A Radiometric Assay Method for Aromatase Activity Using [1β-^{3}H]16α-Hydroxyandrostenedione

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[1β-^{3}H]16α-Hydroxyandrostenedione (16α-OHA) (715 mCi/mmol) was prepared from commercially available [1β-^{3}H]androstenedione (A) by the microbiological method with *Streptomyces roseochromogenes* and its structure and purity were determined by chromatographic and reverse isotope dilution methods. When [1β-^{3}H]16α-OHA was incubated with human placental microsomes and reduced nicotinamide adenine dinucleotide phosphate (NADPH), ^3H_2O-release into the medium was dependent upon protein concentration and incubation time. An apparent K_m and V_{max} of the microsomal aromatase for the [1β-^{3}H]substrate were 650 nM and 34 pmol/min/mg protein, respectively. In this assay, aromatase activity could be determined as low as 0.1 nmol estrogen formation/min/mg protein. 3-Deoxyandrostenedione, a potent competitive inhibitor of the A aromatization, also blocked the 16α-OHA aromatization in a competitive manner with K_i of 15 nM.

Keywords [1β-^{3}H]16α-hydroxyandrostenedione; microbiological transformation; *Streptomyces roseochromogenes*; estriol biosynthesis; human placental microsome; aromatase; radiometric assay; tritiated water; inhibitor

Aromatase is a unique cytochrome P-450 monooxygenase complex and catalyzes the synthesis of estrongens from androgens with a 4-en-3-one structure.[1] The principal estrogen secreted by human placenta is estriol (E_3), whereas the ovary and the fat secrete estradiol and estrone (E_1), respectively. It is now believed that a single enzyme species catalyzes the aromatization independent of the difference in D-ring substitution of the androgens in all tissues.[1e,f,2]

Potential aromatase inhibitors, which have currently been developed for use in the management of breast cancer, are generally tested utilizing aromatase present in human placenta as a source of activity and androst-4-ene-3,17-dione (androstenedione, A) as a substrate. In this assay, aromatase activity is usually determined based on tritiated water released from [1β-^{3}H]A during aromatization. However, 16α-hydroxyandrostenedione (16α-OHA) is a principal precursor of estrogen production in the placenta.[1e,f,3] Therefore, use of 16α-OHA instead of A in the above assay system is promising for the development of inhibitors which are more effective in clinical applications. Radiometric assay with [1β,2β-^{3}H]-labeled substrate,[4] chemically synthesized, and high-performance liquid chromatographic assay with electrochemical detectors[3,5] have previously been reported for the determination of 16α-OHA aromatization with placental microsomes. However, they could not be used for the inhibition study of aromatase because of their low sensitivities.

We report here the preparation of [1β-^{3}H]16α-OHA, which has high specific activity, by means of microorganism and the development of a sensitive method for the aromatase assay using the labeled substrate.

Results

The conversion of non-labeled A by a strain of *Streptomyces roseochromogenes* was initially explored. Thin-layer chromatography (TLC) of the crude product obtained after 3 d incubation at 27 °C showed two polar spots of the products along with that of the substrate (Fig. 1). The major product, isolated by TLC and recrystallization, was completely identical to the authentic 16α-OHA in every respect (30% yield). On the other hand, the minor product, which is less polar than 16α-OHA, was not isolated and further study of it was not carried out.

We then tried to prepare a radioactive form of the 16α-hydroxy steroid. Incubation of [1β-^{3}H]A (715 mCi/mmol) with the microorganism similar to the above followed by purification with TLC and high-performance liquid chromatography (HPLC) afforded the desired [1β-^{3}H]16α-hydroxy compound in 10% of radiochemical yield. The

