

Synthesis and Biochemical Aromatization of 14 β - and 13 α -Androst-4-en-3-ones^{1,2)}

TOSHIO NAMBARA, KYOKO YAMANOUCHI
and YOSHIKO KOBAYASHI

Pharmaceutical Institute, Tohoku University³⁾

(Received November 12, 1968)

Requirements of the configuration at C-14 and C-13 for the aromatization of C₁₉ steroids by human placenta have been investigated. For this purpose 14 β - and 13 α -androst-4-en-3-ones were synthesized as the substrate (see Chart 2). The C/D-*cis*-linked steroids as well as androsta-4,14-diene-3,17-dione were transformed into the 17-hydroxylated estrogens, when incubated with the placental 10000 $\times g$ supernatant in the presence of NADPH and oxygen. The relative yield of Kober chromogens produced from these C₁₉ steroids was found to be lower than that of androst-4-ene-3,17-dione having normal C/D-ring juncture as listed in Table I.

It is well known that the androgenic steroids can be aromatized to the phenolic estrogens with loss of C-19 methyl group by mammalian tissue.⁴⁾ The mechanism of this biotransformation is now sufficiently substantiated as illustrated in Chart 1.⁵⁾ These arguments, however, have been directed mainly to the steroids possessing the normal configuration with respect to the ring fusion. In 1966 Walop, *et al.* and Dellácqua, *et al.* explored the dependence of the biochemical aromatization of C₁₉ steroids on the stereochemistry at C-8, C-9 and C-10.^{6,7)} In connection with our studies on the metabolism of the modified steroids, it seemed to be attractive to examine whether or not the Δ^4 -3-oxosteroids with the unusual configuration at C-14 or C-13 could be aromatized by similar mechanism in human placental tissue.

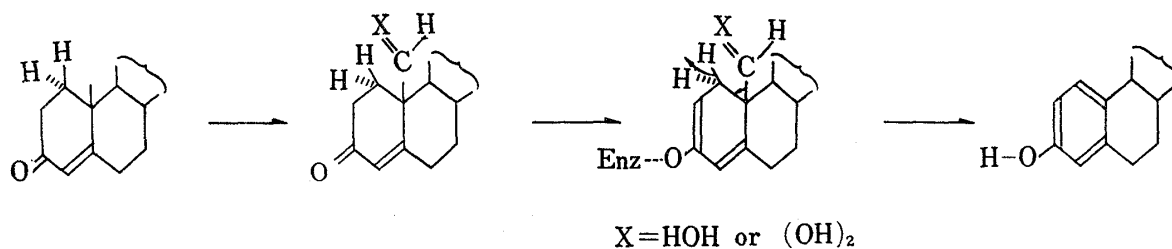


Chart 1. Mechanisms for Ring A Aromatization of C₁₉ Steroids

For this purpose the 14 β - and 13 α - Δ^4 -3-oxosteroids were required as the substrate. Hence the initial project was focused to the synthesis of 14 β -androst-4-ene-3,17-dione. In

- 1) This paper constitutes Part XXVI of the series entitled "Analytical Chemical Studies on Steroids"; Part XXV: T. Nambara, M. Numazawa and H. Takahashi, *Chem. Pharm. Bull.* (Tokyo), **17**, 1725 (1969).
- 2) The following trivial names are used: estrone, 3-hydroxyestra-1,3,5(10)-trien-17-one; estradiol, estra-1,3,5(10)-triene-3,17 β -diol; estradiol-17 α , estra-1,3,5(10)-triene-3,17 α -diol.
- 3) Location: Aobayama, Sendai.
- 4) K.J. Ryan, *J. Biol. Chem.*, **234**, 268 (1959).
- 5) T. Morato, K. Raab, H.J. Brodie, M. Hayano and R.I. Dorfman, *J. Am. Chem. Soc.*, **84**, 3764 (1962); J.D. Townsley and H.J. Brodie, *Biochemistry*, **7**, 33 (1968); H.J. Brodie, G. Possanza and J.D. Townsley, *Biochim. Biophys. Acta*, **152**, 770 (1968).
- 6) J.N. Walop and N. De Lange, *Biochim. Biophys. Acta*, **130**, 249 (1966).
- 7) S. Dellácqua, S. Mancuso, G. Eriksson and E. Diczfalusy, *Biochim. Biophys. Acta*, **130**, 241 (1966).

