The Hydrogenation of α-Hydroxymethylene-ketone Derivatives to α-Hydroxymethyl-ketone Derivatives with a Cell-Free System of *Streptomyces cinereocrocatus*

Taiko ODA, Rumiko SATO, and Yoshihiro SATO*

Kyoritsu College of Pharmacy, Shibakoen 1-chome, Minato-ku, Tokyo 105, Japan. *Received July 26, 1988*

2′,3′-Dihydro-5′-formylgriseofulvin (3), oxymetholone (5), and 3β-acetoxy-16-acetoxymethylene-5-androsten-17-one (9) were used as the substrates of a cell-free system from *Streptomyces cinereocrocatus* NRRL 3443. The results indicated that *S. cinereocrocatus* contains enzymatic activities which reduce the above three α-hydroxymethylene-ketones to the corresponding α-hydroxymethyl-ketones.

Keywords cell-free system; *Streptomyces cinereocrocatus* NRRL 3443; oxymetholone; enzymatic conversion; hydrogenation; stereospecific; α-hydroxymethylene-ketone

In the preceding paper[1] of this series, the stereochemistry of enzymatic transformation of 5′-formylgriseofulvin (**1**) to 5′α-hydroxymethylgriseofulvin (**2**) by a cell-free system of *Streptomyces cinereocrocatus* was elucidated.

In this work, we selected the three kinds of α-hydroxymethylene-ketone derivatives (**3**, **5**, and **9**) as substrates, in order to survey the substrate range of the cell-free system of *S. cinereocrocatus*, and incubations were performed under the same conditions as described previously.[2] Compound **3** corresponds to the 2′,3′-dihydroderivative of **1**. Compounds **5** and **9** are steroids with an α-hydroxymethylene-ketone group at the A and D rings, respectively. The results indicated that all of the enzymatic transformation products were α-hydroxymethyl-ketone derivatives.

Results and Discussion

Enzymatic Transformation of 2′,3′-Dihydro-5′-formylgriseofulvin by a Cell-Free System of *Streptomyces cinereocrocatus* Firstly, in order to examine the enzymatic activity, 2′,3′-dihydro-5′-formylgriseofulvin (**3**) was synthesized from 5′-formylgriseofulvin (**1**) by catalytic hydrogenation. After incubation of **3** with a cell-free system under the same conditions as described previously,[2] the transformation product was separated by column chromatography on silica gel and purified by recrystallization from acetone. Mass spectrometric (MS) analysis (molecular weight: *m/z* 384) of the product (**4**) indicated that dihydrogenation of the substrate had occurred. Analysis of the proton nuclear magnetic resonance (^{1}H-NMR) spectrum of **4** revealed loss of the 5′-hydroxymethylene proton (8.17 ppm) and 7′-hydroxy proton (15.20 ppm) signals of **3**, and methylene proton signals of a hydroxymethyl group at 3.58 and 4.08 ppm newly appeared. Thus, **4** was identified as a 5′-hydroxymethyl derivative. The configuration of the 5′-hydroxymethyl group of the product was determined to be 5′α by comparison of the MS, ^{1}H-NMR and melting point data with those of an authentic sample (**4**) which was synthesized by the catalytic hydrogenation of 5′α-hydroxymethylgriseofulvin (**2**).[1] The formation of 2′,3′-dihydro-5′α-hydroxymethylgriseofulvin (**4**) indicates that the cell-free system of *S. cinereocrocatus* has the capacity to reduce 2′,3′-dihydro-5′-formylgriseofulvin (**3**) as well as 5′-formylgriseofulvin (**1**).

