# Estrogen Biosynthesis: Metabolism of [16,16,19-$^2H_3$][19-$^3$H]19-Oxoandrost-4-ene-3,17-dione by a Placental Microsomal Preparation

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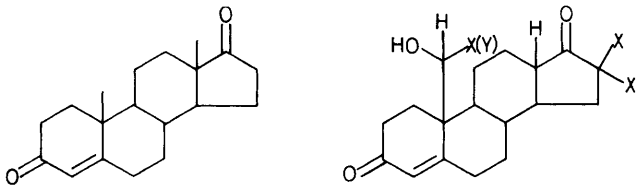
Incubation of the title compound with a microsomal placental preparation gave in addition to the expected metabolites several new products, three of which were identified as [16,16-$^2H_2$]19-norandrost-4-ene-3,17-dione, [16,16-$^2H_2$]19-norandrosta-4,6-diene-3,17-dione, and [16,16,19-$^2H_3$][19-$^3$H]19-hydroxyandrosta-4,6-diene-3,17-dione.

The biosynthesis of estrogens from androgen (**1**) is thought to proceed through stages (a—e): (a) (**1**) → 19-hydroxy[1] (**2**); (b) → 19,19-dihydroxy[2] (**3**); (c) → 19-oxo[2] (**4**); (d) → intermediate-[**X**] [oxygenated-(**4**)];[3] (e) → estrone (**5**). It was suggested that the reactions take place on the aromatase enzyme system as a continuous process.[4] Each of the oxidative transformations[5] (a, b, and d) requires a mole of oxygen and a mole of NADPH for a total of three moles of each. We showed that the first C-19 hydroxylation (a) proceeds with an isotope effect in the retention mode.[6] The second C-19 hydroxylation (b) involves the stereospecific displacement of the 19-*pro-R* hydrogen atom[7—9] of (**2**). The resulting diol (**3**) is then dehydrated (c) with the loss of the oxygen atom introduced in the second hydroxylation[10,2] to yield (**4**).

The 19-oxo-(**4**) is presumed to be converted, using the third mole of oxygen and of NADPH, to an intermediate [**X**] which in turn yields[6] estrone (**5**). In this context, Akhtar *et al.* and we ourselves have proven that an oxygen atom of the 'third' mole of oxygen is incorporated in the formic acid derived from C-19 extruded in the aromatization process.[3,10—12] It was proposed that the third mole of oxygen and of NADPH are utilized for the conversion of 19-oxo-(**4**) to one of the following: (i) 1β-hydroxy-(**4c**),[11] (ii) 2β-hydroxy-(**4b**)[12] (no $^{18}$O), or (iii) 19-peroxy-19-hydroxy-(**7**).[10] Obviously, to be valid, any of the proposed intermediates must account for the incorporation of an oxygen atom of the 'third' mole of oxygen in the formic acid derived from C-19. The nature of the hypothetical [**X**] is not settled. However, we have demonstrated that [2β-$^{18}$O][19$^3$H]-2β-hydroxy-19-oxoandrostene-3,17-dione (**4b**) (50% 2β-$^{18}$O), which was thought[12] to be intermediate [**X**], is not an estrogen precursor, since on aromatization it gave [$^3$H]formic acid (90% of $^3$H) devoid[13,3] of $^{18}$O.

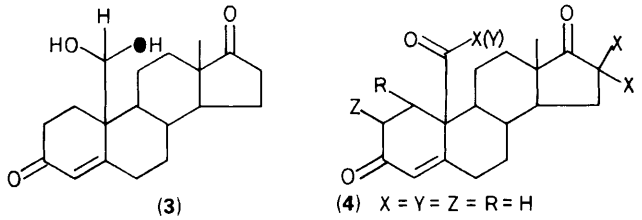
The argument for the intermediacy of 2β-hydroxy (**4b**) was to a large extent based on the observation that following reductive termination ($NaCNBH_3$) of incubations of radioactive androst-4-ene-3,17-dione in the presence of large amounts of (**4b**; no isotopes), a small amount of radioactive tetrol was trapped.[12,14] In essence there is no evidence linking



