Biosynthesis of Estrogens. Estr-5(10)-ene-3,17-dione: Isolation, Metabolism and Mechanistic Implications

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The 16-$^2H_2$ title compound **5b** constituted a significant amount of the non-aromatic metabolites recovered from incubations of 3,17-dioxo-[16,16,19-$^2H_3$]androst-4-en-19-al **1** with placental aromatase. For the evaluation of the role of compound **5b** in the elaboration of estrogens, its transformations at pH 6.5 and 7.2 in the presence and absence of microsomal placental aromatase were investigated. In the presence of the aromatase at pH 6.5, estrogens (6.8%), products of isomerization of the double bond [$\Delta^{5(10)} \rightarrow \Delta^4$] and products of reduction of the carbonyl groups were formed. When the incubation was carried out at pH 7.2, products similar to those obtained above were isolated but in different yields. Noticeably more estrogens (22.7%) and less of the reduced products were formed. Additionally, at pH 7.2, 10β-hydroxy-[16,16-$^2H_2$]estr-4-ene-3,17-dione **4a** was obtained.

In the absence of the aromatase, which was replaced with bovine albumin at both pH 6.5 and 7.2, [16,16-$^2H_2$]estr-4-ene-3,17-dione **3a** and its 10β-hydroxy derivative **4a** were formed in large amounts and were the only products detected.

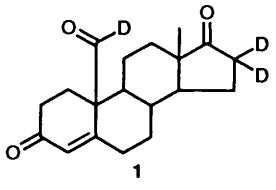
The ramifications of our observations in the context of estrogen biosynthesis are discussed.

In the course of systematic investigation of the metabolism of 3,17-dioxo-[16,16,19-$^2H_3$]androst-4-en-19-al **1** by placental microsomal aromatase,[1,2] we have isolated dideuteriated estrone **2a**, estradiol **2d**, estr-4-ene-3,17-dione **3a**, estra-4,6-di-ene-3,17-dione **3b**, and 10β-hydroxyestr-4-ene-3,17-dione **4a**, together with several trideuteriated C_{19}, and dideuteriated C_{18} metabolites. In continuation of these studies, we have now identified a compound which, following attempted conversion into a methyloxime-trimethylsilyl ether (MO-TMS), gave a product whose mass spectrum (GLC–MS) slowed *m/z* 332 (20%), 331 (18), 317 and 316 (each 10), 301 (72), 300 (100) and 270 and 269 (each 55), *etc.* The mass spectrum indicated that the unknown is very likely the bis-MO of [16-$^2H_2$]estr-(ξ)-ene-3,17-dione. The GLC retention time and mass spectrum of the bis-MO metabolite differed from that of authentic protiated **3a** bis-MO, which showed *m/z* 330 (100%), 317 and 316 (each 5%), 301 (68%) and 300 (79%), *etc.* Note the differences in abundance of bis-MO molecular ions *m/z* 332 (20%) of the deuteriated metabolite and *m/z* 330 (~ 100%) of the protiated form of 4-ene analogue **3a**.

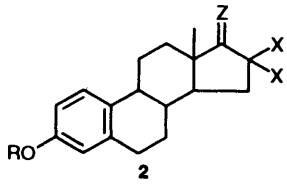
Cleavage of the C-10(19) bond is an obligatory step in the biosynthesis of estrogens from androgens. It was proposed that the scission results in a C-10 radical or cation[3] **7** (see below). Consequently, we have considered the possibility that the C-4 double bond of species **7** isomerized to C-5(10) to yield compound **5b**. To test this hypothesis, authentic samples of protiated (**5a**) and two samples of 16,16-dideuterio (**5b**) compounds were prepared and their syntheses are described in the Experimental section.

The mass spectra of bis-MO of authentic deuteriated (**5b**) and protiated (**5a**) diones (except for a shift by two mass units) were identical with the MS of bis-MO of the metabolite, which established its structure.

We[2] and others have shown[3,4] that 10β-hydroxyestr-4-ene-3,17-dione **4e** is not an estrogen precursor.[5] It was demonstrated that [16,16-$^2H_2$]estr-4-ene-3,17-dione **3a** is metabolized to estrone by placental aromatase in lower yield than is its C_{19} analogue.[6] This led to the conclusion that compound **3a** is not the *preferred* substrate for estrogen biosynthesis by *placental*

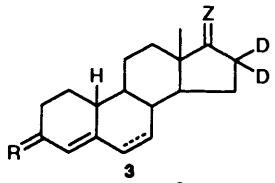


1



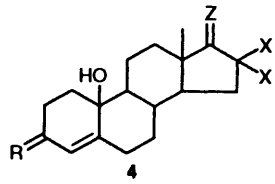
2

a; R = H, Z = O, X = D
b; R = Me, Z = O, X = H
c; R = Me, Z = O, X = D
d; R = H, Z = β-OH,H X = D
e; R = Me, Z = β-OH,H X = D



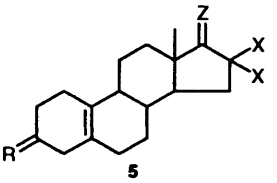
3

a; R = Z = O (no Δ^6)
b; R = Z = O (Δ^6)
c; R = ξ-OH,H, Z = β-OH,H
d; R = ξ-OH,H, Z = β-OH,H (Δ^6)



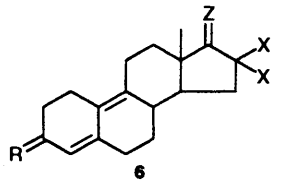
4

a; R = Z = O, X = D
b; R = ξ-OH,H Z = β-OH,H, X = D
c; R = O, Z = β-OH,H X = D
d; R = O, Z = β-OH,H X = H
e; R = Z = O, X = H
f; R = ξ-OH,H Z = β-OH,H, X = H



5

a; R = Z = O, X = H
b; R = Z = O, X = D
c; R = β-OH,H Z = β-OH,H, X = D
d; R = α-OH,H Z = β-OH,H, X = D



