Estrogen Biosynthesis: 2β-Hydroxy-19-oxoandrost-4-ene-3,17-dione Revisited

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Current evidence is consistent with the view that biosynthesis of estrogens by human microsomal placental aromatase proceeds through several different pathways. One of the proposed mechanisms of aromatization of androgens involves the intermediacy of 2β-hydroxy-19-oxoandrost-4-ene-3,17-dione **1a**. It is shown that incubation of 2β[^{18}OH]-**1b** with placental aromatase gave HC^{18}OOH which differs from previous observations. The incorporation of isotopic oxygen in the formic acid is consistent with the view that one of the alternative routes of estrogen elaboration may involve a 2β,19-dioxygenated androgen.

Variations in the stereochemical course of estrogen elaboration are now firmly established.[1-3] The aromatization of androst-4-ene-3,17-dione **2a** and testosterone **2b** proceeds in three oxidative stages each requiring a mole of oxygen and a mole of NADPH.[4] The process is initiated by C-19 hydroxylation (first mole of oxygen) to yield 19-hydroxy[5] **3**, and the reaction was shown to proceed with retention.[6] It was proposed that compound **3** undergoes a second C-19 hydroxylation (second mole of oxygen) to give C-19 diol **4** the formation of which involves the stereospecific abstraction of the 19-*pro-R* hydrogen atom[7-9] of **3**. The diol **4** is thought to lose a molecule of water to yield 19-aldehyde **5**. In the dehydration process the oxygen atom introduced in the second C-19 hydroxylation is eliminated. Finally the aldehyde is oxidatively aromatized (third mole of oxygen) and C-19 is extruded as formic acid. The formic acid contains an atom of oxygen derived from the third mole of oxygen.[7-11a]

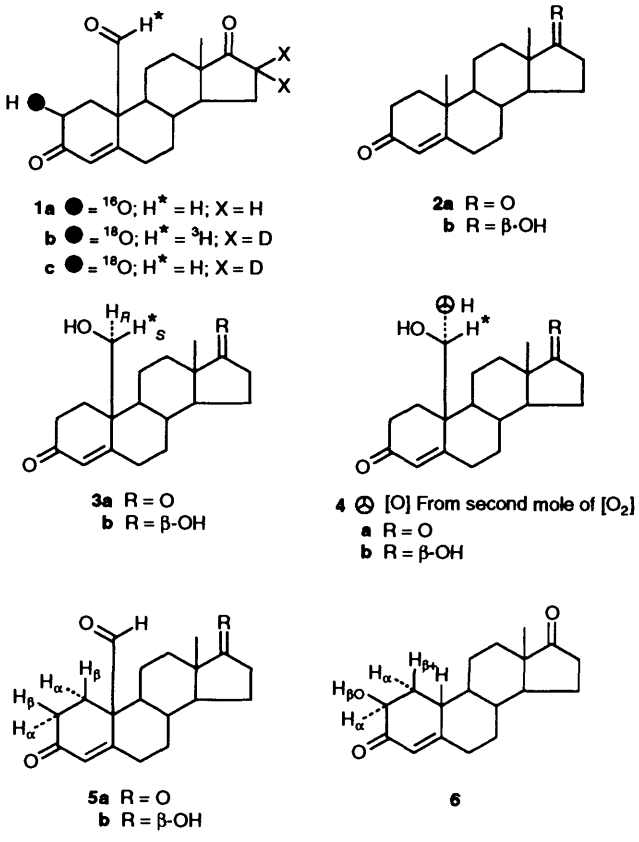
In the case of androst-4-ene-3,17-dione **2a** and its C-19 oxygenated analogues **3a** and **5a** the 1β and 2β hydrogen atoms are eliminated.[12-15] The situation for 17β-hydroxy androgens, testosterone[2] **2b** and its C-19-oxo analogue[1] **5b** is different. In this instance only the 1β hydrogen atom is abstracted stereospecifically while C-2 hydrogen atoms are eliminated non stereospecifically. The 2β or 2α hydrogen atoms are lost in variable amounts[2] of up to 6:5 2β-H:2α-H ratio.[1]

By necessity, in the absence of the 10β-methyl group, the oxidative requirements for the aromatization of estr-4-ene-3,17-dione **6** by placental P-450$_{aromatase}$ must be different. However, here again the formation of estrone proceeds with the stereospecific elimination of 1β-hydrogen[16] atom but the loss of the C-2 hydrogen atom is not stereospecific.[17,18,3]

Controversy persists concerning the mode of utilization of the third mole of oxygen in the process of scission of the C-10(19) bond of 19-oxo **5** and the ensuing aromatization of ring-A. Several mechanisms for the last stage(s) of estrogen biosynthesis were proposed. We will limit our discussion to the three, which are under current consideration.

