Synthesis of and Reactivity Studies with 19-Peroxide-Androstenedione Derivatives: Analogues of a Proposed Aromatase Intermediate

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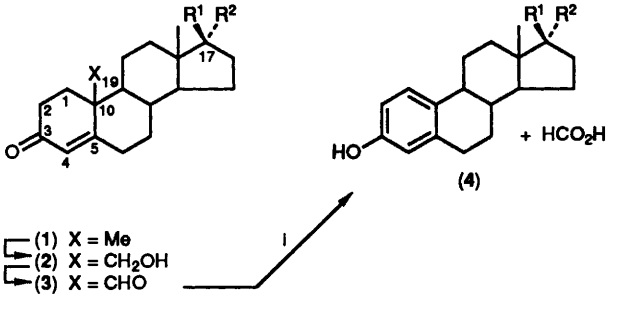
Human placental aromatase is a cytochrome P 450 enzyme system which converts steroidal androgens to steroidal estrogens. Three sequential oxidative steps are involved in the conversion, the first two leading to 19-hydroxy (**2**) and 19-oxo intermediates (**3**), respectively. The mechanism of the third step has remained elusive although one proposal which has remained consistent with experimental data involves formation and aromatization of a 19,19-hydroxyferric peroxide (**5**) intermediate. This study discusses the synthesis of 19,19-methoxy hydroperoxide (**12**) *via* ozonolysis, evidence for its formation, and studies of its reactivity under different conditions. Another approach was used to synthesize 19,19-hydroxy peroxide derivatives *via* the 19-aldehyde (**3b**). The reactivity of these derivatives was also explored. An analogous reaction involving ^{18}O-HOOH was carried out to investigate the mechanistic details of an unprecedented intramolecular epoxidation reaction. None of the peroxide derivatives were converted into estrogen or estrogen derivatives under the conditions examined, and the possible implications for the aromatase reaction are discussed.

Placental aromatase is an enzyme complex which catalyses the conversion of steroidal androgens [testosterone (**1a**), androstenedione (**1b**)] into steroidal estrogens [estradiol (**4a**), estrone (**4b**)]. It has received attention from a diverse group of workers in the biomedical sciences. The selective inhibition of aromatase has been sought as a means to lower estrogen levels in patients with estrogen-dependent tumours including breast cancer.[1] In addition, aromatase is a cytochrome P 450 enzyme system and has attracted interest from members of the haeme-protein field.[2] As the chemical reaction performed by aromatase is complex and interesting, it has been studied intensively as a problem of bioorganic chemistry.

Many aspects of the aromatase reaction have been determined (see Scheme 1). Androstenedione (**1b**), testosterone



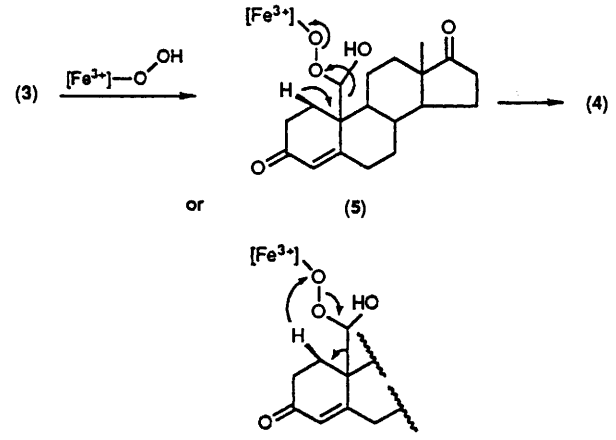
Scheme 1. *Reagents and conditions:* i, NADPH, O_2; **a**, R^1 = OH, R^2 = H; **b**, $R^1 = R^2 = O$.

(**1a**), and 16α-hydroxytestosterone are all direct substrates for aromatase with similar values for k_{cat} but quite different values for K_m.[3] It is postulated that two successive hydroxylations at the angular 19-methyl group lead to 19-hydroxy (**2**) and 19-oxo (**3**) intermediates although direct oxidation of (**2**) to (**3**) is also a possibility. Both reactions are thought to be classical cytochrome P 450 type hydroxylations with one equivalent of molecular oxygen and NADPH consumed in each step.[4] Only the first oxygen equivalent consumed is incorporated into the aldehyde suggesting that there is a stereospecific dehydration of the presumed *gem*-diol intermediate.[5] In the third and last step, another oxidation is thought to occur with consumption of a third equivalent of molecular oxygen and NADPH.[4] The 1β-H is lost to the aqueous medium[6] while C-19 along with one of its original hydrogens is incorporated into formic acid.[7] Interestingly, there is substrate-dependent stereoselectivity with regard to 2-H loss.[8] That is, whereas androstenedione (**1b**) shows highly stereoselective 2β-H loss, testosterone (**1a**) (a 17β-hydroxy compound) is processed with significant 2α-H removal. This substrate-dependent stereoselectivity has been interpreted as evidence supporting the role of the enzyme in 2-H removal.[8b] The third equivalent of oxygen is also incorporated into formic acid.[9] Furthermore, the O-3 is retained throughout the reaction suggesting that Schiff base formation at C-3 is not required in the third step.[10]

Despite these and other findings, the chemical nature of the third step remains elusive. Several theories have been shown to be unlikely including 2β-hydroxylation,[11] 4,5-epoxidation,[12] Baeyer–Villiger type oxygen insertion,[9] and any mechanism involving water incorporation into formic acid. An early proposal which has remained consistent with all known aromatase data and P 450 mechanistic theory was made by Akhtar.[13] It features attack on the 19-aldehyde (**3b**) by the ferric peroxide as shown in Scheme 2. The resultant hydroxyferric peroxide species (**5**) could decompose by hydride shift,[13] proton transfer,[14] or single electron pathways[15] (not shown) to the aromatic steroid and formic acid. Akhtar's theory could be interpreted as stating that collapse of compound (**5**) is spontaneous, with no requirement for catalytic assistance from active site residues. Given the limitations of present technology, such a theory is still difficult to validate rigorously. We thought that a useful approach might be to study the chemical reactivity of analogues of the proposed intermediate (**5**).[16]