6

a; R = Z = O, X = H
b; R = ξ-OH, H, Z = β-OH,H, X = D
c; R = O, Z = β-OH,H, X = H
d; R = O, Z = β-OAc,H, X = H
e; R = ξ-OH,H Z = β-OH,H, X = H

Table 1 Products of incubation of [16,16-$^2H_2$]estr-5(10)-ene-3,17-dione **5b** with microsomal placental aromatase at pH 6.5 (Exp. 1) and pH 7.2 (Exp. 2)[a]

[16,16-$^2H_2$]Steroid formed	Products (%) Exp. 1	Exp. 2
(1) Estr-5(10)-ene-3β,17β-diol **5c**	65.3	38.0
(2) Estr-(ξ)ane-3β,17β-diol	6.4	9.0
(3) Estr-5(10)-ene-3α,17β-diol **5d**	1.8	2.2
(4) Estra-5(10),6-diene-3β,17-diol[b] **11a**	2.9	8.0
(5) Estr-4-ene-3,17-dione **3a**	2.3	4.4
(6) 17β-Hydroxyestr-4-en-3-one **3c**	10.5	11.9
(7) Estrone **2a**	1.0	10.2
(8) Estradiol **2d**	5.8	12.5
(9) 10β-Hydroxyestr-4-ene-3,17-dione **4a**		4.5
(10) Estr-4-ene-3ξ,10β,17β-triol **4b**	< 1.0	< 1.0
(11) 10β,17β-Dihydroxyestr-4-en-3-one **4c**	< 1.0	< 1.0

[a] See Experimental section for conditions of incubation. [b] Tentatively assigned structure.

Table 2 Products of incubation of [16,16-$^2H_2$]estr-5(10)-ene-3,17-dione **5b** at pH 6.5 (Exp. 1) and pH 7.2 (Exp. 2) with the full complement of cofactors except that placental aromatase was replaced by bovine albumin[a]

[16,16-$^2H_2$]Steroid formed	Products (%) Exp. 1	Exp. 2
(1) Estr-5(10)-ene-3,17-dione **5b** recovered	20.3	34.3
(2) Estr-4-ene-3,17-dione **3a**	53.5	57.9
(3) 10-Hydroxyestr-4-ene-3,17-dione **4a**	26.3	8.3

[a] See Experimental section for conditions of incubation.

aromatase.[6] Thus, it is unlikely that free or enzyme-bound estr-4-ene-3,17-dione **3a** is an obligatory intermediate of estrogen biosynthesis from substrate **1** by placental aromatase.

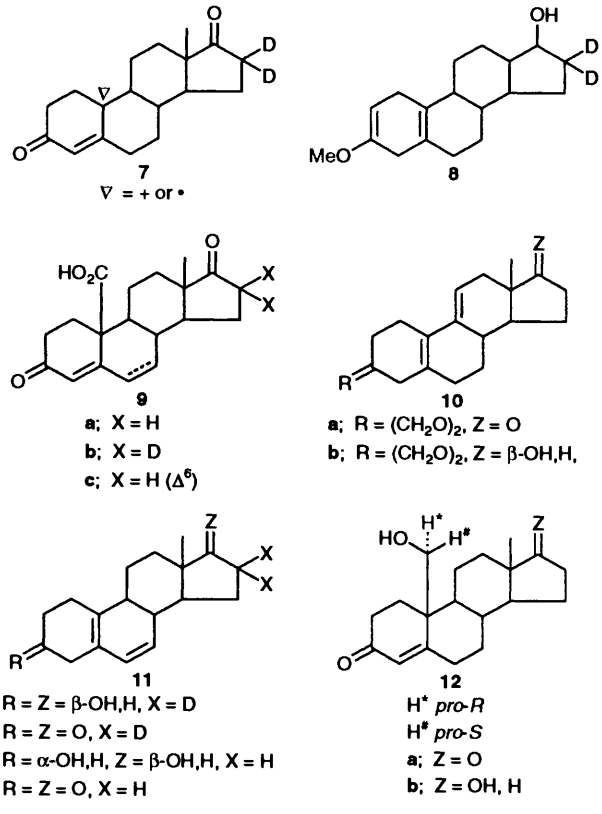
In the course of the current investigations it was noticed that [16,16-$^2H_2$]estr-5(10)-ene-3,17-dione **5b** was consistently produced in incubations of the 19-al **1** with aromatase and is a significant component of the recovered non-aromatic metabolites. It was therefore of obvious interest to assess the role of compound **5b** in estrogen formation and to evaluate its metabolism. To this end compound **5b** was incubated under the usual experimental conditions at pH 6.5 and 7.2 in the presence and absence of placental aromatase. The results of studies in the presence of placental aromatase are summarized in Table 1.

Under both experimental conditions six major metabolic transformations of compound **5b** were noted: (a) reduction of C-17 carbonyl; (b) reductions of C-3 carbonyl; (c) reduction of the olefinic bond; (d) isomerization of $\Delta^{5(10)} \longrightarrow \Delta^4$; (e) 10β-hydroxylation; (f) aromatization.

At pH 6.5 (Exp. 1) reductive processes (a–c) and isomerization of the double bond (d) predominate. It is worthy of note that the product of reduction of both C-3 and -17 carbonyls (diol **5c**) (Table 1, entry 1) is the most abundant metabolite formed (~ 65%). The accumulation of large amounts of the C-3 epimeric 3ξ,17β-diols (Table 1, entries 1 and 3) tends to indicate that reduction of the C-3 carbonyl occurred early in the incubation, which stabilized the 5(10)-double bond and diminished its isomerization to C-4. In Experiment 1 at pH 6.5, 10-hydroxy compounds were detected in trace amounts (Table 1, entries 10, 11). Only small amounts of [16,16-$^2H_2$]estrone **2a** (1%) and of [16,16-$^2H_2$]estradiol **2d** (5.8%) (Table 1, entries 7 and 8) were obtained for a total estrogen production of ~ 7%. At present we cannot differentiate whether or not the accumulated estradiol is derived from the initially formed estrone or if the reduction of the C-17 ketone occurred at an intermediate stage of the biosynthesis.[7]

The transformations at pH 7.2 (Table 1; Exp. 2) were significantly different in that the reductive processes were less dominant. Although diol **5c** was still the major metabolite, it represented only 38% of the recovered products.

