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Synthesis of Deuterium-labelled Estriol, 16α-Hydroxyestrone and Estriol 16-Glucuronide *via* 2,4,16α-Tribromoestrone as Internal Standards for Mass Fragmentography

MITSUTERU NUMAZAWA,* MASAO NAGAOKA, and MIEKO OGATA

*Tohoku College of Pharmacy, 4-1 Komatsushima-4-chome,
Sendai, Miyagi 983, Japan*

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Synthesis of 1,3,5(10)-estratriene-3,16α,17β-triol-d_6 and -d_7 (**4**), 3,16α-dihydroxy-1,3,5(10)-estratrien-17-one-d_5 (**5**) and sodium 3,17β-dihydroxy-1,3,5(10)-estratrien-16α-yl-β-D-glucopyranosuronate-d_6 (**8**) is described. Treatment of 2,4,16α-tribromo-3-hydroxy-1,3,5(10)-estratrien-17-one (**1**) with sodium hydroxide-OD in deuterium oxide-pyridine under controlled conditions gave the [16β-^{2}H]16α-hydroxy-17-one (**2**). The ketol **2** was converted into the triol-d_6 (**4**) *via* sodium borodeuteride reduction in the presence of palladium chloride. Similar treatment of the 17-ethyleneacetal of **2** followed by acid hydrolysis gave compound **5**-d_5. Reaction of **2** with methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranosuronate using silver carbonate as a catalyst yielded the 16-monoglucuronide acetate methyl ester (**6**). The reductive removal of the bromines of **6** and subsequent alkaline hydrolysis gave the glucuronide-d_6 (**8**). Mass spectrometric analysis showed compounds **4**, **5**, and **8** to have good isotopic purity.

Keywords——controlled alkaline hydrolysis; deuterium labelling: 2,4,16α-tribromoestrone; 16α-hydroxyestrone; estriol; estriol 16-glucuronide

Combined gas chromatography-mass spectrometry (GC-MS) has recently been applied for quantitative evaluation of steroid hormones in biological fluids,[1] and deuterium-labelled compounds are essential as carriers and internal standards. Previously, hydrolysis of steroid conjugates was required prior to gas chromatographic analysis but recent reports[1,2] have demonstrated that all major steroid glucuronides can be analyzed intact by capillary GC when suitably protected by derivatization.

16α-Hydroxy estrogens, estriol (**4**)[3] and 16α-hydroxyestrone (**5**), are major estrogens in pregnancy and are mainly found as the 16-glucuronides in the urine[4] and the plasma.[5] The quantitative importance of the estrogens and the glucuronides in pregnancy and non-pregnancy is a well known.[4-6]

We recently developed a controlled stereospecific alkaline hydrolysis of 16-bromo-17-oxo androgens[7] as well as a hydrolytic method for the synthesis of several 16α-hydroxy-17-oxo steroids.[8] We report here an efficient synthesis of deuterium-labelled estriol (**4**), 16α-hydroxyestrone (**5**) and estriol 16-glucuronide (**8**) with good isotopic purity. Labelling experiments were undertaken with the intention of placing deuterium atoms at positions 2, 4, 16 and 17 of the estrogens. Previous work[8c,d] on the synthesis of 16α-hydroxy estrogens involved the controlled hydrolysis of the readily available 2, 4, 16α-tribromoestrone (**1**) to the 16α-hydroxide (**2**), which can be subsequently hydrodebrominated to estriol (**4**) or regiospecifically converted to the 16-glucuronide derivative **6**. The particular advantages in this procedure center on the possibility of achieving the desired deuterium-labelling during the steps of controlled hydrolysis, reductive removal of bromine and reduction of the carbonyl function.

Results and Discussion

When 2,4,16α-tribromoestrone (**1**) was subjected to controlled hydrolysis with 2.5 eq of sodium hydroxide-OD in deuterium oxide and pyridine, [16β-^{2}H]2,4-dibromo-16α-hydroxyestrone (**2**) with better than 98 atom% isotopic purity was obtained in quantitative yield. Reduction of the 17-carbonyl group and removal of the 2- and 4-bromine atoms of the ketol **2** were achieved simultaneously with sodium borodeuteride in methanol-OD in the presence of palladium chloride[9] (reaction time: 1 h) to give deuterated estriol (**4**) (d_6-species 62%) in high yield. The triol **4** contained two deuteriums more than expected from the synthetic pathways (Chart 1). When a longer time (24 h) was employed in the reaction, another deuterium was incorporated in the triol **4** (d_7-species 81%) (Table I). The deuteriums unexpectedly labelled were assigned to the 6- and 9α-positions on the basis of proton magnetic resonance (^{1}H-NMR) evidence. In the preparation of the ketol **4**, the benzilic hydrogens should be exchanged for deuteriums as reported in catalytic deuterations of estrone methyl ether.[10]

