Synthesis of Deuterium-Labeled 16α,19-Dihydroxy C_{19} Steroids as Internal Standards for Gas Chromatography-Mass Spectrometry

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[2β,7,7,16β-$^2H_4$]16α,19-Dihydroxyandrost-4-ene-3,17-dione (14) and [7,7,16β-$^2H_3$]3β,16α,19-trihydroxyandrost-5-en-17-one (16), with high isotopic purity, respectively, were synthesized from unlabeled 3β-(*tert*-butyldimethylsiloxy)-androst-5-ene-17β-yl acetate (1). The deuterium introduction at C-7 was carried out by reductive deoxygenation of the 7-keto compound 3 with dichloroaluminum deuteride and that at C-2β and/or C-16β by controlled alkaline hydrolysis of 16-bromo-17-ketone 11 or 12 with NaOD in D_2O and pyridine. [7,7-$^2H_2$]3β-Hydroxyandrost-5-en-17-one (6), obtained from compound 1 by a five-step sequence, was converted to compound 14 or 16 by an eight-step or seven-step sequence, respectively. The labeled steroids 14 and 16 are useful as internal standards for gas chromatography-mass spectrometry analysis of the endogeneous levels.

Keywords deuterium labeling; dichloroaluminum deuteride; reductive deoxygenation; controlled alkaline hydrolysis; deuterated sodium hydroxide; 16α,19-dihydroxyandrost-4-ene-3,17-dione; 3β,16α,19-trihydroxyandrost-5-en-17-one; internal standard; gas chromatography-mass spectrometry

19-Hydroxyandrost-4-ene-3,17-dione (19-OHA) has been reported to be an amplifier of aldosterone action and in itself a hypertensive steroid.[1] The concentrations of 19-OHA and androst-4-ene-3,17-dione (A) in plasma are raised in patients with hypertensive disease of pregnancy when compared with normotensive pregnant women at the same time of gestation.[2] At least part of the 19-OHA and A are derived from the placenta. 19-OHA is well known as an intermediate of estrone biosynthesis from A.[3] It has been suggested that in hypertensive disease of pregnancy the capacity of the placenta for the formation of estrogen from 19-OHA is reduced, leading to a rise in the concentration of 19-OHA and A.[4]

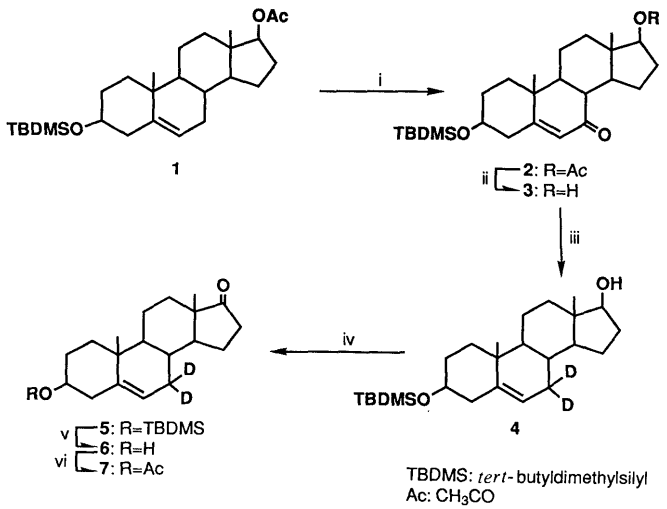
Estriol (E_3), the major estrogen in pregnancy, essentially originates in the placenta during aromatization of 16α-hydroxylated C_{19} steroid precursors, 16α-hydroxy A and 3β,16α-dihydroxyandrost-5-en-17-one.[5] The aromatization reaction to produce E_3 proceeds through their 19-hydroxylated intermediates, similar to the aromatization of A.[5c,d] It is suggested from these findings that the 16α,19-dihydroxy steroids may be found as steroid metabolites in the plasma of pregnant woman and may be involved in the regulation of blood pressure in pregnant subjects.

We are interested in the identification and quantitative analysis of the 16α,19-dihydroxy steroids in human, and therefore report here the synthesis of their deuterated forms as internal standards for gas chromatography-mass spectrometry.

