Enzymic aromatization of deuterium labelled testosterone and androst-4-ene-3,17-dione

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The preparation and aromatization by human placental microsomes of androst-4-ene-3-ones labelled with deuterium at C-4, C-6α, C-6β, C-16, and C-19 is described. Deuterium nmr spectra are reported for these compounds and for a sample of a 1,2-dideuterated androst-4-ene-3-one; the latter is formed nonstereospecifically by reduction of the C-1(2) double bond of a $\Delta^{1,4}$-diene-3-one. Aromatization by placental microsomes occurs with retention of deuterium at C-4 and C-6, but with some loss from C-16. The aromatization of 19-d_1 and 19,19,19-d_3 steroids in the presence of 16,16-d_2 steroids has been carried out to determine the deuterium isotope effect for the oxidative removal of C-19. The values obtained (k_H/k_D for 19-d_1 = 2.3, k_H/k_D for 19,19,19-d_3 = 3.2) are a combination of primary and secondary effects, but suggest that oxidation at C-19 is a rate-limiting reaction of the biosynthetic sequence.

The fungus *Pellicularia filamentosa* NRRL 2727, reported to hydroxylate androst-4-ene-3,17-dione at C-19, gave only the products of hydroxylation at C-11α, C-11β, and C-14.

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On décrit la préparation et l'aromatisation par les microsomes du placenta humain, d'androstène-4 ones-3 marquées au deutérium en C-4, C-6α, C-6β, C-16 et C-19. On rapporte les spectres de rmn du ^2H de ces composés ainsi que celui d'un échantillon d'androstène-4 one-3 dideutéré en positions 1 et 2. On a obtenu ce dernier de façon non stéréospécifique par la réduction de la double liaison en C-1(2) d'une Δ-diène-1,4 one-3. L'aromatisation par les microsomes du placenta se produit avec une rétention de configuration des deutérium en C-4 et en C-6 mais avec quelques pertes au niveau du C-16. On a réalisé l'aromatisation des stéroides deutérés 19-d_1 et 19,19,19-d_3 en présence de stéroides 16,16-d_2 dans le but de déterminer l'effet isotopique du deutérium lors de l'élimination oxydante de C-19. Les valeurs obtenues (k_H/k_D pour 19-d_1 = 2,3, k_H/k_D pour 19,19,19-d_3 = 3,2) sont une combinaison d'effets isotopiques primaires et secondaires mais elles suggèrent que l'oxydation au niveau de C-19 est l'étape déterminante de cette série de réactions biosynthétiques.

Le champignon *Pellicularia filamentosa* NRRL 2727 responsable de l'hydroxylation de l'androstène-4 dione-3,17 au niveau du C-19 donne seulement des produits d'hydroxylation en C-11α, C-11β et C-14.

[Traduit par le journal]

Introduction

In spite of the intense effort which has been expended over the last two decades on investigation of the later stages of the biosynthesis of estradiol (estra-1,3,5(10)-triene-3,17β-diol, **1***a*) and estrone (estra-1,3,5,(10)-triene-3-ol-17-one, **1***b*), several aspects of the conversion of testosterone (androst-4-ene-17β-ol-3-one, **2***a*) or androst-4-ene-3,17-dione (**2***b*), the C_{19} substrates of the aromatase enzyme system, to the estrogens, remain obscure. The established route for this conversion by human placental microsomes is presented in Fig. 1 (1–6).

The key features of this pathway are (*i*) the intermediacy of the 19-alcohol **21** and aldehyde **22**, and their formation by the action of a monooxygenase type enzyme utilizing molecular oxygen and NADPH (two equivalents); (*ii*) the apparent involvement of a third equivalent of oxidizing potential (O_2/NADPH); and (*iii*) the release of C-19 of the substrate as formic acid, the stereochemistry of oxidation at C-19 being that shown in Fig. 1.

Less well defined from a mechanistic standpoint are the following; firstly, the function of the third oxidizing equivalent and the pathway for conversion of **22** to **1***a* or **1***b*. Pathways have been postulated which involve 1β hydroxylation to give intermediate **23** (Fig. 2) (7–9), 2β hydroxylation to give **24** (10, 11), enzymic "Baeyer–Villiger" oxidation to give the ester **25** (12), or epoxidation to **26** (13, 14). The possible involvement of a Δ^5 steroid via a 5,6 epoxide has also been suggested (13). The proposed pathways for the aromatization of the aldehyde **22** are based on the experimental observations of the release of C-19 as formate (Fig. 1), and a stereospecific loss of hydrogen from C-1β and C-2β (15–18). Secondly, the relative rates of the steps shown in Fig. 1 have been the subject of some discussion, complicated by the possible distinction between "free" and enzyme-bound intermediates, which now seems to indicate that the transformations occur at a single enzyme site, with little dissociation of intermediates from the enzyme, and that hydroxylation at C-19 is rate limiting in the overall pathway (5, 6, 19).

Although the stereochemistry of loss of hydrogen from C-1 and C-2 of substrate during aromatization has been investigated (15–18), the possible fate of the hydrogens attached to C-4 and C-6 has

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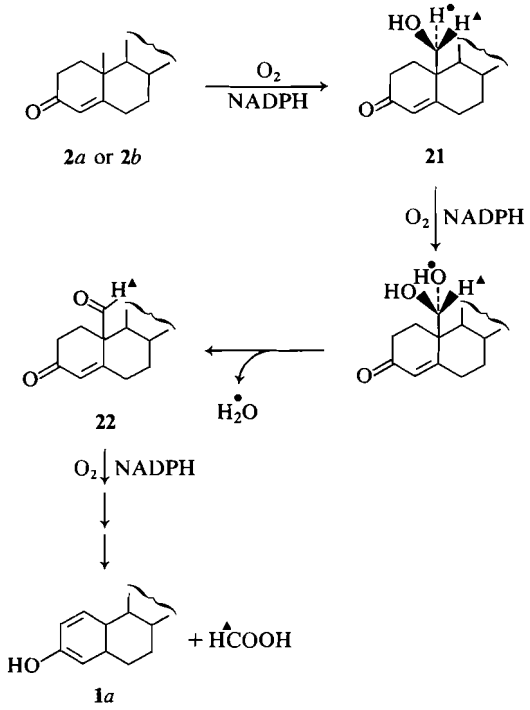


FIG. 1. Established mechanism of the aromatization of C_{19} steroids (1–6)

not hitherto been considered. Several of the mechanisms proposed for the aromatization of **22** have included intermediates in which the Δ^4 double bond is rearranged to the $\Delta^{5(10)}$ position (1, 13) or the $\Delta^{5(6)}$ position (13), and so a knowledge of the fate of the C-4 and C-6 hydrogens of **2***a* or **2***b* during conversion to the estrogens is desirable. In addition, the use of C-19-deuterium labelled steroids enables the kinetic isotope effect, k_H/k_D at C-19, for the aromatization to be determined. This information may provide additional evidence for the identity of the rate-limiting step of the overall process, and for the nature of the oxidation reaction occurring at C-19.