Results and Discussion

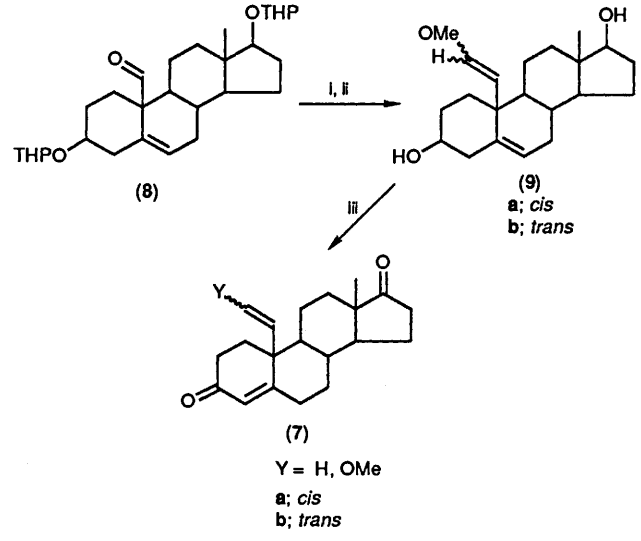
We studied first the 19,19-methoxy hydroperoxide derivative (**12**). This compound possesses some of the key features of (**5**)



Scheme 2.

and was considered a realistic synthetic target. Ozonolysis of an olefin in the presence of methanol is known to result in a carbonyl at one carbon terminus and a methoxy hydroperoxide at the other.[17] As these reactions can be effected at −78 °C, the chances of actually forming and observing the peroxide (**12**) from the corresponding 10β-vinyl compound (**6**) seemed good.[18] However, when the olefin (**6**) was subjected to ozonolysis (4:1 CH_2Cl_2–MeOH, $NaHCO_3$, −78 °C) the aldehyde (**3b**) was formed directly in 53% yield without reductive work-up, suggesting that primary ozonide fragmentation occurred predominantly in the undesired direction. Despite this unwanted fragmentation, the experiment demonstrated that selective ozonolysis of the 10β-vinyl group *versus* the electron-poor 4,5-double bond could in fact be achieved.

Based on literature precedents, it was expected that the methoxyvinyl derivative (**7**) might undergo cleavage in the desired mode.[19] Therefore, we synthesized the methoxyvinyl compounds (**7a**) and (**7b**) as a mixture of double bond isomers



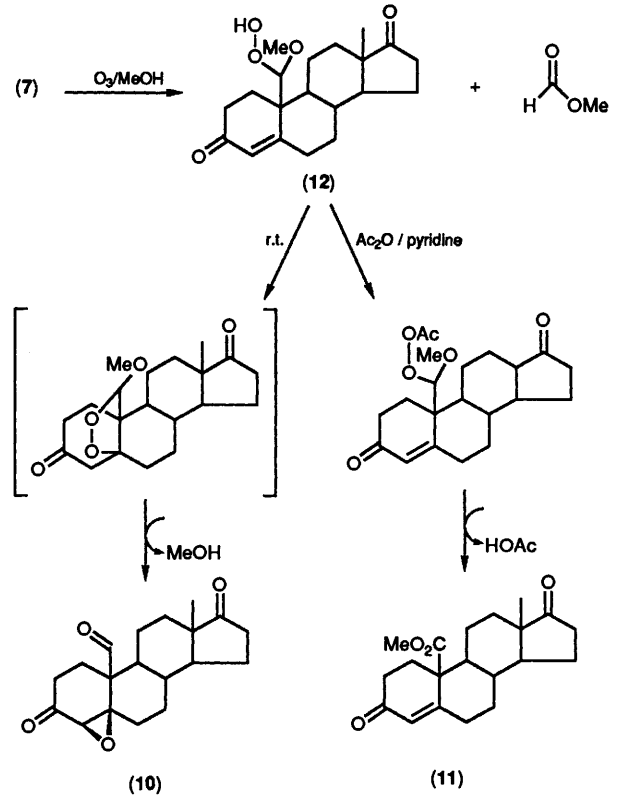
Scheme 3. *Reagents and conditions:* i, $MeOCH_2PPh_3Cl$, 2.0 M Bu^nLi, THF, reflux; ii, MeOH, PPTS, room temp.; iii, cyclohexanone, aluminium isopropoxide, toluene, reflux.

as shown in Scheme 3. Reaction of the protected aldehyde (**8**) with the lithium ylide of (methoxymethyl)triphenylphosphonium chloride in THF under reflux, followed by treatment with pyridinium toluene-4-sulphonate (PPTS)–MeOH to cleave selectively the THP protective groups, afforded the methoxyvinyl diol as a 1:1 mixture of double bond isomers (**9a, b**) in 65% overall yield after flash chromatography.[20] Oppenauer oxidation of this diol mixture (**9a, b**) resulted in an 89% yield of the desired methoxyvinyl compounds (**7a, b**) and is noteworthy for the stability of the methoxyvinyl moiety to the reaction conditions. Individual oxidation of the isomers (**9a**) and (**9b**) obtained by HPLC allowed characterization of each of the *Z*- and *E*-compounds (**7a**) and (**7b**), although the 1:1 mixture of (**7a, b**) was actually used in subsequent experiments.