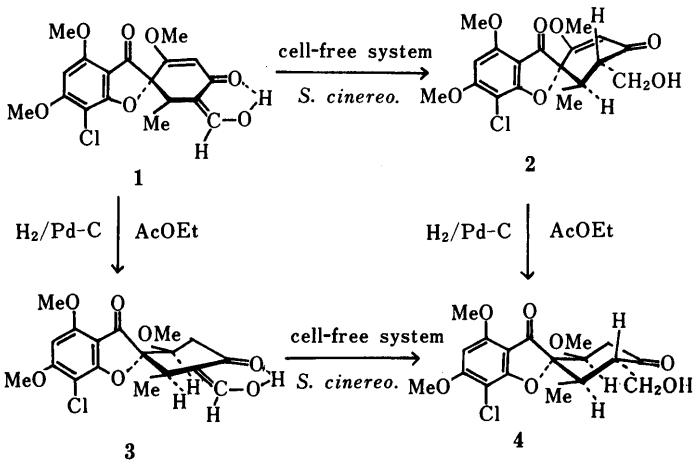
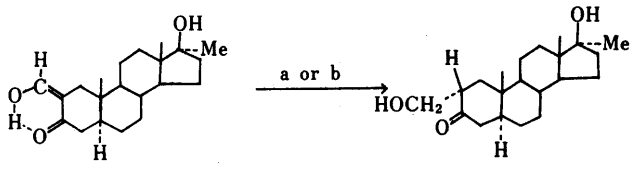


Chart 1

Enzymatic Transformation of Oxymetholone by a Cell-Free System of *Streptomyces cinereocrocatus* Oxymetholone (**5**) was selected as a substrate because it has an α-hydroxymethylene-ketone group on the six-membered A ring of a steroid skeleton, and has biological activity.[3] The incubation conditions were the same as described above, and the transformation product (**6**) was separated by column chromatography on silica gel, and purified by recrystallization from acetone. The molecular weight (*m/z* 334) of **6** was consistent with dihydrogenation of the substrate. Analysis of the ^{1}H-NMR spectrum of **6** revealed that the 2-hydroxymethylene proton signal (8.63 ppm) and its hydroxy proton signal (14.37 ppm) of **5** had disappeared and the methylene proton signals of a hydroxymethyl group in the 3.60—3.74 ppm region newly appeared in the product (**6**). Thus **6** was identified as a 2-hydroxymethyl derivative. The configuration of the 2-hydroxymethyl group of **6** was determined to be α, since the coupling constants between a proton at C-2 and protons at C-1 were 13.0 and 6.1 Hz under the irradiation of the methylene protons of the 2-hydroxymethyl group of **6**. The results indicated that a cell-free system of *S. cinereocrocatus* can reduce oxymetholone (**5**) to 17β-hydroxy-2α-hydroxymethyl-17α-methyl-5α-androstan-3-one (**6**). Moreover, the above result is reminiscent of the result of Manson *et al.*,[4] who found that 2-hydroxymethylene-3-keto steroids were transformed to 2α-hydroxymethyl derivatives by microbial reduction with *Rhizopus stolonifer*. On the

other hand, treatment of **5** with sodium borohydride in methyl alcohol afforded a product which was determined to be **6** by MS and ^{1}H-NMR analyses.