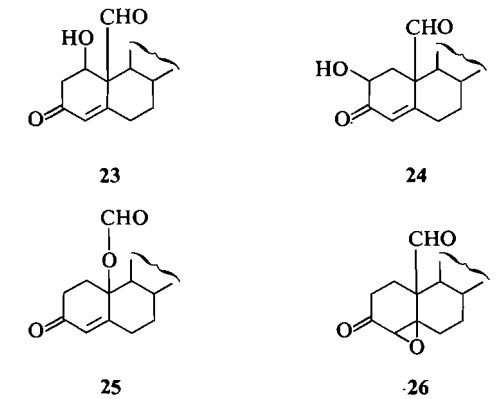


FIG. 2. Proposed intermediates in the conversion of **22** to **1***a*

Results and Discussion

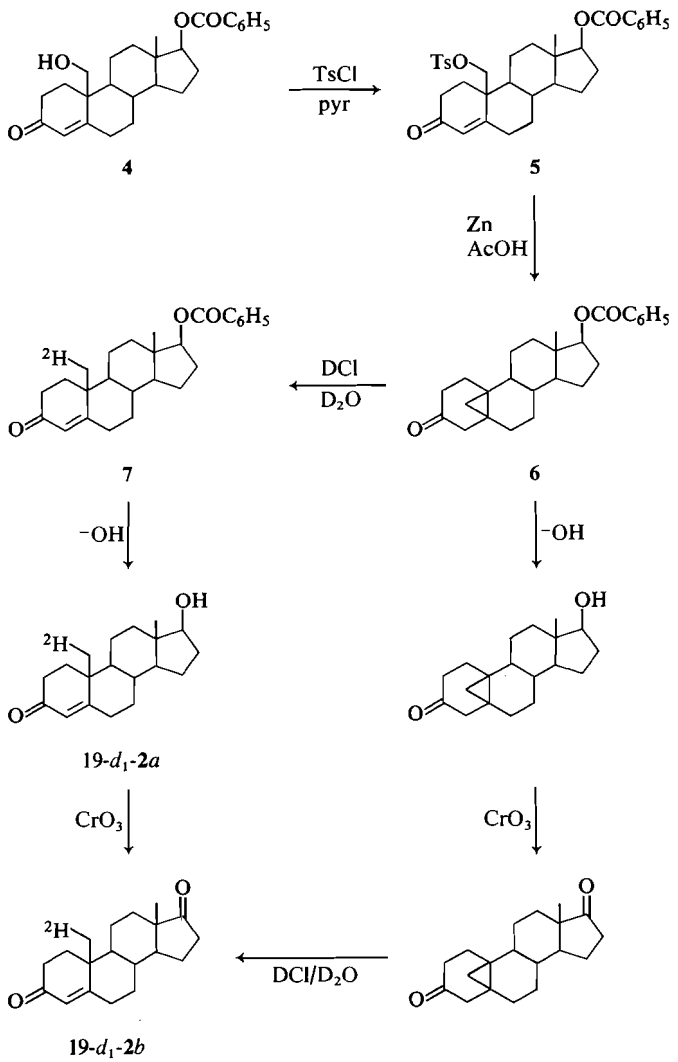
The deuterium labelled steroids used in this study are listed in Table 1, and were prepared as outlined in the Experimental. The preparation of substrates labelled at C-2β, C-4, C-6α, and C-6β was straightforward, and ^2H nmr spectra of the products (Table 1) confirmed the essential integrity of the label. The presence of a small amount of additional deuterium in 4-d_1-**2***b*, presumably at C-6β, and in 6β-d_1-**2***b*, is apparent from both mass spectral and ^2H nmr data, but does not affect the conclusions discussed below. The ^2H nmr chemical shifts observed are consistent with those reported for analogous ^2H (20) and ^3H (21) labelled steroids. The 19-d_1 labelled steroids were prepared by two routes (see Experimental). The material obtained by route A (Scheme 1) was of slightly higher isotopic enrichment and was subsequently used for biotransformation. The 19,19,19-d_3 labelled testosterone was synthesized (Scheme 2) by a procedure similar to that of Baba *et al.* (22), and was obtained in high isotopic purity.

Synthesis of 1β,2β-d_2-testosterone was undertaken for comparative purposes. Reduction of androsta-1,4-diene-17β-ol-3-one (**27**) by deuterium gas gave a low yield of **2***a*. Although the sample was chemically pure and the predominant labelled species was dideuterated, ^2H nmr spectral analysis revealed the presence of deuterium in at least four different locations in **2***a*. The spectra were not sufficiently well resolved to allow quantification of the results, but a recent ^3H nmr study (21) of the product of reduction of **27** by tritium gas identified the presence of label at 1β (36.6%), 2β (2.3%), 1α,2α (12.3%), and 1β,2β (48.8%). Our results confirm that this reduction is indeed nonstereospecific. The substrates "specifically" labelled at C-1β which were originally used to demonstrate stereospecific removal of the C-1β hydrogen during estrogen biosynthesis were made by catalytic reduction of a $\Delta^{1(2)}$ bond (15), but more recent work with C-1α labelled material has confirmed the stereospecific loss of the C-1β hydrogen in this process (23).