Ozonolysis of the 1:1 mixture of (**7a**) and (**7b**) (4:1 CH_2Cl_2–MeOH, $NaHCO_3$, −78 °C) resulted in rapid consumption of starting material (TLC). Interestingly, although the reaction mixture showed strong UV absorption when first spotted on a TLC plate, no UV absorbing components were observed after the plate was eluted. This suggested the initial formation of an unstable adduct which still contained the conjugated ketone moiety. After removal of the excess ozone an aliquot of the mixture was allowed to warm to room temperature, when a major non-UV absorbing product was isolated. This proved to be the 4β,5β-epoxy aldehyde (**10**) (43% yield after flash chromatography). Identification of the known epoxide (**10**) was based on spectroscopic and chromatographic comparison with authentic material.[12b] The ^1H NMR spectrum of crude product showed no evidence for the 4α-epoxy isomer (<2%). Furthermore, estrone could not be detected by HPLC analysis (<0.5%).

The remaining portion of the ozonolysis mixture, while still below 0 °C, was concentrated to remove the methanol. Pyridine–acetic anhydride was added in large excess at −78 °C, and the mixture was allowed to warm to room temperature. After work-up, one major UV absorbing component was isolated in 25% yield. This proved to be the methyl ester (**11**) as shown by spectroscopic and chromatographic comparison with an authentic sample.[21] In addition, the 4β,5β-epoxy aldehyde (**10**) was isolated in 12% yield. Analysis by HPLC indicated estrone or estrone acetate had not been formed (<0.5%).

A mechanistic proposal (Scheme 4) which accounts for the above results is shown below. It is postulated that the relatively unstable methoxy hydroperoxide (**12**) is formed initially. As the



Scheme 4. Mechanistic rationale for the ozonolysis results.

mixture is warmed, an intramolecular Michael addition of the hydroperoxide terminal oxygen to the 4-ene-3-one system, followed by peroxide bond cleavage affords exclusively the 4β,5β-epoxide (**10**). On the other hand, when the methoxy hydroperoxide (**12**) reacts with acetic anhydride, acetylation of the hydroperoxide functionality activates the terminal oxygen as a leaving group, preventing Michael addition, and leading to methyl ester (**11**) formation with elimination of acetic acid.

To further validate this proposal, a separate experiment was performed in which the ozonolysis mixture, derived from (**7**) as described above, was treated with dimethyl sulphide either at −78 °C or after warming to room temperature for 15 min. Analysis by HPLC indicated that the reaction treated at −78 °C contained both the 19-aldehyde (**3b**), (27%) and 4β,5β-epoxide (**10**), (14%). In contrast, a reaction mixture treated after 15 min contained no detectable aldehyde (**3b**) but a good yield of (**10**), (41%). A portion treated 30 min after warming to room temperature, and an untreated portion each showed about 40% of (**10**), and no aldehyde (**3b**). These experiments suggest that the half-life of the peroxide (**12**) at room temperature is under 15 min.

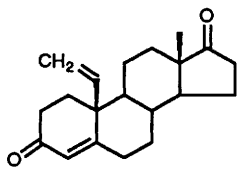
Variable-temperature 400 MHz ^{1}H NMR spectra were obtained for the ozonolysis reaction mixture derived from the methoxyvinyl compound (**7**) in CD_2Cl_2–CD_3OD solution. The ozonolysis reaction was performed in these deuteriated solvents and the solution was rapidly transferred to an NMR tube at −78 °C and placed in the probe which had been pre-chilled to −60 °C. Initial spectra showed a singlet at 5.8 ppm [corresponding to the vinyl C(4)-H proton] and a singlet at 4.95 ppm [the C(19)-H proton], both of which were attributed to the methoxy hydroperoxide intermediate (**12**). As the sample was warmed to 0 °C, the above signals due to (**12**) began to fade, and new peaks at 9.9 ppm (the 19-H proton) and 3.0 ppm [the C(4)-H proton] attributed to compound (**10**) concomitantly appeared. After warming to room temperature, the signals for compound (**12**) had disappeared and the signals due to (**10**) had become prominent. Singlets at 8.0 and 3.7 ppm, presumably representing protons from methyl formate, an expected side product, were visible throughout the warming process.

In order to verify the intramolecularity of the epoxidation reaction, a crossover experiment was carried out. To an ozonolysis reaction mixture containing (**12**) at −78 °C, androstenedione (**1b**) was added. No 4,5-epoxyandrostanedione could be detected by HPLC (<0.6%) after warming the mixture to room temperature. The absence of crossover provides further evidence for the proposed mechanistic theory.

The above experiments suggest that the methoxy hydroperoxide (**12**) is initially formed as an unstable adduct. The acetylation of compound (**12**) increases the leaving group potential of the terminal oxygen. Such a modification might simulate the electron withdrawing potential of Fe^{III}, and should provide a model for the proton-transfer variant of the Akhtar mechanism. As no estrogen was formed in this model reaction, we were led to examine conditions which might mimic a homolytic peroxide cleavage. A convenient method to cleave alkyl hydroperoxides to alkoxy radicals involves the use of Fe^{II}, according to Fenton's procedure.[22] Furthermore, addition of cupric salts to such mixtures can sometimes lead to higher yielding elimination reactions *via* organocopper intermediates.[17] Thus compound (**12**) was generated as before, and treated with excess ferrous sulphate or ferrous sulphate–cupric acetate suspensions at low temperature. In neither case was estrone formation detected (less than 2%) although many unidentified components were evident (TLC). From the ferrous sulphate–cupric acetate reaction mixture, an 8% yield of methyl ester (**11**) was obtained. The production of (**11**) can be explained by initial formation of the alkoxy radical followed by hydrogen atom loss from C-19.

A complementary set of model studies was then initiated, aimed at the synthesis of 19,19-hydroxy peroxides. The approach we chose was addition of hydrogen peroxide to 19-aldehydes. The advantages of this approach include: (i) the compounds generated more closely resemble the Akhtar hydroxy peroxide intermediate, in that a hydroxy rather than a methoxy group would be present; (ii) the use of nucleophilic attack better imitates the proposed nucleophilic attack by ferric peroxide; (iii) the mildness of this method could be applicable to cases involving more complex functionality such as A-ring dienol groups.