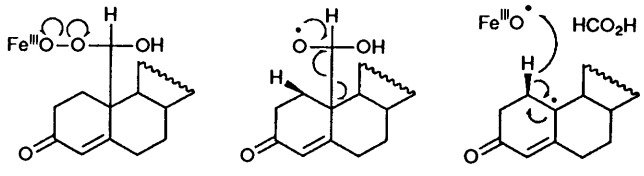
Akhtar *et al.*[7-9] proposed that the peroxy entity Fe^{III}–O_2H (derived from the third mole of oxygen) adds at C-19 of aldehyde **5a** or **5b** to yield **7**. Homolytic cleavage of the peroxy moiety results in alkoxyl radical **8** the fragmentation of which gives formic acid and the tertiary radical **9**. Loss of the 1β-hydrogen atom provides the 4,1(10)-diene system of estrogens.

This hypothesis satisfactorily explains the incorporation of an atom of the 'third mole of oxygen' in the extruded formic acid and for the production of significant amounts estrenes **10**–**13** in incubations of 19-oxo **5a** with microsomal placental aromatase.[3,19,20] Without doubt the major route of stabilization of



radical **9** is the formation of estrogens. However competing pathways are also operating. Thus, acquisition by **9** of [H*] or [HO*] will yield **10** and **12**, respectively. Similarly, homolytic isomerization of C-4 double bond of **9** to C-5(10) and addition of a hydrogen at C-4 will provide **11**. The tentatively identified estra-5(10),6-diene-3β,17β-diol **13** could be derived from the previously isolated 19-oxoandrosta-4,6-diene-3,17-dione[19] or *via* dehydrogenation of **11**.

A revised version of a mechanism outlined by Osawa and Shibata[21] was proposed by Covey *et al.*[22-24] They postulated that 19-diol **4** is the substrate for the third oxygenation in which the 1β-hydrogen is abstracted to give the C-1 radical complex **14**. The radical **14** collapses as indicated to the diene orthoformate complex **15**. The hydroxy group of the orthoformate containing the oxygen atom introduced in the second hydroxylation of C-19 must now be positioned in the vicinity of the 2β-



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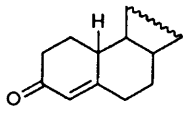

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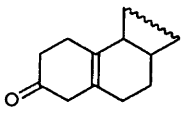

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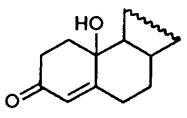

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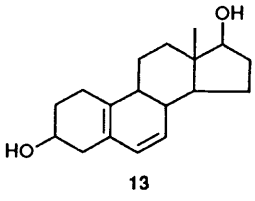

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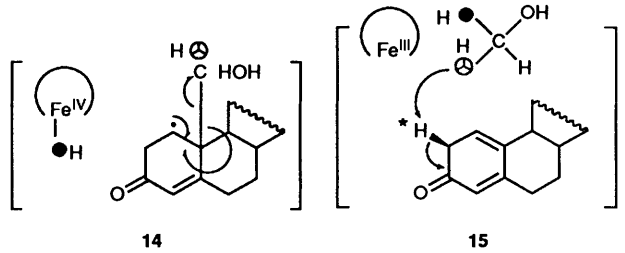
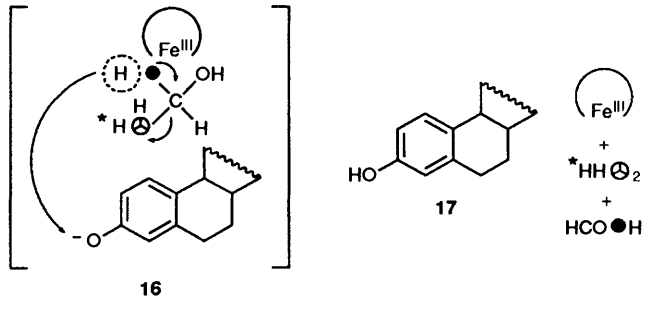


14 15

⊛ = [O] From second mole of [O_2]
● = [O] From third mole of [O_2]