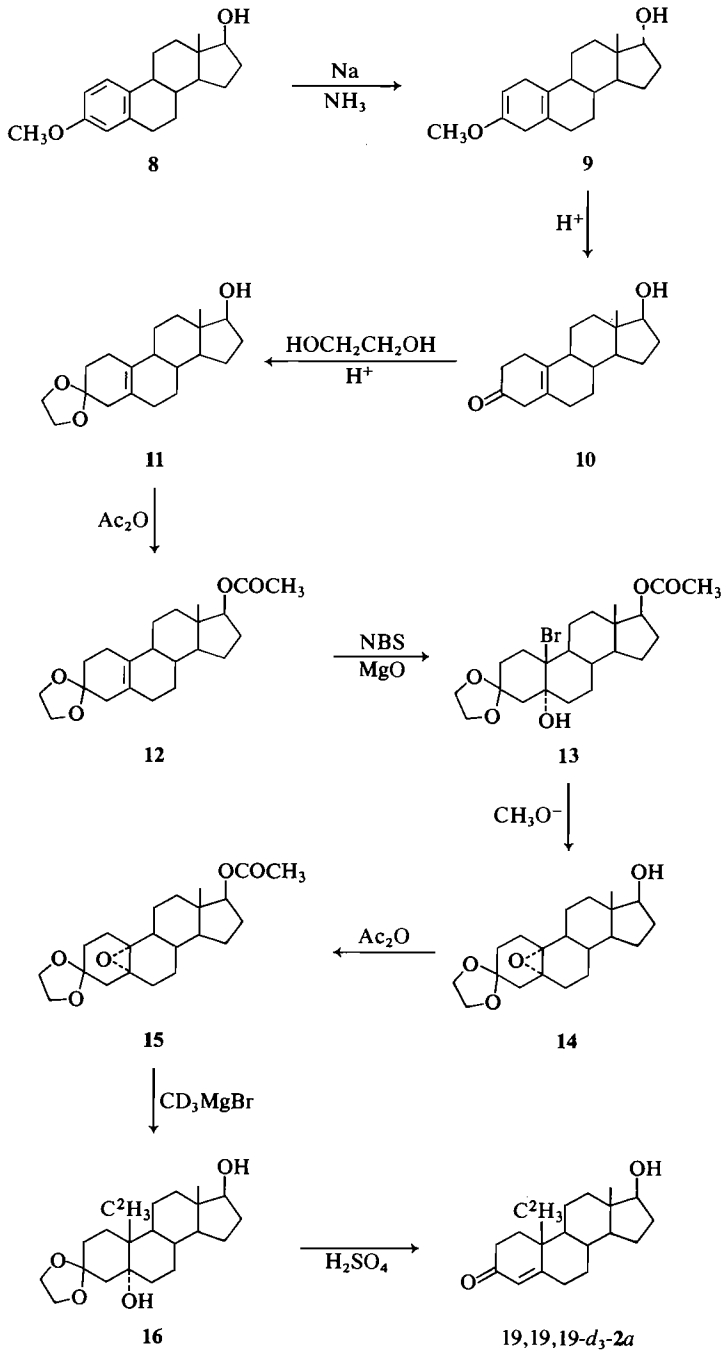
By assignment of different chemical shifts to axial and equatorial deuterium (20) and tritium (21), the presence of two deuteron signals in the ^2H nmr spectra of steroids labelled at C-16 (Table 1) confirms the presence of stereochemically distinct deuterons. If ring D were predominantly in the planar or the envelope conformations then both C-16 deuterons would be virtually equivalent and only one signal would be expected. If ring D were conformationally mobile, a single average resonance might be expected. However, if ring D were

TABLE 1. Isotope content and ^2H nmr chemical shifts for **2***a* and **2***b*

Compound	Isotope content (%)									δ(ppm)
	d_8	d_7	d_6	d_5	d_4	d_3	d_2	d_1	d_0	
2β-d_1-**2***a*						7	9	38	46	2.19
4-d_1-**2***b*						2	22	58	18	2.39(minor), 5.59
6α-d_1-**2***b*						1	4	87	8	2.27
6β-d_1-**2***b*						1	18	70	10	2.34
19-d_1-**2***a* (Route A)						3	6	69	22	1.20
19-d_1-**2***b* (Route A)						4	9	68	19	1.19
19-d_1-**2***a* (Route B)						7	12	62	19	1.19
19-d_1-**2***b* (Route B)						9	10	60	21	1.19
1β,2β-d_2-**2***a*				7	8	10	28	15	32	1.28, 1.66, 2.05, 2.23
16,16-d_2-**2***a*					1	6	63	6	24	
16,16-d_2-**2***b*					1	5	54	33	7	1.95, 2.32
19,19,19-d_3-**2***a*						>99				1.19
2,2,4,6,6,16,16-d_7-**2***b*	1	24	36	24	9	2	2	1	1	1.90(br), 2.25(br), 5.59



SCHEME 1. Preparation of 19-d_1-**2***a* and 19-d_1-**2***b*

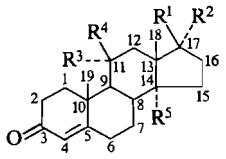


SCHEME 2. Preparation of 19,19,19-d_3-2*a*

predominantly in the half-chair conformation, both pseudo-axial and pseudo-equatorial deuterons would be present. This compares favourably with X-ray crystallographic data (24) where, in the crystalline state, ring D adopts a conformation intermediate between the half-chair and envelope forms.

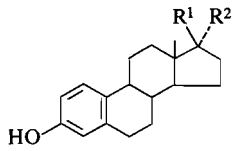
The results of the aromatization of deuterium labelled **2***a* and **2***b* are presented in Table 2. Both substrates are transformed by the same enzyme, at the same site (25, 26), and in both cases only a single product of aromatization (estradiol, **1***a*) is obtained. We have therefore used both 17β-alcohol and 17-keto substrates in the present study.

