Synthesis of 19-Functionalised Derivatives of 16α-Hydroxy-testosterone: Mechanistic Studies on Oestriol Biosynthesis

David E. Stevenson, J. Neville Wright (in part), and Muhammad Akhtar*

Department of Biochemistry, Medical and Biological Sciences Building, Bassett Crescent East, Southampton SO9 3TU, U.K.

The 19-hydroxy and 19-oxo derivatives of 16α,17β-dihydroxy-androst-4-en-3-one (**9a**) and (**9b**) have been synthesised and shown to be intermediates in the biosynthesis of oestriol in the human placenta; O_2 is incorporated into formic acid released from C-19 of the 19-oxo compound (**9b**).

The mechanism through which the aromatic ring-A of the redox-pair comprising oestrone–oestradiol (**5a**)–(**5b**) is biosynthesised from androgens has been the subject of investigation in recent years. It is known that the conversion androstenedione (**1a**) → oestrone (**5a**) by the human placental aromatase-complex involves three oxidative steps, each requiring 1 mole of both O_2 and NADPH.[1] The first step in the transformation is C-19 hydroxylation which occurs with overall retention of stereochemistry to form (**2a**).[2—4] The second hydroxylation involving the H_{re} of (**2a**) gives the *gem*-diol (**3a**),[5—8] which, upon dehydration, produces the 19-aldehyde (**4a**).[9] This aldehyde (**4a**) is subsequently aromatised with the consumption of the 'third' mole of O_2 and NADPH and the 19-carbon is ejected as formic acid.[5,9] In this last transformation one oxygen atom from the 'third' mole of O_2 is incorporated into the formic acid.[9]

This finding has eliminated several possible mechanistic pathways for the C-10–C-19 cleavage step,[9,10] but, the labelling studies do not conclusively prove any single mechanism. Hence it is important to establish whether the chemical processes shown in Scheme 1 represent a general phenomenon by studying other examples of the same generic reaction.

Quantitatively, oestriol is the most important oestrogen produced in human pregnancy but its biosynthesis has not yet been subjected to mechanistic scrutiny. This neglect is primarily due to the difficulty of synthesis and of suitably labelling the putative intermediates of the pathway. Their synthesis, starting from dehydroisandrosterone, (**6**) Scheme 2, requires multistep and often tedious manipulations at the regions shown by arrows.

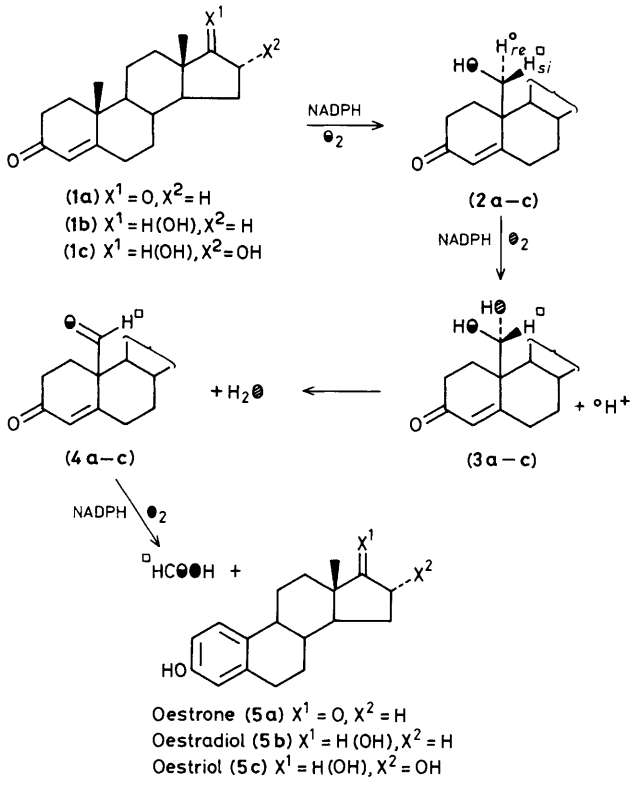
These problems have now been circumvented using a protocol (Scheme 2) in which the 6,19-epoxide (**7a**) was prepared by the Barton reaction[11] and converted, *via* the bromide (**7b**), into the 3-oxo-16,17-diol (**8a**) essentially by the method of Numazawa *et al.*[12] Opening of the 6,19-epoxy group of (**8a**) yielded the 19-hydroxy derivative of 16-hydroxy-testosterone (**9a**), whereas opening of the epoxy group of the diformate (**8b**) followed by oxidation and deprotection yielded the 19-oxo derivative (**9b**). The order in which the three centres were manipulated was crucial to the success of the synthesis. Tritium and deuterium labelling at C-19 was carried out by reducing the diformyl derivative of the 19-aldehyde (**9c**) with NaB^2H_4 or NaB^3H_4.