order to transform the common α -configuration at C-14 into β , the method worked out by St. André, *et al.* was utilized starting from $3\beta,14\alpha$ -dihydroxyandrost-5-en-17-one.⁸⁾ The first attempt, the partial hydrogenation of androsta-4,14-diene-3,17-dione (VII) without disturbance of the Δ^4 -double bond, resulted in failure. An alternative method involving protection of the double bond with halogen also encountered with the difficulties in the subsequent elaboration. Accordingly conversion of $3\beta,14\alpha$ -dihydroxyandrost-5-en-17-one (Ia) into the iso-steroid was undertaken. When the 3-tosylate (Ib) derivable from Ia was refluxed with potassium acetate, the 6β -hydroxy-3,5-cyclo derivative (IIa) was provided in satisfactory yield. Usual acetylation followed by dehydration with potassium bisulfate in acetic anhydride gave the corresponding Δ^{14} -unsaturated compound (III). On catalytic hydrogenation over palladium-on-charcoal hydrogen attacked the Δ^{14} -double bond from the β -side of the molecule to yield 6β -acetoxy-3,5-cyclo- 14β -androst-17-one (IVb). Hydrolysis of IVb with methanolic potassium hydroxide gave the 6β -hydroxy derivative (IVa), which on treatment with sulfuric acid was led to 3β -hydroxy- 14β -androst-5-en-17-one (V). Oppenauer oxidation accompanied with the migration of the double bond furnished the desired 14β -androst-4-ene-3,17-dione (VI) in satisfactory yield.

17 -Oxygenated 13α -androst-4-en-3-ones (IXa, b, c) were prepared according to the procedure of Bots.⁹⁾ In addition the Δ^4 -3-oxosteroid possessing the double bond at C-14 to C-15 was also synthesized. Reduction of androsta-4,14-diene-3,17-dione (VII) with sodium borohydride and subsequent oxidation with activated manganese dioxide gave 17β -hydroxyandrosta-4,14-dien-3-one (VIII) as was expected.