The values in Tables 1 and 2 indicate that



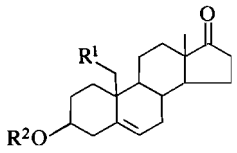
2

a R^1 = OH, R^2 = R^3 = R^4 = R^5 = H
b R^1 + R^2 = O, R^3 = R^4 = R^5 = H
c R^1 + R^2 = O, R^3 = OH, R^4 = R^5 = H
d R^1 + R^2 = O, R^3 = R^5 = H, R^4 = OH
e R^1 + R^2 = O, R^3 = R^4 = H, R^5 = OH



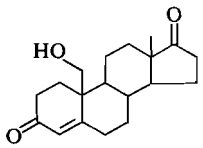
1

a R^1 = OH, R^2 = H
b R^1 + R^2 = O

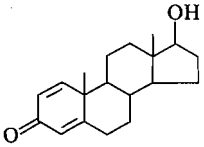


3

a R^1 = R^2 = H
b R^1 = OH, R^2 = H
c R^1 = H, R^2 = Ac
d R^1 = OTr, R^2 = Tr



21



27

aromatization occurs with no loss of label from C-4 or C-6β. There is an apparent loss of some label from the C-6α position, the reason for which is not clear; its stereochemistry is inconsistent with a loss by enolization, in which major loss from C-6β should occur (27). The degree of retention is high, however, and this, together with the retention at C-4 and C-6β, eliminates from consideration any mechanism of aromatization in which the substrate may bind to the enzyme as a $\Delta^{3,5}$ enol (13) with loss of the C-6 hydrogen to the environment. The alternative binding as a $\Delta^{2,4}$ enol is consistent with the specific loss of the axial C-2β hydrogen (17), and thus appears to be the preferred mode of attachment of substrate to the enzyme (7, 15).

Transformations of substrates labelled at C-16 were carried out for control purposes to enable their use as internal rate standards in the determination of isotope effects at C-19 (*vide infra*). Surprisingly, there was a small but consistent loss of label from C-16 during aromatization. Recovered starting material in these experiments showed little or no loss of deuterium. In accord with this, substrate **2***b* labelled at all enolizable positions (2, 2, 4, 6, 6, 16, 16) also showed a greater deuterium loss on aromatization than could be accounted for by the known loss of the C-2β deuterium atom, and aromatizations of **2***b* in 50% and 95% D_2O produced estradiol with deuterium label appreciably greater than that of recovered substrate. The location of exchange of label was not determined in the latter experiments, but by inference is predominantly C-16, since previous work and the results discussed above rule out significant exchange at C-2, -4, or -6. Engel and co-workers (6) have reported appreciable loss of deuterium from C-17α of testosterone during conversion to estradiol by placental microsomes, and have demonstrated that reversible oxidation–reduction at C-17 may occur at any of the intermediate stages of the pathway shown in Fig. 1. We therefore attribute loss of label from C-16 to enolizable exchange, the loss from labelled **2***a* occurring at the carbonyl level at C-16.

In order to determine isotope effects at C-19, equimolar proportions of testosterone labelled at C-16 and at C-19 were incubated with placental microsomes, and the resulting estradiol assayed for deuterium. The deuterium content of product was then used to calculate the deuterium isotope effect for the oxidative removal of C-19 by the procedure of McMullen and Thode (28). The calculations were complicated by the loss of deuterium from C-16 discussed above, and so in order to establish the validity of the method, testosterone labelled at C-16 was co-incubated with unlabelled material, and deuterium content of product, corrected for the expected loss from C-16, was used to calculate the amounts of estradiol produced from labelled and unlabelled substrate (predicted ratio 1.0). The values obtained (1.02 ± 0.05 and 1.03 ± 0.05) demonstrate that correction of the observed deuterium content of product for the expected loss from C-16 could be confidently carried out in experiments with mixed C-16 and C-19 labelled substrates to give accurate values for the deuterium isotope effect at C-19. The values so obtained are listed in Table 2. These are intermolecular "mixed-labelling" effects which circumvent the problem of differential activities of individual microsome preparations. As the enzyme preparations used in studies on aromatization are not purified to homogeneity, individual batches can vary considerably in activity. This must be taken into account in studies which use only the absolute reaction velocities in order to study the kinetics of this system. In addition, during the preparation of labelled substrate, trace amounts of contaminants may be present which may act as inhibitors of the enzyme. In the mixed-labelling studies, such an inhibitor, if present, would be expected to act equally on the

TABLE 2. Transformations of labelled **2***a* and **2***b* by placental microsomes

Substrate	Recovered substrate (% label)[a]									Estra-1,3,5(10)-triene-3,17β-diol (**1***a*) (% label)								k_H/k_D[b]
	d_8	d_7	d_6	d_5	d_4	d_3	d_2	d_1	d_0	d_7	d_6	d_5	d_4	d_3	d_2	d_1	d_0	
4-d_1-**2***b*						9	13	58	19					8	9	59	23	
															18	58	24	
6α-d_1-**2***b*						2	3	88	8						8	73	19	
6β-d_1-**2***b*						3	17	72	9						14	69	16	
						3	16	74	8						18	64	19	
															13	68	19	
16,16-d_2-**2***a*					3	7	60	7	23						54	23	23	
															51	22	27	
															51	20	29	
16,16-d_2-**2***b*						3	52	32	11						46	36	18	
					1	3	50	37	10						48	36	16	
2,2,4,6,6,16,16-d_7-**2***b*	2	21	35	26	11	3	1	1	1	5	15	26	20	13	7	7	8	
	3	21	32	21	9	5	2	3	3[d]	4	17	26	16	17	7	7	6	
19,19,19-d_3-**2***a* + 16,16-d_2-**2***a*					2	36	37	8	17						47	14	38	3.0 ±0.2
					5	35	35	8	16						49	16	35	3.9 ±0.2
															49	10	42	2.8 ±0.2
19-d_1-**2***a*[e] + 16,16-d_2-**2***a*														7	39	10	44	2.3 ±0.1
														6	39	10	45	2.3 ±0.1
2*a* + 16,16-d_2-**2***a*[c]														11	29	10	51	1.02±0.05
														10	31	8	52	1.03±0.05
2*b* in 95% D_2O						5	5	14	76						12	30	58	
							6	10	84						10	22	68	
2*b* in 50% D_2O							2	5	93						9	20	71	

[a] Values are accurate to ±2%.
[b] Value calculated for **2***a* → **1***a* (28).
[c] For control purposes.
[d] Isolated as androst-4-ene-17β-ol-3-one.
[e] Route A material (see Table 1).

conversion of both labelled and unlabelled substrate.