Deprotection yielded the labelled 19-alcohol (**9a**), R = ^{2}H-

Table 1. Incorporation of $^{18}O_2$ or retention of 19-^{18}O in formic acid.

Compound	Gas phase	Relative intensities of peaks due to benzyl ester of $^2HCO_2H$ (*m/z* 137)	$^2HC^{18}OOH$ (139)	$^2HC^{18}O^{18}OH$ (141)	% Incorporation (inc.) or retention (ret.)
[19-^{2}H]-19-oxo-(**9b**), *H = ^{2}H	$^{16}O_2$	99.5	0.25	0.25	—
[19-^{2}H]-19-oxo-(**9b**), *H = ^{2}H	$^{18}O_2$ (99%)	8.8	83.6	7.6	(83.6 inc.)
[19-^{2}H, 19-^{18}O]-19-oxo-(**9b**), *H = ^{2}H, R = ^{18}O[a]	$^{16}O_2$	23.2	72.5	4.3	(82.5 ret.)

[a] 42% excess ^{2}H, 88% excess ^{18}O.

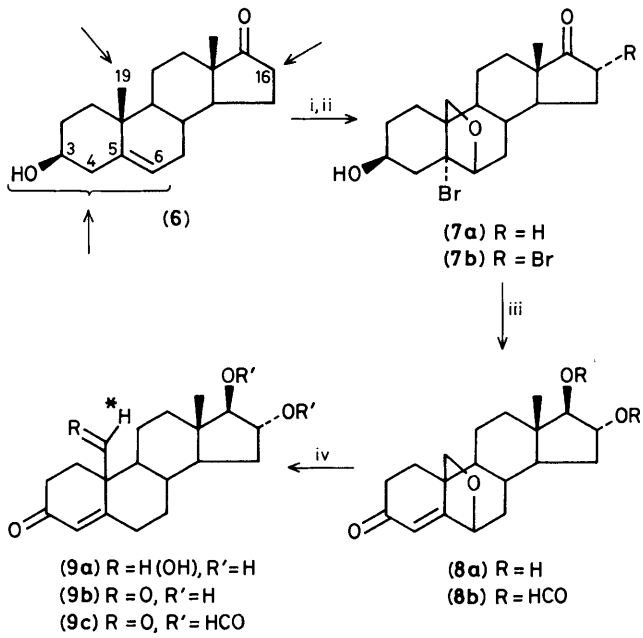


Scheme 1. Pathway for the biosynthesis of oestrone–oestradiol.

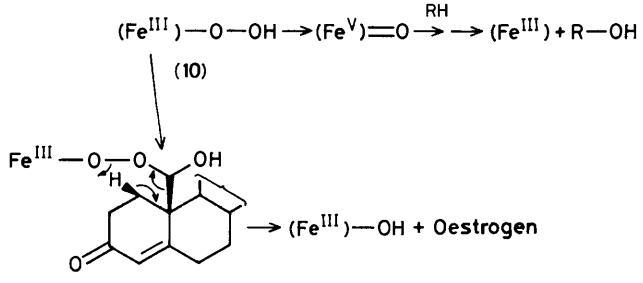
(OH) or ^{3}H(OH). Our previous work has shown that the major part of the label introduced by reduction of the steroidal 19-aldehyde goes into the H_{si} position.[6] The [19-^{2}H, 19-^{18}O]-aldehyde (**9b**) *H = ^{2}H, R = ^{18}O, was prepared from (**9c**) using the sequence of reactions previously developed[9] for the corresponding 16-desoxy derivative.