Enzymatic Transformation of the Acetate of 3β-Hydroxy-16-formyl-5-androsten-17-one by a Cell-Free System of *Streptomyces cinereocrocatus* 3β-Hydroxy-16-formyl-5-androsten-17-one (**7**) was selected as a further substrate, since it has an α-hydroxymethylene-ketone group on a five-membered ring. Compound **7** was synthesized from dehydroepiandrosterone acetate (**8**) by treatment with sodium methoxide in methyl formate, and its molecular weight (*m/z* 316) was consistent with the hydroxymethylene derivative of **8**. Analysis of the ^{1}H-NMR spectrum of **7** revealed that it showed signals at 7.07 and 9.77 ppm, indicating that **7** exists in two forms, the 16-hydroxymethylene and 16-formyl compounds, in deuteriochloroform, in a ratio of about 2 : 1. Moreover, the acetate (**9**) of **7** was used as a substrate since it dissolves in acetone. The incubation of **9** afforded a product which was separated by column chromatography on silica gel and purified by recrystallization from methanol. Analysis of the ^{1}H-NMR spectrum of the product revealed that the 16-hydroxymethylene proton (8.12 ppm) and methyl proton signals of two acetyl groups (2.23 and 2.04 ppm) of the substrate (**9**) had disappeared and methylene proton signals of a hydroxymethyl group newly appeared at 3.75 and 3.92 ppm. Thus, the enzymatic transformation product (**10**) was concluded to be the 16-hydroxymethyl derivative. The configuration of the 16-hydroxymethyl group was determined to be 16α, since no nuclear Overhauser effect (NOE) was observed between the methylene protons of the 16-hydroxymethyl group and the 18-methyl protons. On the other hand, the ultraviolet (UV) maximum of **10** at 240 nm, suggested a 3-keto-4-ene derivative. In a comparison of **10** and androst-5-ene-3,17-dione (**11**), the UV spectra and the chemical shifts of the 4-proton, and 18- and 19-methyl protons in the ^{1}H-NMR spectra showed high coincidence. Therefore, **10** was determined to be 16α-hydroxymethyl-4-androstene-3,17-dione. Moreover, treatment of **7** with sodium borohydride in methyl alcohol afforded a reduction product (**12**). Since NOE was not observed between the methylene protons of the 16-hydroxymethyl group and the 18-methyl protons of **12** in the NOE difference spectrum, **12** was determined to be the 16α-hydroxymethyl derivative. The present results demonstrate that the micro-organism, *Streptomyces cinereocrocatus* NRRL 3443, has enzymatic activities to reduce the three α-hydroxymethylene-ketones to the corresponding α-hydroxymethyl-ketones. This enzyme(s) may be applicable for the stereospecific transformation of other compounds which are not readily susceptible to chemical reagents.



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a : cell-free system of *S. cinereocrocatus*
b : $NaBH_4$ in MeOH

Chart 2

Experimental

Apparatus All melting points were obtained on a Shimadzu MM2 micro-melting point apparatus, and are uncorrected. ^{1}H-NMR spectra and NOE difference spectra were obtained at 270 MHz on a JEOL JNM-GX 270 FT NMR spectrometer. All ^{1}H-NMR data were recorded in deuteriochloroform and are reported as parts per million downfield from Me_4Si (δ=0). Abbreviations used: s=singlet, d=doublet, br=broad, m=multiplet, dd=doublet of doublets, ddd=doublet of doublets of doublets, q=quartet. The MS and high-resolution MS (High MS) were recorded on a JEOL JMS-DX303 mass spectrometer at 70 eV ionizing potential. The UV spectra were recorded on a JEOL UVIDEC 430B double-beam spectrophotometer. Column chromatography was performed with Kanto Kagaku silica gel (100 mesh). The thin layer chromatography (TLC) plates (precoated TLC plates, Silica Gel 60F-254, Merck)

