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Conversion of an Androgen Epoxide into 17β-Estradiol by Human Placental Microsomes†

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ABSTRACT: Three androgen epoxides, 17β-hydroxy-4β,5-oxido-5β-androstan-3-one (II), 3β,19-dihydroxy-5,6β-oxido-5β-androstan-17-one 3-acetate (VIII), and 19-hydroxy-4β,5-oxido-5β-androstane-3,17-dione (V), were synthesized and subsequently evaluated as potential precursors in the biosynthesis of estrogens by incubation with human placental microsomes. One of these epoxides (V) was converted into 17β-estradiol, whereas the other two were metabolized to unidentified products. The possible intermediacy of an androgen epoxide in the biosynthesis of estrone and of 17β-estradiol is discussed and a mechanism is proposed for the aromatization process.

The placental aromatization of the A ring of androgens requires NADPH and oxygen (Ryan, 1958, 1959) and both 19-hydroxy-4-androstene-3,17-dione (XII, R = OH; R′ = H) (Meyer, 1955; Ryan, 1959; Longchampt *et al.*, 1960; Morato *et al.*, 1961; Wilcox and Engel, 1965) and 19-oxo-4-androstene-3,17-dione (XII, R, R′ = O) (Axelrod *et al.*, 1965; Akhtar and Skinner, 1968; Oh and Tamaoki, 1971) have been shown to be involved as intermediates. The same cofactors are required for the aromatization of 19-norandrogens (Townsley and Brodie, 1968) and androgens with an oxygen function at C-19 (Morato *et al.*, 1961) and there is evidence (Meigs and Ryan, 1971; Bellino and Osawa, 1974) that several mixed function oxidases may be involved in the aromatization process. After the introduction of the oxygen at C-19 there remain, in chemical terms, only two operations to be effected for estrogen formation. One is the removal of the hydrogen atoms at C-1 and C-2 and the other is the expulsion of the angular substituent at C-10. Although it is well established (Morato *et al.*, 1962; Brodie *et al.*, 1969; Fishman *et al.*, 1969; Osawa and Spaeth, 1971) that the C-1 and C-2 hydrogen atoms are stereospecifically lost from the β side of the molecule, the actual mechanism for the final stages of the aromatization of the A ring is still not clear. Morato *et al.* (1962) have suggested that aromatization of 19-oxoandrogens may involve a substituent at C-1 or concerted removal of the 1β-hydrogen atom with expulsion of the angular group at C-10. Experimental results with various androgen derivatives (Townsley and Brodie, 1968; Brodie *et al.*, 1969) have not confirmed these views.

Recently, Morand *et al.* (1974) proposed that epoxidation of the double bond in the A ring (XIII) or the B ring (XI) of androgens may play a role in the biological aromatization process. This paper describes the synthesis of some potential androgen epoxide precursors and the results of incubation of these compounds with human placental microsomes.

Experimental Procedure

Materials. Solvents used for extractions and chromatography were reagent grade. Testosterone, 17β-estradiol, es-

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trone, NADPH, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of Placental Microsomal Fractions. All procedures were carried out at 4°. Human placentas, obtained immediately after delivery, were immersed in ice-cold aqueous 0.15 M potassium chloride, then blotted dry, and put through a meat grinder. The tissue was then homogenized in a Waring Blendor for 1 min with 1.5 vol of buffer solution containing 0.25 M sucrose and 0.05 M sodium phosphate (pH 7.0). The homogenate was centrifuged for 30 min at 10,000*g;* the supernatant was decanted and centrifuged for 60 min at 105,000*g*. The resulting microsomal pellet was suspended in a volume of homogenizing buffer to give a protein concentration of 35 mg/ml. The microsomal suspensions were stored frozen. Protein was determined by the method of Lowry *et al.* (1951).