The isolation of an unknown estradiene-3,17-diol, which was later *tentatively* identified as compound **11a** (Table 1, entry 4), was of interest. Structures [16,16-$^2H_2$]estra-4,9-diene-3ξ,17β-diol **6b**, [16,16-$^2H_2$]estra-4,6-diene-3ξ,17β-diol **3d** and [16,16-$^2H_2$]estra-5(10),6-diene-3ξ, 17β-diol **11a** were considered for the unknown. Elaboration of structure **6b** could be rationalized in a mechanistically plausible manner, whereby elimination (concerted or stepwise) of the C-9 hydrogen atom occurred in the course of the C-10 → C-4 double-bond isomerization to give diene dione **6a** (X = D). Reduction of the carbonyls would yield the diene diol **6b**. The 4,6-diene diol **3d** could be derived from the previously isolated[1] estra-4,6-diene-3,17-dione **3b** or from the aldehyde **14** as shown in Fig. 1. Alternatively, 6(7)-dehydrogenation of substrate **5b**, and reduction of both carbonyls, would also yield diene diol **11a**.



The syntheses of reference samples **6e**, **3d** (protiated) and **11c** are described in the Experimental section. The GLC–MS spectra of authentic bis-TMS of **6e** and **3d** (protiated) differed from that of the bis-TMS of the metabolite. In contrast, the GLC–MS of bis-TMS of the reference estra-5(10),6-diene-3α,17β-diol **11c** *matched* that of the bis-TMS of the metabolite (shifted by two mass units) *except* for differences in the relative intensities of several peaks. Therefore, it is reasonable to assume that the metabolite is estra-5(10),6-diene-3β,17β-diol **11a** (Table 1; entry 4). Very likely, the observed peak intensity differences between the two spectral profiles reflect the expected differences upon fragmentation of the 3α- and 3β-epimers.

The total amount of $\Delta^{5(10)} \longrightarrow \Delta^4$ double-bond isomerization can be calculated as the sum of entries 2,5,6 and 9 (Table 1). It follows that at pH 6.5 (Exp. 1) ~ 19% and at pH 7.2 (Exp. 2) ~ 30% of the substrate was isomerized. As will be seen later,

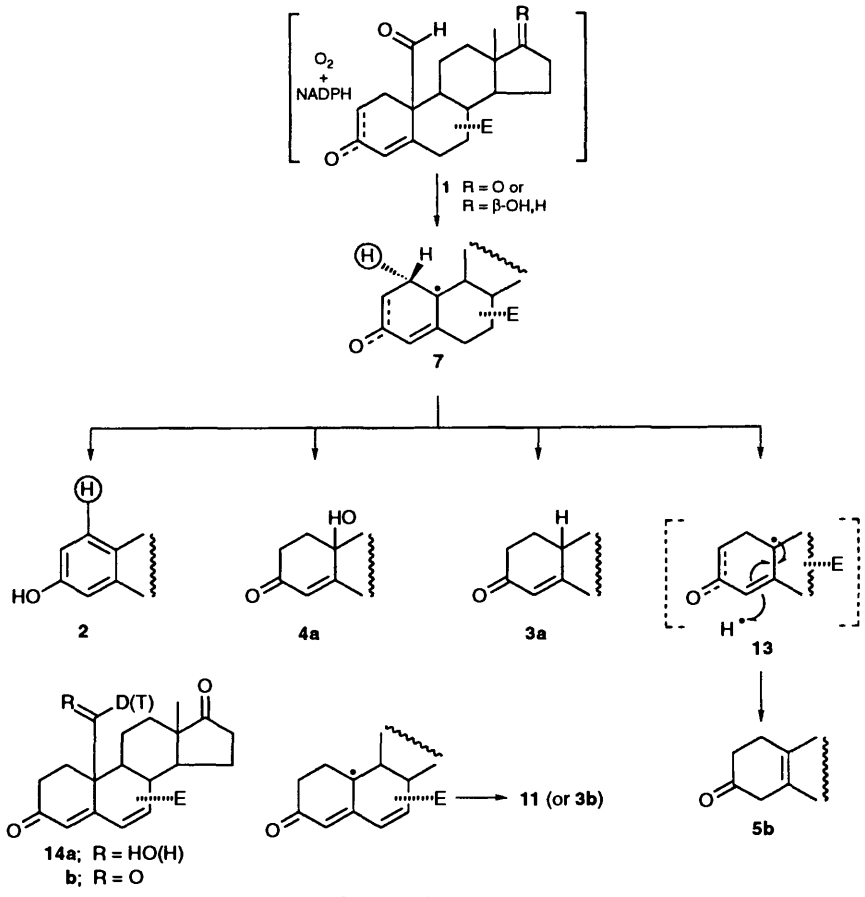


Fig. 1 ('E' = enzyme)

the increased yield of isomerized products at pH 7.2 (Exp. 2) is not necessarily the result of the higher pH at which the incubation was carried out. In Experiments 1 and 2, 17β-hydroxyestr-4-en-3-one (Table 1, entry 6) accumulated in larger amounts than did estr-4-ene-3,17-dione (Table 1, entry 5). In contrast to the incubation at pH 6.5, where only ~7% of estrogens were produced at pH 7.2, ~23% of estrogens were formed. This could be expected since it is well established that the rate of aromatization at lower pH-values is considerably slower.