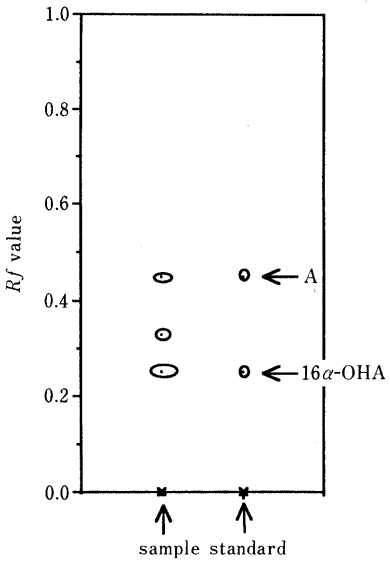

Fig. 1. Thin-Layer Chromatogram of Products Obtained by Incubation of Non-labeled A with a Strain of *Streptomyces roseochromogenes*

Solvent, hexane : AcOEt = 1 : 2.

TABLE I. Reverse Isotope Dilution Analysis of [1β-^{3}H]16α-OHA[a]

Crystallization		Crystalline	
No.	Solvent	Weight (mg)	Specific activity (dpm/mg)
1	Methanol	28	2734
2	Methanol	18	2735
3	Acetone	10	2698
4	Acetone	6	2701

a) [1β-^{3}H]16α-OHA (1.156×10^5 dpm) was mixed with 42 mg of non-labeled 16α-OHA and then recrystallized repeatedly from MeOH or acetone.

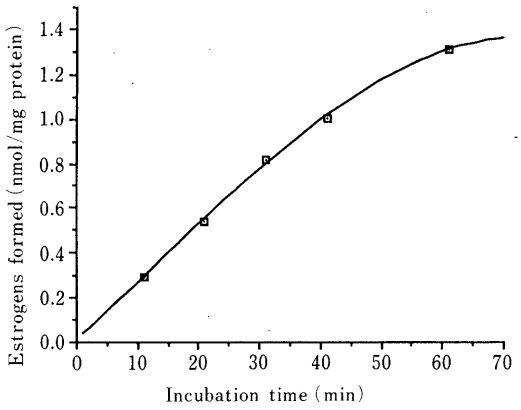

Fig. 2. Effect of Incubation Time on Aromatization of [1β-^{3}H] 16α-OHA

The radioactive steroid (6 μM) was incubated with 200 μg of the placental microsomal protein in the presence of NADPH over various time periods. The estrogen formed was obtained by measuring an amount of ^3H_2O released in the medium. Each point is a mean of two determinations.

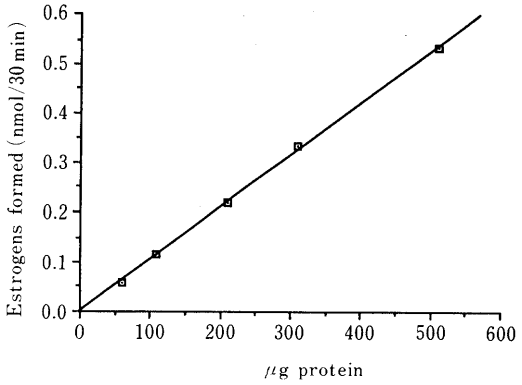

Fig. 3. Effect of Protein Concentration on Aromatization of [1β-^{3}H] 16α-OHA

The radioactive steroid (6 μM) was incubated at various protein concentrations for 30 min in the presence of NADPH. The estrogen formed was obtained as described in Fig. 2. Each point is a mean of two determinations.

labeled steroid was finally identified by the reverse isotope dilution method (Table I) and its radiochemical purity was more than 98%.

^3H_2O-release from [1β-^{3}H]16α-OHA into the medium during the aromatization was determined essentially according to Siiteri and Thompson: ^3H_2O was separated from the radioactive steroids by shaking the incubation mixture with $CHCl_3$.[6] The ^3H_2O-release was initially examined as a function of incubation time or enzyme concentration. Aromatase activity linearly increased up to 40 min of incubation time (Fig. 2) or up to 500 μg of protein of the placental microsomes (Fig. 3), respectively, in a similar manner as described previously.[5] Kinetic constants of the microsomal aromatase were then determined using a 30-min incubation time and 200 μg of protein. When the concentration of the radioactive substrate was changed to between 0.5 and 6 μM, a typical saturation curve was obtained (Fig. 4a). A Lineweaver-Burk plot of the velocity against the concentration of the substrate gave an apparent K_m of 650 nM and V_{max} of 34 pmol/min/mg protein (Fig. 4b). In this assay, about 0.1 nmol estrogen formation/min/mg protein could be reliably measured.