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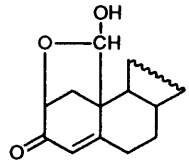
17

hydrogen atom for its stereospecific removal. Following the abstraction of the 2β-hydrogen atom this oxygen is then eliminated in the dehydration of the orthoformate to formic acid **16**. Only now are the products released from the aromatase to yield estrogen **17**, formic acid and water. The scheme has several drawbacks. It postulates that throughout, the complexes **14** → **15** → **16** must be held by the enzyme until the aromatization process is completed. The requirement for the specific positioning of the orthoformate within the complex **15** for the oxygen atom introduced in the second hydroxylation to abstract the 2β-hydrogen atom and then be eliminated in the dehydration to formic acid is most unusual. Also the proposed[22-24] intrinsic stereospecific elimination of the 2β-hydrogen atom contradicts the proven lack of stereospecificity in the abstraction of C-2 hydrogen atoms in the aromatization of 17β-hydroxylated androgens and estren-4-ene-3,17-dione.[1-3]

Finally, within the framework of this mechanism the formation of estrenes[3,19,20] cannot be explained rationally.

Fishman *et al.*[25-28] suggested that the third mole of oxygen is utilized for the 2β-hydroxylation of **5** to give **1** which, in turn, is released from the enzyme complex and then aromatizes non-enzymatically. In the revised 2(19)-hemiacetal form **18** the



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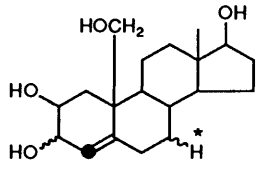
mechanism accommodates the requirement for the incorporation of the C-2 oxygen atom derived from the third mole of oxygen in the extruded formic acid. The 2β-hydroxy-19-oxo **1** was not isolated or detected in incubations of androgens with placental microsomal aromatase. However, it was shown that it aromatizes very rapidly even in the absence of the enzyme.

Support for the intermediacy of **1** was inferred from an experiment in which a mixture of [1,2-^{3}H; 4-^{14}C]androst-4-ene-3,17-dione and a large amount of **1a** was incubated with placental aromatase. Following reductive termination of the incubation ($NaCNBH_3$) androst-4-ene-2β,3β,17β,19-tetrol **17a** (purified and counted as acetate) containing 0.08% of ^{14}C was isolated.[25,26] We have incubated a mixture of [7-^{3}H]-testosterone and **1a** and as above terminated the reaction with $NaCNBH_3$. After extensive TLC and HPLC purification of the acetylated metabolites 3α and 3β, 2β, 17β, 19-tetrol acetates containing 0.02% and 0.07% tritium, respectively, were isolated.[11] These results support the view that small amounts of 2β,19-dioxygenated analogues of **2a** are very likely formed in incubations of androgens **2a** and **2b** with aromatase.

To evaluate the incorporation of the C-2 oxygen atom of **1** in the extruded formic acid we used the difficult to prepare [2β-^{18}O; 19-^{3}H]-2β-hydroxy **1b**. Our initial efforts to synthesize the doubly labelled **1b** were only marginally successful and produced very small amounts of the needed substrate.[10,29,30] This was used in a preliminary study in which **1b** was incubated[6] in Tris-buffer at pH 7.4 in the presence and absence of the placental aromatase. The samples of formic acid recovered from the two incubations were converted into benzyl formates[31,7] and shipped to Professor G. Spiteller in Germany for GC–MS analysis. The benzyl formates from both experiments were devoid[11b] of oxygen-18 which led us to conclude that the route *via* 2β-hydroxy-19-oxoandrost-4-ene-3,17-dione is 'not an obligatory pathway of estrogen biosynthesis'.[10]