Control incubations of compound **5b** were carried out at pH 6.5 and 7.2 exactly as above except that the microsomal placental aromatase was replaced by bovine albumin. The results of the control experiments are shown in Table 2.

The formation of significant amounts of 10β-hydroxy compound **4a** (Table 2; entry 3) in the absence of aromatase is worthy of note. The product is clearly an artifact, whose formation could be rationalized in terms of a radical mechanism. The photochemical (365 nm) generation of H_2O_2 from O_2 and NADH has been demonstrated.[8,9] Dissociation of H_2O_2 would give hydroxyl radicals, which could then abstract a C-4 hydrogen from the steroid, thereby forming the delocalized radical 7. Radical 7 could, in turn, react at C-10 with a hydrogen-atom source to give compound **3a**, or with a hydroxyl radical to give the vinylogous acyloin **4a**. Alternatively, compound **4a** could have arisen by reaction of O_2 with radical 7, followed by reduction of the resulting C-10 peroxy radical. Compound **3a** could also have been formed by way of a dienolate intermediate followed by protonation at C-10. However, an ionic oxidative mechanism for the conversion of ene dione **5a** into compound **4a** seems less likely. In any event, it is clear that formation of compound **4a** is not aromatase dependent and that it is not an estrogen precursor.[2-4]

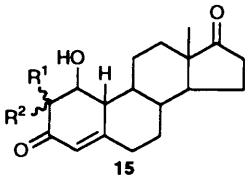
In the control experiments, the estr-4-ene-3,17-dione **3a** (Table 2; entry 2) was produced in 53.5% at pH 6.5 and 57.9% at pH 7.2. The total amount of $\Delta^{5(10)} \longrightarrow \Delta^4$ isomerization in the control experiments can be calculated as 79.7% at pH 6.5 and 66.2% at pH 7.2. It is apparent that, in the control experiments, larger amounts of the 5(10)-en-3-one **5b** were isomerized to 4-en-3-one. This is not surprising since, in the absence of the enzyme, the 3-ketone was not reduced and the β(γ)-double bond could shift into conjugation with the 3-carbonyl. Worthy of note is that more substrate was isomerized at pH 6.5 (79.7%) than at pH 7.2 (66.2%), which may suggest that isomerization is accelerated at a lower pH.

Currently it is recognized that the elaboration of estrogens from androst-4-ene-3,17-dione[10-13] and testosterone[7,14] and their respective C-19 oxygenated analogues, proceeds by different routes. The aromatization of androgens involves three oxidative[15] stages catalysed by $P\text{-}450_{aromatase}$. Each oxidative step requires 1 mole of oxygen and 1 mole of NADPH.[15] The process starts with C-19 hydroxylation of the androgen substrate[16] (first mole of oxygen) to give 19-alcohol **12**. The hydroxylation proceeds with retention.[17] A second hydroxylation at C-19 is thought to give a hypothetical C-19 diol[3,16] (second mole of oxygen). The 19-*pro-R* hydrogen atom of the parent 19-alcohol is displaced by the second hydroxy.[18-21] Dehydration of the gem-diol, with the loss of the oxygen atom derived from the second mole of oxygen,[3,16] yields the 19-al androgen **1** (17-ketone, or 17β-hydroxy). Finally, the aldehyde is aromatized with the use of the third mole of oxygen and NADPH to give estrone. In the process, C-19 is extruded as formic acid which incorporates an atom of oxygen from the third mole of oxygen.[3,18] In the case of androst-4-ene-3,17-dione,[10-13] and its 19-oxo analogue[7] **1** the aromatization involves the stereospecific elimination of the 1β and 2β

hydrogen atoms. However, in the case of testosterone,[14] and its 19-oxo analogue[7] only the 1β-hydrogen is eliminated stereospecifically. In contrast, the elimination of the C-2 hydrogen atoms is *not stereospecific* and either the 2β or the 2α hydrogen atom can be lost in variable amounts of up to 6:5 2β-H/2α-H ratio.[7,14]

In the absence of C-19 aromatization of estr-4-ene-3,17-dione **3a** (no deuterium) must proceed by a different route. It was shown that incubation of dione **3a** (no deuterium) with placental aromatase proceeded with the loss of the 1β-hydrogen atom[6] to yield estrone. For studies of the fate of the C-2 hydrogen atoms of compound **3a** (no deuterium) the epimeric 2α- and 2β-[^{3}H]estr-4-ene-3,17-diones were prepared.[22,23] The methodology of the preparation of the labelled estrenes assures the location (at C-2) and stereochemical integrity of the isotopes.[22,23] Incubation of the [2β-^{3}H]estr-4-ene-3,17-dione with placental aromatase gave estrone with the loss of 79% of tritium. In contrast, estrone obtained from the [2α-^{3}H]-epimer had lost 20% of the tritium.[23] It is evident that in each instance 20% of the biosynthesis of estrone proceeded *via* the abstraction of the 2α-hydrogen (tritium) atom. These results show that aromatization of estrene-dione **3a** (no deuterium) is not stereospecific in respect to elimination of the C-2 hydrogen atom.*

In the incubations of the 2α and 2β tritiated estradiones the 1β-hydroxy-[2-^{3}H]estra-4-ene-3,17-diones **15** were also formed. The production of compounds **15** from 2α[^{3}H] and 2β[^{3}H] substrates was accompanied by the loss of 19.8 and 22.3% of tritium, respectively. The hydroxylation of C-1 is a side reaction of the aromatase not related to the mechanism of estrogen biosynthesis. The loss of label is most likely the result of enolization of the C-3 ketone associated with the hydroxylation process. It can be inferred that the steric direction of the enolization was determined by a tritium isotope effect as evidenced by the loss of ~20% of tritium irrespective of the C-2 stereochemistry of the isotope. It is accepted that the same aromatase complex is operating for the above discussed substrates.[6,7] However, as indicated previously, aromatization[5] of C_{18}-estrene **3a** and of 17-keto and 17β-hydroxy androgens[1] proceeds by three different routes.