Previous studies on intermolecular isotope effects of cytochrome (cyt) P-450-dependent hydroxylations have generally shown deuterium isotope effects of 2 or less. The benzylic hydroxylation of phenylethane by liver microsomes was determined to proceed with a deuterium isotope effect of 1.8 (29). The 7β-hydroxylation of estr-4-ene-3,17-dione by *Botryodiplodia malorum* was found to have an isotope effect (k_H/k_D) of 1.4 (30), and the 21-hydroxylation of pregn-4-ene-3,20-dione was determined to proceed with an intramolecular isotope effect of 1.3 (31), whereas no isotope effect was observed in the 3-hydroxylation of estra-1,3,5(10)-triene-2,17β-diol when examined by mixed-labelling studies (32).

All of the above hydroxylations are presumed to involve cyt P-450. Some hydroxylations, however, are not mediated by carbon monoxide-sensitive cyt P-450; this appears to be the case for 19-hydroxylation during aromatization (33). Another such hydroxylation, the 7α-hydroxylation of taurodeoxycholic acid was determined to proceed with a tritium isotope effect of 3.8 (34), equivalent to a deuterium isotope effect of 2.5 (based on $(k_H/k_D)^{1.44} = k_H/k_T$).

The 16α-hydroxylation of pregn-5-ene-3β-ol-20-one and the 24-hydroxylation of 5β-cholestane-3α,7α,21α-triol were determined to proceed with a deuterium isotope effect of 3 to 4. These two processes appear to be insensitive to inhibition by carbon monoxide (35). Conney *et al.* (36) suggested a correlation between the degree of sensitivity toward carbon monoxide and the nature of the rate-limiting step in hydroxylation.

The rate-limiting step in these reactions has been identified in some cases as decomposition of the cyt P-450 – O_2-substrate complex to products (37). It has also been suggested (31, 37) that hydroxylations occurring at unactivated positions proceed by an oxene-type mechanism involving direct insertion of activated oxygen into a C—H bond. The analogous chemical insertion of carbene into a C—H or C—D bond proceeds with an observed isotope effect of 1.8 (38). This mechanism is consistent with the low observed isotope effects, and the retention of configuration observed in such processes (39, 40).

The results from the incubation of a 1:1 mixture of the 19-d_1 and 16,16-d_2 labelled steroids verified the presence of an isotope effect ($k_H/k_D = 2.3$) operating at the level of 19-hydroxylation. The complexity of this enzymic process makes unambiguous interpretation of the apparent isotope effect difficult. As expected, the effect observed for the 19-d_1 steroid was less than that for the 19-d_3 substrate. This may be the result of an isotope effect operating for a single hydroxylation at C-19 or the result of cumulative primary and secondary isotope effects incurred during both initial hydroxylation and subsequent hydroxylation (alcohol to aldehyde) at C-19. Equilibrium effects could result in the reduction of the intrinsic isotope effect of an early step in the reaction sequence, but in the absence of evidence for reversibility of any of the steps of the process, this would seem unlikely.

It is apparent from the observed isotope effects that a carbon–hydrogen bond cleavage during oxidation at C-19 is at least a partially rate-determining process in aromatization. This is consistent with previous proposals (6, 19) concerning the rate-determining step of aromatization.

It was our original intention to compare the isotope effect of aromatization with that observed for hydroxylation of **2***b* at C-19 by the fungus *Pellicularia filamentosa* (*Corticium microsclerotia*) NRRL 2727, a micro-organism reported to produce the desired alcohol **21** in high yield from **2***b* (41). However, in our hands this fungus yielded only products of C-11α, -11β, and -14 hydroxylation. Hasegawa and Takahashi (42) previously identified the 11α- and 11β-hydroxylated products from the incubation of pregn-4-ene-17α,21-diol-3,20-dione with another fungus of the same family, *Corticium sasakii*, and Takahashi (43) only found the 11β-hydroxylated product upon the incubation of the same substrate with *P. filamentosa*. It therefore appears that earlier claims of C-19 hydroxylation of C_{19} steroids by *P. filamentosa* are unsubstantiated, and that the microbial 19-hydroxylation of **2***a* or **2***b* is unknown (44, 45).

Experimental

Apparatus, Materials, and Methods

Melting points were determined on a Kofler heating stage and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer 237B spectrometer. Proton nmr spectra were obtained with a Varian A-60 or Bruker WP-60 at 60 MHz using $CDCl_3$ or DMSO-d_6 as solvent and TMS as internal standard, ^{13}C nmr spectra with a Bruker WP-60 at 15.18 MHz using $CDCl_3$ as solvent and TMS as internal standard, and ^2H nmr spectra at McMaster University, Hamilton, Ont., with a Bruker WH-90 at 13.8 MHz using CS_2 as solvent and $CDCl_3$ or acetone-d_6 as external standard. Mass spectra were obtained with an AEI MS30 spectrometer. Isotopic composition was determined by repetitive scanning of the relevant ions and application of the appropriate corrections for natural abundances; values are accurate to ±2%. Column chromatography was performed on silica gel (60–200 mesh), thin layer chromatography on Merck silica gel 60 F-254 (0.25 mm), and preparative layer chromatography on Merck silica gel F-254 (2.0 mm). Microanalyses were performed by the Guelph Chemical Laboratories, Guelph, Ontario. Human placental microsomes were obtained and

stored as previously described (46). *Pellicularia filamentosa* NRRL 2727 was maintained on slopes composed of malt agar (1.5%), tomato paste (2.0%), and oatmeal (2.0%).

Preparation of Substrates

Androst-4-ene-3,17-dione (**2***b*), androst-5-ene-3β-ol-17-one (**3***a*), androst-4-ene-17β-ol-3-one (testosterone) (**2***a*), estra-1,3,5(10)-triene-3-ol-17-one (estrone) (**1***b*), estra-1,3,5(10)-triene-3,17β-diol (estradiol) (**1***a*), androst-4-ene-3,17-dione-11β-ol (**2***d*), androst-4-ene-17β,19-diol-3-one, 17-benzoate (**4**), and androst-5-ene-3,19-diol-17-one (**3***b*) were commercial samples.

Authentic samples of androst-4-ene-3,17-dione-11α-ol, (**2***c*) and androst-4-ene-3,17-dione-14α-ol (**2***e*) were available in our laboratories from incubations of **2***b* with *Rhizopus arrhizus* ATCC 11145 (47) and *Mucor griseocyanus* ATCC 1207 (48), respectively.

