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Preparation of [1,2-³H,4-¹⁴C]16 α -Hydroxyandrostenedione and Its Use for Radiometric Determination of Human Placental Aromatase Activity

MITSUTERU NUMAZAWA,^{*,a} MASACHIKA TSUJI,^a CAROL YARBOROUGH,^b
and YOSHIO OSAWA^{*,b}

*Tohoku College of Pharmacy,^a 4-1 Komatsushima-4-chome, Sendai 983, Japan
and Medical Foundation of Buffalo, Inc.,^b 73 High Street,
Buffalo, NY 14203, U.S.A.*

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[1,2-³H, 4-¹⁴C]16 α -Hydroxyandrostenedione (**4**) (³H, 3.20 mCi/mmol; ³H/¹⁴C = 222) was synthesized from commercially available [1,2-³H, 4-¹⁴C]dehydroepiandrosterone (**1**) through bromination at C-16 α of the 17-ketone **1** and controlled alkaline hydrolysis of the 16 α -bromoketone **3**, obtained from the brominated product **2** by 8N CrO₃ oxidation followed by *p*-toluenesulfonic acid treatment, as key reactions. The tritium distribution of the labeled 16 α -ketol **4** was determined by chemical and biochemical methods to be 47% at C-1 α , 18% at C-2 α , and 35% at the β -side of C-1 and C-2. When the labeled ketol **4** was incubated with human placental microsomes and reduced nicotinamide adenine dinucleotide phosphate, the rate of ³H₂O release into the medium was dependent upon protein concentration and incubation time. Aromatase activity obtained by the radiometric assay was comparable to that determined by the high-performance liquid chromatographic method.

Keywords—[1,2-³H, 4-¹⁴C]16 α -hydroxyandrostenedione; chemical synthesis; human placenta; aromatase; aromatization; estriol biosynthesis; tritiated water; radiometric assay

Aromatase is capable of catalyzing the synthesis of estrogens from androgens. There are certain structural variations in the androgen precursors for naturally occurring estrogen.¹⁾ Two distinct aromatases (I and II) which have different substrate specificities and cytochrome P-450s have recently been separated in soluble form from human term placenta by Osawa's group.²⁾ The difference in the substrate specificity lies in the D-ring structure.

Aromatase II activities have usually been determined by radiometric methods based upon the stereospecific removal of tritium from [1 β ,2 β -³H]androstenedione³⁾ to ³H₂O during aromatization.⁴⁾ This assay is highly sensitive, potentially accurate and speedy. On the other hand, we⁵⁾ have recently developed a sensitive high-performance liquid chromatographic (HPLC) method using 16 α -hydroxyandrostenedione as a substrate for the determination of aromatase I activities. However, this chromatographic method is not as simple and speedy as the ³H₂O release method. Thus it seems to be important to develop a convenient and rapid radiometric ³H₂O assay method for the determination of aromatase I activities. We planned to synthesize [1,2-³H, 4-¹⁴C]16 α -hydroxyandrostenedione (**4**) and to validate its use for the aromatase activity determination of human placental microsomes.

Results and Discussion

[1,2-³H, 4-¹⁴C]16 α -Hydroxyandrostenedione (**4**) was chemically synthesized essentially according to our previous report.⁶⁾ [1,2-³H]Dehydroepiandrosterone (DHEA, **1**) was mixed with [4-¹⁴C]DHEA and diluted with non-labeled DHEA. The double labeled DHEA (³H/

Chart 1

TABLE I. Determination of Radiochemical Purity [1,2-³H,4-¹⁴C]Compound 4 by the Reverse Isotope Dilution Method^{a)}

No.	Crystallization From	Specific activity (dpm/mg)		³ H/ ¹⁴ C
		³ H	¹⁴ C	
1	Acetone	67550	302	224
2	Acetone	67400	305	221
3	MeOH-H ₂ O	67620	305	222

a) [1,2-³H,4-¹⁴C]Compound 4 (³H, 1.36 × 10⁶ dpm; ¹⁴C, 6.14 × 10³ dpm) was diluted with non-labeled compound 4 (20 mg) and repeatedly recrystallized.