15

a; R^1 = T, R^2 = H
b; R^1 = H, R^2 = T

We have documented that, in incubations of compound **1** with microsomal placental aromatase, in addition to large amounts of estrogens, significant amounts of estrenes **5b**, **3a**, **3b**, **4a** and **11** were produced. The metabolism of estr-5(10)-ene-3α,17β-diol by a stallion testis preparation has also been investigated.[24]† With the exception of the 10-hydroxy compounds, similar metabolic products to ours were observed. The formation of estrogens and estrenes can be rationalized by assuming that scission[18,25] of the C-10(19) bond (third monoxygenation) results in a C(10) radical or cation **7** as shown in Fig. 1. The alternative proposal that the third monoxygenation is used for the abstraction of a 1β-hydrogen radical and is followed by homolytic cleavage of the C-10(19) bond to give estrone[26-28] cannot accommodate the consistent production of substantial amounts of estrenes.

If indeed radical (or cation) **7** is formed its *predominant* reaction pathway is *via* stereospecific enzyme-assisted loss of the 1β-hydrogen atom to yield estrone **2** (Fig. 1). However, competing reaction routes are also operating. Hence, acquisition of [HO] or [H] (radicals or ions) by species **7** at C-10 will yield product **4a** or product **3a**, respectively. Similarly, isomerization of the C-4 double bond [ionic or homolytic (Fig. 1; **13**)] and acquisition of [H] (radical or ion) at C-4 will give product **5b**. A C-6(7) dehydrogenation of compound **5b** and reduction of both carbonyls will give the metabolite tentatively identified as having structure **11a**. Alternatively, compound **11a** could be derived from the previously isolated[1] 19-hydroxy- or 19-oxo-androsta-4,6-diene-3,17-dione (**14a** or **14b**, respectively, Fig. 1).

The observed differences in the abstraction of the C-2 hydrogen atoms in the *aromatization* of 17-keto and 17β-hydroxy C_{19} substrates can be rationalized by assuming that enolization of the C-3 ketone occurs at different stages of estrogen biosynthesis. In the case of androstenedione, the postulated (enzyme-bound) precursors **12a**, **1** and the as yet unknown 'final' intermediate *are subject to severe 1,3-diaxial repulsion* between the 2β-hydrogen atom and the functionalized 19-carbon atom. Should enolization of the 3-ketone occur at a stage prior to the cleavage of the C-10(19) bond, stereospecific elimination of the 2β-hydrogen atom will relieve the system of its internal 1,3-diaxial strain. In the case of testosterone, enolization may be taking place after the C-10(19) bond was severed. In this instance, the 1,3-diaxial repulsion is absent and the driving force for the stereospecific extrusion of the 2β-hydrogen atom was eliminated. Consequently, enolization proceeds with the removal of either 2α or 2β hydrogen atoms. The observation[23] that aromatization of estr-4-ene-3,17-dione proceeds with the abstraction of 2β-hydrogen atoms (80%) and 2α-hydrogen atoms (20%) provides support for this hypothesis.

Experimental

TLC (0.25 mm thick) and preparative layer (PLC) (1.0 mm thick) chromatography was carried out using precoated plates [silica gel 60 (HF 254 + 366)] (Alltech Inc., Newark, DE, USA). The plates were developed with the indicated solvents. For high-performance liquid chromatography (HPLC) an instrument equipped with (1) Waters Co. Model 510 twin pump and an automatic gradient controller; (2) Micromeritics Co. Model 788 Dual Variable Detector, and (3) Houston Instrument Co. Omni Recorder was used. Throughout, an Alltech Nucleosil Silica 5μ (4 mm/25 cm) column was used. The column was eluted with the indicated solvent at the rate of 1 cm^3 min^{-1} with the detector set at 240 and 280 nm. Routine ^{1}H NMR spectra were recorded on a Varian EM-390 spectrometer for solutions in C^2HCl_3. Several NMR spectra were recorded at 500 MHz JEOL GSX 500, 400 MHz (Bruker AM 400 WB), or at 300 MHz (Varian Unity 300). *J* Values are given in Hz. UV spectra were measured with a Beckman DU-70 spectrometer.

Syntheses of Steroidal Substrates.—[16,16-$^2H_2$]*Estrone methyl ether* **2c**. A mixture of estrone 3-methyl ether **2b** (1.2 g, 4.2 mmol), tetraheptylammonium bromide (1.4 g), 40% sodium deuterioxide in deuterium oxide (3 cm^3), deuterium oxide (99.9%; 10 cm^3) and toluene (20 cm^3) was stirred in a round-bottomed flask[29,30] at 60–70 °C for 4 h. The products were recovered with chloroform and the extract was washed (water), dried (Na_2SO_4), and concentrated to give a residue. Following column chromatography (silica) title compound **2c** (1.1 g) was recovered. R_f and HPLC retention time (t_R) data of deuteriated (**2c**) and protiated (**2b**) species were the same; δ_H($CDCl_3$; 300

* The authors of ref. 23 concluded on the basis of the loss of 80% of the 2β-hydrogen (tritium) atoms that the aromatization proceeds stereoselectively. They did not consider the 20% loss of the 2α-hydrogen (tritium) atoms, which revealed a lack of stereospecificity.

† We thank one of the referees for bringing ref. 24 to our attention.

MHz) 0.91 (3 H, 13-Me), 2.90 (3 H, m, 6-H_2 and 9-H), 3.78 (3 H, s, OMe), 6.65 (1 H, d, *J* 2.7, 4-H), 6.74 (1 H, dd, *J* 2.7 and 8.4, 2-H) and 7.22 (1 H, d, *J* 8.7, 1-H); *m/z* (MO) 286 (M^+, 100%), 258, 227 and 201.