Results and Discussion

The labeled 16α,19-dihydroxy steroids **14** and **16** were synthesized from [7,7-$^2H_2$]3β-hydroxyandrost-5-en-17-one (**6**) essentially according to the previously reported method[6] involving the addition of hypobromous acid, "hypoiodite reaction" (lead tetraacetate and iodine), and stereospecific alkaline hydrolysis of a 16-bromoketone as key reactions. We initially prepared the labeled steroid **6**, as a starting material, from 3β-(*tert*-butyldimethylsiloxy)androst-5-en-17β-yl acetate (**1**) (Chart 1). Reaction of steroid **1**, having two hydroxyl groups protected by acetyl and *tert*-butyldimethylsilyl (TBDMS) groups, with *tert*-butylhydroperoxide and pyridinium dichromate in the presence of celite[7] at room temperature, gave the 7-oxo derivative **2** in high yield. After alkaline hydrolysis of the acetate **2**, reductive deoxygenation of the 17β-hydroxy-7-keto-product **3** with dichloroaluminum deuteride (generated from lithium aluminum deuteride and aluminum chloride in ether)[8] yielded the [7,7-$^2H_2$]-labeled steroid **4** (78%). Jones oxidation of compound **4** followed by deprotection of the TBDMS group at C-3 with acid, and subsequent acetylation with acetic anhydride and pyridine, produced steroid **7**. Mass spectrometry (MS) analysis of compound **7** showed it to have very high isotopic purity with more than 95 atom% of the d_2-form (Table I) which is better than that (about 87 atom%) previously reported for the deoxygenation of 3β,17β-diacetoxyandrost-5-en-7-one.[9]

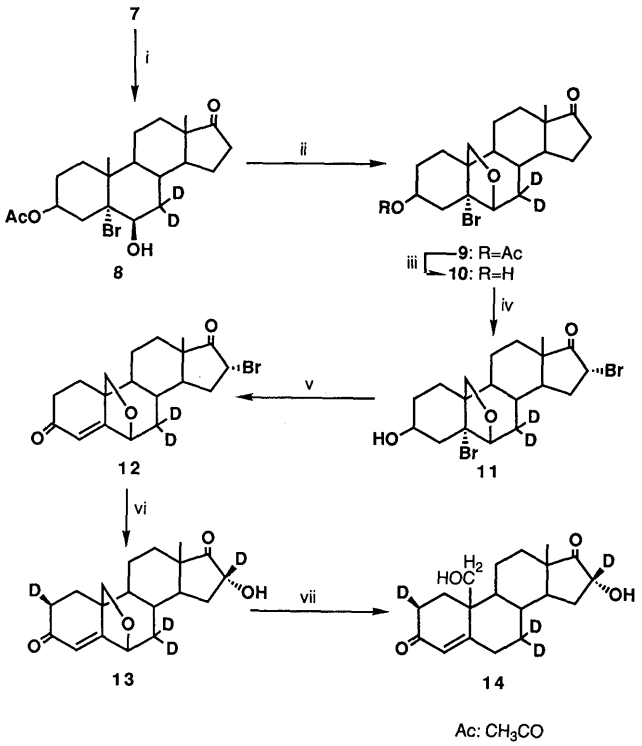
Treatment of the [7,7-$^2H_2$]3-acetate **7** with *N*-bromoacetamide (NBA) in the presence of 0.28 M $HClO_4$ in dioxane yielded the bromohydrin **8** (72%) which was



reagent i) pyridinium dichromate, *tert*-butylhydroperoxide, celite, benzene
ii) K_2CO_3, MeOH iii) $LiAlH_4$, $AlCl_3$, ether iv) Jones reagent, acetone
v) dil.HCl, THF, isopropanol vi) Ac_2O-pyridine

Chart 1

subsequently converted to the 6β,19-epoxide **9** with the "hypoiodite reaction" in high yield (Chart 2). After hydrolysis of the acetate **9** with K_2CO_3, the product, 3β-ol **10**, was subjected to bromination with 3 mol eq of $CuBr_2$ followed by Jones oxidation and HCl treatment to afford the 16α-bromo-4-ene-3-keto steroid **12** (44% from **10**). Hydrolysis of the bromoketone **12** with NaOD under controlled conditions, using D_2O–pyridine as a solvent,[10] gave stereospecifically the 16α-ketol **13**, in high yield, with deuterium atoms at the 2β, 7, and 16β positions. Reductive cleavage of the epoxide **13** with zinc dust in acetic acid and isopropanol led to the formation of the desired steroid **14**. MS analysis showed that compound **14** consists of 62.3 atom% of d_4 as a main species in which the d_0 and d_1 species were not detected; also, that deuterium atoms were not significantly replaced by hydrogens during the reduction (Table I). We have previously reported[10] that a deuterium atom is quantitatively incorporated in the C-16β position of a 16α-hydroxy-17-keto steroid upon treatment of the corresponding 16α-bromoketone with NaOD under controlled conditions. On



