

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

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2',3'-Dihydro-5'-formylgriseofulvin (3), oxymetholone (5), and 3 β -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 paper¹⁾ 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 (¹H-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, ¹H-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).

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 ¹H-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 17 β -hydroxy-2 α -hydroxymethyl-17 α -methyl-5 α -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 2 α -hydroxymethyl derivatives by microbial reduction with *Rhizopus stolonifer*. On the

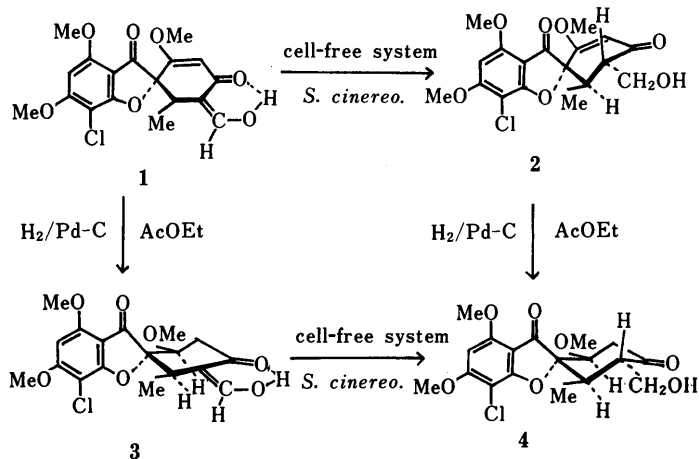


Chart 1

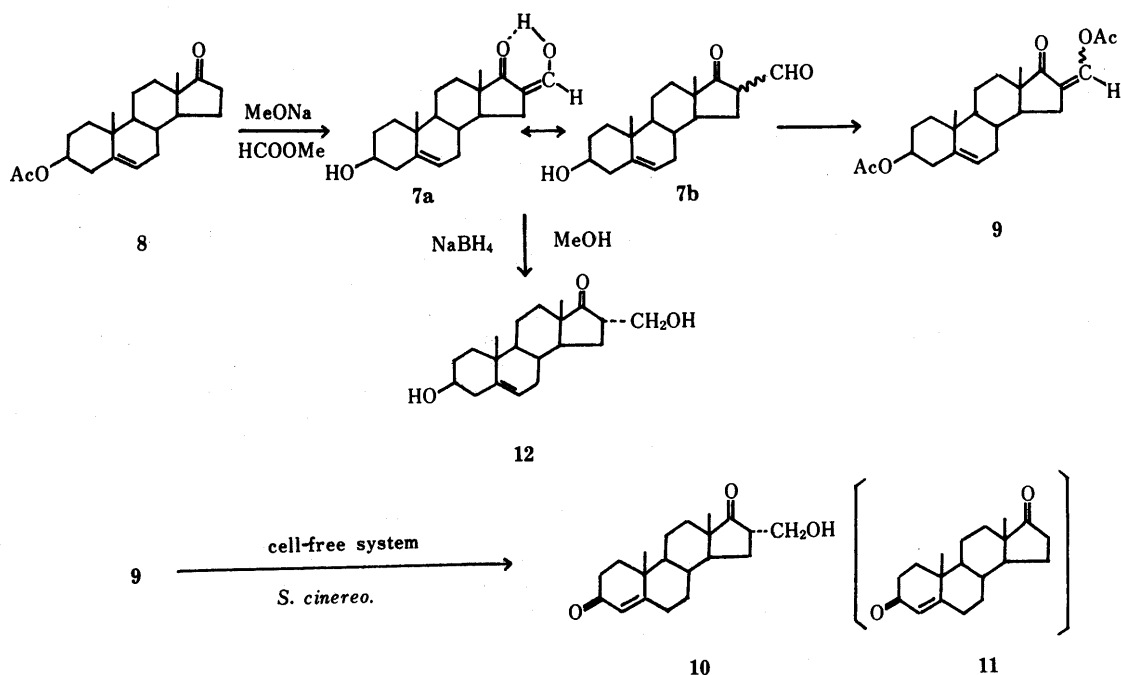
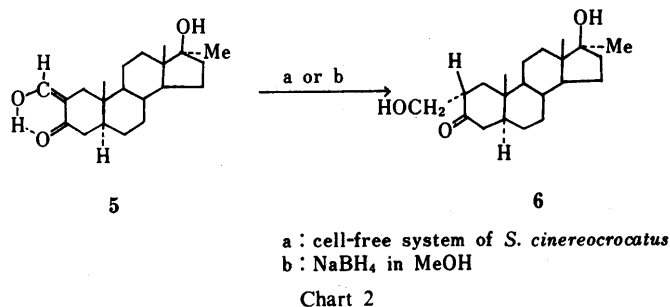
other hand, treatment of **5** with sodium borohydride in methyl alcohol afforded a product which was determined to be **6** by MS and $^1\text{H-NMR}$ analyses.

Enzymatic Transformation of the Acetate of 3β -Hydroxy-16-formyl-5-androsten-17-one by a Cell-Free System of *Streptomyces cinereocrocat* 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 $^1\text{H-NMR}$ 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 $^1\text{H-NMR}$ 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 trans-

formation 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 $^1\text{H-NMR}$ 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 cinereocrocat* 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.