[16,16-$^2H_2$]*Estradiol 3-methyl ether* **2e**. A mixture of ketone **2c** (1.00 g, 3.49 mmol) and $NaBH_4$ (50 mg, 1.31 mmol) in methanol (10 cm^3) was stirred for 2 h at room temperature. Most of the methanol was removed in a stream of nitrogen and the residue was partitioned between chloroform and water. Evaporation of the dried organic extract gave title compound **2e** (850 mg). The crude product was recrystallized from methanol, m.p. 97–98 °C; $\delta_H(CDCl_3$; 300 MHz) 0.81 (3 H, 13-Me), 2.85 (3 H, m, 6-H_2 and 9-H), 3.78 (3 H, s, OMe), 6.63 (1 H, d, *J* 2.7, 4-H), 6.71 (1 H, dd, *J* 2.7 and 8.4, 2-H) and 7.24 (1 H, d, *J* 8.5, 1-H).

[16,16-$^2H_2$]*Estra*-2,5(10)-*diene*-3,17-*diol* 3-*methyl ether* **8**. To liquid ammonia (90 cm^3) were added sequentially a solution of compound **2e** (830 mg, 2.92 mmol) in anhydrous diethyl ether (75 cm^3) and small pieces of lithium wire (850 mg total). The reaction was allowed to proceed for 10 min, then absolute ethanol (10 cm^3) was added dropwise (10–20 min). After the removal of ammonia, ice-cold water was carefully added. The product was extracted with diethyl ether, washed successively with Claisen's alkali [KOH (35 g) in methanol–water (4:1, 125 cm^3)], water and saline, and dried (Na_2SO_4). Removal of solvent gave a glass (705 mg), which was crystallized (absolute ethanol) to give title compound **8**, m.p. 118–119 °C; $\delta_H(CDCl_3)$ 0.77 (3 H, s, 13-Me), 2.66 (4 H, br m, 1- and 4-H_2), 3.24 (3 H, s, OMe), 3.66 (1 H, s, 17α-H) and 4.62 (1 H, t, *J* 3, 2-H).

[16,16-$^2H_2$]*Estr*-5(10)-*ene*-3,17-*dione* **5b**. (*a*) A solution of compound **8** (50 mg, 0.17 mmol) in dichloromethane (5 cm^3) was treated with pyridinium chlorochromate (PCC) (100 mg) at ambient temperature. After 15 min, diethyl ether (10 cm^3) was added and the mixture was filtered through a short Celite column. The eluate and column washings (diethyl ether) were combined and concentrated to give a residue. Following PLC fractionation [EtOAc–cyclohexane (1:1)] compounds **5b** (13 mg, 28%) and **2c** (20 mg, 41%) were obtained. GLC–MS analysis indicated that the isolated title compound **5b** was homogeneous and that the product is essentially 100% di-deuteriated; $\delta_H(CDCl_3$; 300 MHz) 0.84 (3 H, 13-Me), 2.39 (4 H, m, 1- and 6-H_2), 2.63 (1 H, d, J_{gem} 21.0, 4β-H^{31}) and 2.74 (1 H, d, J_{gem} 21.0, 4α-H^{31}); *m/z* (MO-TMS) 332 [$(M + 2)^+$, 20%], 317 {[$M + 2) - 15]^+$, 10} and 301 {[$(M + 2) - 31$], 100}.

(*b*) A solution of [16,16-$^2H_2$]-19-carboxylic acid **9b**[29,30,32] (10 mg, 0.0316 mmol) in pyridine (0.5 cm^3) was refluxed for 30 min. Removal of pyridine under reduced pressure and PLC fractionation of the residue [EtOAc–cyclohexane (1:1)] gave title compound **5b** (6.8 mg, 80%).

Estr-5(10)-*ene*-3,17-*dione* **5a**.[32] A solution of the 19-acid **9a**[32] (100 mg, 0.316 mmol) in pyridine (5 cm^3) was refluxed for 30 min. Then the pyridine was removed under reduced pressure and the residue was fractionated by PLC [EtOAc–cyclohexane (1:1)] to give title compound **5a** (70 mg, 82%); HPLC [propan-2-ol–isooctane* (1:9)] t_R 6 min; $\delta_H(CDCl_3$; 500 MHz) 0.9 (3 H, s, 13-Me), 2.46 (4 H, br m, 1- and 6-H_2), 2.70 (1 H, d, *J* 20.5, 4β-H^{31}) and 2.8 (1 H, d, *J* 20.5, 4α-H^{31}).

[16,16-$^2H_2$]Estr-5(10)-ene-3α,17β-diol **5d** was prepared by treatment of a methanolic solution of 3α-hydroxy-[16,16-$^2H_2$]estr-5(10)-ene-17-one with sodium borohydride as described.[33]

Estr-5(10)-ene-3ξ,17β-diol was prepared by treatment of a methanolic solution of 17β-hydroxyestr-5(10)-en-3-one with sodium borohydride as described.[33]

* 2,2,4-Trimethylpentane.

17β-*Hydroxyestra*-5(10), 9(11)-*dien*-3-*one* 3-*ethylene ketal* **10b**. To a solution of ketone **10a**[34] (100 mg, 0.34 mmol) in methanol (5 cm^3) was added $NaBH_4$ (15 mg), and the mixture was stirred for 15 min at ambient temperature. Then water (5 cm^3) was added and the product was recovered (chloroform) and processed in the conventional manner to give title compound **10b** (100 mg, 98%); $\delta_H(CDCl_3)$ 0.7 (3 H, m, 13-Me), 3.74 (1 H, t, *J* 8, 17-H), 3.95 [4 H, s, $(OCH_2)_2$] and 5.56 (1 H, m, 11-H); *m/z* (TMS) (388, M^+ not detected), 343 $(M - 45)^+$, 10%], 328 (6); 313 (6), 297 (100) and 269 (13).