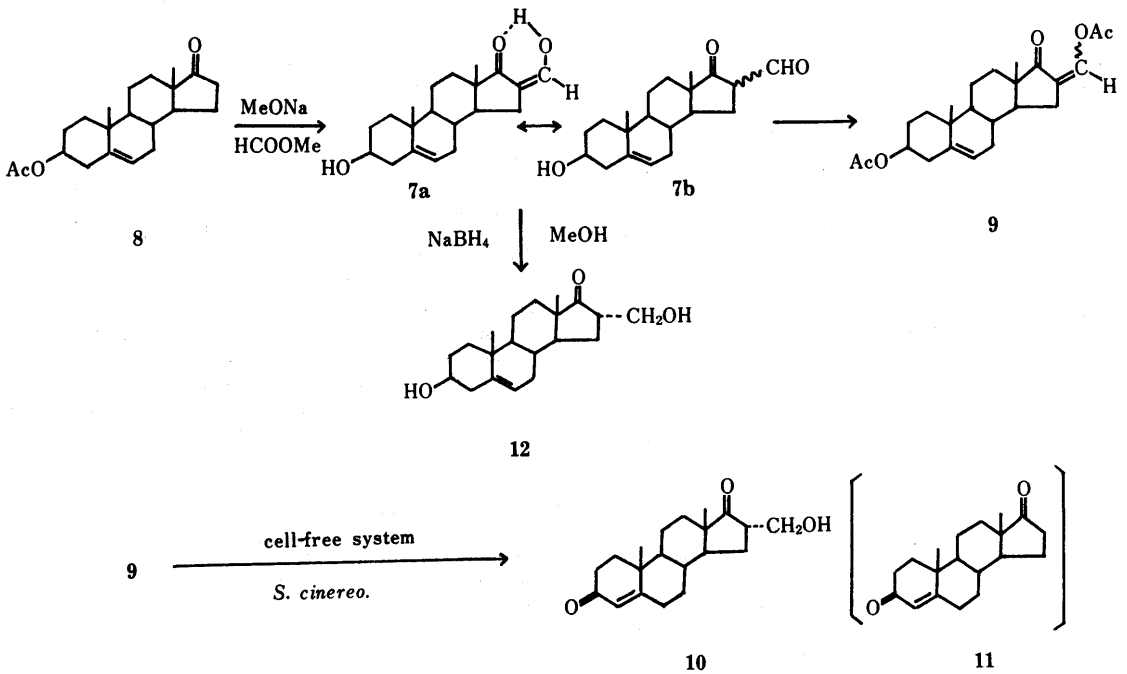

Chart 3

were developed in benzene–acetone (7:3, v/v). The compounds were visualized under UV light and/or by spraying with concentrated H_2SO_4 and heating on an electric heater. pH values were recorded on a LAB-O-MATE (Beckman-Toshiba, Ltd.).

Materials Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Boehringer Mannheim GmbH. All other reagents were purchased from commercial sources and were of analytical grade.

Preparation of the Cell-Free System The preparation of the cell-free system of *S. cinereocrocatus* was undertaken as described in the previous report.[2]

Determination of Protein Protein concentration of the cell-free extract was determined by the method of Lowry *et al.*[5] with bovine serum albumin as a standard.

Synthesis of 2′,3′-Dihydro-5′-formylgriseofulvin (3) as a Substrate A suspension of 5% palladium-charcoal catalyst (100 mg) in an ethyl acetate solution (100 ml) of 5′-formylgriseofulvin (**1**) (200 mg, 0.5 mmol) was shaken under a stream of hydrogen at atmospheric pressure and at room temperature. The hydrogenation was stopped after 65 min, the catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. The residue, which was found to be a 73:27 mixture of the starting material and a product by ^{1}H-NMR analysis, was chromatographed on silica gel (20 g). 1) Elution with benzene–methylene chloride (60:40) and recrystallization of the product from benzene gave 2′,3′-dihydro-5′-formylgriseofulvin (**3**) as colorless needles, mp 88—89 °C. *Anal.* Calcd for $C_{18}H_{19}ClO_7 \cdot C_6H_6$: C, 62.54; H, 5.47. Found: C, 61.35; H, 5.25. MS *m/z*: 382 (M^+) (for the ^{35}Cl-compound), 350, 255, 254, 214 (base peak). ^{1}H-NMR δ(ppm): 1.05 (3H, d, J=6.6 Hz, 6′-CH_3), 2.93 (1H, dd, J=18.7, 7.1 Hz, 3′α-H), 3.11 (1H, dd, J=18.7, 10.6 Hz, 3′β-H), 3.20 (1H, br d, J=6.8 Hz, 6′α-H), 3.35 (3H, s, 2′β-OCH_3),3.95 (3H, s, 4-OCH_3), 3.96 (1H, dd, J=10.7, 7.2 Hz, 2′α-H), 4.02 (3H, s, 6-OCH_3), 6.09 (1H, s, 5-H), 7.36 (5.2H out of 6H, s, benzene of solvate), 8.17 (1H, d, J=6.6 Hz, =C<u>H</u>OH), 15.20 (1H, d, J=6.6 Hz, =CHO<u>H</u>). 2) Elution with benzene–methylene chloride (50:50) and recrystallization of the product from benzene gave the starting material (**1**), as pale yellow needles, mp 196—197 °C.