Incubation Procedure. Incubations were carried out essentially as described by Meigs and Ryan (1971). Incubation mixtures contained the following: 100 μmol of sodium phosphate (pH 7.0); 10 μmol of NADPH; 100 μmol of glucose 6-phosphate; 14 units of glucose-6-phosphate dehydrogenase; and 300 μg of steroid substrate in 150 μl of 95% ethanol. The final volume was 2.5 ml. Incubations were carried out in air at 37° for 35 min. When it was necessary to obtain quantities of steroid product sufficient for rigorous identification, large scale incubations were carried out in which incubation components were increased by a factor of 50 and the final incubation volume was 125 ml.

Extraction and Isolation of Steroid Products. Incubations were terminated by extraction of the incubation medium with an equal volume of chloroform (three times). The chloroform extracts were pooled, washed with water, and dried over magnesium sulfate. In preliminary experiments 17β-estradiol or estrone formation was determined by thin-layer chromatography of a portion of the chloroform extract on silica gel G (Macherey-Nagel and Co., Düren) in the solvent system benzene–acetone (4:1) or benzene–ethyl acetate (7:3). Steroids were visualized by spraying with 2% sulfuric acid in ethanol and their mobilities compared to those of authentic 17β-estradiol and estrone. In the large scale incubations, products were isolated by thin-layer chromatography and a small portion of the chromatogram was sprayed with 2% sulfuric acid to locate the steroids. Material with an R_F corresponding to that of authentic 17β-estradiol was eluted from the silica gel with methanol and further characterized by ultraviolet absorption (Perkin-Elmer double beam spectrophotometer), gas–liquid chromatography (Carlo-Erba GLC, 6-ft G.E. SE-30 column), and mass spectrometry (A.E.I. MS 902S double-focusing high resolution mass spectrometer). The diacetate of 17β-estradiol was prepared by treatment with pyridine and acetic anhydride and the diheptafluorobutyrate was prepared by the method of Challis and Heap (1970).

Synthesis of Androgen Epoxides. GENERAL. Melting points were determined on a Hoover Uni-Melt apparatus and are uncorrected. Infrared and nuclear magnetic resonance spectra were recorded on a Beckman IR-8 spectrophotometer and on a Varian HA-100 spectrometer, respectively. A Durrum-Jasco automatic spectropolarimeter, Model ORD-5, and a Perkin-Elmer 141 recording polarimeter were used to determine optical rotatory dispersion curves and optical rotations, respectively. Reactions were monitored by thin-layer chromatography (pre-coated plates, SIL G-25 UV_{254}, Macherey-Nagel Co., Düren) and sulfuric acid was used as a spraying agent. For column chromatographies an L.K.B. 3400 fraction collector was used with SilicaR (200–325 mesh) as the adsorbent.

17β-HYDROXY-4β,5-OXIDO-5β-ANDROSTAN-3-ONE (II). This epoxide was prepared by treatment of a methanolic NaOH solution of testosterone (I) with a 30% hydrogen peroxide solution (Ringold *et al.*, 1956). A pure sample, crystallized from methanol, had mp 153–155° (lit. mp 157–158°); nuclear magnetic resonance (nmr) ($CDCl_3$) 364 (t, H-17), 296 Hz (s, H-4).

3β,19-DIHYDROXY-5-ANDROSTEN-17-ONE 3-ACETATE (VIIa). 3β-Hydroxy-5-androsten-17-one acetate (III) was converted to the 6β,19-oxide (IV) by the method of Kalvoda *et al.* (1963). Treatment of this compound in refluxing ethanol with zinc powder gave 3β,19-dihydroxy-5-androsten-17-one 3-acetate (VIIa) (Tadanier, 1963; Morand and Van Tongerloo, 1973); mp 158–160°; lit. mp 157–158°.