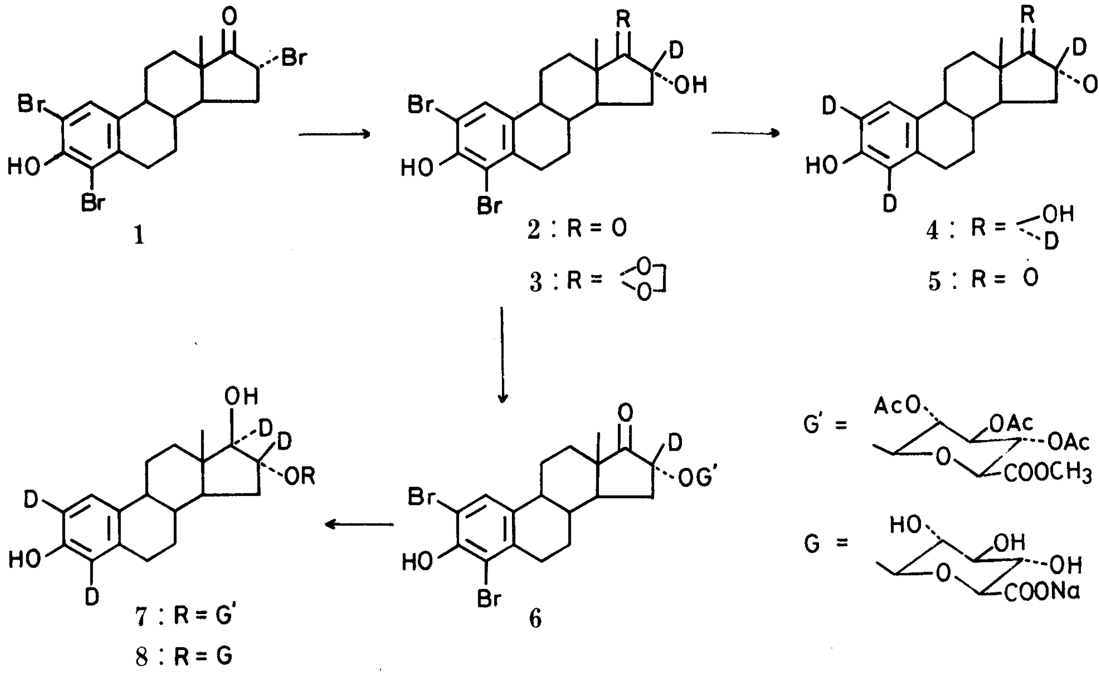

Chart 1

Table I. Isotope Analysis of 16α-Hydroxy Estrogens by Mass Spectrometry

Compound	Deuterium content (%)							
	d_0	d_1	d_2	d_3	d_4	d_5	d_6	d_7
4 (exptl. 1)[a]	0	0	0	0	9	25	62	4
4 (exptl. 2)[b]	0	0	0	0	1	4	14	81
5	0	0	0	6	11	67	16	0
8	0	0	0	0	8	21	68	3

a) Reaction time: 1 h.
b) Reaction time: 24 h.

Treatment of the [16β-^{2}H]-ketol **2** with ethylene glycol in the presence of *p*-toluenesulfonic acid gave the 17-ethylene acetal **3** in good yield. Hydrodebromination of [16β-^{2}H]-compound **3** with sodium borodeuteride as described above followed by cleavage of the carbonyl protecting group with acid afforded the desired labelled 16α-hydroxyestrone (**5**) with good isotopic purity (d_5-species 67%), but this also contained two deuteriums more than expected. The structures of the deuterated compounds **4** and **5** were identified by comparison with authentic samples and the isotope contents were obtained by MS analysis.