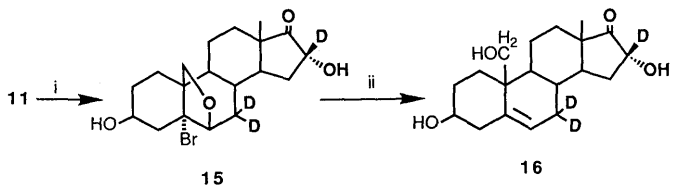
reagent i) NBA, $HClO_4$, dioxane ii) $Pb(OAc)_4$, I_2, cyclohexane iii) K_2CO_3, MeOH iv) $CuBr_2$, MeOH v) Jones reagent, acetone and then HCl vi) NaOD, D_2O, pyridine vii) Zn dust, isopropanol, AcOH

Chart 2

TABLE I. Deuterium Contents of Labeled 16α,19-Dihydroxy Steroids[a]

Compound		m/z	d_0	d_1	d_2	d_3	d_4	d_5	d_6
7	(M^+-60)	270	0	3.1	95.7	1.2	0	0	0
13	(M^+)	316	0	0	6.8	8.4	63.6	21.2	0
14	(M^+)	318	0	0	6.7	12.6	62.3	18.4	0
14[b]	(M^+-30)	432	0	0	6.6	10.6	62.4	19.4	0
16	(M^+-18)	302	0	0	5.2	92.4	2.4	0	0
16[b]	(M^+-90)	446	0	0	5.1	92.6	2.3	0	0

a) Steroids were directly analyzed by MS. *b*) After conversion to the trimethylsilyl ether derivative, deuterium content was obtained by a GC-MS system.



reagent i) NaOD, D_2O, pyridine ii) Zn dust, EtOH

Chart 3

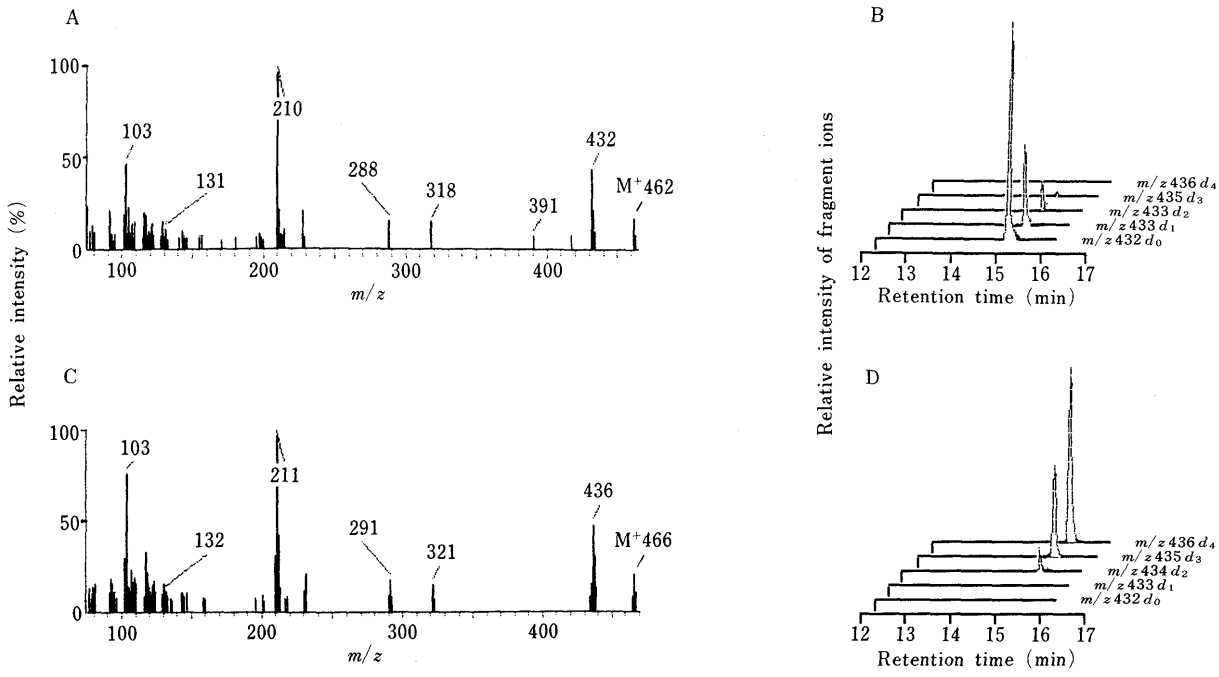


Fig. 1. Mass Spectra and Mass Fragmentography of the Trimethylsilyl Derivatives of Compound **14** (C and D) and Standard (Non-labeled) Steroid (A and B)