3β,19-DIHYDROXY-5,6β-OXIDO-5β-ANDROSTAN-17-ONE 3-ACETATE (VIII). *m*-Chloroperbenzoic acid (560 mg, 3.25 mmol) dissolved in methylene chloride (15.0 ml) was added to a solution of 3β,19-dihydroxy-5-androsten-17-one 3-acetate (VIIa) (913 mg, 2.63 mmol) in methylene chloride (15.0 ml) and the reaction mixture was left in the dark at room temperature for 24 hr. After washing with aqueous solutions of sodium sulfite and sodium bicarbonate the methylene chloride solution was dried and the solvent removed under reduced pressure. Crystallization of the crude product from methylene chloride–ether gave two crops (470 and 108 mg) of pure epoxide (VIII) as white needles: mp 159–160°; $[\alpha]^{25}_D$ ($CHCl_3$) + 45°; $\nu_{CHCl_3}^{max}$ 3560 (OH), 1745 (CO), 1252 cm^{-1} (COC); nmr ($CDCl_3$) 482 (m, H-3, $W_{1/2}$ = 9.0 Hz), 420 (d, H-19, J = 5.5 Hz), 356 (t, 19-OH), 310 (d, H-6, J = 1.0 Hz), 202 Hz (s, OAc); ($CDCl_3$–D_2O) 482 (m, H-3), 391 (q, H-19, J = 5.5 Hz), 310 Hz (d, H-6, J = 1.0 Hz).

19-HYDROXY-4-ANDROSTENE-3,17-DIONE (VI). Hydrolysis of 3β,19-dihydroxy-5-androsten-17-one 3-acetate (VIIa) in methanol containing potassium carbonate gave 3β,19-dihydroxy-5-androsten-17-one (VIIb) (Tadanier, 1963): mp 214°; lit. mp 213–219° dec. A solution of this diol (VIIb) (882 mg, 2.90 mmol) in toluene (8.8 ml) and freshly distilled cyclohexanone (2.6 ml) was heated and 2.0 ml of solvent removed by distillation. To the refluxing solution, aluminum isopropoxide (176 mg) dissolved in dry toluene (2.0 ml) was added and heating was continued for 10 min. The reaction was stopped by adding water (2.0 ml) and the cooled reaction mixture was diluted with benzene (40 ml). The solution was washed with dilute HCl and then with water. After drying the solution ($MgSO_4$), the solvent was removed under reduced pressure and the crude product crystallized from methanol (661 mg). The compound obtained in this manner was identified as 19-hydroxy-4-androstene-3,17-dione (VI) (Bowers *et al.*, 1962); mp 166–167°; lit. mp 168–170°.

19-HYDROXY-4β,5-OXIDO-5β-ANDROSTANE-3,17-DIONE (V). A solution of 19-hydroxy-4-androstene-3,17-dione (VI) (200 mg, 0.66 mmol) in methanol (20.0 ml) was cooled to 0° and aqueous solutions of 4N NaOH (0.8 ml) and 30% hydrogen peroxide (0.8 ml) were then added. The reaction mixture was maintained at 0° for 16 hr, water (30 ml) was added, and most of the methanol removed under reduced pressure. Extraction with chloroform and working up in the usual manner gave the crude epoxide (206 mg). Crystallization from methanol gave pure 19-hydroxy-4β,5-

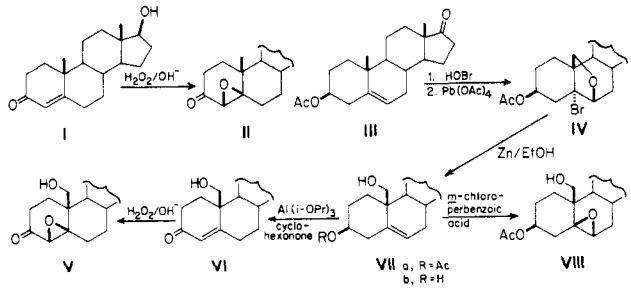


FIGURE 1: Synthesis of androgen epoxides.