When the ketol **2** was treated with 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranosuronate and silver carbonate in dry benzene, the 16-monoglucuronide triacetate methyl ester (**6**) was regiospecifically obtained as the sole product in good yield. Compound **6** was converted into estriol 16-glucuronide acetate methyl ester (**7**) in the same way as described for the synthesis of compound **4** and then, finally, deuterium-labelled estriol 16-glucuronide (**8**) was obtained by hydrolysis of the acetate methyl ester **7** with sodium hydroxide in aqueous methanol. Amberlite XAD-2 resin was effective for the isolation of the deuterated glucuronide **8** in high yield. The overall yield of compound **8** from the starting tribromide **1** was 60% and its isotopic purity was satisfactory (Table I).

In addition to its simplicity and high yield, this synthesis offers a high deuterium-content without possible contamination by the natural form (d_0-species) and the deuterated estrogens **4**, **5**, and **8** are suitable as internal standards for mass fragmentography with high sensitivity. Furthermore, the controlled alkaline hydrolysis of 16-bromoketones should be very effective for deuterium-labelling of other 16α-hydroxy steroids. A quantitative GC-MS analysis of the estrongens in biological fluids is under way to further investigate their physiological importance.

Experimental

Melting points were measured on a Yanagimoto melting-point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Shimadzu IR 400 spectrometer as KBr pellets. NMR spectra were obtained with a JEOL PMX 60 spectrometer at 60 MHz with tetramethylsilane as an internal standard. MS were measured on a Hitachi RMU-7 spectrometer and a JEOL 01SG-2 spectrometer.

2,4,16α-Tribromo-3-hydroxy-1,3,5(10)-estratrien-17-one (1)——Compound **1** was synthesized according to the method previously reported.[8c,d)]

[16β-^{2}H]2,4-Dibromo-3,16α-dihydroxy-1,3,5(10)-estratrien-17-one (2)——Sodium metal (91 mg) was carefully added to a mixture of D_2O (10 ml) and pyridine (29 ml) and then **1** (1 g) was dissolved in the solution. The mixture was allowed to stand at room temperature for 2 h. After this time, the mixture was poured into 1% HCl solution (200 ml) and the precipitate was collected by filtration, washed with water and dried. The solid was crystallized from acetone to give **2** (780 mg, 89%) as colorless needles, mp 204—206 °C (lit.,[8c,d)] 205—206 °C). IR $_{\max}^{\text{KBr}}$ cm^{-1}: 3300 and 3450 (OH), 1729 (ketone). ^{1}H-NMR ($CDCl_3$): 0.97 (3H, s, 18-CH_3), 7.36 (1H, s, 1-H). MS: d_0 2%, d_1 98%.

2,4-Dibromo-17,17-ethylenedioxy-1,3,5(10)-estratriene-3,16α-diol (3)——A mixture of non-labelled **2**[8c,d)] (450 mg), ethylene glycol (3 ml) and toluene (50 ml) was slowly distilled for 10 min to remove traces of water. *p*-TsOH·H_2O (13 mg) was added and the mixture was heated under reflux for 7 h with a water separator. A 5% $NaHCO_3$ solution (100 ml) was added to the cooled mixture and the organic layer was washed twice with water, dried (Na_2SO_4) and evaporated to give a solid residue. The residue was crystallized from acetone to give non-labelled **4** (372 mg, 75%) as colorless needles, mp 194—197 °C. IR $_{\max}^{\text{KBr}}$ cm^{-1}: 3450 (OH). ^{1}H-NMR ($CDCl_3$): 0.87 (3H, s, 18-CH_3), 4.01 (4H, m, 17-OCH_2CH_2O–), 4.27 (1H, m, 16β-H), 5.83 (1H, br s, 3-OH), 7.37 (1H, s, 1-H). *Anal.* Calcd for $C_{20}H_{24}Br_2O_4$: C, 49.20; H, 4.96; Br, 32.73. Found: C, 49.36; H, 4.88; Br, 32.55.

[16β-^{2}H]-Compound 3——Deuterated ketal **3** was obtained from [16β-^{2}H]ketol **2** as above in 68% yield. mp 195—198 °C. This was identical with the non-labelled analog **3** by mixture melting point determination and thin layer chromatographic comparison. MS: d_0 2%, d_1 98%.

1,3,5(10)-Estratriene-3,16α,17β-triol-2,4,6,9α,16β,17α-d_6 and -d_7 (4)——$NaBD_4$ (80 mg) was added to a solution of **2** (200 mg) and $PdCl_2$ (180 mg) in MeOD (15 ml). The mixture was then stirred under nitrogen at 0 °C for 1 or 24 h. After extraction with AcOEt (50 ml × 2), the organic layer was washed with 5% $NaHCO_3$ solution and water, dried (Na_2SO_4), and evaporated to give a solid residue. Crystallization of the residue from MeOH–AcOEt gave **4** (120 mg, 92%) as colorless needles, mp 278—284 °C (lit.,[11)] 278.5—284 °C). MS: a) 1 h reaction time: d_4 9%, d_5 25%, d_6 62%, d_7

4%; b) 24 h reaction time: d_4 1%, d_5 4%, d_6 14%, d_7 81%.