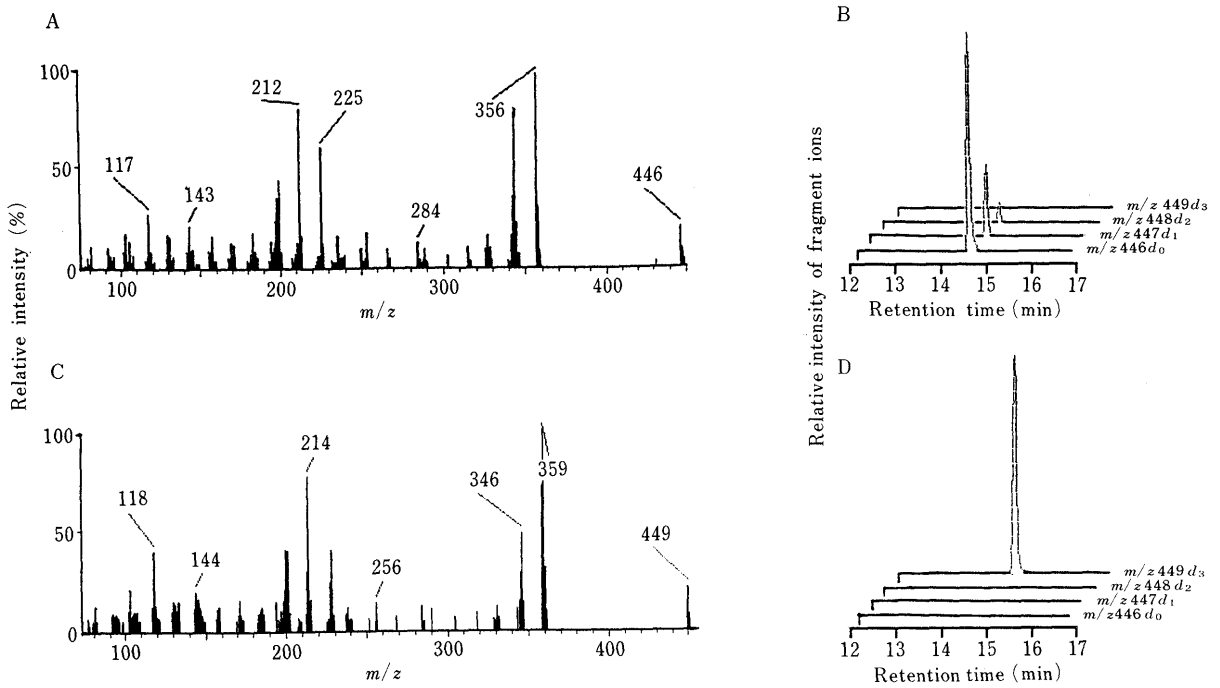


Fig. 2. Mass Spectra and Mass Fragmentography of the Trimethylsilyl Derivatives of Compound **16** (C and D) and Standard (Non-labeled) Steroid (A and B)

the other hand, since a 4-ene-3-ketone system having a 6β,19-epoxide substituent enolizes solely towards the C-2 position, another deuterium should be incorporated principally into the C-2β position through enolization[11] during the alkaline hydrolysis. The deuterium labeling was also established by ^{1}H-NMR analysis of steroid **14**; a signal of the 16β-proton at 4.40 ppm was not detected and a signal intensity of the 2β-proton at 2.28 ppm corresponded to about 0.3H, although the 2α-proton signal could not be identified. This also supports the finding that d_5-species (18.4%) would correspond to a [2,2,7,7,16β-$^2H_5$]-labeled molecule.

The bromoketone **11** was hydrolyzed under controlled conditions using NaOD as described above to yield [7,7,16β-$^2H_3$]16α-ketol **15**, of which treatment with zinc dust in EtOH afforded another desired compound **16** (49% from **11**) (Chart 3). The isotope purity of [7,7,16β-$^2H_3$]-steroid **16**, determined by MS analysis, was d_4, 2.4; d_3, 92.4; d_2, 5.2; and d_1 and d_0, 0%; a signal of the 16β-proton (δ 4.33 ppm) was not detected by ^{1}H-NMR spectroscopy.

The isotopic purity of steroids **14** or **16** is sufficient for use as an internal standard for GC-MS analysis of endogeneous plasma contents. The deuterium atoms, especially those labeled at the C-2 position, were not liberated, to a significant extent, during the pretreatment procedure (extraction and chromatography) for their assays. The mass spectra and mass fragmentograms of trimethylsilyl derivatives of compounds **14** and **16** are shown in Figs. 1 and 2, respectively (the detection limit: about 50 pg in every case). Our preliminary study of three subjects showed that pregnant women with 38—40 weeks gestation have serum levels of about 150 pg/ml of non-labeled (natural) steroid **14**, and the other natural steroid **16** could not be detected. Following more extensive research, a detailed discussion of our finding will be made.

Experimental

General Methods Melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. Infrared (IR) spectra were determined on a Perkin Elmer FT-IR 1725X spectrophotometer. ^{1}H-NMR spectra were obtained with a JEOL GX 400 (400 MHz) spectrometer using tetramethylsilane as an internal standard. Mass spectra were measured on a JEOL JMS-DX 303 spectrometer (direct mode) and a Hewlett Packard 5970B GC-MS system.