At the outset, we were encouraged by the finding that hydrogen peroxide reacted rapidly with 19-oxoandrostenedione (**3b**) *in the absence of strong base*, to generate the 4β,5β-epoxide (**10**) at 4 °C, in 64% yield after HPLC. In contrast the 19-methyl (**1b**), and 19-hydroxy analogues (**2b**) failed to react with



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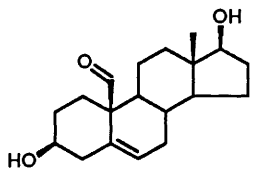

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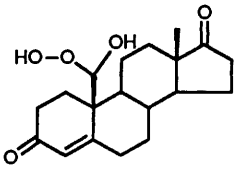


(13)

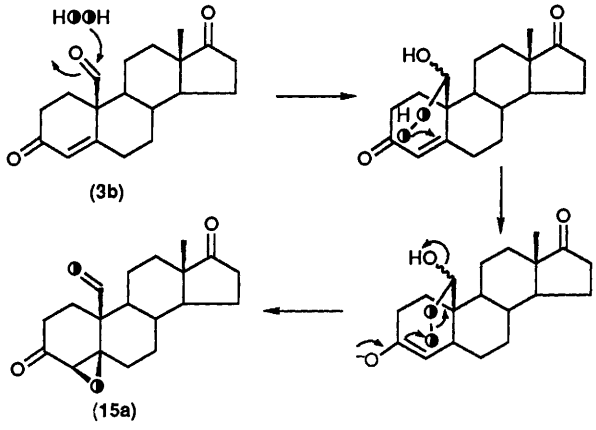
hydrogen peroxide under similar conditions. These results are consistent with the initial reversible formation of 19,19-hydroxy hydroperoxide (**13**) followed by intramolecular attack of the terminal oxygen of the hydroperoxide on the 4-ene-3-one system as seen for the methoxy hydroperoxide (**12**). Supporting this idea is the fact that t-butyl hydroperoxide fails to react with (**3b**) under similar conditions. In this case, the 19-t-butyl peroxide adduct would not be expected to add to the α,β-unsaturated ketone in a nucleophilic sense. It is important to note that extended reaction of t-butyl hydroperoxide with (**3b**) also resulted in no detectable estrone formation after several days. It should also be mentioned that the ^{1}H NMR spectrum of dihydroxy-19-aldehyde (**14**) in CD_3OD and HOOH (aqueous) showed no detectable diminution of the 19-aldehyde hydrogen signal compared to the signal for the 6-vinyl hydrogen. This suggests that the equilibrium between the 19-aldehyde and 19,19-hydroxy hydroperoxide forms lies predominantly towards the 19-aldehyde under these conditions.

To further investigate the possibility of covalent assistance in the epoxidation of 19-oxoandrostenedione (**3b**) by HOOH, a similar reaction was carried out with a 20% aqueous solution of 50%-^{18}O-HOOH (random distribution of label). The reaction was quenched just before completion (15 min) to minimize the risk of exchange of the 19-aldehyde oxygen with the oxygen of water. Mass spectral analysis of the labelled product (**15a**) for ^{18}O-content was performed. Three possible theoretical outcomes are shown in the Table. Column A shows the expected ^{18}O distribution for a mechanism where no covalent assistance occurs. Column B depicts the expected outcome for a covalently assisted pathway, followed by 'concerted' breakdown of the cyclic peroxide (Scheme 5). Column C shows the anticipated outcome for a mechanism involving covalent assistance followed by 'stepwise' breakdown of the cyclic peroxide, and random dehydration of the *gem*-diol that is transiently formed (Scheme 6).

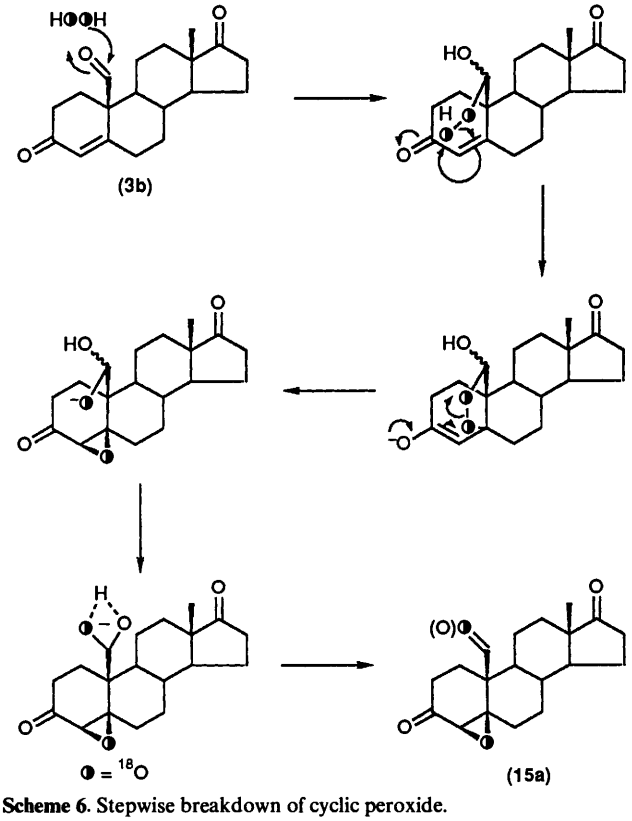
Table. Theoretical and experimental ^{18}O levels (%) in labelled epoxide (**15**).

	A*	B	C	D	E
0-^{18}O	50	25	37.5	37.5	40.0
1-^{18}O	50	50	50	50.9	51.4
2-^{18}O	0	25	12.5	11.6	8.7

* Columns: A, theoretical outcome for no covalent assistance by 19-oxo group; B, theoretical outcome for covalent assistance, concerted breakdown of peroxide and dehydration; C, theoretical outcome for covalent assistance, stepwise breakdown of peroxide and dehydration; D, experimental result for product (**15a**) of reaction of (**3b**) with ^{18}O-HOOH, estimated error is ±2% on all values; E, experimental result for (**15b**) [obtained after exposure of (**15a**) to ^{16}O-HOOH reaction and workup conditions], estimated error is ±2% on all values.