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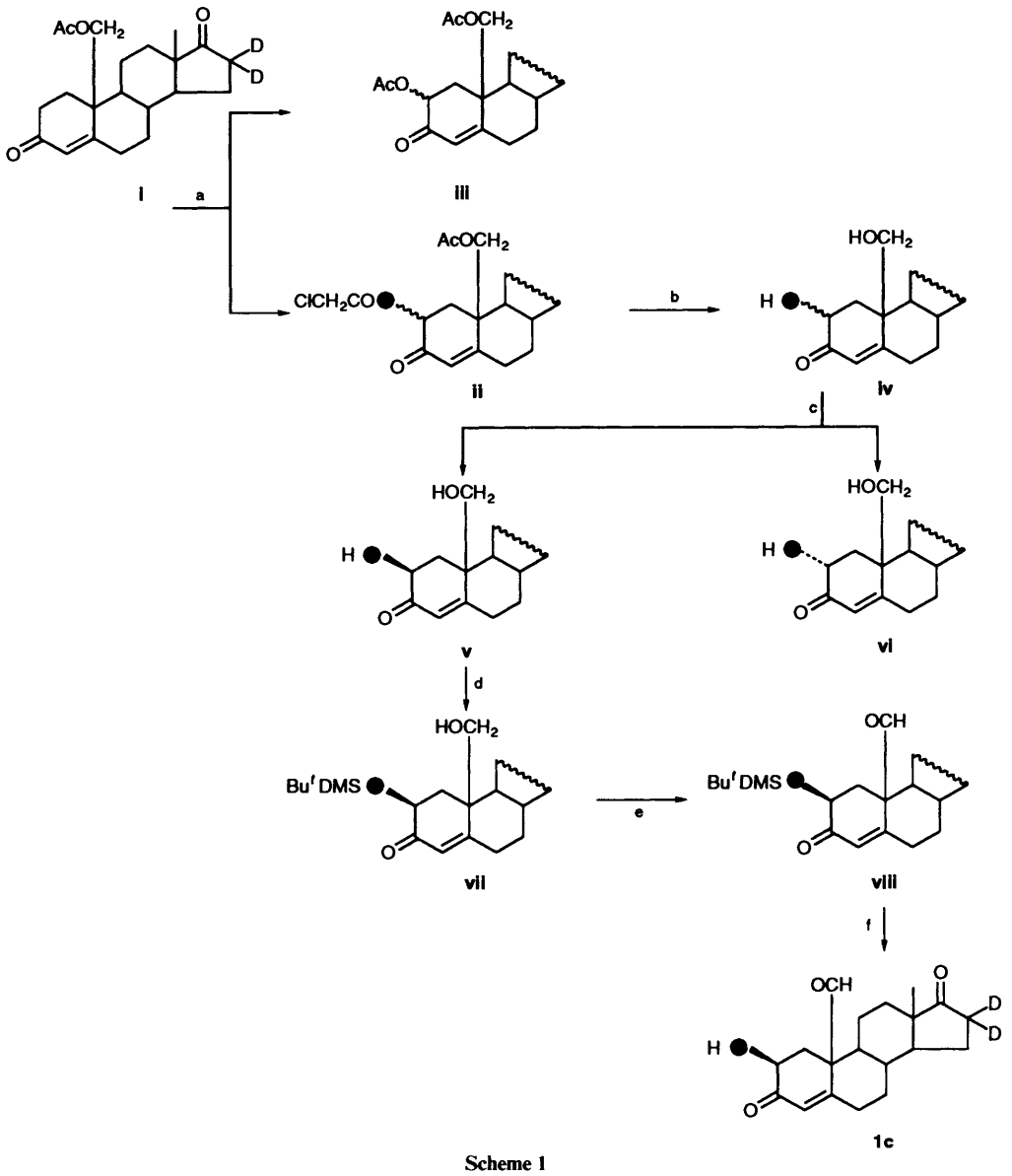
a ● = ^{14}C; H* = H;
b ● = ^{12}C; H* = ^{3}H;

However, there remained an inherent inconsistency related to the trapping of the small amount of [^{14}C]-tetrol **19a** and [^{3}H]-tetrols **19b** in reductively terminated incubation experiments. The logical assumption of these observations was that some of the radioactive substrates **2a** and **2b** were converted into 2β,19-dihydroxy or 2β-hydroxy-19-oxo analogues, respectively. These, in turn, in the course of the work-up with $NaCNBH_3$ were reduced to 3α- and 3β-tetrols **19**. In this context the biosynthetic



Scheme 1

formation of some 2β-hydroxy-19-oxo **1** seemed possible. And while it was apparent that the compound is not an obligatory estrogen precursor its participation in an alternate pathway could not be excluded. Under the circumstances we thought that our preliminary observations on the lack of incorporation of the oxygen of the 2β-hydroxy of **1c** into the formic acid should be re-examined.

The scarcity of 2β-hydroxy-19-oxo **1** and particularly of the [^{18}O]-labelled analogue was a formidable obstacle impeding the study of the biosynthetic role of the compound. It was obvious that without an adequate supply of the substrate a thorough investigation of the problem was not feasible. Consequently we first concentrated on the development of a more efficient synthesis of the required substrate. The general outline of the synthesis, the details of which will be published elsewhere,[32] are shown in Scheme 1.