3β-(*tert*-Butyldimethylsiloxy)androst-5-en-17β-yl Acetate (1) Non-labeled 17β-ol (**4**) (10 g, 24.7 mmol), obtained according to the previously reported method,[12] was dissolved in pyridine (100 ml) and acetic anhydride (50 ml). The solution was allowed to stand at room temperature overnight and poured into ice-water (2 l). The precipitates were collected by filtration, dried under a vacuum, and recrystallized from acetone to give **1** (10.5 g, 95%) as colorless prisms, mp 140—141 °C. IR (KBr): 1734 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s, $OSiMe_2$), 0.83 (3H, s, 18-Me), 0.90 (9H, s, $OSi(Me_2)CMe_3$), 1.02 (3H, s, 19-Me), 2.04 (3H, s, 17-OCOMe), 3.54 (1H, m, 3α-H), 4.64 (1H, m, 17α-H), 5.36 (1H, m, 6-H). *Anal.* Calcd for $C_{27}H_{46}O_3Si$: C, 72.59; H, 10.38. Found: C, 72.71; H, 10.30.

3β-(*tert*-Butyldimethylsiloxy)-17β-acetoxyandrost-5-en-7-one (2) *tert*-Butylhydroperoxide (11.6 ml, 144 mmol) was added dropwise at 10 °C over a period of 20 min to a stirred mixture of steroid **1** (10.2 g, 22.9 mmol), celite (35 g), pyridinium dichromate (43 g, 115 mmol), and benzene (350 ml), and the reaction mixture was stirred at room temperature for 24 h. The resulting mixture was diluted with AcOEt (200 ml), filtered through a pad of celite and washed with AcOEt (200 ml). The filtrate was washed with 5% $NaHCO_3$ solution and water, dried (Na_2SO_4), and the solvent was evaporated to yield the crude product which was recrystallized from acetone to afford **2** (8.1 g, 77%) as colorless needles, mp 183—184 °C. ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s, $OSiMe_2$), 0.81 (3H, s, 18-Me), 0.90 (9H, s, $OSi(Me_2)CMe_3$), 1.20 (3H, s, 19-Me), 2.03 (3H, s, 17β-OCOMe), 3.64 (1H, m, 3α-H), 4.66 (1H, m, 17α-H), 5.71 (1H, s, 6-H). *Anal.* Calcd for $C_{27}H_{44}O_4Si$: C, 70.39; H, 9.63. Found: C, 70.35; H, 9.83.

3β-(*tert*-Butyldimethylsiloxy)-17β-hydroxyandrost-5-en-7-one (3) 2.5% K_2CO_3 solution (89 ml) was added to a solution of compound **2** (8.1 g, 17.7 mmol) in MeOH (320 ml) and the mixture was heated under reflux for 2 h. After this time, the mixture was neutralized with 5% HCl, concentrated under reduced pressure, poured into ice-water (600 ml) saturated with NaCl, and extracted with AcOEt (600 ml × 2). The combined organic layers were washed with 5% $NaHCO_3$ solution and water, then dried (Na_2SO_4). Evaporation of the solvent gave a solid

product which was recrystallized from acetone to yield **3** (6.6 g, 90%) as colorless plates, mp 166—167 °C. IR (KBr) 3432 (OH), 1672 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.07 (6H, s, $OSiMe_2$), 0.78 (3H, s, 18-Me), 0.91 (9H, s, $OSi(Me_2)C\underline{Me}_3$), 1.22 (3H, s, 19-Me), 3.37—3.94 (2H, m, 3α-H, 17α-H), 5.72 (1H, s, 6-H). *Anal.* Calcd for $C_{25}H_{42}O_3Si$: C, 71.72; H, 10.11. Found: C, 71.86; H, 10.12.

[7,7-$^2H_2$]3β-(*tert*-Butyldimethylsiloxy)androst-5-en-17β-ol (4) $LiAl^2H_4$ (2.0 g, 47.6 mmol) in dry ether (100 ml) under nitrogen was carefully treated with $AlCl_3$ (19 g, 142.8 mmol) in dry ether (300 ml) with cooling. The mixture was stirred for 30 min under reflux, cooled to room temperature and compound **3** (6.6 g, 15.8 mmol) in dry tetrahydrofuran (THF) (100 ml) was added dropwise to the mixture. After heating under reflux for 90 min, the reaction mixture was cooled and water was carefully added, diluted with AcOEt (500 ml). The organic layer was washed with 1% HCl, 5% $NaHCO_3$ solution, and water, successively, then dried (Na_2SO_4). Evaporation of the solvent afforded a solid which was recrystallized from acetone to give **4** (5.0 g, 78%) as colorless prisms, mp 172—173 °C (lit.[12] 171—172 °C for non-labeled form). IR (KBr): 3371 (OH) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s, $OSiMe_2$), 0.76 (3H, s, 18-Me), 0.89 (9H, s, $OSi(Me_2)C\underline{Me}_3$), 1.01 (3H, s, 19-Me), 3.48 (1H, m, 3α-H), 3.65 (1H, m, 17α-H), 5.31 (1H, d, J=1.5 Hz, 6-H).