Scheme 5. Concerted breakdown of cyclic peroxide.



Scheme 6. Stepwise breakdown of cyclic peroxide.

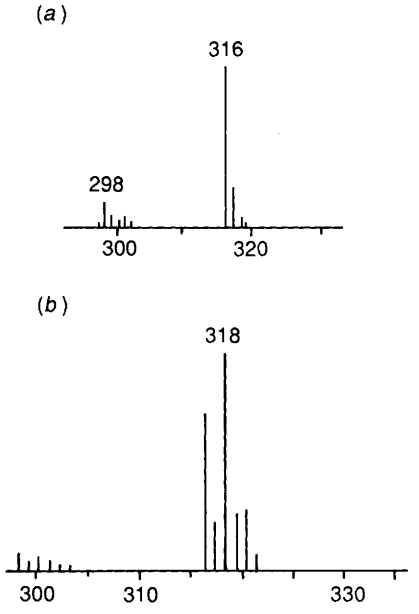


Figure. (*a*) EIMS partial spectrum of unlabelled 4β,5β-epoxy-19-oxoandrostane-3,17-dione (**10**); (*b*) EIMS partial spectrum of ^{18}O-4β,5β-epoxy-19-oxoandrostane-3,17-dione (**15a**).

In principle, dehydration of the *gem*-diol could occur with a stereochemical preference for loss of a pro-*R* or pro-*S* hydroxy group, but random loss seemed most likely. The experimental results (Column D, Table; see also Figure) are in closest agreement with the 'stepwise' breakdown model (Column C, Table). A control experiment was carried out to examine the potential for exchange of the aldehyde carbonyl oxygen with water under the reaction and work-up conditions. Compound (**15a**) was resubmitted to a 100%-^{16}O-HOOH reaction exactly like the ^{18}O-HOOH reaction and (**15a**) was re-isolated as (**15b**). Given the estimated experimental error (±2%) for these measurements, the re-isolated labelled epoxide (**15b**) showed no significant loss of label (see Column E, Table). Although more complex mechanisms are possible, the reaction of (**3b**) with HOOH to form (**10**) can be explained plausibly by a process involving covalent assistance, followed by stepwise breakdown. The incorporation of ^{18}O at C-19 validates the mechanistic proposal for the ozonolytic results of (**7**) described earlier.

Conclusions

Alternative ozonolytic and nucleophilic approaches were used to synthesize and study 19-peroxide derivatives of androstenedione as analogues of a proposed aromatase intermediate. In none of the cases examined could estrogen derivatives be detected. In the absence of a blocking group (acetyl, t-butyl) on the terminal peroxide oxygen, intramolecular Michael addition was the predominant reaction outcome. There are now at least four documented examples of various C-19 linked nucleophilic groups that appear to undergo rapid, intramolecular 1,4-addition to the 4-ene-3-one system. They include thiol,[23] hydroxy,[5] peroxy, and the carbanion [24] of a sulphonium ylide. The labelled oxygen experiments suggest that, in our case, the primary mode of breakdown of the postulated cyclic peroxide is a stepwise process (see Scheme 6).

The collapse of the acetyl peroxide grouping described above, as well as the Fe^{II} catalysed breakdown of (**12**), both involve loss of the 19-H to produce methyl ester (**11**) rather than the 1-H loss which would be necessary for C-10–C-19 fragmentation. The enzyme could enhance the reactivity of the 1-H by prior (or

concomitant) enolization of the 3-ketone. Such enolization would also prevent Michael-type addition to the 4-ene-3-one system. As mentioned, indirect evidence suggesting enzymic assistance in 2-H removal has recently been obtained.[8b] An aromatase model reaction based on a 2,4-dienol species has recently been developed in this laboratory,[16b] and a mechanistic analysis of this reaction will be reported in due course.

Experimental

General.—IR spectra were recorded on a Perkin-Elmer 710B instrument. UV spectra were obtained on a Perkin-Elmer Lambda 3B machine. ^{1}H NMR spectra were measured in $CDCl_3$ solution and referenced to $CHCl_3$ (7.26 ppm) using an IBM FT-80 spectrometer or a Varian XL-400 spectrometer. Flash chromatography was performed according to the method of Still,[25] using Baker flash silica gel. HPLC was performed on Beckman or Waters instruments using a normal phase Whatman Partisil 10 semi-preparative column and refractive index detection. TLC was performed using fluorescence indicator Macherey–Nagel D-5160 plates. All compounds and solvents were commercially available reagent grade unless otherwise noted. Mass spectra were recorded on a VG70S instrument or an LKB-9000 instrument. A Welsbach T-ozonator was employed with a 90 V setting. The ^{18}O-labelled hydrogen peroxide was obtained from ICON, Summit, New Jersey.

Conversion of 10β-*Vinylestr-4-ene-3,17-dione* (**6**) *into* 19-*Oxoandrostenedione* (**3b**).—The vinyl compound[20] (**6**) (20.0 mg, 0.067 mmol) was dissolved in a magnetically stirred 4:1 CH_2Cl_2–MeOH solution (2.4 ml) containing anhydrous $NaHCO_3$ (*ca.* 3 mg) in a 10 ml 3-necked round bottom flask. The mixture was cooled to −78 °C in a solid CO_2–acetone bath and ozone was bubbled in slowly (about 1 bubble s^{-1}) *via* a Pasteur pipette. The reaction was followed by TLC (60% EtOAc–hexanes) and nearly complete disappearance of starting material occurred after 70 min, with concomitant appearance of a more polar product. Excess ozone was removed under a stream of N_2 for 10 min, and the cooling bath was removed and the reaction allowed to warm to room temperature. The reaction mixture was concentrated *in vacuo* and taken up in methylene dichloride, filtered through glass wool and purified by HPLC (55% EtOAc–hexanes) to afford (**3b**) (10.6 mg, 0.0353 mmol, 53%) which was identical to the known material[26,8b] based on comparison of the TLC, HPLC, IR, UV, ^{1}H NMR, and MS data.