The following substrates were prepared by published procedures (47) and gave satisfactory spectral and analytical data: androst-4-ene-3,17-dione, 4-*d*, (4-*d*-**2***b*), androst-4-ene-3,17-dione, 6α-d_1, (6α-d_1-**2***b*), androst-4-ene-3,17-dione, 2,2,4,6,6,16,16-d_7, and androst-4-ene-3,17-dione, 16,16-d_2.

Androst-4-ene-17β-ol-3-one, 19-d_1*, (19-*d_1*-2a) and Androst-4-ene-3, 17-Dione, 19-*d_1 *(19-*d_1*-2b) (Scheme 1)*

Route A: From Androst-4-ene-17β,19-diol, 17-Benzoate (4)

Androst-4-ene-17β,19-diol-3-one, 17-Benzoate, 19-Tosylate (5)

Toluene *p*-sulphonyl chloride (2.9 g) was added to a solution of androst-4-ene-17β,19-diol-3-one, 17-benzoate (**4**) (2 g) (Maybridge Chemical Co., U.K.) in pyridine (5 mL). The mixture was allowed to stand at room temperature overnight and then poured into ice water. The resultant precipitate was collected by filtration and treated briefly with refluxing ether (40 mL). Filtration of the mixture afforded the product (2.41 g, 88%), mp 142–147°C, which was used directly in the preparation of **6**.

5β,19-Cycloandrostan-17β-ol-3-one, 17-Benzoate (6)

The tosylate **5** (1.4 g) was dissolved in aqueous acetic acid (350 mL, 50%) and zinc dust (17 g, Shawinigan) added to the solution. The resulting suspension was stirred and refluxed for 90 min, then filtered. The zinc was washed with methanol and the total filtrate evaporated. The residue was crystallized from acetone–ether, and recrystallized from methanol to give the product (0.4 g, 41%), mp 184–186°C; ^{1}H nmr included signals at δ 0.50 (2H, s, C-19H), 0.97 (3H, s, C-18H), 2.55 (2H, br s, C-4H), 4.9 (1H, t, C-17H), and 7.3–8.3 (5H, m, aromatic H) ppm; ms *m/e* (%): 392 (6), 287 (3), 270 (13), 255 (6), 105 (100). *Anal.* calcd. for $C_{26}H_{32}O_3$: C 79.59, H 8.16%; found: C 79.26, H 8.42%.

Androst-4-ene-17β-ol-3-one, 17-Benzoate, 19-d_1 *(7)*

Conversion of **6** to **7** was performed by a modification of the procedure of Rakhit and Gut (49). To a solution of **6** (540 mg) in dried dioxan (10 mL) was added D_2O (80 mg) and DCl (1 mL, 38% in D_2O). The mixture was refluxed for 24 h, and the solvents then removed under vacuum. The crude residue was purified by column chromatography (benzene/ether gradient elution) affording **7** (460 mg, 85%), mp 187–190°C (lit. (50) mp 190°C); ^{1}H nmr included signals at δ 0.99 (3H, s, C-18H), 1.21 (2H, s, C-19H), 4.9 (1H, t, C-17H), 5.8 (1H, s, C-4H), and 7.3–8.3 (5H, m, aromatic H) ppm.

Androst-4-ene-17β-ol-3-one, 19-d_1 *(19-*d_1*-2a)*

A solution of **7** (0.17 g) in methanolic sodium hydroxide (5 mL, 5%) was refluxed for 1 h. The mixture was cooled, neutralized with acetic acid, and worked up in the usual way to give 19-d_1-**2***a* (0.12 g, 96%), mp 152–154°C (lit. (50) mp 154°C); ^{1}H nmr included signals at δ 0.81 (3H, s, C-18H), 1.22 (2H, s, C-19H), 3.75 (1H, t, C-17H), and 5.9 (1H, s, C-4H) ppm; ms *m/e* (%): 289 (28), 273 (5), 247 (28), 125 (100). Isotopic content: see Table 1.

Androst-4-ene-3,17-dione, 19-d_1 *(19-*d_1*-2b)*

Jones' oxidation of 19-d_1-**2***a* (0.16 g) afforded 19-d_1-**2***b* (0.12 g, 76%); ^{1}H nmr included signals at δ 0.92 (3H, s, C-18H), 1.22 (2H, s, C-19H), and 5.8 (1H, s, C-4H) ppm; ^{2}H nmr: see Table 1; ms *m/e* (%): 287 (100), 272 (14), 269 (13), 259 (20), 245 (96). Isotopic content: see Table 1.

Route B. From Androst-4-ene-3,17-dione, 19-ol (27) (Scheme 1)

An analogous series of reactions and conditions to those of the route described above for the conversion of **4** into **7** (Scheme 1) was used to convert the 19-ol **27** into androst-4-ene-3,17-dione, 19-d_1 (19-d_1-**2***b*). Alternatively, 19-d_1-**2***b* was available by the following procedure.

5β,19-Cycloandrostan-17β-ol-3-one

The benzoate **6** (0.5 g) was dissolved in methanolic sodium hydroxide (15 mL, 5%) and the solution refluxed for 1 h. Conventional work-up gave the product (81%), which was used in the next step without further purification.

5β,19-Cycloandrostan-3,17-dione

Jones' oxidation of the aforegoing alcohol (0.3 g) afforded the product (0.24 g, 81%), identical in all respects with an authentic sample (49).

Androst-4-ene-3,17-dione-19-d_1*, (19-*d_1*-2b)*

The cyclopropyl ketone was treated with DCl in D_2O by the procedure described above for the conversion of **6** into **7**. The product was then refluxed briefly in 5% methanolic sodium hydroxide to ensure removal of any enolizable deuterium. Conventional work-up afforded 19-d_1-**2***b* identical in physical and spectral properties with an authentic sample of unlabelled material. Isotope content is presented in Table 1.

Androst-4-ene-17β-ol-3-one, 19-d_1*(19-*d_1*-2a)*

The conversion of 19-d_1-**2***b* into 19-d_1-**2***a* was performed by a published procedure (51). The product was purified by preparative layer chromatography and obtained in 60% yield. Isotope content is presented in Table 1.

Androst-4-ene-17β-ol-3-one, 19,19,19-d_3 *(19,19,19-*d_3*-2a) (Scheme 2)*

The procedure followed was similar to that of Baba *et al.* (22).