Experimental

Apparatus All melting points were obtained on a Shimadzu MM2 micro-melting point apparatus, and are uncorrected. $^1\text{H-NMR}$ spectra and NOE difference spectra were obtained at 270 MHz on a JEOL JNM-GX 270 FT NMR spectrometer. All $^1\text{H-NMR}$ data were recorded in deuteriochloroform and are reported as parts per million downfield from Me_4Si ($\delta=0$). 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)



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. cinereocrocutus* 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 $^1\text{H-NMR}$ 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 $\text{C}_{18}\text{H}_{19}\text{ClO}_7 \cdot \text{C}_6\text{H}_6$: 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). $^1\text{H-NMR}$ δ (ppm): 1.05 (3H, d, $J=6.6$ Hz, 6'- CH_3), 2.93 (1H, dd, $J=18.7$, 7.1 Hz, 3'-H), 3.11 (1H, dd, $J=18.7$, 10.6 Hz, 3'-H), 3.20 (1H, brd, $J=6.8$ Hz, 6'-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'-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, $=\text{CHOH}$), 15.20 (1H, d, $J=6.6$ Hz, $=\text{CHOH}$). 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 paper²⁾ 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 $\text{C}_{18}\text{H}_{21}\text{ClO}_7$: 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. $^1\text{H-NMR}$ 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'-H), 2.81 (1H, dd, $J=13.0$, 5.6 Hz, 3'-H), 3.23 (1H, brddd, $J=12.9$, 6.1, 4.6 Hz, 5'-H), 3.29 (3H, s, 2'- β - OCH_3), 3.37 (1H, dd, $J=13.0$, 12.8 Hz, 3'-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'-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 (17 β -hydroxy-2-hydroxymethylene-17 α -methyl-5 α -androstan-3-one), mp 180–182°C (lit., mp 178–181°C).⁶⁾ *Anal.* Calcd for $\text{C}_{21}\text{H}_{32}\text{O}_3$: C, 75.86; H, 9.70. Found: C, 75.90; H, 9.70. MS m/z : 332 (M^+), 274, 174, 79 (base peak). $^1\text{H-NMR}$ 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, brd, $J=14.2$ Hz, 1-H), 2.2–2.3 (2H, m, 4 α , 4 β -H), 2.34 (1H, brd, $J=14.2$ Hz, 1-H), 8.63 (1H, brs, 2- CHOH), 14.37 (1H, brs, 2- CHOH).

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 0.5N HCl and then extracted with CHCl_3 (70 ml $\times$ 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 $^1\text{H-NMR}$ 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 17 β -hydroxy-2 α -hydroxymethyl-17 α -methyl-5 α -androstan-3-one (6) as colorless needles, mp 199–200°C. *Anal.* Calcd for $\text{C}_{21}\text{H}_{34}\text{O}_3$: C, 75.40; H, 10.25. Found: C, 75.52; H, 10.19. MS m/z : 334 (M^+), 316, 277, 154 (base peak). $^1\text{H-NMR}$ 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, 4 α -H), 2.35 (1H, dd, $J=14.2$, 14.2 Hz, 4 β -H), 2.58 (1H, m, 2 β -H), 2.70 (1H, dd, $J=8.3$, 5.3 Hz, 2- CH_2OH), 3.60–3.74 (2H, m, 2- CH_2OH).

Transformation of Oxymetholone (5) to the 2 α -Hydroxymethyl Derivative (6) by a Cell-Free System The incubation and separation were carried out essentially as described in the previous paper²⁾ 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 17 β -hydroxy-2 α -hydroxymethyl-17 α -methyl-5 α -androstan-3-one, mp 199–200°C. The MS and $^1\text{H-NMR}$ data were identical with those of a standard sample (6).

Synthesis of 3 β -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. 3 β -Acetoxy-5-androsten-17-one (8) (lg, 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 0.5N HCl. The precipitate (421 mg) was collected by filtration and recrystallized from CHCl_3 to give an isomer mixture (320 mg) of 3 β -hydroxy-16-hydroxymethylene-5-androsten-17-one (7a) and 16-formyl-3 β -hydroxy-5-androsten-17-one (7b) as colorless needles, mp 235–236°C. MS m/z : 316 (M^+), 298, 69 (base peak). $^1\text{H-NMR}$ 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, 3 α -H), 5.39 (1H, brs, 6-H), 7.07 (0.7H, s, $=\text{CHOH}$), 9.77 (0.3H, s, $=\text{CHO}$). 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 3 β -acetoxy-16-acetoxymethylene-5-androsten-17-one (9), as colorless needles, mp 193–194°C. *Anal.* Calcd for $\text{C}_{24}\text{H}_{32}\text{O}_5$: C, 71.97; H, 8.05. Found: C, 72.08; H, 7.98. MS m/z : 386 ($\text{M}^+ - \text{CH}_3$), 340 (base peak), 298, 69. $^1\text{H-NMR}$ 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, 3 α -H), 5.42 (1H, brd, $J=4.62$ Hz, 6-H), 8.12 (1H, dd, $J=2.97$, 1.98 Hz, 16- CHOCOCH_3).

Transformation of 3 β -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 paper²⁾ except that 30 mg of 3 β -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 16 α -hydroxymethyl-4-androstene-3,17-dione (10) as colorless needles, mp 186–187°C. UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm(ϵ): 240 (281,600). $^1\text{H-NMR}$ 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 $\text{C}_{20}\text{H}_{28}\text{O}_3$: 316.2039. Found: 316.2064.

Reduction of an Isomer Mixture of 16-Formyl-3 β -hydroxy-5-androsten-17-one (7b) and 3 β -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 0.5N HCl and then extracted with CHCl_3 (60 ml $\times$ 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 3 β -hydroxy-16 α -hydroxymethyl-5-androsten-17-one (12) as colorless needles, mp 208–209°C. MS m/z : 318 (M^+) (base peak), 300, 285, 91. $^1\text{H-NMR}$ 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, 3 α -H), 3.75

(1H, dd, $J=10.89, 6.26$ Hz, 16-CH₂OH), 3.89 (1H, dd, $J=10.89, 5.61$ Hz, 16-CH₂OH), 5.38 (1H, br s, 6-H).

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