10β-(*Z- and* E-2-*Methoxyethylene*)*estr-5-ene-3β,17β-diol* (**9a, b**).—To a magnetically stirred suspension of (methoxymethyl)-triphenylphosphonium chloride (30 g, 88 mmol) in freshly distilled THF (100 ml) under argon at room temperature was gradually added BuLi in hexane (2M; 30 ml, 60 mmol) over 5 min *via* a syringe, whereupon the reaction mixture turned deep red. After the mixture had been stirred for 30 min, the aldehyde (**8**) (3.7 g, 7.9 mmol) was added to it in THF (20 ml). After 2 min, the reaction mixture was heated to gentle reflux for 95 min in an oil bath. After this time, the reaction appeared essentially complete (TLC) with conversion into a less polar component. The mixture was cooled to room temperature, quenched with aqueous $(NH_4)_2SO_4$ (1M; 3 ml), diluted with aqueous 1M $(NH_4)_2SO_4$ (400 ml), and extracted twice (300 ml, 150 ml) with CH_2Cl_2. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure to yield a crude dark red oil. The oil was triturated with hexanes (3 ×, 300 ml total) and the organic solution was filtered through glass wool and concentrated under reduced pressure. The residue (12 g) was flash chromatographed (220 g SiO_2, gradient from 7.5% EtOAc–hexanes to 10% EtOAc–hexanes) to afford methoxyvinyl bis-THP ether (2.9 g) as a pale yellow oil. The oil was dissolved in dry THF (40 ml) and MeOH (180 ml) and pyridinium toluene-4-sulphonate (2.1 g, 8.4 mmol) was added at room temperature with stirring under N_2. The reaction appeared to be complete after 8 h (TLC). The reaction mixture was concentrated under reduced pressure, dissolved in EtOAc (500 ml), and the organic layer was washed twice with water (100 ml). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to afford a white solid composed of (**9a**) and (**9b**) as a 1:1 mixture based on the ^{1}H NMR spectrum (1.7 g, 5.1 mmol. 65% total yield).[20] For the purposes of characterization, a small amount of this mixture was separated by HPLC (4 ml min^{-1}, 67% EtOAc–hexane). *Z*-Isomer (**9a**), t_R 14 min; δ_H(80 MHz) 5.92 (1 H, d, *J* 7.3 Hz, MeOC*H*=CHR), 5.35 (1 H, br d, *J* 4.9 Hz, 6-H), 3.90 (1 H, d, *J* 7.3 Hz, MeOCH=C*H*R), 3.51 (3 H, s, CH_3OR), and 0.73 (3 H, s, 18-H); ν_{max}($CHCl_3$) 3 600 and 1 660 cm^{-1}; *m/z* (EIMS) 332 (M^+) (Found: *m/z* 332.2356. Calc. for $C_{21}H_{32}O_3$: *m/z* 332.2351). *E*-isomer (**9b**) t_R 15 min; δ_H(80 MHz) 6.09 (1 H, d, *J* 12.9 Hz, MeOC*H*=CHR), 5.53 (1 H, br d, *J* 3.8 Hz, 6-H), 4.52 (1 H, d, *J* 12.9 Hz, MeOCH=C*H*R), 3.55 (3 H, s, CH_3OR), and 0.67 (3 H, s, 18-H); ν_{max}($CHCl_3$) 3 600 and 1 660 cm^{-1}; *m/z* (EIMS) 332 (M^+) (Found: *m/z* 332.2359. Calc. for $C_{21}H_{32}O_3$: *m/z* 332.2351).

10β-(*Z- and* E-2-*Methoxyethylene*)*ester-4-ene-3,17-dione* (**7a**) *and* (**7b**).—To a stirred solution of a 1:1 mixture of diols (**9a**) and (**9b**) (200 mg, 0.602 mmol) in dry toluene (150 ml) was added cyclohexanone (6.0 ml, 5.7 g, 58 mmol) under N_2. The mixture was refluxed for 1 h, using a Dean–Stark apparatus, and 9 ml of solvent was removed. After the mixture had been cooled below boiling point, aluminium isopropoxide (1.2 g, 5.9 mmol) was added and refluxing was continued for 2.5 h. The mixture was cooled to room temperature and partitioned between EtOAc (500 ml) and $NaHCO_3$ (satd.) (150 ml). The organic phase was further washed $NaHCO_3$ (satd.) (150 ml) and the organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The crude residue was flash chromatographed (100 g SiO_2, gradient from 20% to 40% EtOAc–hexanes) to afford a 1:1 mixture of (**7a**) and (**7b**) as a colourless oil (175 mg, 0.534 mmol, 89% total yield).