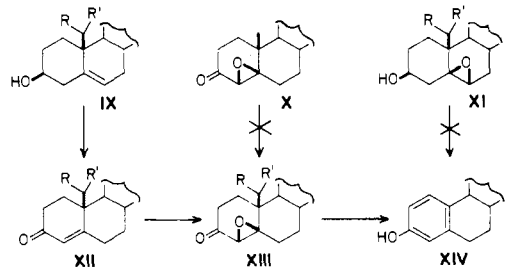


FIGURE 2: Postulated pathway for the biosynthesis of estrone and estradiol (R = OH, R′ = H; R = R′ = OH; R, R′ = O).

oxido-5β-androstane-3,17-dione (V) (137 mg): mp 185°; $[\alpha]^{25}_D$ +247°; nmr ($CDCl_3$) 393 (q, H-19, J = 5.5 Hz), 298 Hz (s, H-4); optical rotatory dispersion (CH_3OH) $[\Phi]_{450}$ +1020°, $[\Phi]_{329}$ +5350°, $[\Phi]_{306}$ 0°, $[\Phi]_{282}$ −4205°.

Anal. Calcd for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23. Found: C, 71.76; H. 8.28.

Results

In order to determine whether androgen epoxides could act as precursors in the biosynthesis of estrone and 17β-estradiol, it was decided to synthesize the three following compounds (see Figure 1): a 4β,5β-epoxide with the C-10 angular methyl group intact (II), a 5β,6β-epoxide with a C-19 hydroxyl group (VIII), and a 4β,5β-epoxide with a C-19 hydroxyl group (V). 17β-Hydroxy-4β,5-oxido-5β-androstan-3-one (II) was prepared directly from testosterone (I) (Ringold *et al.*, 1956) while the two other epoxides were prepared by a series of reactions starting with 3β-hydroxy-5-androsten-17-one acetate (III). The latter was transformed into the 6β,19-oxide (IV) (Kalvoda *et al.*, 1963) which could then be opened by treatment with zinc to give the known 3β,19-dihydroxy-5-androsten-17-one 3-acetate (VIIa) (Tadanier, 1963). Under the orienting influence of the C-19 hydroxyl group (Henbest and Wilson, 1957; Morand and Kaufman, 1971), the reaction of II with peracid gave exclusively the 5β,6β-epoxide (VIII). The β configuration of the epoxide group was confirmed by examination of the nuclear magnetic resonance spectrum (see Experimental Section for details) which shows the same type of stereochemical dependence of vicinal H-C-O-H coupling constants observed in the analogous compound in the cholestane series (Fraser *et al.*, 1969). Hydrolysis of the monoacetate (VIIa) gave the diol (VIIb) which was converted in good yield to the ketone (VI) by Oppenauer oxidation. Treatment of the latter with hydrogen peroxide in the presence of base gave the epoxide V whose structure was confirmed by the "reversed Cotton effect" observed in its optical rotatory dispersion curve (Djerassi *et al.*, 1965).

Incubation of 17β-hydroxy-4β,5-oxido-5β-androstan-3-one (II) and extraction of the medium (see Experimental Section) indicated that no detectable amount of estrone or of 17β-estradiol had been formed. However, most of the precursor had been metabolized and the presence of three substances was observed, all of which were more polar than 17β-estradiol on thin-layer chromatography. Similarly, the 19-hydroxy-5β,6β-epoxide (VIII) was found to be metabolized during the incubation period but neither estrone nor 17β-estradiol could be detected. When 17β-hydroxy-4β,5-oxido-5β-androstane-3,17-dione (V) was incubated under the same conditions used for the two other epoxides (II and VIII), thin-layer chromatography indicated disappearance of the precursor and a major spot corresponding to 17β-estradiol. In large scale control experiments without epoxide V in the incubation medium, no trace of 17β-estradiol or of estrone could be detected. All three epoxides were unchanged when incubated in the biological medium containing all the cofactors except the placental microsomes.