Estra-2,5(10)-diene-3,17β-diol, 3-Methyl Ether (9) (52)

Isopropanol (30 mL) and a solution of estra-1,3,5(10)-triene-3,17β-diol, 3-methyl ether (5.0 g) in THF (30 mL) were added to redistilled ammonia (80 mL). To this mixture, sodium metal (total 4.5 g) was added in portions. After the addition was complete and all of the sodium consumed (ca. 1 h), methanol (20 mL) was added. The ammonia was then allowed to evaporate and the THF subsequently removed by distillation under reduced pressure. The precipitated product was collected, washed with water, and dried to give a product (5.0 g, 99%), mp 110–112°C (lit. (53) mp 118–119.5°C) of sufficient purity to be useable directly in the next step; ^{1}H nmr included signals at δ 0.77 (3H, s, C-18H), 2.69 (4H, m, C-1 and C-4H), 3.65 (1H, t, C-17H), 3.66 (3H, s, OCH_3), and 4.7 (1H, m, C-2H) ppm.

Estr-5(10)-ene-17β-ol-3-one (10)

The enol ether **9** (5.0 g) was dissolved in a mixture of THF (25 mL), water (12 mL), and sulphuric acid (96%, 1.5 mL), and the resulting solution stirred at room temperature for 45 min. Ammonium sulphate was then added until the solution became saturated, and the product was extracted into dichloromethane. The extract was washed (saturated $NaHCO_3$/$(NH_4)_2SO_4$), dried, and evaporated to afford the product **10** (4.10 g). Crystallization from ethyl acetate/hexane gave 3.84 g, mp 178–181°C (lit. (54) mp 181–182°C); ^{1}H nmr included signals at δ 0.79 (3H, s,

C-18H), 2.47 (4H, m, C-1 and C-2 H's), 2.75 (2H, m, C-4H), and 3.65 (1H, t, C-17H) ppm.

5α-Estran-17β-ol-3-one-5α,10α-oxide, 17-Acetate, 3-Cyclic Ethylene Ketal (15)

Conversion of the ketone **10** to the title compound **15** as outlined in Scheme 2 was carried out precisely as described (22).

5α-Androstan-5,17β-diol-3-one, 3-Cyclic Ethylene Ketal (16)

The reagent CD_3MgBr (1 *M* in THF) was prepared by condensing CD_3Br (2.0 g, 99.5 at.% ^2H), by means of a cold-finger condenser, into an ice-cooled reaction flask containing dry THF (20 mL) and magnesium turnings (0.5 g). To this reagent was added dropwise a solution of the epoxide **15** (0.5 g) in dry THF (5 mL). The solution was refluxed for 2 h and then cooled, whereupon 10 mL 1.5 *M* NH_4Cl solution was added dropwise. The resultant mixture was diluted with water, extracted with methylene chloride, and washed successively with 3 *N* HCl, 5% $NaHCO_3$, and water. Drying over Na_2SO_4 followed by evaporation yielded 490 mg crude product. Purification by column chromatography (benzene/ether gradient elution) yielded 260 mg product (55%), identified by comparison with an authentic sample of unlabelled material prepared by reaction of (5,6α)-epoxytestosterone ethylene ketal with lithium aluminum hydride.

Androst-4-ene-17β-ol-3-one, 19,19,19-d_3 (19,19,19-d_3-2a)

The ketal **16** (0.26 g) was dissolved in a mixture of methanol (7 mL) and sulphuric acid (0.7 mL, 1 *M*), and the solution refluxed for 30 min. The solution was then cooled and diluted with ether and water and the organic layer removed. The aqueous layer was re-extracted with ether and the combined organic extract washed (5% $NaHCO_3$, water), dried, and evaporated to yield 180 mg of crude product. Column chromatography followed by preparative layer chromatography afforded 75 mg of pure product, identified by spectral and mp comparison with an authentic sample of unlabelled material. Isotope content is presented in Table 1.

Androst-4-ene-17β-ol-3-one, 16,16-d_2 (16,16-d_2-2a) (Scheme 3)
Androst-5-ene-17β-ol-3-one, 3-Cyclic Ethylene Ketal (17)

Androst-4-ene-17β-ol-3-one (**2***a*, 1.5 g) was dissolved in a mixture of dry benzene (80 mL), ethylene glycol (1.5 g), and *p*-toluene sulphonic acid (100 mg), and the solution refluxed for 24 h under a Dean–Stark trap. Conventional work-up gave 1.43 g (82%) product.

Androst-5-ene-3,17-dione, 3-Cyclic Ethylene Ketal (18)

The above ketal (1.4 g) was dissolved in pyridine (15 mL) to which Sarett reagent was added (15 mL, 0.1 g CrO_3 per mL). The mixture was stirred for 20 h and then diluted with dichloromethane. The resulting mixture was washed (5% HCl, water), dried, and evaporated to yield crude **18** (900 mg), which was purified by column chromatography and then crystallized from ethyl acetate/hexane to afford the title compound (0.6 g, 43%), mp 175–180°C (lit. (55) mp 197–198°C); ^1H nmr included signals at δ 0.90 (3H, s, C-18H), 1.08 (3H, s, C-19H), 3.98 (4H, s, $-OCH_2$), and 5.4 (1H, m, C-6H) ppm.

Androst-5-ene-3,17-dione, 3-Cyclic Ethylene Ketal, 16,16-d_2 (19)

A solution of **18** (0.5 g) in dry benzene (10 mL) was added to a solution of tetra *n*-butyl ammonium bromide (0.5 g) in basic D_2O (5% NaOD, 10 mL), and the resulting mixture stirred and refluxed for 20 h. Normal work-up afforded **19** (0.45 g), identified by comparison with an authentic sample of **18**.

Androst-5-ene-17β-ol-3-one, Cyclic Ethylene Ketal, 16,16-d_2 (20)

A solution of the ketal **19** (0.45 g) in benzene/ether (1:1, 25 mL) was added to a suspension of lithium aluminum hydride (0.45 g) in ether (30 mL) and the mixture refluxed for 3.5 h. Conventional work-up afforded **20** (0.4 g, 88%), identified by comparison with an authentic sample of **17**.