In other experiments, each diol isomer (**9a**) and (**9b**) was oxidized separately as described for the mixture to provide pure (**7a**) and (**7b**). Both (**7a**) and (**7b**) were shown to be pure by HPLC: *Z*-isomer (**7a**) HPLC (42% EtOAc–hexane, 4 ml min^{-1}) t_R 18 min; δ_H(80 MHz) 5.99 (1 H, d, *J* 7.2 Hz, MeOC*H*=CHR), 5.79 (1 H, s, 4-H), 4.19 (1 H, d, *J* 7.2 Hz, MeOCH=C*H*R), 3.51 (3 H, s, CH_3OR), 0.88 (3 H, s, 18-H); ν_{max}($CHCl_3$) 1 735, 1 660, and 1 610 cm^{-1}; λ_{max}(MeOH) 240 nm (ε 15 000); (Found: *m/z* 328.2029. Calc. for $C_{21}H_{28}O_3$: *m/z* 328.2038). *E*-isomer (**7b**) HPLC (42% EtOAc–hexane, 4 ml min^{-1}) t_R 20 min; δ(80 MHz) 6.12 (1 H, d, *J* 13.0 Hz, MeOC*H*=CHR), 5.87 (1 H, s, 4-H), 4.83 (1 H, d, *J* 13.0 Hz, MeOCH=C*H*R), 3.55 (3 H, s, CH_3OR), and 0.88 (3 H, s, 18-H); ν_{max}($CHCl_3$) 1 735, 1 660, and 1 610 cm^{-1}; λ_{max}(MeOH) 237 nm (ε 14 000) (Found: *m/z* 328.2045. Calc. for $C_{21}H_{28}O_3$: *m/z* 328.2038).

Ozonolysis of (**7**) *followed by warming to Room Temperature.*—A 1:1 mixture of methoxyvinyl isomers (**7**) (17.0 mg, 0.0518 mmol) in 4:1 CH_2Cl_2–MeOH (2.7 ml) containing anhydrous $NaHCO_3$ (*ca.* 2 mg) was cooled to −78 °C and ozone was bubbled slowly (*ca.* 1 bubble s^{-1}) through the solution until starting material had been consumed (about 1 h; TLC). The excess of ozone was removed under a stream of argon, and the mixture was allowed to warm to room temperature. After 1 h, the mixture was concentrated under reduced pressure and the major component was purified by flash chromatography (4 g SiO_2, 30% EtOAc–hexanes) to afford

the pure epoxide (**10**) (7.3 mg, 0.0231 mmol, 45%) as a white solid. Compound (**10**) was identical with the known compound (**10**)[12b] based on comparison of the TLC, HPLC, ^{1}H NMR, IR, and MS data.

Ozonolysis of (**7**) *followed by warming or Treatment with Acetic Anhydride–Pyridine.*— Ozonolysis of (**7**) (20.0 mg, 0.0610 mmol) was performed as described above. From the crude mixture, 25% was removed and allowed to warm to room temperature, while the remaining 75% was concentrated to near dryness at below 0 °C and then treated with 3:1 pyridine–acetic anhydride (20 ml) at −78 °C and then allowed to warm to room temperature. The aliquot which was not treated with acetic anhydride–pyridine afforded (**10**) (2.1 mg, 0.066 mmol, 43% yield) and contained <2% of 19-oxoandrostane-3,17-dione 4α,5α-epoxide (^{1}H NMR) and less than 0.5% estrone (**4b**) (HPLC, 44% EtOAc–hexane).

The aliquot which was treated with acetic anhydride–pyridine was concentrated under reduced pressure and subjected to HPLC analysis (44% EtOAc–hexane). Less than 0.5% estrone (**4b**) (or estrone 3-acetate) was observed. The two major products were methyl ester (**11**) (3.8 mg, 0.0114 mmol, 25% yield) and epoxide (**10**) (1.8 mg, 0.0057 mmol, 12% yield). Compound (**11**) was identical with the known compound[21] based on TLC, HPLC, ^{1}H NMR, IR, UV, and MS data. Compound (**10**) was identical with the known material[12b] based on TLC, HPLC, ^{1}H NMR, IR, and MS data.

Ozonolysis of (**7**) *followed by Treatment with Dimethyl Sulphide after Varying Lengths of Time.*—Ozonolysis of (**7**) (20.0 mg, 0.0610 mmol) was performed as described above. Aliquots of 0.5 ml (from 2.5 ml total reaction volume) were removed and quenched with dimethyl sulphide (0.1 ml, 85 mg, 1.4 mmol) at zero, 15, and 30 min after the ozonolysis mixture had been warmed to room temperature. A fourth aliquot was not quenched with dimethyl sulphide and allowed to warm to room temperature for several hours. After concentration under reduced pressure, the aliquots were analysed by HPLC (40% EtOAc–hexane). The zero time fraction contained enone (**3b**) (27% yield) and epoxide (**10**) (14% yield). The 15 min, 30 min, and unquenched fractions contained 41, 35, and 43% yields respectively of epoxide (**10**) with no detectable enone (**3b**) (<2%). The enone (**3b**) was identical with the known material[26,8b] based on TLC, HPLC, ^{1}H NMR, IR, and UV data.

Ozonolysis of (**7**) *followed by Treatment with Androst-4-ene-3,17-dione* (**1b**).—Ozonolysis of (**7**) (20.0 mg, 0.061 mmol) was performed as described above and one-half of the reaction mixture was transferred to a vial containing androstenedione (**1b**) (7.5 mg, 0.026 mmol) in CH_2Cl_2 (1 ml) which was cooled to −78 °C. After 1 min, the mixture was warmed to room temperature and after 1 h, the products were analysed by HPLC (40% EtOAc–hexane). Neither 4α,5α- nor 4β,5β-epoxyandrostane-3,17-dione could be detected (less than 1%).

Variable Temperature ^{1}H *NMR Experiment.*—Ozonolysis of (**7**) (20.0 mg, 0.061 mmol) was performed as described above except that CD_2Cl_2–CD_3OD was used as the reaction solvent. Approximately 0.5 ml of the reaction mixture was transferred to an NMR tube at −78 °C and the tube placed in the 400 MHz NMR probe which had been pre-chilled to −60 °C. Spectra were obtained (16 transients) every 5–10 min as the temperature was successively raised to −40 °C, −20 °C, 0 °C, and +20 °C over the course of 1 h (see text for the results).