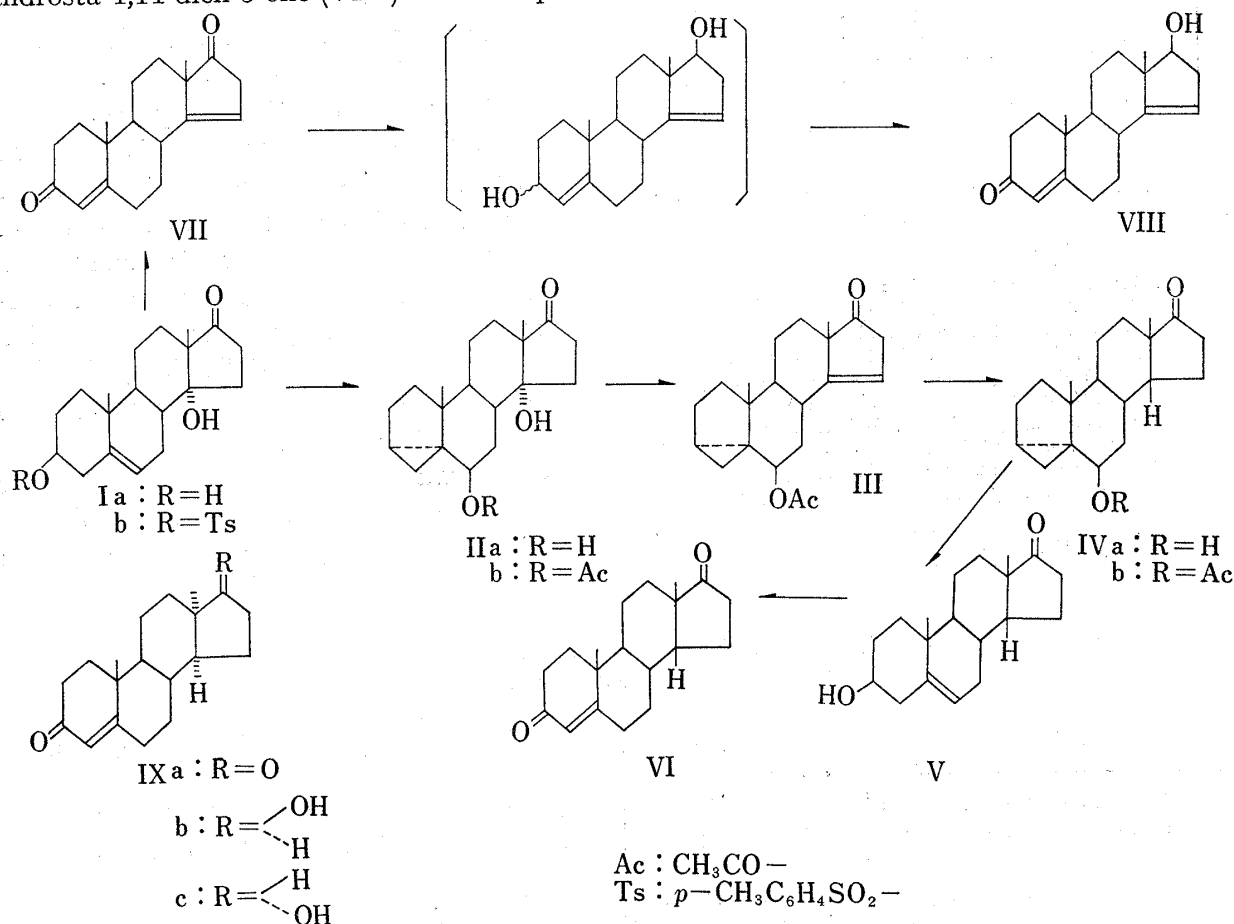


Chart 2

8) A.F. St. André, H.B. MacPhillamy, J.A. Nelson, A.C. Shabica and C.R. Scholz, *J. Am. Chem. Soc.*, **74**, 5506 (1952).

9) J.P.L. Bots, *Rec. Trav. Chim.*, **77**, 1010 (1959).

For the biotransformation experiment the enzyme system consisting of the 10000×g supernatant of human placental tissue in the presence of NADPH and oxygen was used. At the end of the incubation time the mixture was extracted with organic solvent and the phenolic fraction was then separated. In order to obtain an evidence for the occurrence of aromatization a portion of the phenolic fraction was submitted to thin-layer chromatography (see Fig. 1). Staining with both conc. sulfuric acid and Folin-Ciocalteu's reagent demonstrated the formation of the phenolic steroids together with reduction of the 17-ketone. As for 13 α -androst-4-ene-3,17-dione (IXa) the detectable amount of bioconversion product was identified as 13 α -estradiol but not as the 17 α -epimer. In addition two epimeric 17-hydroxy-13 α -androst-4-en-3-ones (IXb, c) were also aromatized to the corresponding 13 α -estradiols. Although no definite characterization could be attained with the C-17 epimeric 14 β -estradiols, the aromatized steroid derived from 14 β -androst-4-ene-3,17-dione (VI) showed the identical

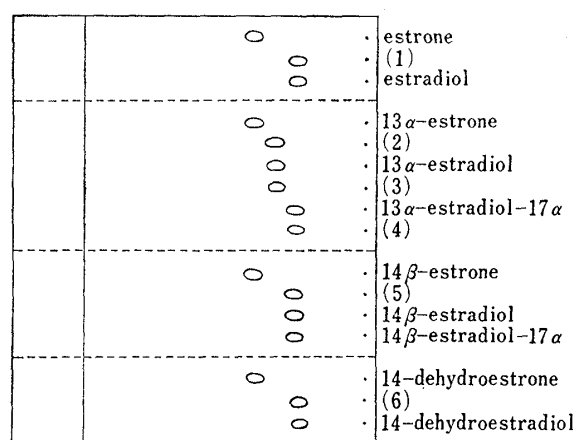


Fig. 1. Thin-Layer Chromatogram of Bio-transformation Products derived from Δ^4 -3-Oxosteroids