17β-*Hydroxyestra*-4,9-*dien*-3-*one* **6c**. A solution of compound **10b** (80 mg, 0.25 mmol) in acetic acid (1 cm^3) was stirred and cooled in an ice-bath, then perchloric acid (70%; 0.1 cm^3) was added. The mixture was removed from the ice-bath and allowed to warm up to room temperature while being stirred (10 min). The reaction was again cooled in ice and water (1 cm^3) was added. The products were recovered with diethyl ether, washed successively with water, 5% aq. sodium hydrogen carbonate and water, dried, and concentrated to give a residue (70 mg). PLC fractionation [EtOAc–cyclohexane (2:1)] gave the title compound **6c** (23 mg) and a mixture of products containing mainly the acetate **6d**. For compound **6c**: δ_H 0.92 (3 H, s, 13-Me), 3.68 (1 H, t, *J* 7.5, 17-H) and 5.7 (1 H, s, 4-H); *m/z* (MO-TMS) 373 (M^+, 100), 358 [$(M - 15)^+$, 4], 341 [$(M - 32)^+$, 11], 283 (11) and 268 [$(283 - 15)^+$, 12].

Estra-4,9-*diene*-3ξ,17β-*diol* **6e**. A solution of ketone **6c** (22 mg, 0.08 mmol) in methanol (3 cm^3) was treated with $NaBH_4$ (5 mg). After 15 min, water (2 cm^3) was added and the product was recovered (chloroform) to give, after the usual work-up, the diene-diol **6e** (21 mg, 95%); $\delta_H(CDCl_3)$ 0.84 (3 H, s, 13-Me), 3.62 (1 H, t, *J* 8, 17-H), 4.25 (1 H, m, 3-H) and 5.38 (1 H, s, 4-H); *m/z* (TMS) 418 (M^+, 61%), 329, [$(M - 89)^+$, 8]; 299 [(329 − 30), 6], 259 (19), 195 [(299 − (89 + 15), 25] and 182 (100).

10β,17β-*Dihydroxyestr*-4-*en*-3-*one* **4d**.[2,35] To a solution of compound **4e**[2,35] (7 mg) in methanol (2 cm^3) was added $NaBH_4$ (2 mg). After 2 min, the reaction was terminated and water (0.5 cm^3) was added. The products were recovered (chloroform) and processed to give a residue (7 mg). PLC fractionation [EtOAc–cyclohexane (3:1)] gave the title diol **4d** (4 mg, 57%) and triol **4f** (2 mg, 28%).

For diol **4d**: λ_{max}(MeOH) 240 nm; $\delta_H(CDCl_3)$ 0.90 (3 H, s, 13-Me), 3.70 (1 H, t, *J* 7.5, 17-H) and 5.70 (1 H, br s, 4-H); *m/z* (MO-TMS) 463 (M^+, 54%), 432 [$(M - 31)^+$, 100] and 342 [$(M - 31 - 90)^+$, 23].

Estr-4-*ene*-3ξ,10β,17β-*triol* **4f**. A solution of compound **4e** (5 mg, 0.117 mmol) and $NaBH_4$ (3 mg) in methanol (2 cm^3) was stirred for 30 min. Then water (0.5 cm^3) was added and the products were recovered (chloroform) to give a residue (5 mg) containing the crude triol **4f**. GLC–MS analysis showed that two of the three major products (GLC t_R 15.85 and 16.61 min) had molecular ion *m/z* 508, consistent with the assigned structure. For the t_R 15.85 min peak [5.1% of Total Ion Current (TIC)]; *m/z* (TMS) 508 (M^+, 19%), 418 [$(M - 90)^+$, 17], 392 (100), 328 (5) and 302 [$(292 - 90)^+$, 13]. For the t_R 16.61 min peak (71.1% of TIC); *m/z* (TMS) 508 (M^+, 24%), 418 [$(M - 90)^+$, 20], 392 (100), 328 [$(M - 90 - 90)^+$, 6] and 302 [$(292 - 90)^+$, 18].

Two other GLC peaks (t_R 16.05 min and 16.41 min; 2.7% and 20.5% of TIC, respectively) had *m/z* 510 as the molecular ion, consistent with conjugate reduction of the enone prior to reduction of the 3-carbonyl group.

3,17-*Dioxoandrosta*-4,6-*dien*-19-*oic acid* **9c**.[36] A sample of 19-hydroxyandrost-4-ene-3,17-dione **12a** was treated with *p*-chloranil, as described by Alvarez and Watt,[37] to give 19-hydroxyandrosta-4,6-diene-3,17-dione ([19-$^1H_2$]**14a**; not enz. bound). Following recrystallization from EtOAc, 19-hydroxyandrosta-4,6-diene-3,17-dione ([19-$^1H_2$]**14a**; not enz. bound) was obtained, m.p. 197–198.5 °C (lit.,[37] 195–196 °C).

A solution of the alcohol **14a** (147 mg, 0.49 mmol) in acetone (10 cm^3) was cooled in an ice-bath, then Jones' reagent was added in aliquots (10 × 50 mm^3).[36] The mixture was allowed to warm up to 15 °C and was held at that temperature for 20 min. The reaction mixture was poured into 10% aq. Na_2CO_3. The neutral fraction, containing mainly aldehyde[38] (**1**; 6-ene; no deuterium), was recovered (CH_2Cl_2) and the aqueous phase was acidified (6 mol dm^{-3} HCl). Following extraction with CH_2Cl_2 and evaporation of the solvent, a semicrystalline residue of the title acid **9c** (89 mg, 58%) was obtained. Following recrystallization from diethyl ether–hexane, the product had m.p. 123–125 °C; δ_H($CDCl_3$; 400 MHz) 0.93 (3 H, s, 13-Me), 5.90 (1 H, s, 4-H), 6.27 (1 H, dd, *J* 9.8 and 1.5, 6-H) and 6.36 (1 H, dd, *J* 9.9 and 2.5, 7-H) (carboxyl proton not observed).