Transformation of 2′,3′-Dihydro-5′-formylgriseofulvin (3) to 2′,3′-Dihydro-5′α-hydroxymethylgriseofulvin (4) by a Cell-Free System The incubation and separation were carried out essentially as described in the previous paper[2] except that 30 mg of 2′,3′-dihydro-5′-formylgriseofulvin (**3**) was used as a substrate in the cell-free system (150 ml). Column chromatography of the residue on silica gel (7 g) and recrystallization of the product from acetone gave 4.2 mg of 2′,3′-dihydro-5′α-hydroxymethylgriseofulvin (**4**), mp 195—196 °C. *Anal.* Calcd for $C_{18}H_{21}ClO_7$: C, 56.18; H, 5.50. Found: C, 56.27; H, 5.52. MS *m/z*: 384 (M^+) (for the ^{35}Cl-compound), 255 (base peak), 130. ^{1}H-NMR δ(ppm): 0.94 (3H, d, J=6.6 Hz, 6′-CH_3), 2.29 (1H, dd, J=8.3, 6.3 Hz, 7′-OH), 2.43 (1H, dq, J=12.9, 6.6 Hz, 6′α-H), 2.81 (1H, dd, J=13.0, 5.6 Hz, 3′α-H), 3.23 (1H, br ddd, J=12.9, 6.1, 4.6 Hz, 5′β-H), 3.29 (3H, s, 2′β-OCH_3), 3.37 (1H, dd, J=13.0, 12.8 Hz, 3′β-H), 3.58 (1H, ddd, J=12.0, 8.3, 4.6 Hz, 7′-H), 3.84 (1H, dd, J=12.8, 5.6 Hz, 2′α-H), 3.98 (3H, s, 4-OCH_3), 4.03 (3H, s, 6-OCH_3), 4.08 (1H, ddd, J=12.0, 6.3, 6.1 Hz, 7′-H), 6.11 (1H, s, 5-H).

The Synthesis of 2′,3′-Dihydro-5′α-hydroxymethylgriseofulvin (4) as a Standard Sample 2′,3′-Dihydro-5′α-hydroxymethylgriseofulvin was synthesized from 5′α-hydroxymethylgriseofulvin as described previously.[1]

The Extraction of Oxymetholone (5) Anadrol tablets (200 mg in 40 tablets; Shionogi) were suspended in water (600 ml). The insoluble material was obtained by filtration and dissolved in $CHCl_3$ (100 ml) and the $CHCl_3$ extract was washed with water, dried (Na_2SO_4), and concentrated *in vacuo* (yield, 128 mg). The extracted product was recrystallized from acetone to give colorless needles of oxymetholone (17β-hydroxy-2-hydroxymethylene-17α-methyl-5α-androstan-3-one), mp 180—182 °C (lit., mp 178—181 °C).[6] *Anal.* Calcd for $C_{21}H_{32}O_3$: C, 75.86; H, 9.70. Found: C, 75.90; H, 9.70. MS *m/z*: 332 (M^+), 274, 174, 79 (base peak). ^{1}H-NMR δ(ppm): 0.79 (3H, s, 18-CH_3), 0.87 (3H, s, 19-CH_3), 1.22 (3H, s, 17-CH_3), 1.15—1.85 (17H, m, methylenes, methines, and 17-OH), 1.99 (1H, br d, J=14.2 Hz, 1-H), 2.2—2.3 (2H, m, 4α, 4β-H), 2.34 (1H, br d, J=14.2 Hz, 1-H), 8.63 (1H, br s, 2-C<u>H</u>OH), 14.37 (1H, br s, 2-CHO<u>H</u>).