Substrates used for incubation:

- (1) androst-4-ene-3,17-dione
- (2) 13 α -androst-4-ene-3,17-dione (IXa)
- (3) 17 β -hydroxy-13 α -androst-4-en-3-one (IXb)
- (4) 17 α -hydroxy-13 α -androst-4-en-3-one (IXc)
- (5) 14 β -androst-4-ene-3,17-dione (VI)
- (6) androsta-4,14-diene-3,17-dione (VII)

adsorbent: silica gel G (E. Merck AG)

developing solvent: hexane-ethyl acetate (7:3)

detection: conc. H₂SO₄, Folin-Ciocalteu's reagent

R_f value with these authentic samples. These findings are of interest in that the human placenta is capable of transforming the C/D-*cis*-linked 17-ketosteroid into the 17-hydroxy derivative. It is also noteworthy that 17 α -hydroxy-13 α -steroid (IXc) is converted to the estrogen with retention of α -configuration of 17-hydroxyl group. The presence of Δ^{14} -double bond also did not substantially affect the biochemical aromatization of Δ^4 -3-oxosteroid.

Then, quantitative assay was carried out with the four representatives of the different series according to Ittrich's method employing the modified Kober reaction.¹⁰⁾ All the biotransformation products exhibited the characteristic coloration of the estrogens, whose absorption maximum appeared at 538.5 m μ .¹¹⁾ The relative yield of the Kober chromogen produced from the above-mentioned substrates was expressed as a percentage of that of androst-4-ene-3,17-dione. As can

be seen in Table I the bioconversion rate of the modified steroids appears to be lower than that of the normal series.

TABLE I. Relative Yield of Kober Chromogens Produced from Δ^4 -3-Oxosteroids

Substrate	Yield ^{a)}	Substrate	Yield ^{a)}
Androst-4-ene-3,17-dione	100	13 α -Androst-4-ene-3,17-dione (IXa)	28
14 β -Androst-4-ene-3,17-dione (VI)	28	Androsta-4,14-diene-3,17-dione (VII)	38

a) Expressed as a percentage of that of androst-4-ene-3,17-dione.
Mean of determination values obtained with duplicate samples.

10) G. Ittrich, *Z. Physiol. Chem.*, **312**, 1 (1958); *idem*, *Acta Endocrinol.*, **35**, 34 (1960).

11) The 14 β -, 13 α - and 14-dehydro-estrogens exhibit the same coloration (λ_{\max} 538.5 m μ) as natural estrogens by the modified Kober reaction under these conditions (T. Nambara and K. Yamanouchi, unpublished data).

It has already been demonstrated that among the unnatural stereoisomers with respect to C-8, C-9 and C-10 an aromatization takes place only with $8\beta,9\beta,10\beta$ -steroids as with normal series ($8\beta, 9\alpha, 10\beta$), not occurring to any demonstrable extent with the other series. These results are indicative of the significance of 8β -hydrogen and β -oriented C-10 methyl group, where an initial step of biotransformation takes place. Recently, Knuppen, *et al.* reported the interesting fact that 14α -hydroxyandrost-4-ene-3,17-dione can readily be aromatized to the C-14-oxygenated estrogen by human placenta.¹²⁾ The authors' observation together with the previous finding would strongly suggest that the structural factors far removed from the site of aromatization do not significantly affect the enzyme system.

It is hoped that these investigations will provide the valuable informations regarding the metabolic fate of the modified steroid hormones as well as the aromatizing enzyme-substrate interaction.

