Conversion of 19-oxo[2 β -³H]androgens into oestrogens by human placental aromatase

An unexpected stereochemical outcome

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Aromatase is a cytochrome *P*-450 enzyme that catalyzes the conversion of androgens into oestrogens via sequential oxidations at the 19-methyl group. Despite intensive investigation, the mechanism of the third step, conversion of the 19-aldehydes into oestrogens, has remained unsolved. We have previously found that a pre-enolized 19-al derivative undergoes smooth aromatization in non-enzymic model studies, but the role of enolization by the enzyme in transformations of 19-oxoandrogens has not been previously investigated. The compounds 19-oxo[2 β -³H]testosterone and 19-oxo[2 β -³H]androstenedione have now been synthesized. Exposure of either of these compounds to microsomal aromatase, in the absence of NADPH, for an extended period led to no significant ³H loss or epimerization at C-2, leaving open the importance of an active-site base. However, in the presence of NADPH there was an unexpected substrate-dependent difference in the stereoselectivity of H loss at C-2 in the enzyme-induced aromatization of 19-oxo[2 β -³H]testosterone versus 19-oxo[2 β -³H]androstenedione. The aromatization results for 17 β -ol derivative 19-oxo[2 β -³H]testosterone correspond to about 1.2:1 2 β -H/2 α -H loss from unlabelled 19-oxotestosterone. In contrast, aromatization results for 19-oxo[2 β -³H]androstenedione correspond to at least 11:1 2 β -H/2 α -H loss from unlabelled 19-oxoandrostenedione. This substrate-dependent stereoselectivity implies a direct role for an enzyme active-site base in 2-H removal. Furthermore, these results argue against the proposal that 2 β -hydroxylation is the obligatory third step in aromatase action.

INTRODUCTION

There has been a longstanding interest in the mechanism of placental aromatase since the discovery of this enzymic activity by Meyer in the 1950s (Meyer, 1955*a,b*). This cytochrome *P*-450 enzyme system catalyses the conversion of steroidal androgens [testosterone (**1a**, Scheme 1); androstenedione (**1b**)] to steroidal oestrogens [oestradiol (**4a**); oestrone (**4b**)]. Interest in the aromatase mechanism has been generated from two main sources. Because of the well-proven role of oestrogens in promoting some forms of neoplastic growth, a vigorous effort to design aromatase inhibitors rationally has been undertaken (Brodie, 1985). This effort has resulted in several promising compounds (Johnston & Metcalf, 1984), although for most of these their detailed mode of action remains unsolved. Furthermore, the chemical uniqueness and complexity of the aromatase reaction has provided a strong enticement for mechanistic enzymologists.

Several mechanistic features of the aromatase-catalysed reaction have been established. Three oxidative steps are involved, each apparently requiring one molar equivalent of NADPH and molecular oxygen (Siiteri & Thompson, 1975) (see Scheme 1). The first two steps are stereospecific hydroxylations at the C-19 methyl group of the androgen (Arigoni *et al.*, 1975; Osawa *et al.*, 1975; Caspi *et al.*, 1986). The 19-ol (**2**) is initially formed and then converted into the 19-al (**3**), presumably after stereospecific dehydration of the corresponding *gem*-diol (Beusen *et al.*, 1987). The 19-al (**3**) is oxidatively converted into oestrogen (**4**) and