To validate the radiometric assay, [1β-^{3}H]16α-hydroxy

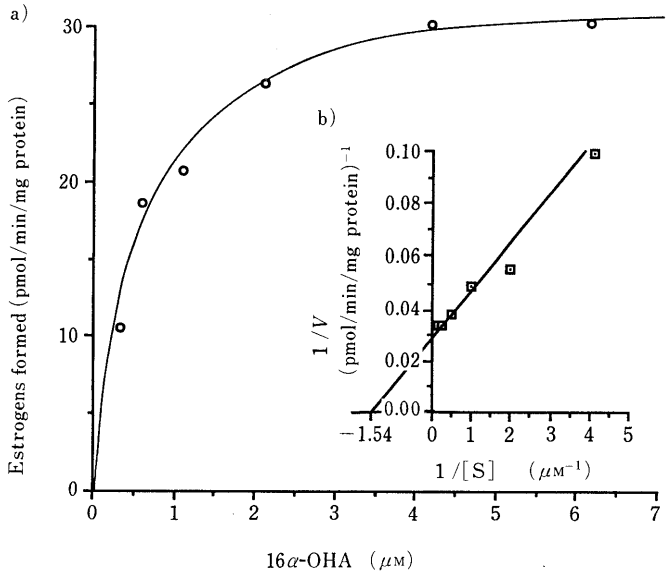

Fig. 4. Lineweaver–Burk Plot of Aromatization of [1β-^{3}H] 16α-OHA

The microsomes (200 μg protein) and 30-min incubation time were employed and each point is a mean of two determinations.

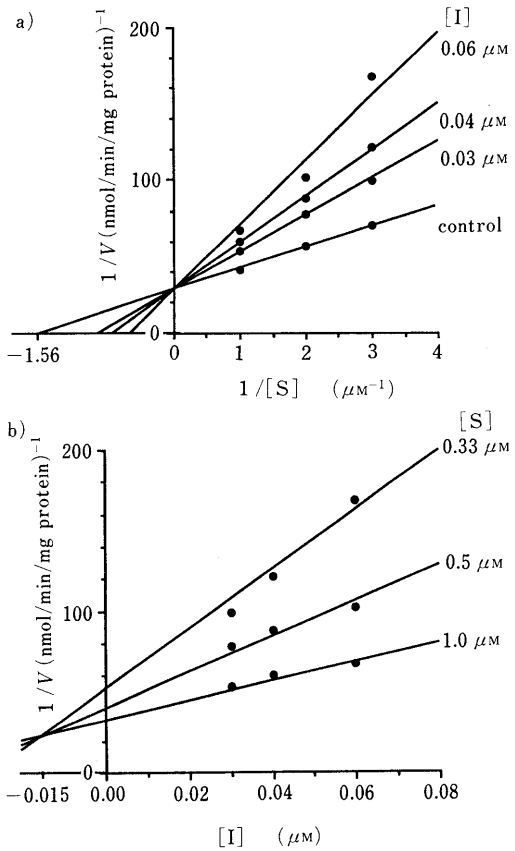

Fig. 5. Lineweaver–Burk Plot (a) and Dixon Plot (b) to Determine the Apparent Inhibition Constant for 3-Deoxy A

steroid was employed as a substrate of aromatase for the inhibition study. 3-Deoxyandrostenedione (3-deoxy A),[7] a potent inhibitor of the A aromatization, prevented the 16α-OHA aromatization with the placental microsomes in a competitive manner with K_i of 15 nM, obtained by analysis of Dixon plot (Fig. 5).