[7,7-$^2H_2$]3β-(*tert*-Butyldimethylsiloxy)androst-5-en-17-one (5) Jones reagent (7.2 ml) was added to a solution of compound **4** (4.96 g, 12.2 mmol) in acetone (320 ml) and the mixture was stirred at 5 °C for 5 min. After the addition of MeOH (5 ml) to decompose the excess reagent, the resulting solution was poured into chilled water (3 l) saturated with NaCl. The precipitates were collected by filtration, dried under a vacuum, and recrystallization of the solid from MeOH gave **5** (3.84 g, 75%) as colorless prisms, mp 147—148 °C (lit.[12] 146—147 °C reported for non-labeled form). IR (KBr): 1748 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.06 (6H, s, $OSiMe_2$), 0.885 (3H, s, 18-Me), 0.893 (9H, s, $OSi(Me_2)C\underline{Me}_3$), 1.03 (3H, s, 19-Me), 3.49 (1H, m, 3α-H), 5.34 (1H, d, J=1.8 Hz, 6-H).

[7,7-$^2H_2$]3β-Hydroxyandrost-5-en-17-one (6) Compound **5** (3.65 g, 9.03 mmol) was dissolved in isopropanol (104 ml) and then THF (70 ml), then 3 M HCl (33 ml) was added to the solution and stirred at room temperature for 2 h. After this time, the mixture was diluted with AcOEt (600 ml), washed with 5% $NaHCO_3$ solution, and with saturated NaCl solution, and dried (Na_2SO_4). Evaporation of the solvent gave a solid which was recrystallized from MeOH to yield **6** (2.44 g, 93%) as colorless needles, mp 136—137 °C (lit.[13] 140—141 °C and 152—153 °C, dimorphous, reported for non-labeled form). IR (KBr): 3449 (OH), 1741 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, s, 18-Me), 1.04 (3H, s, 19-Me), 3.54 (1H, m, 3α-H), 5.38 (1H, d, J=1.8 Hz, 6-H).

[7,7-$^2H_2$]3β-Acetoxyandrost-5-en-17-one (7) A solution of compound **6** (2.0 g, 6.89 mmol) in pyridine (20 ml) and acetic anhydride (10 ml) was allowed to stand at room temperature overnight. After the usual work-up, the product obtained was recrystallized from MeOH to give **7** (1.87 g, 82%) as colorless needles, mp 165—167 °C (lit.[14] 167—169 °C for non-labeled form). IR (KBr): 1740, 1727 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, s, 18-Me), 1.05 (3H, s, 19-Me), 2.04 (3H, s, 3β-OCOMe), 4.60 (1H, m, 3α-H), 5.40 (1H, s, 6-H).

[7,7-$^2H_2$]5α-Bromo-6β-hydroxy-3β-acetoxyandrostan-17-one (8) NBA (1.4 g, 10.1 mmol) was added to a solution of compound **7** in 17 ml of dioxane containing 0.28 M $HClO_4$ (0.8 ml). The mixture was stirred at room temperature in the dark for 1 h, poured into ice-cold 10% $Na_2S_2O_3$ solution (500 ml), and extracted with AcOEt (300 ml × 3). The combined organic layers were washed with 5% $NaHCO_3$ solution and water and dried (Na_2SO_4). Concentration of the solvent under reduced pressure to about 50 ml yielded a solid product which was collected by filtration and recrystallized from acetone to afford **8** (1.65 g, 72%) as colorless needles, mp 179—182 °C (lit.[15] 173—175 °C for non-labeled form). IR (KBr): 3506 (OH), 1742, 1732 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, s, 18-Me), 1.35 (3H, s, 19-Me), 2.04 (3H, s, 3β-OCOMe), 4.23 (1H, s, 6α-H), 5.48 (1H, m, 3α-H).