Initial biochemical experiments were performed using the 19-tritiated, 19-hydroxy, and 19-oxo steriods [*cf.* (**9a**) and (**9b**)]. 0.5 μmol of the steroid was incubated in air for 1 h with 50 mg of microsomal protein in the presence of an NADPH regenerating system. The results showed that all the ^{3}H from C-19 of the aldehyde was released as formate, whereas the distribution of radioactivity between ^3H_2O and formate in the aromatisation of the 19-alcohol was in the ratio 21 : 79. This ratio shows that in this case, as has been shown before for the corresponding conversion in the oestrone biosynthetic pathway,[9] the oxidation reaction (**2c**) → (**3c**) occurs involving H_{re}. Chromatographic analysis of steriods extracted from a similar incubation of (**9a**) with tritium in the steroid nucleus showed the conversion of (**9a**) into oestriol.

The status of oxygen in the conversion of the aldehyde (**9b**) into oestriol (**5c**) was studied using two incubation protocols in parallel. In one, [19-^{2}H, 19-^{18}O]-aldehyde (**9b**) *H = ^{2}H, R = ^{18}O, 1 mg, was incubated with placental microsomes (250 mg), in Tris-maleate buffer (10 ml) containing an NADPH regenerating system for 30 min under $^{16}O_2$, while in the other, [19-^{2}H]-aldehyde (**9b**)*H = ^{2}H, 1 mg, was similarly treated but in the presence of $^{18}O_2$.[9] In both cases about 40—60 μg of sodium formate were produced. These samples were converted into benzyl formate and analysed by g.l.c.–mass spectroscopy.[9] Table 1 shows that the values for retention of the original C-19 oxygen of the aldehyde, as indicated by the formate produced, and the incorporation of one oxygen atom from O_2 into formate are more than 80% of that predicted by the sequence shown in Scheme 1 (see the status of the oxygen atoms marked by ◒ and ●).



Scheme 2. Synthesis of 19-oxo-16α-hydroxyandrogens. *Reagents and conditions:* i, Ac_2O–pyridine; *N*-bromoacetamide, $HClO_4$; $Pb(OAc)_4$, I_2, *hv*; K_2CO_3–aq. MeOH; ii, $CuBr_2$, dry MeOH, reflux (**7a**) → (**7b**); iii, Jones reagent; 2 mol K_2CO_3–aq. Me_2CO; 1.1 mol $NaBH_4$, MeOH, −21 °C, 15 min; iv, 1,1′-carbonyl di-imidazole, HCO_2H; Zn, AcOH–H_2O reflux; pyridinium chlorochromate; $NaHCO_3$, aq. MeOH.



Scheme 3. Mechanism showing the dual role of a P-450–peroxide species (**10**) in hydroxylation and C–C bond cleavage steps.

Cumulatively, these results show that 16α-hydroxytestosterone is converted into oestriol *via* its 19-hydroxy and 19-oxo derivatives and that the overall molecular changes occurring during the transformation are identical to those already established for the conversion of androstenedione into oestrone. The present work, validating the conversion (**4c**) → (**5c**) with one atom of oxygen incorporated into the expelled formate,[9] provides a further basis for considering such an event in the mechanism of C-10–C-19 bond cleavage.

An earlier proposal in which the third mole of O_2 was suggested to produce 2β-hydroxy-19-oxo-androstenedione, which fragmented non-enzymatically producing the aromatic ring-A of oestrone[13,14] has been weakened by the recent work of Caspi *et al.*[15] These authors failed to show the transfer of the oxygen from the 2β-hydroxy group of the aforementioned compound into formate as would be expected (*vide supra*) had this compound been an obligatory intermediate in oestrogen biosynthesis. The mechanism originally proposed by us[9] and further elaborated in Scheme 3 thus remains a strong contender. According to this proposal the third mole of oxygen is involved in the formation of an enzyme–peroxide

species that attacks the C-19 aldehyde causing elimination as shown in Scheme 3. An Fe^{III}–peroxide species is a postulated intermediate in the catalytic cycle of cytochrome P-450 enzyme.[16] It is this type of system that is considered to be involved in the first two hydroxylation reactions of Scheme 1. Our proposed mechanism requires only that the reaction of the peroxide with the aldehyde is faster than the normal hydroxylation reaction which which would otherwise follow through an enzyme–mono-oxygen species (Scheme 3).

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