Discussion

Microbiological transformation of [1β-^{3}H]A with *Streptomyces roseochromogenes*, which is known to introduce a hydroxyl group at C-16 of pregnenolone,[8] its 3-succinate,[9] or dehydroepiandrosterone 3-sulfate,[10] yielded the corresponding 16α-hydroxide with a high specific activity, and its structure and purity were determined by TLC, HPLC, and the reverse dilution method along with a cold experiment with the non-labeled steroid.

Kinetic studies on the aromatization of 16α-OHA and its 17β-hydroxy derivative (16α-hydroxytestosterone) in the placental microsomes have shown that aromatization of the 17β-ol is practically negligible in the presence of the 17-ketone (16α-OHA) in the feto-placental unit and therefore almost all of the E_3 in human pregnancy comes from the aromatization of 16α-OHA followed by the reduction of 16α-hydroxy E_1, the initial aromatized product.[1e,f,3] Moreover, the overall molecular changes occurring during the transformation of 16α-OHA into 16α-hydroxy E_1 are identical with those already established for the conversion of A into E_1, in which C-19 and 1β-proton are eliminated as formic acid and water, respectively, to produce the estrogen.[11] Osawa's group[1f,12] has recently reported that when the purified and reconstituted aromatase is incubated with A, the major product is E_1, with some formation of 1β- and 2β-hydroxy A. This suggests that some of the ^3H_2O released from [1β-^{3}H]16α-OHA during incubation may be due to the 1β-hydroxylation of the steroid by aromatase. Considering these, the ^3H_2O-release method with [1β-^{3}H]-16α-OHA should be very useful for the determination of aromatase activity in human placental microsomes. This assay was simple and rapid and its sensitivity was high enough for the inhibition study of the enzyme.

The inhibition study of the placental aromatase was, for the first time, carried out using [1β-^{3}H]16α-OHA as a substrate. The potent inhibitor of the A aromatization, 3-deoxy A, also prevented the 16α-OHA aromatization in a competitive manner. The K_i/K_m ratio is extremely smaller than that obtained with [1β-^{3}H]A (15/650 for 16α-OHA *vs.* 37/60 for A[7b]). This would essentially depend on more than ten-times higher K_m for 16α-OHA compared to that for A and suggests that new information concerning the structure–activity relationship of aromatase inhibitors would be attained using 16α-OHA as a substrate, instead of A, in the inhibition study.

Experimental

Materials and General Methods *Streptomyces roseochromogenes* IFO-13080 was obtained from the Institute for Fermentation Osaka (Osaka, Japan). [1β-^{3}H]A (27.5 Ci/mmol; ^{3}H-distribution, 74—79% at 1β) was purchased from New England Nuclear (Boston, U.S.A.) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) from Kohjin Co., Ltd. (Tokyo, Japan). 3-Deoxy A and 16α-OHA were synthesized according to the methods[13,14] previously reported.

The melting point was measured on a Yanagimoto melting point apparatus and is uncorrected. The proton nuclear magnetic resonance (^{1}H-NMR) spectrum was recorded with a JEOL GX 400 (400 MHz) spectrometer using tetramethylsilane ($\delta=0.00$) as an internal standard. TLC was performed with E. Merck pre-coated TLC plated, Silica gel 60F-254, layer thickness 0.25 mm. The HPLC system consisted of a Waters 6000A pump and U6K injector and a Soma UV detector S-310A (240 nm).

Fermentation The microbiological transformation was carried out essentially according to the method[9] previously reported in a medium consisting of (grams per liter of distilled water): Meat extract 2.5 g, peptone 5 g; yeast extract 1 g, glucose 10 g, NaCl 1 g, $MgSO_4\cdot 7H_2O$ 0.5 g. The cells of strain IFO-13080 maintained in an agar slant were inoculated into 100 ml of the medium in 500 ml Erlenmeyer flasks and cultured on a rotary shaker (200 rpm) at 27 °C. After incubation for 3 d, 2 ml of the incubation medium was transferred to 100 ml of the fresh medium and incubated as described above.