[7,7-$^2H_2$]5α-Bromo-3β-acetoxy-6β,19-epoxyandrostan-17-one (9) A mixture of lead tetraacetic acid (6.0 g, 13.5 mmol), $CaCO_3$ (3.0 g, 30 mmol), and cyclohexane (400 ml) was heated under reflux for 20 min; compound **8** (1.6 g, 3.38 mmol) and iodine (1.7 g, 6.71 mmol) were then added simultaneously. The mixture was heated under reflux for 1 h and then filtered through a bed of celite, which was washed with hot cyclohexane. The filtrate was washed with 15% $Na_2S_2O_3$ solution (250 ml) and water and dried (Na_2SO_4). After removal of the solvent, the solid product was recrystallized from acetone–hexane to give **9** (1.31 g, 91%) as colorless prisms, mp 190—191 °C (lit.[16] 187—188 °C for non-labeled form). IR (KBr): 1737 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, s, 18-Me), 2.04 (3H, s, 3β-OCOMe), 3.74 (1H, d, J=8.4 Hz, 19-H_a), 3.97 (1H, d, J=8.4 Hz, 19-H_b), 4.11 (1H, s, 6α-H), 5.20 (1H, m, 3α-H).

[7,7-$^2H_2$]5α-Bromo-3β-hydroxy-6β,19-epoxyandrostan-17-one (10) 2.5% K_2CO_3 solution (13.5 ml) was added to a solution of compound **9** (1.25 g, 2.93 mmol) in MeOH (56 ml). The mixture was heated under reflux for 1 h, poured into ice-cold 5% HCl solution (300 ml), and extracted with AcOEt (300 ml × 3). The combined organic layers were washed with 5% $NaHCO_3$ solution and water and dried (Na_2SO_4). After the usual work-up, the solid product obtained was recrystallized from acetone to yield **10** (1.0 g, 89%) as colorless needles, mp 189—191 °C (lit.[17] 188 °C for non-labeled form). IR (KBr): 3446 (OH), 1740 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.91 (3H, s, 18-Me), 3.73 (1H, d, J=8.4 Hz, 19-H_a), 3.96 (1H, d, J=8.4 Hz, 19-H_b), 4.12 (1H, s, 6α-H), 4.15 (1H, m, 3α-H).

[7,7-$^2H_2$]5α,16α-Dibromo-3β-hydroxy-6β,19-epoxyandrostan-17-one (11) A solution of compound **10** (950 mg, 2.47 mmol) and $CuBr_2$ (1.67 g, 7.47 mmol) in 60 ml of MeOH was heated under reflux for 8 h. After evaporation of the solvent to about 30 ml, the mixture was poured into water (500 ml) and the precipitates were collected by filtration, dried under a vacuum, and then dissolved in 100 ml of AcOEt. After removal of the insoluble material by filtration, the organic layer was further washed with water and dried (Na_2SO_4). Evaporation of the solvent afforded a solid which was recrystallized from acetone to yield **11** (760 mg, 66%) as colorless needles, mp 206—208 °C (lit.[6] 197—198 °C for non-labeled form). IR (KBr): 3446 (OH), 1742 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 0.95 (3H, s, 18-Me), 3.71 (1H, d, J=8.4 Hz, 19-H_a), 3.97 (1H, d, J=8.4 Hz, 19-H_b), 4.12 (1H, s, 6α-H), 4.16 (1H, m, 3α-H), 4.54 (1H, d, J=7.0 Hz, 16β-H).

[7,7-$^2H_2$]16α-Bromo-6β,19-epoxyandrost-4-ene-3,17-dione (12) Compound **11** (200 mg, 0.43 mmol) was dissolved in 60 ml of acetone. Jones reagent (0.36 ml) was added dropwise to this solution with stirring at 0 °C and the mixture continued to be stirred at 0 °C for another 5 min. After this time, MeOH (2 ml) and 35% HCl (0.4 ml) were added; the mixture was stirred at room temperature overnight, poured into ice water (500 ml), and extracted with AcOEt (400 ml × 2). The combined organic layers were washed with 5% $NaHCO_3$ solution and water and dried (Na_2SO_4). Evaporation of the solvent gave a solid, which was recrystallized from acetone to afford **12** (110 mg, 67%) as colorless needles, mp 217—219 °C (lit.[10] 220—221 °C reported for non-labeled form). IR (KBr): 1747, 1677 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.01 (3H, s, 18-Me), 3.55 (1H, d, J=8.1 Hz, 19-H_a), 4.21 (1H, d, J=8.1 Hz, 19-H_b), 4.54 (1H, d, J=7.0, 16β-H), 4.75 (1H, s, 6α-H), 5.86 (1H, s, 4-H).