Estra-4,6-diene-3ξ,17β-diol **3d** (*no deuterium*). A solution of compound **9c** (83 mg) in HOAc (1 cm^3) was heated at reflux (15 min).[36] The volatile components were removed under reduced pressure, then toluene (10 cm^3) was added and the resulting mixture was concentrated to give a residue of crude dione **3b**; λ_{max}(MeOH) 282 nm (lit.,[33] λ_{max} 282 nm).

$LiAl(OBu^t)_3$ (150 mg) was added to a solution of the dione **3b** in diethyl ether (2 cm^3) and the mixture was stirred at room temperature for 2 h. The reaction was terminated with aq. 5% acetic acid (5 cm^3). The product was extracted with diethyl ether and, after evaporation, the residue was purified by silica gel flash chromatography (10–35% EtOAc–hexane) to give title compound **3d**; λ_{max}(MeOH)/nm 233, 240 and 248; δ_H($CDCl_3$; 400 MHz) 0.82 (3 H, s, 13-Me), 3.68 (1 H, t, 17-H), 4.34 (1 H, m, 3-H), 5.45 (1 H, s, 4-H), 5.68 (1 H, d, 6-H) and 5.99 (1 H, dd, 7-H); *m/z* (TMS) 418 (M^+, 100%), 328 [(M − 90), 4], 287 (7), 223 (4), 194 (15), 182 (13) and 129 [(M − 289), 9].

Estra-5(10),6-diene-3α,17β-diol **11c**. A solution of acid **9c** (75 mg) in dry pyridine (1 cm^3) was heated at 110 °C for 10 min.[36] The mixture was cooled to room temperature and the solvent was evaporated off (~ 10 mmHg; 25 °C bath). The remaining traces of pyridine were removed by co-distilling the residue with toluene (2 × 3 cm^3). The residual oil was immediately purified by silica gel flash chromatography (10–30% EtOAc–hexane) to give dione **11d** (23 mg) as an oil; λ_{max}(MeOH)/nm 267 (lit.,[36] λ_{max} 268 nm).

Compound **11d** was reduced with $LiAl(OBu^t)_3$ in diethyl ether as described for compound **3d**, to give, after work-up and flash chromatography (15–40% EtOAc–hexane), the title diol **11c**. Crystallization from acetone–hexane gave a product with m.p. 158–160 °C (lit.,[37] 165–166 °C); two further recrystallizations did not alter the m.p.; λ_{max}(MeOH)/nm 266; δ_H($CDCl_3$, 400 MHz) 0.78 (3 H, s, 13-Me_3), 3.70 (1 H, t, 17-H), 3.95 (2 H, m, 3-H + OH) and 5.70 (2 H, br s, 6- and 7-H); *m/z* (TMS) 418 (M^+, 24%), 329 (32), 328 [(M − 90), 100], 238 [(M − 90 − 90), 39], 225 (50), 209 (52), 197 [(M − 129 − 90 − 2), 61], 195 (39), 144 (56) and 129 (57).

Incubation Experiments.—Aromatase activity. The activity of the aromatase was tested with [1,2-^{3}H]testosterone purchased from DuPont NEN, Boston, MA. The distribution of tritium in the testosterone (determined by the manufacturer by tritium NMR spectroscopy) was as follows: 1α 13%; 1β 48%; 2α 9%; 2β 30%. Based on the amount of the released tritiated water, 54% of the incubated testosterone was aromatized.

Incubation procedure of steroids with microsomal placental aromatase at the required pH-values. To a solution of NADPH (6.25 mg), glucose 6-phosphate (10.5 mg), dithiothreitol (2.3 mg), glucose-6-phosphate dehydrogenase (130 mm^3; 1 μkat mol^{-1}) in Tris-malate buffer (pH 7.2; 3 cm^3) a preparation of microsomal placental aromatase (500 mm^3) was added. The pH of the mixture was adjusted to the desired value with 1 mol dm^{-3} NaOH. Then a solution of the appropriate steroid (500 μg) in ethanol (50 mm^3) was added and the mixture was incubated (with reciprocal shaking) for 1 h at 37 °C in the air. The products were recovered with $CHCl_3$ (3 × 25 cm^3) and processed in the conventional manner to give a crude residue. The residue was purified by PLC [EtOAc–cyclohexane (3:1)]. Except for the region of the starting line, the silica was scraped from the plate and extracted [$CHCl_3$–MeOH (9:1)]. The extract was evaporated to give a crude residue, which was anslysed by GLC–MS as described below.

Control incubations were carried out as above except that the aromatase was replaced with bovine serum albumin (100 mg).

Mass Spectrometry.—Sample derivatization for GLC–MS analyses. To an aliquot of an incubation residue (or of a synthetic sample), a 2% solution of methoxylamine hydrochloride (Sigma Co., St. Louis, MO 63178) in pyridine (100 mm^3) was added and the mixture was stored (16 h) at room temperature. The pyridine was removed in a stream of nitrogen and trimethylsilylimidazole (Pierce Chemicals, Rockford, IL 61105) (100 mm^3) was added. The reaction was allowed to proceed (15 min) at 60 °C, then excess of reagents was removed by Lipidex chromatography (Lipidex-5000, Packard Instrument Co., Downers Grove, IL 60515).

GLC–MS analyses.[39] An HP-5970 instrument equipped with a mass-selective detector and a 15 meter DB1 column (J&W Scientific, Folsom, CA 95630) was used. The samples were introduced by splitless injection at 50 °C, and following a 3-min hold the oven was taken to its starting temperature of 210 °C at the rate of 27 °C min^{-1}. The temperature was then increased linearly by 3 °C min^{-1} to a final temperature of 320 °C. Mass spectra were acquired by continuously scanning over the range 100–600 amu. GLC–MS analysis of compound **5b** (bis-MO) derivatized under the described conditions showed no evidence of degradation and isomerization. A single chromatographic peak was obtained and compound **3a** (bis-MO) was not detected. The mass spectrum of compound **5b** (bis-MO) showed that the product is homogenous and almost exclusively dideuteriated.

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