TABLE II. Determination of ³H- to-¹⁴C Ratio of Estrogens Formed from [1,2-³H,4-¹⁴C]Compound 4 with Human Placental Microsomes

No.	Crystallization From	Specific activity (dpm/mg)		³ H/ ¹⁴ C
		³ H	¹⁴ C	
Estriol ^{a)}				
1	MeOH	13440	89	151
2	MeOH	13140	90	146
3	MeOH-H ₂ O	13200	91	145
2,4-Dibromoestriol triacetate ^{b)}				
1	Acetone	6610	61	108
2	Acetone	6077	59	103
3	MeOH	6300	60	105

a) [1,2-³H,4-¹⁴C]Compound 4 (³H/¹⁴C=222) was incubated with human placental microsomes in the presence of NADPH at 37 °C for 2 h as described in the text. 16α-Hydroxyestrone (3 mg) was added to the extract. The steroid fraction was reduced with NaBH₄ and then isolated by TLC as estriol. b) Estriol was brominated with Br₂ in MeOH to give 2,4-dibromoestriol, which was subsequently acetylated with Ac₂O-pyridine, yielding the triacetate.

¹⁴C = 340) was first brominated with CuBr₂ to give the 16α-bromo derivative **2** which was then oxidized with 8 N CrO₃ and subsequently treated with *p*-toluenesulfonic acid, yielding the 4-ene-3-keto derivative **3**. [³H, ¹⁴C]Compound **3** was hydrolyzed with 1.2 eq of NaOH in aqueous pyridine to give ³H- and ¹⁴C-labeled compound **4** (21% from compound **1**; specific activity, ³H 3.20 mCi/mmol; ³H/¹⁴C = 222, this ratio is equivalent to a 35% loss of the tritium from DHEA which could be attributed to the C-2 position). The labeled 16α-ketol **4** was identical with authentic non-labeled compound, and its radiochemical purity was established to be more than 98% by thin-layer chromatography (TLC) and reverse isotope dilution analysis (Table I).

The distribution of tritium in the double labeled 16α-ketol **4** was initially determined by chemical and biochemical methods. After incubation of **4** with human placental microsomes

and reduced nicotinamide adenine dinucleotide phosphate (NADPH), 16 α -hydroxyestrone obtained was reduced with NaBH₄ and then estrogen products⁷⁾ were isolated by TLC as estriol. Estriol was purified to a constant ³H/¹⁴C ratio by recrystallization with carrier (Table II). With this technique it was determined that 35% of the tritium in the substrate was lost as ³H₂O during aromatization (³H/¹⁴C decreased from 222 to 145). Assuming that aromatization mechanism is completely stereospecific for the 1 β ,2 β -hydrogen removal,^{4,8)} the loss of 35% tritium during conversion of the ketol **4** into estriol can be attributed to the amount of tritium located in the 1 β and 2 β -positions. The tritium at C-2 and C-1 in estriol corresponds to the tritium located at C-2 α and C-1 α of the ketol **4**. Displacement of the C-2 hydrogen would give the amount of tritium located at the C-2 α position. Bromination of estriol with Br₂ in MeOH followed by acetylation with acetic anhydride in pyridine gave 2,4-dibromoestriol triacetate (the ³H/¹⁴C ratio decreased from 145 to 105: this is equivalent to a 18% loss of the tritium from the labeled ketol **4**). The ³H/¹⁴C ratios of estriol and 2,4-dibromoestriol triacetate show that the tritium of [1,2-³H, 4-¹⁴C]compound **4** is distributed 47% at C-1 α , 18% at C-2 α , and 35% at the β -side of C-1 and C-2. However, the fact that the 2 β -axial hydrogen is more labile than the 2 α -hydrogen and is preferentially eliminated by the enolization of steroidal 4-en-3-ones under basic and acidic conditions^{8d,9)} suggests that tritium located at the

Fig. 1. Effect of Incubation Time on the ³H₂O Formation

[1,2-³H, ¹⁴C]16 α -Hydroxyandrostenedione (9.1 μ M) was incubated with the placental microsomes (1.1 mg protein/ml) in the presence of NADPH over various time periods. The ³H₂O formation was determined as described in the text.

Fig. 2. Effect of Protein Concentration on the ³H₂O Formation

[1,2-³H, ¹⁴C]16 α -Hydroxyandrostenedione (9.1 μ M) was incubated at various protein concentrations for 60 min in the presence of NADPH. The ³H₂O formation was analyzed as described in the text.