Experimental

Synthesis of Substrates¹³⁾

3 β ,14 α -Dihydroxyandrost-5-en-17-one 3-*p*-Toluenesulfonate (Ib)—To a solution of 3 β ,14 α -dihydroxyandrost-5-en-17-one⁸⁾ (Ia) (100 mg) in pyridine (3 ml) was added *p*-toluenesulfonyl chloride (100 mg) under ice-cooling, and the reaction mixture was stirred at 0° for 43 hr. The solution was poured into ice-water and the resulting precipitate was filtered, washed with H₂O and dried. Recrystallization from aq. acetone gave Ib (113 mg) as colorless prisms. mp 144–148°, $[\alpha]_D^{25}$ –33.4° ($c=0.10$). Anal. Calcd. for C₂₆H₃₄O₅S: C, 68.10; H, 7.47. Found: C, 68.41; H, 7.72.

6 β ,14 α -Dihydroxy-3 α ,5 α -cycloandrostan-17-one (IIa)—To a solution of Ib (98 mg) in 80% aq. acetone (5 ml) was added freshly fused AcOK (150 mg) and the solution was refluxed for 4 hr. After evaporation of the solvent, the residue was extracted with AcOEt, washed with H₂O and dried over anhydrous Na₂SO₄. On usual work-up the crude product obtained was submitted to preparative thin-layer chromatography (TLC) using benzene-ether (2:8) as developing solvent. The adsorbent corresponding to the spot (*R_f* 0.14) was collected and eluted with acetone. Recrystallization of the eluate from aq. MeOH gave IIa (59 mg) as colorless needles. mp 96–98°, $[\alpha]_D^{25}$ +98.4° ($c=0.11$). Anal. Calcd. for C₁₉H₂₈O₃·½H₂O: C, 72.81; H, 9.33. Found: C, 72.67; H, 9.45.

6 β ,14 α -Dihydroxy-3 α ,5 α -cycloandrostan-17-one 6-Acetate (IIb)—Usual acetylation of IIa with Ac₂O and pyridine followed by recrystallization from acetone-hexane gave IIb as colorless plates. mp 175–177°, $[\alpha]_D^{25}$ +94.4° ($c=0.10$). Anal. Calcd. for C₂₁H₃₀O₄: C, 72.80; H, 8.73. Found: C, 73.04; H, 8.71.

6 β -Hydroxy-3 α ,5 α -cycloandrost-14-en-17-one Acetate (III)—To a solution of IIb (570 mg) in Ac₂O (6.5 ml) was added freshly fused KHSO₄ (660 mg) and the solution was heated at 95–100° for 1.5 hr. The reaction mixture was diluted with AcOEt and washed with 5% NaHCO₃ solution, H₂O and dried over anhydrous Na₂SO₄. After usual work-up the residue obtained was chromatographed on acid-washed Al₂O₃ (10 g). Elution with hexane and hexane-benzene (5:5) and recrystallization of the eluate from MeOH gave III (186 mg) as colorless needles. mp 129–132.5°, $[\alpha]_D^{25}$ +42.4° ($c=0.11$). Anal. Calcd. for C₂₁H₂₈O₃: C, 76.79; H, 8.59. Found: C, 76.66; H, 8.34.

6 β -Hydroxy-3 α ,5 α -cyclo-14 β -androstan-17-one Acetate (IVb)—A solution of III (37 mg) dissolved in EtOH (5 ml) was shaken with 5% Pd/C (40 mg) under a stream of H₂ at room temperature for 24 hr. After removal of catalyst by filtration, the filtrate was concentrated to give the crystalline product. Recrystallization from EtOH gave IVb (28 mg) as colorless plates. mp 172–174°, $[\alpha]_D^{25}$ +41.9° ($c=0.11$). Anal. Calcd. for C₂₁H₃₀O₃: C, 76.32; H, 9.15. Found: C, 76.57; H, 9.38.

6 β -Hydroxy-3 α ,5 α -cyclo-14 β -androstan-17-one (IVa)—To a solution of IVb (23 mg) in MeOH (1.8 ml) was added 12.5% K₂CO₃ solution (1.2 ml) and the resulting solution was refluxed for 30 min. After evaporation of the solvent, the residue was extracted with CHCl₃, washed with 5% HCl, H₂O and dried over anhydrous Na₂SO₄. On usual work-up the crude product was recrystallized from MeOH to give IVa (18 mg) as colorless needles. mp 167–168°, $[\alpha]_D^{25}$ +42.2° ($c=0.11$). Anal. Calcd. for C₁₉H₂₈O₂: C, 79.12; H, 9.79. Found: C, 79.09; H, 9.90.