formic acid. One of the original C-19 methyl H atoms (Caspi *et al.*, 1986) and the first and third equivalents of O₂ consumed are recovered in the formic acid (Akhtar *et al.*, 1982). On the basis of the work of Fishman, Brodie and co-workers the 1 β and 2 β -H atoms have been considered to be stereospecifically lost to the aqueous medium (Brodie *et al.*, 1969; Fishman & Guzik, 1969; Fishman *et al.*, 1969). The notion that one cytochrome *P*-450 enzyme carries out all three oxidative steps, and that 19-ol (**2**) and 19-al (**3**) are true intermediates, has been strongly substantiated by the recent progress in aromatase purification (Kellis & Vickery, 1987*a*). Furthermore, androstenedione (**1b**), testosterone (**1a**) and 16 α -hydroxytestosterone are all direct substrates for aromatase, with similar catalytic centre activities (*k*_{cat} values), but widely different *K*_m values (Kellis & Vickery, 1987*a*). The most definitive work in this regard was performed by Simpson and co-workers, who have recently cloned and expressed the human placental aromatase cDNA in COS cells (Corbin *et al.*, 1988). They showed that one protein catalyses all three oxidative steps. Furthermore, they find no evidence for more than one human aromatase gene (Means *et al.*, 1989).

One of the major unanswered questions about aromatase concerns the nature of the third oxidative step. Proposals suggesting 4,5-epoxidation (Morand *et al.*, 1975; Mastalerz & Morand, 1982), Baeyer–Villiger-type oxygen insertion (Akhtar *et al.*, 1982) and 2 β -hydroxylation (Hahn & Fishman, 1984; Caspi *et al.*, 1984) have all been shown to be unlikely, although the results obtained by Caspi *et al.* (1984) have recently been

Abbreviations used: PPTS, pyridinium *p*-toluenesulphonate; h.r.m.s., high-resolution mass spectrometry; e.i.m.s., electron-impact mass spectrometry; THP, tetrahydropyran; HOMO2DJ, two-dimensional homonuclear ¹H n.m.r. with *J*-resolved coupling; n.O.e., nuclear Overhauser enhancement; PCC, pyridinium chlorochromate; d_x, fraction of molecules containing *x* ³H atoms.

Scheme 1.

a: R = OH, R' = H; b: R, R' = O; i = NADPH, O₂.

challenged (Fishman & Hahn, 1987). Recently, Watanabe & Ishimura (1989) have proposed 10 β -hydroxyoestr-4-ene-3,17-dione formation, in spite of earlier work which showed that this compound is not processed by aromatase (Townsend *et al.*, 1966; Covey *et al.*, 1984). Of the remaining leading candidates, one was originally proposed by Akhtar *et al.* (1976) and restated in various forms (Akhtar *et al.*, 1981; Stevenson *et al.*, 1985), most recently as a radical mechanism (Stevenson *et al.*, 1988). The central feature of the Akhtar mechanism is nucleophilic attack of the haem ferric peroxide on the 19-aldehyde group to afford the 19,19-hydroxyperoxide intermediate (see Scheme 2). Decomposition of this intermediate either by proton transfer (as shown), hydride shift or radical pathways could yield oestrogen and formic acid, with or without enzymic assistance. An attractive aspect of the Akhtar mechanism is that it is consistent with the oxygen-incorporation pattern. Nevertheless, classical cytochrome *P*-450 reactions are generally accepted to involve the iron oxo (ferryl) species as the reactive oxidant (Ortiz de Montellano, 1986). It can be argued in the case of aromatase that the electrophilic aldehyde would be intercepted by the reactive ferric peroxide before the latter had a chance to fragment to the iron oxo species. Akhtar and co-workers have discussed the potential generality of this theory to rationalize the mechanisms of several cytochrome *P*-450 desmolases (Stevenson *et al.*, 1988).

Previously, we synthesized steroidal 4-ene-3-one analogues of the Akhtar peroxide intermediate (Cole & Robinson, 1986). No oestrogen products could be detected under a variety of different non-enzymic conditions. In contrast, reaction of peroxides with a 2,4-dienol-19-oxo compound (**6**) led to the formation of the corresponding oestrogen derivative in high yield (Cole & Robinson, 1988). Presumably this non-enzymic aromatization results from more facile 1 β -H removal (and carbon-carbon bond cleavage) in that an aromatic product is directly produced (Cole & Robinson, 1988).