Androst-4-ene-17β-ol-3-one, 16,16-d_2 (16,16-d_2-2a)

The above ketal **20** (0.4 g) was dissolved in a mixture of methanol (6 mL) and dilute sulphuric acid (6%, 1 mL) and the solution refluxed for 30 min, diluted with water, and the product extracted into ether. The extract was dried and evaporated, and the residue crystallized from acetone/hexane to give 16,16-d_2-**2***a* (0.3 g, 86%), mp 149–151°C (lit. (50) mp 154°C). Isotope content is listed in Table 1.

Androst-4-ene-17β-ol-3-one, 2β-d, (2β-d_1-2a)

A solution of androst-4-ene-17β-ol-3-one (**2***a*, 0.25 g) in dry benzene (5 mL) was added to a solution of tetra *n*-butyl ammonium bromide (0.25 g) in basic D_2O (5% NaOD, 5 mL), and the mixture stirred at room temperature for 45 h. Conventional work-up afforded the title compound (0.18 g). Isotope content is listed in Table 1.

Androst-4-ene-17β-ol-3-one, 1β,2β-d_2 (1β,2β-d_2-2a)

A mixture of androsta-1,4-diene-17β-ol-3-one (**27**) (0.2 g) and 10% Pd/C (0.2 g) in ethyl acetate (10 mL) was stirred under deuterium gas at atmospheric pressure for 6 min. Conventional work-up afforded 0.2 g crude product, which was subjected to preparative layer chromatography (4% methanol in ether as solvent) to give the desired product (40 mg, 20%), identified by comparison with an authentic sample of unlabelled material. Isotope content is listed in Table 1. A reaction time of 25 min resulted in the production of 5β-androstan-17β-ol-3-one as major product.

Androst-4-ene-3,17-dione-19-ol (21)

Method A. From Androst-5-ene-3β,19-diol-17-one (3b) by Oppenauer Oxidation (56)

A solution of androst-5-ene-3β,19-diol-17-one (**3***b*, 0.5 g) (Bader Chemical Co.) and aluminum isopropoxide (0.5 g) in toluene (40 mL) and cyclohexanone (10 mL) was refluxed for 10 min. Work-up and chromatography afforded a 6–15% yield of **21** in several experiments; ^1H nmr included signals at δ 0.92 (3H, s, C-18H), 3.90 (2H, ABq, C-19H), and 5.7 (1H, s, C-4H) ppm.

Method B. From 3b via bis Trityl Ether (3d) (57)

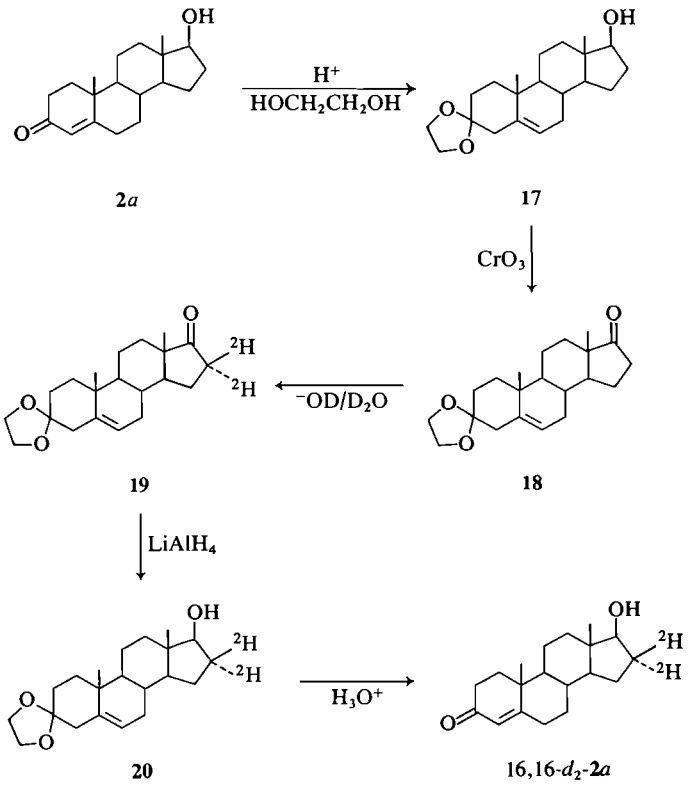
Conversion of **3***c* to the ether **3***d* was performed as described (58). The product was obtained in 92% yield. Triphenylmethylfluoroborate (1.3 g) (59) was added to a solution of **3***d* (2.93 g) in dichloromethane. The mixture was stirred for 12 h, then washed ($NaHCO_3$), dried, and evaporated to yield 1.6 g crude product. This was dissolved in a mixture of ethanol (100 mL), benzene (25 mL), and dilute sulphuric acid (2 *N*, 10 mL) and the mixture refluxed for 4 h. The product was isolated by extraction and purified by column chromatography to give **21** in 35% yield, mp 163–167°C (lit. (60) mp 164–166°C).

Method C. From Androst-5-ene-3β-ol-17-one, 3-Acetate (3c)

The conversion of **3***c* to **21** was performed as described (60) in an overall yield of 18%. The last step described in ref. 60 proceeded in acceptable yield in our hands only with the use of MCB zinc dust as a reagent.

Incubation with P. filamentosa *NRRL 2727*

The fungus was grown in 1-L Erlenmeyer flasks as described (41). After 3 to 4 days of fungal growth at room temperature, androst-4-ene-3,17-dione (**2***b*) in ethanol solution (80 mg in 0.5 mL ethanol per flask) was added, and growth allowed to continue for a further 8–12 h. The medium and mycelia were then separated by filtration, and each extracted with dichloromethane. The combined extract was dried and evaporated, and



SCHEME 3. Preparation of 16,16-d_2-2*a*

the residue subjected to column chromatography (benzene/ether gradient elution) to give recovered starting material (typically 30–40%), androst-4-ene-3,17-dione-11α-ol (**2***c*, typically 35%), androst-4-ene-3,17-dione-11β-ol (**2***d*, typically 15%), and androst-4-ene-3,17-dione-14α-ol (**2***e*, typically 10%), all of which were identical in all respects with corresponding samples of authentic material.

Incubations with Placental Microsomes

Incubations of androst-4-ene-3,17-dione (**2***b*) and androst-4-ene-17β-ol-3-one (**2***a*) with a microsomal suspension from human placentae were performed as previously described (46). Isolated yields of estra-1,3,5(10)-triene-3,17β-diol were typically 5–10%. Isotope content of substrates and products are presented in Table 2.

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