3 β -Hydroxy-14 β -androst-5-en-17-one (V)—To a solution of IVa (270 mg) in AcOH (5 ml) was added conc. H₂SO₄ (5 drops) and the solution was allowed to stand at room temperature overnight. The reaction mixture was diluted with ether, washed with 5% NaHCO₃, H₂O and dried over anhydrous Na₂SO₄. The yellow crystalline product obtained was dissolved in 5% methanolic KOH (30 ml) and refluxed for 30 min.

12) R. Knuppen, O. Haupt, and H. Breuer, *Biochem. J.*, **105**, 971. (1967).

13) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were determined in CHCl₃ unless otherwise specified. Absorption spectra were obtained on Hitachi Model EPS-3 spectrophotometer.

After evaporation of the solvent, the residue was diluted with CHCl_3 , washed with H_2O and dried over anhydrous Na_2SO_4 . On usual work-up the crystalline product was obtained. Recrystallization from acetone gave V (215 mg) as colorless needles. mp $160\text{--}162^\circ$, $[\alpha]_D^{25} + 72.6^\circ$ ($c=0.05$). *Anal.* Calcd. for $\text{C}_{19}\text{H}_{28}\text{O}_2$: C, 79.12; H, 9.79. Found: C, 79.38; H, 10.12.

14 β -Androst-4-ene-3,17-dione (VI)—To a solution of V (190 mg) in toluene (20 ml) was added cyclohexanone (3 ml), and approximately 3 ml of the solvent was distilled off for removal of H_2O . To the resulting solution was added dropwise the solution of $\text{Al}(\text{iso-PrO})_3$ (160 mg) in toluene (4 ml) over a period of 1 hr, during which the solvent was distilled off slowly. After addition of the saturated solution (6 ml) of Rochelle salt, the mixture was submitted to the steam distillation. Then, the residue was extracted with CHCl_3 , washed with H_2O and dried over anhydrous Na_2SO_4 . After usual work-up the yellow crystalline product was subjected to preparative TLC using benzene-ether (6:4) as developing solvent. The adsorbent corresponding to the spot (R_f 0.40) was collected and eluted with AcOEt . Recrystallization of the eluate from MeOH gave VI (45 mg) as colorless prisms. mp $188\text{--}189.5^\circ$, $[\alpha]_D^{25} + 197.5^\circ$ ($c=0.10$). UV $\lambda_{\text{max}}^{\text{EtOH}}$ $m\mu$ (log ϵ): 240 (4.21). *Anal.* Calcd. for $\text{C}_{19}\text{H}_{26}\text{O}_2$: C, 79.68; H, 9.15. Found: C, 79.75; H, 9.01.

17 β -Hydroxyandrosta-4,14-dien-3-one (VIII)—To a solution of androsta-4,14-diene-3,17-dione⁹⁾ (VII) (47 mg) in MeOH (1.5 ml) was added NaBH_4 (47 mg) portionwise and the resulting solution was allowed to stand at room temperature for 1 hr. After addition of AcOH (1 drop), the reaction mixture was diluted with H_2O , extracted with AcOEt . The organic layer was washed with H_2O and dried over anhydrous Na_2SO_4 . On usual work-up an oily residue was obtained. To a solution of the crude product in CHCl_3 (5 ml) was added the activated MnO_2 (600 mg) and the mixture was shaken at 38° for 25 hr. After removal of MnO_2 by filtration, the filtrate was concentrated to give the semi-crystalline product, which in turn was submitted to preparative TLC using hexane- AcOEt (6:4) as developing solvent. The adsorbent corresponding to the spot (R_f 0.33) was collected and eluted with AcOEt . Recrystallization of the eluate from hexane- CHCl_3 gave VIII (12 mg) as colorless needles. mp $163\text{--}166^\circ$, $[\alpha]_D^{25} + 128^\circ$ ($c=0.02$). UV $\lambda_{\text{max}}^{\text{MeOH}}$ $m\mu$ (log ϵ): 240 (4.27). *Anal.* Calcd. for $\text{C}_{19}\text{H}_{26}\text{O}_2$: C, 79.68; H, 9.15. Found: C, 79.58; H, 9.02.