1) Preparation of Non-labeled 16α-OHA After 24 h incubation, 30 mg of A in EtOH (1 ml) was added to each flask and the incubation was carried out for another 3 d as above. The combined mixture of two flasks were then extracted with AcOEt (200 ml × 3). The organic phase was dried over Na_2SO_4 and evaporated to afford a brown oil which was purified by column chromatography (silica gel, 5 g; hexane : AcOEt = 2 : 1, 1 : 1, and 1 : 2) and preparative HPLC [column, YMC-Pak D-ODS-5 250 × 20 mm i.d. (Yamamura Chemical Lab. Co., Ltd., Kyoto, Japan); mobile phase, MeCN : H_2O = 60 : 40, 6 ml/min]. Steroid eluted at t_R of 4.3 min was recrystallized from MeOH to give 16α-OHA (19 mg, 30%): mp 185—187 °C (lit.[14] 188—190 °C). ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, s, 18-Me), 1.21 (3H, s, 19-Me), 4.39 (1H, d, J = 7.7 Hz, 16β-H), 5.76 (1H, s, 4-H).

2) Preparation of [1β-^{3}H]16α-OHA [1β-^{3}H]A (250 μCi, 100 μg) in 10 μl of EtOH was added to 1 ml of the above incubation medium and incubated similarly as above. After incubation, the steroid was extracted with AcOEt (1 ml × 3). The combined organic phase was evaporated under a N_2 stream to give the residue which was dissolved in acetone (30 μl) and subjected to preparative TLC (hexane : AcOEt = 1 : 2). The area (Rf, 0.25) corresponding to 16α-OHA was scraped off and eluted with AcOEt (10 ml). After evaporation of the solvent, the residue was dissolved in MeOH (25 μl) and subjected to HPLC [column, ERC-ODS-1161 100 × 6-mm i.d. (Erma Optical Works, Ltd., Tokyo, Japan); mobile phase, MeCN : H_2O = 45 : 55, 1 ml/min]. The fraction corresponding to 16α-OHA (t_R, 3.8 min) was collected and the solvent was evaporated under a N_2 stream to give [1β-^{3}H] 16α-OHA (radiochemical yield, 10%).

Reverse Isotope Dilution Method [1β-^{3}H] 16α-OHA (1.156×10^5 dpm) obtained above was mixed with 42 mg of non-labeled 16α-OHA and then repeatedly recrystallized from MeOH and acetone. Specific activities of 16α-OHA obtained as the crystalline were determined.

Enzyme (Aromatase) Preparation Human term placental microsomes (particles sedimenting at 105000 × *g* for 60 min) were obtained as described by Ryan.[15] They were washed twice with 0.5 mM dithiothreitol solution, lyophilized, and stored at −20 °C. No loss of activity occurred over the period of the study. Prior to assay, the lyophilized microsomes were suspended in 67 mM phosphate buffer, pH 7.5. The protein content averaged 0.59 mg protein per 1 mg dry weight, measured by the method of Lowry *et al.*[16] using bovine serum albumin as a reference.

Incubation Conditions Aromatase activity was measured essentially according to the original ^3H_2O-assay procedure of Siiteri and Thompson.[6] The incubations were carried out in a final incubation volume of 1.1 ml as follows: the microsomal preparation (0.5 ml), NADPH (500 μM), [1β-^{3}H] 16α-OHA dissolved in 50% aqueous MeOH (0.1 ml), and sufficient 67 mM phosphate buffer (pH 7.5) to total 1.1 ml. The mixture was incubated at 37 °C, with shaking, in the absence or presence of various concentrations of inhibitor in the air. Incubations were terminated by the addition of 3 ml of $CHCl_3$, and vortexed for 40 s. After centrifugation at 12500 × *g* for 5 min, aliquots (500 μl) were removed from the water phase and added to the scintillation mixture for determination of ^3H_2O production. For inhibition studies, 30 min of the incubation time and 200 μg of the protein were used.

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