However, when the appropriate triol substrate (Figure 1) was reacted with cholesterol oxidase for 4 days, we could detect, at most, only trace yield of the desired 7β-hydroxy-C_{19}-steroid. In an attempt to imitate cholesterol and, thereby, increase the bulk and hydrophobicity of the substrate, we converted the C-17 hydroxyl group into an ester. Our choice of ester group on the cholesterol oxidase substrate was governed by two factors. The ester chain had to be long enough to mimic the cholesterol side chain and, because we found later that the 7β-hydroxy-Δ^4-3-ketone functionality is very sensitive to a variety conditions used for ester hydrolysis, the ester had to be of a type that could be efficiently removed enzymatically. Because Abul-Hajj and Nurieddin in their study of the enzymatic hydrolysis of similar C-17 esters of estradiol[14] had found that caprylate is the longest chained ester of estradiol that is appreciably hydrolyzed by porcine esterase, we chose the C_8-ester and, thus, synthesized 5-androstene-3β7β,17β-triol 17-caprylate **7** as the substrate in the cholesterol oxidase catalyzed synthesis of 7β-hydroxytestosterone (Figure 2).

The substrate **7** for the enzymatic oxidation reaction was synthesized as follows. The hydroxyl group of dehydroepiandrosterone was protected as the *tert*-butyldiphenylsilyl ether in quantitative yield using *tert*-butyldiphenylsilyl chloride (TBDPSCl) in dimethyl formamide (DMF). Reduction of the C-17 ketone using $NaBH_4$ in wet tetrahydrofuran yielded the 17β-alcohol **3**, which was esterified directly with octanoyl chloride in pyridine to give the ester **4** in 82% yield. Allylic oxidation of **4** to the 7-ketone was carried out as previously described[12] using CrO_3-3,5-dimethylpyrazole complex in CH_2Cl_2 to give **5** in 44% yield. Stereoselective reduction of the 7-ketone of **5** using $NaBH_4CeCl_3$ using the procedure of Kumar et al.[13] gave the 7β-hydroxy steroid **6**, which was desilylated with nBu_4NF in THF to give **7** in 70% yield.

Enzymatic oxidation of **7** was carried out according to the procedure of Alexander and Fisher.[10] The steroid was first combined with 1.3 equivalents of hydroxypropyl-β-cyclodextrin in methanol. After the solvent was removed, the residue was dissolved in buffer. The hydroxypropyl-β-cylodextrin is used as a solubilizing agent for the steroid.[15] Oxidation using the enzyme concentrations and conditions suggested by Alexander and Fisher cleanly gave the 7β-hydroxyenone **8** in 89% yield. Confirmation of the desired transformation was provided by the ultraviolet spectrum for **8**, which shows a λ_{max} at 242 nm (ϵ = 10,425). The ^{1}H NMR spectrum of **8** features a signal (ddd, J = 10.7, 9.6 and 5.2 Hz) at δ 3.48 ppm and is consistent in chemical shift and coupling pattern with that of the 7α-proton.

As mentioned above, all of our attempts to remove the C-17 ester group by mild aqueous hydrolysis failed because of the ease at which the C-7 hydroxy group is eliminated to give the $\Delta^{4,6}$-3-ketone. Enzymatic hydrolysis using porcine lipase resulted in smooth conversion of **8** to **9** in 75% yield.

Table 1 ^{13}C Chemical shifts (σ ppm) of testosterone and 7β-hydroxytestosterone[a] (**9**)

Carbon	Testosterone[b]	Compound **9**	Δ[σ] (**9**-Testosterone)
1	35.6	35.6	0
2	33.8	33.9	0.1
3	199.4	199.4	0
4	123.6	124.7	1.1
5	171.0	167.5	3.5
6	32.7	42.2	9.5
7	31.5	74.9	43.4
8	35.0	43.0	8.0
9	53.9	49.8	−4.1
10	38.6	38.0	−0.6
11	21.2	20.5	−0.7
12	37.1	36.3	0.8
13	43.2	43.5	0.3
14	51.1	50.7	−0.4
15	23.8	26.3	2.5
16	30.7	30.7	0
17	81.3	81.1	−0.2
18	11.3	11.1	−0.2
19	17.3	17.3	0

[a]Spectra taken in $CDC1_3$.
[b]Blunt and Slothers[16].

The ^{1}H NMR spectrum of **9** features the absence of the ester group and shows the expected signal (dd, J = 8.5, 8.1 Hz) at δ 3.46 ppm corresponding to the proton (H-17α) geminal to the hydroxyl group. The rest of the ^{1}H NMR spectrum is otherwise similar to that of **8**. The ^{13}C NMR spectrum of **9** is shown in Table 1. Assignment of peaks in the ^{13}C NMR spectrum of **9** was aided by the DEPT spectrum and comparison to the reported ^{13}C NMR spectrum of testosterone.[16] Chemical shift differences between the ^{13}C NMR spectrum of **9** and that reported for testosterone are consistent with those expected for 7β-hydroxytestosterone.

In all, the synthesis of 7β-hydroxytestosterone was achieved in a 17% yield in eight steps from commercially available starting materials. Enzymatic transformations using cholesterol oxidase and porcine lipase were used in the later steps to accommodate chemically sensitive functionality and were essential to the synthesis of this otherwise difficult to obtain steroid.

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