(1)

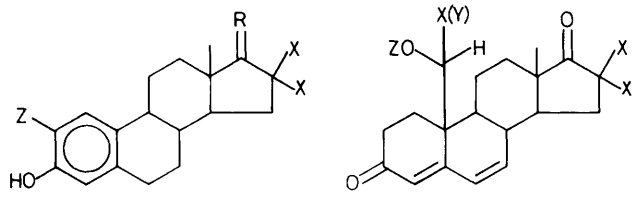
(2) X = Y = H
(2a) X = D, Y = T

D = $^{2}$H
T = $^{3}$H



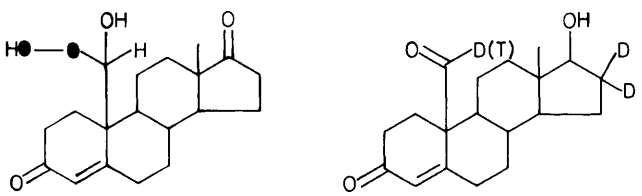
(3)

(4) X = Y = Z = R = H
(4a) X = D, Y = T, Z = R = H
(4b) X = D, Y = T, Z = 2β-$^{18}$OH, R = H
(4c) X = Y = Z = H, R = 1β-OH



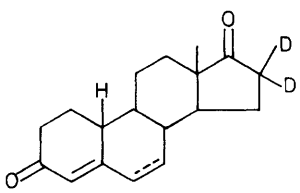
(5) R = O, X = Z = H
(5a) R = O, X = D, Z = H
(5b) R = 17β-OH, X = D, Z = H
(5c) R = 17β-OH, X = H, Z = OH

(6) X = Y = Z = H
(6a) X = D, Y = T, Z = H
(6b) X = Y = H, Z = Bu$^{t}$Me$_2$Si



(7)

(8)



(9) 6(7)-ene
(9a) no 6(7)-ene

the tetrol to (**4b**), which as we have shown is not an estrogen precursor. We argued that the crude microsomal aromatase preparation is capable of numerous reactions, many of which may not be related to estrogen biosynthesis.[14] Hence, the 'unknown' metabolite from which the tetrol was derived reductively ($NaCNBH_3$) is not necessarily an estrogen precursor.

To clarify the scope of transformations of 19-oxo-(**4**) and eventually identify the intermediate [**X**], we investigated the metabolism of (**4**) by a placental microsomal aromatase preparation. In addition to the expected products we isolated several new metabolites, three of which were identified.

Incubations[14] of [16,16,19-$^{2}H_3$][19-$^{3}$H]19-oxoandrost-4-ene-3,17-dione[15] (**4a**) (1 mg; 23.5 μCi of $^{3}$H) with microsomal placental aromatase[11,12,3] (pH 6) were terminated by extraction with chloroform and processed.† H.p.l.c. (5% propan-2-ol–iso-octane) of the residue of zone-A† gave [16,16-$^{2}H_2$]-estrone (**5a**), retention time ($R_t$) 7.8 min [m.s. *m/z* 272 ($M^+$)], and [16,16-$^{2}H_2$]estradiol (**5b**), $R_t$ 11.5 min [m.s. *m/z* 274 ($M^+$)]. The h.p.l.c. $R_t$ and m.s. of the metabolites were identical‡ to those of authentic samples.

The extract of zone-B† was resolved by h.p.l.c. (20% propan-2-ol–iso-octane) into several products. The product showing $R_t$ 8.5 min was identified as [16,16,19-$^{2}H_3$][19-$^{3}$H]-17β-hydroxy-19-oxandrost-4-en-3-one (**8**). The m.s. of the bis-*O*-methyloxime trimethylsilyl (MO-TMS) derivative of (**8**)($M^+$ 435) was identical to that of a (published) authentic sample.[16] The eluate ($R_t$ 5.5 min) was rechromatographed (h.p.l.c. 10% propan-2-ol–iso-octane), $R_t$ 9 min, and identified as estra-1,3,5(10)-triene-2,3,17β-triol (**5c**), m.s. *m/z* 288 ($M^+$). Since the compound was devoid of deuterium it is of endogenous (placental) origin.