Ozonolysis of (**7**) *followed by Treatment with* $FeSO_4$–$Cu(OAc)_2$.—Ozonolysis of (**7**) (14.0 mg, 0.043 mmol) was performed as described above. The reaction was evaporated to near dryness with a jet of N_2 (below 0 °C), and a degassed solution of saturated $Cu(OAc)_2$ in MeOH (3 ml) at −78 °C was added, followed by $FeSO_4 \cdot 7H_2O$ (80 mg, 0.29 mmol) under N_2 with vigorous magnetic stirring. After 30 min at −78 °C, the cold bath was removed and the reaction was stirred at room temperature for 4 h. The resultant mixture was partitioned between water (30 ml) and CH_2Cl_2 (50 ml). The aqueous phase was further extracted with CH_2Cl_2 (30 ml). The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. A ^{1}H NMR spectrum of the crude material indicated that <2% estrone had been formed. Normal phase HPLC purification (42% EtOAc–hexane) afforded methyl ester (**11**) (1.1 mg, 0.0033 mmol, 8% yield) whose identity was verified by comparison with the known material, based on TLC, HPLC, ^{1}H NMR, and MS data.

Conversion of 19-*Oxoandrost-4-ene-3,17-dione* (**3b**) *into* 19-*Oxoandrost-4β,5β-epoxy-3,17-dione* (**10**).—To a solution of enone (**3b**) (15 mg, 0.050 mmol) in MeOH (1 ml) and anhydrous $NaHCO_3$ (*ca.* 3 mg) was added 30% HOOH(aqueous; 0.1 ml, 30 mg, 0.88 mmol). The mixture was vortexed and then allowed to stand at 4 °C for 2 h after which time starting material had disappeared (TLC) and a less polar product had formed. The mixture was transferred to a separating funnel containing CH_2Cl_2 (60 ml) and aqueous 2% sodium thiosulphate (40 ml). The organic phase was dried (Na_2SO_4) and purified by HPLC (40% EtOAc–hexane) to afford pure epoxide (**10**) (10 mg, 0.032 mmol, 64% yield) which showed TLC, HPLC, IR, ^{1}H NMR, and MS data identical with those of the known material.[12b] In addition, the ^{1}H NMR spectrum of the crude product indicated that <5% 4α,5α-epoxide had been formed.

Treatment of 19-*Hydroxyandrost-4-ene-3,17-dione* (**2b**) *with Hydrogen Peroxide.*—To a solution of the enone (**2b**) (5.0 mg, 0.017 mmol) in MeOH (1 ml) containing anhydrous $NaHCO_3$ (*ca.* 3 mg) was added aqueous 30% HOOH (0.1 ml, 30 mg, 0.88 mmol). The mixture was vortexed and allowed to stand at 4 °C for 6 h. The resultant mixture was worked up as described above for the reaction of (**3b**). The ^{1}H NMR spectrum of the crude product indicated that <5% epoxidation had occurred.

Treatment of Androst-4-ene-3,17-dione (**1b**) *with Hydrogen Peroxide.*—To a solution of androstenedione (**1b**) (5.0 mg, 0.017 mmol) in 5:1 MeOH–CH_2Cl_2 (1.2 ml) containing anhydrous $NaHCO_3$ (*ca.* 3 mg) was added aqueous 30% HOOH (0.2 ml, 60 mg, 1.7 mmol). The mixture was vortexed and allowed to stand at 4 °C for 30 h. The mixture was concentrated under a stream of N_2 and a ^{1}H NMR spectrum of the crude material indicated that <5% epoxidation had occurred.

Treatment of 19-*Oxoandrost-4-ene-3,17-dione* (**3b**) *with t-Butyl Hydroperoxide.*—To a solution of the enone (**3b**) (5.0 mg, 0.017 mmol) in MeOH (1 ml) containing anhydrous $NaHCO_3$ (*ca.* 3 mg) was added 70% aqueous Bu^{t}OOH (0.1 ml, 70 mg, 0.78 mmol). The mixture was vortexed and allowed to stand at 4 °C for 3 d. The mixture was shaken with aqueous 3% sodium thiosulphate (40 ml) for 5 min after which the aqueous phase was extracted with CH_2Cl_2 (40 ml). The aqueous phase was further extracted with CH_2Cl_2 (40 ml), and the combined organic phases were dried (Na_2SO_4), and concentrated under reduced pressure. The ^{1}H NMR spectrum of the crude material demonstrated that <5% epoxidation or estrone formation had occurred.

Treatment of 19-*Oxoandrost-4-ene-3,17-dione* (**3b**) *with* 50%-^{18}O-20% HOOH.—The enone (**3b**) (7.0 mg, 0.023 mmol) was dissolved in MeOH (0.6 ml) and anhydrous $NaHCO_3$ (*ca.* 3 mg) was added and the mixture was cooled to 4 °C. To this mixture was added randomly labelled aqueous 20% HOOH (50%-^{18}O,

0.06 ml, 12 mg, 0.33 mmol). After vortexing, the reaction mixture was allowed to stand at 4 °C for 15 min, by which time the reaction was nearly complete (TLC). The mixture was transferred to a separating funnel containing aqueous 2% sodium thiosulphate (60 ml) and extracted with CH_2Cl_2 (3 ×, 90 ml total). The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. Flash chromatography (0.5 g SiO_2, 40% EtOAc–hexanes) afforded pure labelled epoxide (**15a**) (5.5 mg, 0.017 mmol, 74%) as a white solid. The TLC and ^1H NMR properties were identical with the material analogously prepared with unlabelled hydrogen peroxide. Mass spectral analysis for ^{18}O of (**15a**) using the parent ion (M^+) indicated $^{18}O_0$-37.5%, $^{18}O_1$-50.9%, and $^{18}O_2$-11.6%. Resubmission of a portion of labelled epoxide (**15a**) (2.5 mg, 0.0078 mmol) to identical reaction conditions except that unlabelled aqueous 20% HOOH was employed, afforded (**15b**) after purification. MS analysis of (**15b**) indicated $^{18}O_0$-40.0%, $^{18}O_1$-51.4%, and $^{18}O_2$-8.7%.

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