Treatment of 19-acetoxyandrost-4-ene-3,17-dione **i** with [$^{18}O_1$]$ClCH_2CO_2H$–$Mn(AcO)_3$ in dry benzene gave a mixture of [2-^{18}O]-(2ξ)-chloroacetoxy 19-acetates **ii** which was separated by preparative layer chromatography (PLC) from the accompanying unlabelled (2ξ)-diacetates **iii**. Treatment of **ii** with KOH–MeOH yielded the epimeric C-2 alcohols **iv** which were resolved by PLC to diols **v** and **vi**. Selective silylation of **v** provided (2β)-Bu^{t}DMS **vii** which following oxidation with PCC yielded **viii**. Removal of the protective Bu^{t}DMS moiety with aq. HF–CH_3CN gave the required **1c**. Starting from the 19-acetate **i**, without recycling of intermediates and/or optimizing reaction conditions, the overall yield of [^{18}O]-2β-hydroxy **1c** was 3%. The incorporation of isotopic oxygen [^{18}O] in several syntheses varied in the range of 30–48%. The availability of sufficient amounts of **1c** allowed the scaling up and repetition several times of the incubations.

We first incubated a sample of **1c** (33%, 2β-^{18}O)], in Tris buffer (pH 7.2; 37 °C) containing all the cofactors, in the absence of placental microsomal aromatase. The incubation was terminated by acidification (1.25 mol dm^{-3} phosphoric acid; Congo Red), immediately frozen (liquid N_2) and lyophilized. The distillate was made alkaline (1.25 mol dm^{-3} NaOH; phenolphthalein) and again lyophilized. The resulting sodium formate was mailed from the Worcester Foundation in Shrewsbury Massachusetts to the Children's Hospital Oakland Research Institute CA where it was converted into Bu^{t}DMS formate and analysed by GC–MS. The Bu^{t}DMS formate showed a 30% content of [$^{18}O_1$] and indicated a nearly complete (*ca.* 90%) incorporation of the C-2β-isotopic oxygen of **1c** (Table 1; entry 1).

The results were in contrast to our preliminary observations[10,11] and two problems needed attention. First, it was

Table 1 Incorporation of [^{18}O] into formic acid extruded in the aromatization of [2-^{18}O]2β-hydroxy-19-oxoandrost-4-ene-3,17 dione **1c**. The samples of formic acid were analysed as benzyl formates and/or Bu^{t}DMS formates by GC–MS.

Exp.	Substrate **1c** %[^{18}O]	Experimental conditions[a] Enzyme	**1c**/mg	Benzyl formate %[^{18}O]	(% incorp)[b]	Bu^{t}DMS formate %[^{18}O]	(% incorp)[b]
1	33	(−)	4		—	30	(91)
2	33	(+)	4	30	(91)	12	(36)
3	48	(+)	3	20	(42)	2	(4)
4	48	(−)	1.5	14	(29)	0	—
5a[c]	48	(+)	4	10	(21)	9	(19)
5b[c]	48	(+)	4	10	(21)	14	(29)
5c[c]	48	(+)	4	10	(21)	6	(13)

[a] The same medium (pH 7.2) was used in all incubation except that (−) enz. and (+) enz. denote experiments carried out in the absence and presence of microsomal placental aromatase respectively (see Experimental section for details). [b] % of [^{18}O] of substrate **1c** incorporated in the formate ester. [c] Three equal aliquots were taken from the distillate of the lyophilized incubation mixture containing the extruded formic acid. The aliquots were made alkaline with 20 mm^3, 40 mm^3 and 60 mm^3 of 5% aq. NaOH respectively and lyophilized. The resulting samples of sodium formate were converted into the benzyl and Bu^{t}DMS esters.

necessary to define the role if any of the aromatase in the C-2 → C-19 oxygen transfer. Secondly, since previously[10,11] the recovered formic acid was analysed as benzyl formate,[31] the impact of the two methods of esterification of the formic acid on the retention and/or loss of isotopic oxygen had to be evaluated. To this end three incubation experiments of **1c** with placental microsomal aromatase were carried out. The incubations were terminated and processed as above to yield three samples of sodium formate. An aliquot of each sample of the sodium formates was converted into benzyl formate. The remainder of the sodium formates were shipped (together with the benzyl formates) to Oakland CA for preparation of Bu^{t}DMS formates and GC-MS analyses. The results are summarized in Table 1. The large discrepancies in isotopic oxygen content of the samples was revealing and indicated a susceptibility of the formic acid and/or or the esters to oxygen exchange.