The metabolite (**6a**) ($R_t$ 10 min) showed a peak at 240 nm and a more intense peak at 280 nm: 0.28 μCi of $^{3}$H (12 μg). The m.s. gave *m/z* 303 ($M^+$), 271 ($M$ − CDHOH), 253 (271 − 18), 227 (271 − $CD_2CO$), *etc.* The $R_t$ and m.s. of (**6a**) were identical‡ to those of authentic[17] (**6**).

The product showing $R_t$ 12.5 min absorbed at 240 nm: 0.21 μCi of $^{3}$H (9 μg); m.s. *m/z* 305 ($M^+$), 287 ($M$ − 18), 274 [$M$ −(CDHOH + $H_2O$)] and was identified as [16,16,19-$^{2}H_3$][19-$^{3}$H]19-hydroxyandrost-4-ene-3,17-dione (**2a**) since its $R_t$ and m.s. were identical‡ to those of authentic (**2**).

G.c.–m.s. of derivatized (MO-TMS) residue (A)† indicated the presence of several metabolites. One of the derivatized products showed *m/z* for (MO-TMS) 330 ($M^+$), MO-[$^{2}H_9$]TMS unchanged (330), and ethyl oxime-TMS (EO-TMS) 358 ($M^+$; 330 + 28). These results are consistent with the presence of two carboxy groups (no hydroxy) and a calculated molecular weight of 272. The metabolite was identified as [16,16-$^{2}H_2$]19-norandrosta-4,6-diene-3,17-dione (**9**).

A second product showed *m/z* for MO-TMS 332, and for EO-TMS 360. This was proven to be [16,16-$^{2}H_2$]19-norandrost-4-ene-3,17-dione (**9a**).

The formation of 19-norandrogens and steroids with a 4,6-dien-3-one moiety in human placental microsomal aromatase preparations has not been observed previously. While 19-norandrogens are frequently found in tissues and body fluids of different species,[18] only minute amounts were isolated from human follicular fluid.[19] The mechanisms of elaboration of the 19-norandrogens and of the C-6(7) dehydro

† The extract was washed with water, dried ($Na_2SO_4$), and concentrated in a stream of $N_2$. Following preliminary t.l.c. fractionation [20 × 20 cm; $SiO_2$; ethyl acetate–cyclohexane (3:1)] the plate was arbitrarily divided in two zones. Area: (A) above 19-oxo-(**4a**) to the solvent front; and (B) area from starting line to the front of (**4a**). The products of each area were recovered (10% methanol in chloroform) and concentrated. The residues were fractionated on an h.p.l.c. instrument equipped with: (1) Waters Co. Model 510 twin pumps and an automatic gradient controller; (2) Micromeritics Co. Model 788 Dual Variable Detector; and (3) Houston Instrument Co. Omni Scribe Series 5000 recorder. In all instances an Alltech Nucleosil silica 5 μ, 25 cm column was used. The column was eluted with the indicated solvent system at a flow rate of 1 ml min$^{-1}$, with the detector set at 240 and 280 nm. The $R_t$ of 19-oxo (**4a**) was 9 min. The h.p.l.c. eluted products were analysed by electron-impact m.s. and/or g.c.–m.s. of oxime-silyl ethers. Alternatively the recovered (crude) t.l.c. residues (A) and (B) were derivatized (oxime-silyl ethers) and analysed by g.c.–m.s.

‡ The statement means that the spectra were identical except for a shift by three or two mass units for $C_{19}$ or $C_{18}$ metabolites respectively.

products by the microsomal placental aromatase are unknown. The production of 19-nordeoxycorticosterone was shown to involve the stepwise oxidation in the adrenal gland of the 10β-methyl of deoxycorticosterone to 19-hydroxy → 19-oxo → 19-carboxylic acid,[20] while decarboxylation occurs extra-adrenally.[21] It is likely that a similar mechanism may operate in the placental homogenate but in this instance all the required enzymes seem to be in place. No clues are available as yet on the mechanism of C-6-dehydrogenation.

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