TABLE III. Comparison of Radiometric and HPLC Methods for Determination of Aromatization of 16 α -Hydroxyandrostenedione with Human Placental Microsomes

Substrate	Product	Aromatase activity pmol/mg protein/min ^{a)}
[³ H, ¹⁴ C]-Labeled	Tritiated water ^{b)}	86 $\pm$ 8
Non-labeled	16 α -Hydroxyestrone and estriol ^{c)}	94 $\pm$ 7

a) Mean $\pm$ S.D.; *n* = 4. b) Radioactivity of ³H₂O released from the labeled substrate was measured. c) Amounts of the products, 16 α -hydroxyestrone and estriol, were determined by HPLC.

β -side would be principally incorporated at the 1β -position. The distribution at C-1 absolutely depends upon that of the commercial $[1,2-^3\text{H}]\text{DHEA}$.

To confirm that the rate of estrogen formation is equal to that of $^3\text{H}_2\text{O}$ formation, the $[1,2-^3\text{H}, 4-^{14}\text{C}]16\alpha$ -ketol **4** was incubated with placental microsomes in the presence of NADPH. Figure 1 shows a progress curve of the aromatization reaction as a function of time. The amount of tritium that was released and incorporated into $^3\text{H}_2\text{O}$ reached a plateau with 33% relative conversion to estrogens. This value is almost the same as the tritium distribution (35%) at the β -side of C-1 and C-2 of the substrate.

To further validate the $^3\text{H}_2\text{O}$ assay, it was necessary to establish that the rate of $^3\text{H}_2\text{O}$ release depends upon protein concentration. As shown in Fig. 2, the rate of aromatization of 16α -hydroxyandrostenedione as determined by measuring $^3\text{H}_2\text{O}$ release was proportional to protein concentration up to about 2 mg/ml. The $^3\text{H}_2\text{O}$ production also reached a plateau with 34% relative conversion to estrogens, and this also supports the tritium distribution at the β -side of the substrate.

The specific activity of the microsomal aromatase was then determined by employing a 60 min incubation time, 1.1 mg protein/ml, and $9.1\ \mu\text{M}$ labeled substrate, which is a saturated concentration. The specific activity obtained by the radiometric assay was comparable to that obtained by the HPLC method previously developed by us⁵⁾ (Table III).

Considering the stereospecific loss of the 1β and 2β -protons of androstenedione during aromatization with the placental microsomes,¹⁰⁾ the tritium distribution at C-1 of the $[^3\text{H}, ^{14}\text{C}]16$ -ketol **4** is not ideal for a sensitive aromatase activity determination. However, the radiometric assay using this compound is sufficiently sensitive and reliable. The obvious advantages of this method are to be found in the speed and the simplicity, and hence this quick assay should be very useful for the characterization of aromatase I in placental microsomes.

Experimental

Materials— $[1,2-^3\text{H}(\text{N})]\text{Dehydroepiandrosterone}$ (58.6 Ci/mmol) and $[4-^{14}\text{C}]\text{dehydroepiandrosterone}$ (55 mCi/mmol) were obtained from New England Nuclear Corp. (Boston, MA). NADPH was purchased from Sigma Chemical Co. (St. Louis, MO). 16α -Hydroxyandrostenedione and 16α -hydroxyestrone were synthesized according to a previous report.⁶⁾

Synthesis of $[1,2-^3\text{H}, 4-^{14}\text{C}]16\alpha$ -Hydroxyandrostenedione (4**)**— $[1,2-^3\text{H}]\text{Dehydroepiandrosterone}$ (**1**) (18 μg , 3.4 mCi) was mixed with $[4-^{14}\text{C}]\text{compound 1}$ (53 μg , 10 μCi) and diluted with 200 mg of non-labeled compound **1**. A solution of the ^3H - and ^{14}C -labeled compound **1** (200 mg, 0.69 mmol) and 2.1 mmol of CuBr_2 in 10 ml of dry MeOH was heated under reflux for 12 h. After the same workup as previously reported,⁶⁾ the crude bromoketone **2** (260 mg) was obtained as a solid.

A solution of the crude compound **2** (260 mg) in 50 ml of acetone was treated dropwise with 0.2 ml of 8 N CrO_3 solution with stirring below 5°C , and then the solution was allowed to stand for 5 min. After this time, the mixture was poured into ice-water (300 ml) saturated with NaCl. The precipitates (180 mg) were collected by filtration, dried under vacuum, and then dissolved in 5 ml of acetone. *p*-Toluenesulfonic acid monohydrate (15 mg, 0.08 mmol) was added to the solution and the mixture was allowed to stand for 6 h and then poured into water (50 ml) followed by extraction with AcOEt (50 ml $\times$ 2). The organic layer was washed with 5% NaHCO_3 solution and water, dried (Na_2SO_4), and evaporated to give a solid (120 mg) which was submitted to column chromatography (silica gel 6 g, hexane-AcOEt) to give a partially purified $[^3\text{H}, ^{14}\text{C}]16\alpha$ -bromo-4-androstene-3,17-dione (**3**) (95 mg).