13 α -Androst-4-ene-3,17-dione (IXa)—Prepared from 3 β -hydroxy-13 α -androst-5-en-17-one according to the procedure of Bots.⁹⁾ mp $148\text{--}150^\circ$.

17 β -Hydroxy-13 α -androst-4-en-3-one (IXb), 17 α -Hydroxy-13 α -androst-4-en-3-one (IXc)—Prepared from IXa according to the procedure of Bots.⁹⁾ IXb: mp $152\text{--}153^\circ$, IXc: mp $127\text{--}129.5^\circ$.

Incubation with Human Placenta and Extraction Procedure—Human placenta, obtained immediately after delivery, was processed at 4° according to Ryan's procedure.⁴⁾ The placental tissue was teased free of large blood vessels, put through a meat grinder, weighed and homogenized in 0.05M phosphate buffer for 1 min. The homogenate was centrifuged at $10000 \times g$ for 30 min, and the supernatant was separated and stored in a deep freezer. In a typical run the supernatant (5 ml) (9.5 g wet weight of tissue) was incubated with the steroid (100 μg) dissolved in propylene glycol (0.1 ml) and with 0.05M phosphate buffer (pH 7.1) (0.5 ml) containing NADPH (Sigma Chem., Co.) (3 mg) in air at 37° for 1 hr with shaking. The incubation mixture was extracted with CHCl_3 (30 ml \times 3), and the organic phase was evaporated to dryness *in vacuo*. The residue was dissolved in toluene (30 ml) and partitioned with 1N NaOH (30 ml \times 2 and 20 ml \times 1). The alkaline solution was neutralized with conc. HCl and then extracted with ether (50 ml \times 2 and 30 ml \times 1). The combined organic phases were washed with H_2O and dried over anhydrous Na_2SO_4 . After evaporation of solvent the residue obtained was subjected to the analysis.

Thin-Layer Chromatography—A portion of the phenolic fraction separated from the incubation mixture was chromatographed on silicagel G (E. Merck AG) plate using hexane- AcOEt (7:3) and benzene-ether (8:2) as developing solvent. For the staining of the phenolic steroids on the plate both conc. H_2SO_4 and Folin-Ciocalteu's reagent were employed. The typical chromatograms obtained with the former solvent system are illustrated in Fig. 2. The aromatized steroids could also be identified in the same manner with the latter system.

Determination of Kober Chromogens Produced from Δ^4 -3-Oxosteroids—To an ethanolic solution (0.5 ml) of estrogen was added 4% hydroquinone in EtOH (0.5 ml), and the resulting solution was evaporated to dryness under a stream of N_2 . The residue thus obtained was dissolved in 65% H_2SO_4 (2 ml) and was heated on the boiling water bath for 40 min with occasional agitation. After cooling in ice-bath the reaction mixture was diluted with H_2O (2 ml) and then allowed to stand for 15 min. To this solution was added 2% *p*-nitrophenol in tetrachloroethane (4 ml), and the mixture was shaken vigorously for 30 sec and then centrifuged at 3000 rpm for 7 min. The upper layer was discarded and the remaining organic phase was submitted to colorimetric determination. The extinction of the colored solution was read at 538.5 $m\mu$ (λ_{max}) and was corrected according to Allen's method. The relative yield of Kober chromogens was expressed as a percentage of that of androst-4-ene-3,17-dione as listed in Table I.

Acknowledgement The authors express their gratitudes to Dr. Pierre Crabbé, Syntex Research Laboratory, for generous gift of 14 β -estrone and to all the staff of the central analytical laboratory of this Institute for elemental analyses and spectral measurements. Thanks are also due to Dr. M. Hirano, Department of Obstetrics & Gynecology of this Faculty for making available the placenta used in this study. This work was supported in part by a Grant-in-Aid from Yakurikenkyukai, which is gratefully acknowledged.