$NaBH_4$ Reduction of Oxymetholone (5) A solution of oxymetholone (**5**) (332 mg, 1 mmol) and $NaBH_4$ (50 mg, 1.3 mmol) in MeOH (20 ml) was stirred for 100 min at room temperature. The reaction mixture was diluted with ice-water, adjusted to pH 7 with 0.5 N HCl and then extracted with $CHCl_3$ (70 ml × 3). The chloroform solution was washed with water, dried (Na_2SO_4) and concentrated *in vacuo* to give a residue (425 mg). The ratio of the starting material and the product (**6**) in the reaction mixture was found to be 75:25 by ^{1}H-NMR analysis. The mixture in benzene was chromatographed on silica gel (30 g). Elution with benzene–methylene chloride (10:90) and recrystallization of the residue from benzene gave the starting material as colorless needles, mp 180—182 °C. Elution with methylene chloride–methanol (99:1) and recrystallization from $CHCl_3$ gave 17β-hydroxy-2α-hydroxymethyl-17α-methyl-5α-androstan-3-one (**6**) as colorless needles, mp 199—200 °C. *Anal.* Calcd for $C_{21}H_{34}O_3$: C, 75.40; H, 10.25. Found: C, 75.52; H, 10.19. MS *m/z*: 334 (M^+), 316, 277, 154 (base peak). ^{1}H-NMR δ(ppm): 0.88 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 1.21 (3H, s, 17-CH_3), 1.20—2.05 (19H, m, methylenes, methines and 17-OH), 2.10 (1H, dd, J=14.2, 3.6 Hz, 4α-H), 2.35 (1H, dd, J=14.2, 14.2 Hz, 4β-H), 2.58 (1H, m, 2β-H), 2.70 (1H, dd, J=8.3, 5.3 Hz, 2-CH_2O<u>H</u>), 3.60—3.74 (2H, m, 2-C<u>H</u>$_2$OH).

Transformation of Oxymetholone (5) to the 2α-Hydroxymethyl Derivative (6) by a Cell-Free System The incubation and separation were carried out essentially as described in the previous paper[2] except that 30 mg of oxymetholone (**5**) was used as a substrate in the cell-free system (150 ml). Column chromatography of the residue from the incubated mixture on silica gel (20 g) and recrystallization of the material from acetone gave 17β-hydroxy-2α-hydroxymethyl-17α-methyl-5α-androstan-3-one, mp 199—200 °C. The MS and ^{1}H-NMR data were identical with those of a standard sample (**6**).

Synthesis of 3β-Acetoxy-16-acetoxymethylene-5-androsten-17-one (9) as a Substrate Freshly prepared sodium methoxide (600 mg, 11 mmol) was cautiously added, with stirring and external cooling, to 10 ml of methyl formate. 3β-Acetoxy-5-androsten-17-one (**8**) (1g, 2.9 mmol) was added portionwise over a 15-min period and the resultant thick white slurry was stirred at room temperature. After 4 d, the reaction mixture was diluted with ice-water (50 ml), and adjusted to pH 7 with 0.5 N HCl. The precipitate (421 mg) was collected by filtration and recrystallized from $CHCl_3$ to give an isomer mixture (320 mg) of 3β-hydroxy-16-hydroxymethylene-5-androsten-17-one (**7a**) and 16-formyl-3β-hydroxy-5-androsten-17-one (**7b**) as colorless needles, mp 235—236 °C. MS *m/z*: 316 (M^+), 298, 69 (base peak). ^{1}H-NMR δ(ppm): 0.97 (2H, s, 18-CH_3), 0.98 (1H, s, 18-CH_3), 1.04 (1H, s, 19-CH_3), 1.05 (2H, s, 19-CH_3), 1.1—2.5 (18H, m, methylenes and methines), 3.39 (0.2H, dd, J=9.2, 9.2 Hz, 16-H), 3.52 (1H, m, 3α-H), 5.39 (1H, br s, 6-H), 7.07 (0.7H, s, =C<u>H</u>OH), 9.77 (0.3H, s, –C<u>H</u>O). The isomer mixture (243 mg), pyridine (1 ml) and Ac_2O (1 ml) were left to stand overnight at room temperature. The reaction mixture was poured into ice-water, and extracted with chloroform (100 ml). The chloroform extract was washed with water, dried (Na_2SO_4) and concentrated in *vacuo*. The residue (271 mg) was recrystallized from MeOH to give 3β-acetoxy-16-acetoxymethylene-5-androsten-17-one (**9**), as colorless needles, mp 193—194 °C. *Anal.* Calcd for $C_{24}H_{32}O_5$: C, 71.97; H, 8.05. Found: C, 72.08; H, 7.98. MS *m/z*: 386 ($M^+ - CH_3$), 340 (base peak), 298, 69. ^{1}H-NMR δ(ppm): 0.92 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 2.04 (3H, s, 3-$OCOCH_3$), 2.23 (3H, s, 16-$CHOCOCH_3$), 1.10—2.80 (17H, m, methylenes and methines), 4.60 (1H, m, 3α-H), 5.42 (1H, br d, J=4.62 Hz, 6-H), 8.12 (1H, dd, J=2.97, 1.98 Hz, 16-C<u>H</u>OCOCH$_3$).