The crude ^3H - and ^{14}C -labeled compound **3** (95 mg) was dissolved in 5 ml of 75% aqueous pyridine, and 0.30 ml of 1 N NaOH solution was added. The mixture was stirred at room temperature for 2 h and then poured into 50 ml of 5% HCl solution and extracted with AcOEt (50 ml $\times$ 2). The organic layer was washed with 5% HCl and 5% NaHCO_3 solutions and water and dried (Na_2SO_4). After evaporation of the solvent, the residue (76 mg) was submitted to column chromatography (silica gel 4 g, hexane-AcOEt) yielding the crude product **4**. Recrystallization from acetone gave **4** as colorless needles (45 mg, 21% from **1**), mp $187\text{--}188^\circ\text{C}$ (lit.⁶⁾ $188\text{--}191^\circ\text{C}$). $^1\text{H-NMR}$ (CDCl_3) δ : 1.01 (1H, s, 18-Me), 1.22 (3H, s, 19-Me), 5.71 (1H, s, 4-H).

Preparation of Human Placental Microsomes—The placental microsomes were isolated from homogenates of fresh term placenta by differential centrifugation, essentially according to the method of Ryan.¹¹⁾ The $105000\times g$

(60 min) fraction was washed twice with homogenizing buffer and once with water, then freeze-dried and stored desiccated at 4 °C.

Incubation Conditions—The incubations were carried out as follows: the microsomal preparation, NADPH (3.3 μ mol) and [1,2-³H, 4-¹⁴C]compound **4** were dissolved in MeOH (50 μ l) and sufficient 0.067 M phosphate buffer (pH 7.5) to give total volume of 1.8 ml was added. The mixture was incubated at 37 °C with shaking under aerobic conditions, and terminated by addition of 4 ml of CHCl₃, followed by vortexing for 40 s. After centrifugation of the mixture at 2500 rpm for 10 min, aliquots (1.2 ml) were removed from the water phase and added to scintillation mixture for determination of ³H₂O production.⁴⁾ To correct for non-specific release of tritium from the C-2 position and labeled steroids that were not extracted by CHCl₃, control incubations without NADPH were carried out simultaneously. The radioactivity (ca. 0.071% of that of the substrate) found in the aqueous phase from these blanks was subtracted from the radioactivity of ³H₂O formed in the sample tubes.

Isolation and Bromination of Estriol—The CHCl₃ phases obtained from four incubation experiments using 6.1 μ M labeled substrate **4** (³H 1.1 $\times 10^5$ dpm), and 4 mg protein of the microsomes, and incubated for 120 min incubation were combined and 3 mg of 16 α -hydroxyestrone was added to the solution, then the solvent was evaporated off under an N₂ stream. NaBH₄ (3 mg) was added to a solution of the extract in 0.5 ml of MeOH and the mixture was kept at 0 °C for 2 h. The solvent was removed under an N₂ stream. The residue was partitioned between AcOEt (4 ml) and water (2 ml). The organic phase was washed with water, dried (Na₂SO₄) and evaporated, and the residue was submitted to TLC (silica gel 60 F₂₅₄, 0.5 mm of layer thickness, E. Merck; hexane-AcOEt = 1 : 4, v/v). Estriol was eluted with AcOEt. Another 20 mg of estriol was added to the eluate and the product was recrystallized from MeOH to constant specific activity (Table II).

The ³H- and ¹⁴C-labeled estriol (20 mg) obtained above was diluted with carrier to 20 mg and then brominated in MeOH according to the previous report^{8d)} to yield 2,4-dibromoestriol, mp 278–280 °C (lit.¹²⁾ 276–277 °C), which was converted into the triacetate in a usual manner, and then recrystallized to a constant specific activity (Table II).

HPLC Method for Determination of Non-labeled Compound 4 Aromatization—Estrogens, estriol and 16 α -hydroxyestrone, produced by the incubation of non-labeled 16 α -hydroxyandrostenedione with the placental microsomes and NADPH were determined by HPLC essentially according to the previous report.⁵⁾

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