In a large scale incubation with 19-hydroxy-4β,5-oxido-5β-androstane-3,17-dione (V) it was possible to isolate 4.3 mg of material with the same R_F value as 17β-estradiol. Further purification by preparative thin-layer chromatography gave a pure substance which was unequivocally identified as 17β-estradiol in the following manner. An ether solution of the compound isolated showed the characteristic ultraviolet absorption at 280 nm (Fieser and Fieser, 1959) and when half the amount was treated with heptafluorobutyric anhydride, the resulting derivative had the same retention time as the diheptafluorobutyrate of 17β-estradiol on gas–liquid chromatography. The remainder of the unesterified substance was submitted to mass spectral analysis. A mass spectrum, determined at a source temperature of 250°, showed an ion peak at 272 mass units corresponding to the molecular weight of 17β-estradiol. However, the presence of an intense ion peak at 205 could not be explained and it was decided to determine the mass spectrum again after exposing the substance to acetylating conditions. The mass spectrum (source temperature of 250°) of the product obtained showed a molecular ion peak at 356 (molecular weight of 17β-estradiol diacetate, 356) and the same fragment ion peaks (214, M − ketene; 254, M − ketene − acetic acid) (Okerholm *et al.*, 1971) with the corresponding intensities observed for an authentic sample of 17β-estradiol diacetate.

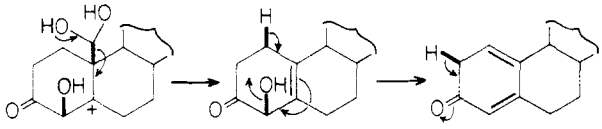
Discussion

The enzymatic conversion of 19-hydroxy-4β,5-oxido-5β-androstane-3,17-dione (V) into 17β-estradiol demonstrates that such an epoxide may, in fact, be involved as an intermediate in the biosynthesis of estrogens. The results reported here also show that although both the epoxide with the C-10 methyl group intact (II) and the 19-hydroxy-5β,6β-epoxide (VIII) are metabolized, they are not converted to estrone or 17β-estradiol. Therefore, if an epoxide is involved, it must be formed *after* oxidation of the Δ^5-3β-alcohol to the Δ^4-3-ketone and functionalization of the methyl group at C-10 (see Figure 2, XIII, R = OH, R′ = H or R, R′ = O). Since 19-hydroxy and 19-oxo androgens with the double bond in the A ring (XII, R = OH, R′ = H; R, R′ = O) are aromatized by placental microsomes extremely rapidly (Wilcox and Engel, 1965), this may explain why epoxides (*e.g.*, XIII, R = OH, R′ = H or R, R′ = O) have so far not been identified in biological incubations of androgen precursors.

The plausibility of an epoxide precursor is enhanced

when one considers the cofactors required in the biological aromatization process, the precedents for epoxide formation in terpene and steroid biosynthesis (Corey *et al.*, 1966; van Tamelen *et al.*, 1966), and the importance of carbonium ions in biogenetic theory (Eschenmoser *et al.*, 1955; Bascoul and Crastes de Paulet, 1970). Also, in terms of reactive sites in the molecule, attack of the C-4,C-5 double bond seems more likely than substitution at C-1 or C-2.

The intermediacy of a 4β,5β-epoxide in the biosynthesis of estrone and estradiol makes it possible to write a mechanism in which the 1β- and 2β-hydrogen atoms are stereospecifically removed (see below). On opening the epoxide,



the carbonium ion which results can be stabilized by removal of the C-10 substituent and concomitant formation of a double bond in the C-5,C-10 position. From models, one observes that an intermediate of this type has its 1β-hydrogen atom in a quasiaxial orientation. Subsequent loss of the 4β-hydroxyl group concerted with a shift of the C-5,C-10 double bond from the α side of the molecule and loss of the 1β proton gives the A ring dienone shown above. Enolization, involving the 2β-hydrogen atom (Corey and Sneen, 1956) gives the aromatic A ring of estrone or 17β-estradiol.

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