Transformation of 3β-Acetoxy-16-acetoxymethylene-5-androsten-17-one (9) by a Cell-Free System The incubation and separation were carried out essentially as described in the previous paper[2] except that 30 mg of 3β-acetoxy-16-acetoxymethylene-5-androsten-17-one (**9**) was used as a substrate in the cell-free system (150 ml). Column chromatography of the residue on silica gel (20 g) and recrystallization of the material from MeOH afforded 16α-hydroxymethyl-4-androstene-3,17-dione (**10**) as colorless needles, mp 186—187 °C. UV λ_{max}^{MeOH} nm(ε): 240 (281,600). ^{1}H-NMR δ(ppm): 0.92 (3H, s, 18-CH_3), 1.22 (3H, s, 19-CH_3), 1.00—2.51 (19H, m, methylenes and methines), 3.75 (1H, m, 20-H), 3.92 (1H, m, 20-H), 5.76 (1H, s, 4-H). High MS Calcd for $C_{20}H_{28}O_3$: 316.2039. Found: 316.2064.

Reduction of an Isomer Mixture of 16-Formyl-3β-hydroxy-5-androsten-17-one (7b) and 3β-Hydroxy-16-hydroxymethylene-5-androsten-17-one (7a) with $NaBH_4$ A suspension of an isomer mixture (**7**) (34 mg, 0.11 mmol) and $NaBH_4$ (5 mg, 0.12 mmol) in MeOH (2 ml) was stirred for 120 min at room temperature. The reaction mixture was diluted with ice-water, adjusted to pH 7 with 0.5 N HCl and then extracted with $CHCl_3$ (60 ml × 3). The $CHCl_3$ solution was washed with water, dried (Na_2SO_4) and concentrated *in vacuo*. The 72:28 mixture of the starting material was chromatographed on silica gel (10 g). Elution with benzene–methylene chloride (10:90) and recrystallization from $CHCl_3$ gave the starting material as colorless needles, mp 235—236 °C. Elution with methylene chloride–methanol (98:2) and recrystallization of the material from MeOH gave 3β-hydroxy-16α-hydroxymethyl-5-androsten-17-one (**12**) as colorless needles, mp 208—209 °C. MS *m/z*: 318 (M^+) (base peak), 300, 285, 91. ^{1}H-NMR δ (ppm): 0.89 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 1.00—2.45 (20H, m, methylenes and methines), 3.53 (1H, m, 3α-H), 3.75

(1H, dd, $J=10.89$, 6.26 Hz, 16-C$\underline{H}_2$OH), 3.89 (1H, dd, $J=10.89$, 5.61 Hz, 16-C$\underline{H}_2$OH), 5.38 (1H, br s, 6-H).

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