Although the results of experiments 1–4 (Table 1) are rather scattered they indicate that oxygen of the 2β-hydroxy of **1c** was incorporated into the extruded formic acid. It is likely that in many instances isotopic oxygen was exchanged in the process of isolation, esterification and/or shipment of the samples for GC–MS analyses. The large differences in [^{18}O] content of benzyl and Bu^{t}DMS esters and especially the absence of isotope in Bu^{t}DMS formate in entry 4 can not be easily rationalized. It is possible that an exchange occurred in the shipment of the sodium formate and the ensuing delay in processing the sample(s). Also of concern was the impact that the 'excess' of NaOH used for neutralization of aqueous formic acid prior to its concentration to sodium formate may have on the exchange of isotopic oxygen. To evaluate this, equal aliquots of the solution of formic acid from the same incubation were treated with 20 mm^3, 40 mm^3 and 60 mm^3 of 5% aq. NaOH respectively and lyophilized and processed as above. The addition of 20 mm^3 of the 5% aq NaOH sufficed to turn the mixture alkaline (phenolphthalein). The results, entries 5a, 5b and 5c (Table 1), indicate that the content of isotopic oxygen of the benzyl formates, although low in comparison to the parent **1c** was not changed by increasing amounts of base. The results for Bu^{t}DMS formates are less consistent and do not indicate a base-related trend.

At present we do not have a satisfactory answer for the inconsistency of the result and particularly the failure to detect the isotopic oxygen in some of the current and previous[10] experiments. While we tried to standardize the laboratory procedures certain factors, *e.g.* shipping samples to Germany[10] or California, were beyond our control. In retrospect, considering the proven lack of stereospecificity of elimination of C-2 hydrogen atoms discussed above, our preliminary conclusion that the 2β-hydroxy **1c** is not an obligatory estrogen precursor[11] proved to be valid but for a different reason.

Experimental

Incubation Procedure of **1c** *With or Without Microsomal Placental Aromatase.*—To a solution of NADPH (25 mg), glucose-6-phosphate (42 mg), dithiothretol (9.2 mg), glucose-6-phosphate dehydrogenase (532 mm^3; 1 μkat mol^{-1}) in Tris-malate buffer (pH 7.2; 8 cm^3) a preparation of microsomal placental aromatase (2 cm^3) was added and the pH of the mixture was adjusted to 7.2 with 1 mol dm^{-3} NaOH. Then a solution of **1c** (4 mg) in ethanol (200 mm^3) was added and the mixture was incubated (with reciprocal shaking) 1 h at 37 °C in the air. The reaction was terminated with 0.74 mol dm^{-3} phosphoric acid (2 cm^3), frozen (liq. N_2) and lyophilized. The distillate was made alkaline with aq. 1 mol dm^{-3} NaOH (phenolphthalein), frozen and again lyophilized to leave a residue containing sodium formate.

Incubations in the absence of the placental microsomal aromatase were carried out as above except that the enzyme preparation was omitted.

When smaller amounts of substrate **1c** were used the overall volume of the incubation medium was reduced proportionally.

Benzyl formate. An aliquot of the sodium formate residue (about 50%) was dissolved in water (200 mm^3) and acidified with 1 mol dm^{-3} HCl to pH 2 and processed by the literature procedure.[31] The concentrated ether extract of the benzyl ester[31] was shipped in a sealed vial for GC–MS analysis to Children's Hospital Oakland Research Institute, Oakland CA.

Bu^{t}DMS formate. The remainder of the sodium formate was shipped to Oakland CA.

To the dry sodium formate powder *N*-methyl-*N*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) (Regis Chemical Co. Morton IL 60053) (20 mm^3) was added and the mixture was heated at 60 °C for 15 min. An aliquot(s) of the mixture (2 mm^3) were taken for GC–MS analysis.

GC–MS analyses of formate esters. An HP-5970 instrument equipped with a mass selective detector and a 15 m DB1 column (i.d. 0.25 mm; 0.25 μm film thickness) (J & W Scientific, Folsom, CA 95630) was used. The samples of formate esters were injected in the split mode and the analyses were carried out isothermally at 70 °C. The temperatures of the injector and interface were 260 and 280 °C respectively.

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