3,16α-Dihydroxy-1,3,5(10)-estratrien-17-one-2,4,6,9α,16β-d_5 (5)——[16β-^{2}H]-Compound **3** (370 mg) was hydrodebrominated in the same manner as above (280 mg of $PdCl_2$, 200 mg of $NaBD_4$, 22 ml of MeOD, 6 h reaction time) to give an oily residue. The oily substance was dissolved in MeOH (60 ml) and then 5% HCl solution (5 ml) was added to the solution. The mixture was allowed to stand at room temperature for 6 h, then poured into water (200 ml). The precipitate was collected by filtration, washed with water and dried. The solid was crystallized from MeOH to afford **5** (250 mg, 81%) as colorless needles, mp 204—206 °C (lit.,[12] 205—207 °C). This was identical with the non-labelled sample by mixture melting point determination and TLC analysis. MS analysis showed the product **6** to contain d_3 6%, d_4 11%, d_5 67%, d_6 16%.

[16β-^{2}H]Methyl 2,4-Dibromo-3-hydroxy-17-oxo-1,3,5(10)-estratrien-16α-yl-2,3,4-tri-*O*-acetyl-β-D-glucopyranosuronate (6)——A solution of methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranosuronate (600 mg) in dry benzene (15 ml) was added dropwise to a stirred solution of **2** (300 mg) in dry benzene (200 ml) containing anhydrous Ag_2CO_3 (800 mg). The mixture was stirred at room temperature for 48 h, additional bromo sugar (300 mg) and catalyst (300 mg) being added to the mixture after a period of 24 h. The precipitate was filtered off and washed with benzene, and the combined filtrate was evaporated to give a viscous amber gum. The gummy substance was chromatographed on a silica gel column (70 g) with hexane–AcOEt to give a solid product. Crystallization of the solid from acetone gave **6** (350 mg, 66%) as colorless needles, mp 173—174 °C (lit.,[8c,d] 172—174 °C), undepressed upon admixture with authentic non-labelled **6**. ^{1}H-NMR ($CDCl_3$): 0.73 (3H, s, 18-CH_3), 2.03, 2.06 and 2.09 (3H, s, pyranose-$OCOCH_3$), 3.76 (3H, s, $COOCH_3$), 4.53 (1H, d, $J=5$ Hz, pyranose-anomeric), 7.36 (1H, s, 1-H). The isotopic purity was 98% by MS analysis.

Methyl 3,17β-Dihydroxy-1,3,5(10)-estratrien-16α-yl-2,3,4-tri-*O*-acetyl-β-D-glucopyranosuronate (7)——Compound **6** (500 mg) was treated with $NaBD_4$ (300 mg) in the presence of $PdCl_2$ (350 mg) as described for the reaction of **2**. The product was crystallized from MeOH to give **7** (360 mg, 92%), mp 229—231 °C (lit.,[13] 228—230 °C). ^{1}H-NMR spectrum of **7** showed that hydrogens at the C-2 and -4 positions had been exchanged for deuterium to the extent of about 95%.

Sodium 3,17β-Dihydroxy-1,3,5(10)-estratrien-16α-yl-β-D-glucopyranosuronate-2,4,6,9α,16β,17α-d_6 (8)——The above acetate methyl ester **7** (200 mg) was dissolved in MeOH (60 ml), and 2 N NaOH solution (4 ml) was added. The reaction mixture was then set aside at room temperature overnight. Subsequently the solution was concentrated to 8 ml under reduced pressure at below 50 °C and poured into ice-cold water (1000 ml). The solution was then passed through a column of Amberlite XAD-2 (3 × 60 cm). After washing of the column with water the adsorbed glucuronide was eluted with 50% aqueous MeOH. The steroid fraction was concentrated to give a solid product. The solid was recrystallized from aqueous MeOH to give **8** (63 mg, 50%) as colorless needles, mp 244—247 °C (lit.,[14] 246—249 °C). FD-MS: d_4 8%, d_5 21%, d_6 68%, d_7 3%.

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