[2,7,7,16β-$^2H_4$]16α-Hydroxy-6β,19-epoxyandrost-4-ene-3,17-dione (13) A solution of compound **12** (90 mg, 0.24 mmol), pyridine (2.5 ml) and D_2O containing NaOH (11.3 mg, 0.28 mmol) was stirred at room temperature for 1 h, poured into 5% HCl (50 ml) saturated with NaCl, and extracted with AcOEt (50 ml × 3). The organic layer was washed with saturated $NaHCO_3$ solution and water, dried (Na_2SO_4), and evaporated to give an oil. Purification of the product with column chromatography (silica gel, 10 g; hexane : AcOEt = 1 : 2) and a subsequent recrystallization from acetone yielded **13** (55 mg, 73%) as colorless prisms, mp 205—207 °C (lit.[10] 207—209 °C reported for non-labeled form). IR (KBr): 3470 (OH), 1751, 1674 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.05 (3H, s, 18-Me), 3.54 (1H, d, J=8.1 Hz, 19-H_a), 4.22 (1H, d, J=8.1, 19-H_b), 4.74 (1H, s, 6α-H), 5.84 (1H, s, 4-H).

[2,7,7,16β-$^2H_4$]16α,19-Dihydroxyandrost-4-ene-3,17-dione (14) Zinc dust (350 mg) was added to a solution of compound **13** (40 mg, 0.13 mmol) in 4 ml of isopropanol and 0.3 ml of acetic acid and the mixture was heated under reflux with stirring. The suspension was filtered and the residue was washed with isopropanol. Removal of the solvent from the combined filtrates afforded an oily residue, which was dissolved in AcOEt (50 ml), washed with saturated NaCl solution, dried (Na_2SO_4), and evaporated to yield the crude product. This was purified by flash chromatography (silica gel, 6 g; hexane : AcOEt = 2 : 7) and recrystallized from acetone to give **14** (26 mg, 63%) as colorless needles, mp 185—186 °C (lit.[10] 189—190 °C reported for non-labeled form). IR (KBr): 3453 (OH), 1745, 1658 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.01 (3H, s, 18-Me), 3.94 (1H, dd, J=5.5, 10.3 Hz, 19-H_a), 4.06 (1H, d, J=10.6 Hz, 19-H_b), 5.96 (1H, d, J=1.1 Hz, 4-H).

[7,7,16β-$^2H_3$]5α-Bromo-3β,16α-dihydroxy-6β,19-epoxyandrostan-17-one (15) NaOH (7.0 mg, 0.41 mmol) solution in D_2O (1.5 ml) was added to a solution of compound **11** (160 mg, 0.34 mmol) in pyridine (5 ml) and the mixture was stirred at room temperature for 1 h. It was then poured

into 5% HCl (65 ml) saturated with NaCl and extracted with AcOEt (65 ml × 3). The organic layer was washed with 5% $NaHCO_3$ solution and water, dried (Na_2SO_4), and evaporated to give an oil. This was purified by column chromatography (silica gel, 9 g; hexane : AcOEt = 3 : 1) and recrystallized from acetone to give **15** (102 mg, 75%) as colorless prisms, 200—202 °C (lit.[10] 197—198 °C reported for non-labeled form). IR (KBr): 3435 (OH), 1752 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.00 (3H, s, 18-Me), 3.72 (1H, d, J=8.4 Hz, 19-H_a), 3.96 (1H, d, J=8.4, 19-H_b), 4.11 (1H, s, 6α-H), 4.14 (1H, m, 3α-H).

[7,7,16β-$^2H_3$]3β,16α,19-Trihydroxyandrost-5-en-17-one (16) A mixture of zinc dust (240 mg), compound **15** (80 mg, 0.20 mmol), and EtOH (6 ml) was heated under reflux with stirring for 3 h. A similar work-up as described in the synthesis of compound **14** gave the crude product, which was recrystallized from acetone to give **16** (42 mg, 65%) as colorless powder, mp 205—207 °C (lit.[10] 199—200 °C reported for non-labeled form). IR (KBr): 3446 (OH), 1734 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.04 (3H, s, 18-Me), 3.59 (1H, m, 3α-H), 3.61 (1H, d, J=11.4 Hz, 19-H_a), 3.89 (1H, d, J=11.4 Hz, 19-H_b), 5.78 (1H, d, J=1.5 Hz, 6-H).

Derivation for GC-MS Analysis The deuterated steroids were dissolved in 30 μl of *N,O*-bis-trimethylsilyltrifluoroacetamide : pyridine (1 : 1) and heated at 60 °C for 1 h. The excess of the reagent was removed under a stream of nitrogen, and the residue was dissolved in 10 μl of anhydrous hexane. One μl portion of the solution was subjected to GC-MS.

GC-MS Conditions Hewlett-Packard 5970B, EI-ionization at 70 eV, column: HP-1 fused silica capillary column crosslinked methyl silicone, 12 m × 0.2 i.d. mm, film thickness 0.3 μm, carrier gas: helium, 50 ml/min, temperature program: initial temperature 50 °C, program rate 35 °C/min to 100 °C and then 18 °C/min to 280 °C and, thereafter, the temperature was maintained at 280 °C.

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