One of the obvious questions provoked by these model studies

concerns the timing and the role of the enzyme in 2 β -H removal. It is quite feasible that the enzyme could by-pass the need for prior enolization (as was required in the model studies) by judicious placement of suitable amino acid side chains which might facilitate 1 β -H removal. Yet, if a peroxide mechanism is involved in the aromatase reaction, the above studies suggest that the enzymic reaction might well be enhanced by prior enolization. One approach to the problem is to examine the enzyme-induced exchangeability of the 2 β -H with the aqueous medium in the absence of turnover. An exchange rate that is greater than, or equal to, the normal substrate turnover rate would argue in favour of the importance of an active-site base. In contrast, the implications of the absence of such rapid exchange are less clear (see below). Although NADPH is required for 2 β -³H release from labelled androstenedione in microsomes (microsomal fractions) from placenta, no work has been reported on the correspondingly labelled 19-aldehyde substrate. One could imagine that the oxidation state of C-19 might be critical in positioning the substrate near the active-site base. We report here efforts to examine the lability of 2 β -³H in 19-oxoandrogens. An unexpected outcome of the present study was the discovery of an unusual substrate-dependent stereospecificity for 2 β -H removal.

MATERIALS AND METHODS

General

I.r. spectra were recorded on a Perkin-Elmer 710B instrument. U.v. spectra were obtained on a Perkin-Elmer Lambda 3B machine. Proton-n.m.r. spectra were acquired on a Varian XL-400 or a Bruker AM-600 spectrometer and referenced to chloroform (7.26 p.p.m.). Flash chromatography was performed as described by Still *et al.* (1978), using Baker flash silica gel. H.p.l.c. was performed on a Waters model 6000 instrument, using a Schoeffel SF770/GM770 u.v. detector and a Waters 740 integrator. A Whatman Partisil 10 analytical h.p.l.c. column was used. T.l.c. was done using fluorescence-indicator Macherey-Nagel D-5160 Duren plates. All compounds and solvents used were commercially available reagent grade unless otherwise noted. Mass spectra were recorded on a VG70S instrument, and ²H levels determined by using the average of at least ten scans; the VG70S isotopic analysis computer program which subtracts theoretical natural-abundance levels was employed. A Sorvall Superspeed RC2-B instrument was used for low-speed centrifugation and an OTD-65 ultracentrifuge was used for high-speed centrifugation, with GSA and A641 rotors respectively. Liquid-scintillation counting was performed on a Beckman LS 7000 instrument.

Scheme 2.

Microsomal preparations

Human placentas obtained immediately after Caesarean delivery were placed on ice and all of the following procedures were done at 4 °C (Ryan, 1959). The cotyledons were dissected away from the fetal membranes from one placenta, and the former (~200 g) were washed with ice-cold potassium phosphate buffer (150 mM-KCl/50 mM-potassium phosphate/1 mM-EDTA, pH 7.3; 400 ml). The washed cotyledons were minced with scissors, suspended in 3 vol. of cold phosphate buffer, and homogenized in a Waring blender in portions for 2 min total at medium speed (with care to assure the mixture stayed cold). The combined homogenates were centrifuged at 16000 *g* for 15 min at 4 °C. The combined pink supernatants were filtered through double-layered cheesecloth to give about 700 ml. The filtrate was then centrifuged at 120000 *g* in portions for 45 min at 4 °C. The pellets were then resuspended in 200 ml of cold potassium phosphate buffer (as described above) using a Dounce homogenizer (ten complete strokes) and the resulting mixture was centrifuged at 120000 *g* for 45 min at 4 °C. The washing process was repeated on the new pellets with homogenization and centrifugation performed identically. The near-colourless supernatant was discarded and the combined pellets were resuspended by Dounce homogenization in the minimum amount of cold phosphate buffer (about 20 ml) and stored in 2 ml cryotubes at -78 °C until needed. No loss in activity was detected after several months.

Protein assay

Protein was determined by using the BCA assay (Smith *et al.*, 1985), with bovine serum albumin as standard. Batch-1 (microsomes from the first preparation) contained about 39 mg of protein/ml. Batch-2 (microsomes from the second preparation) contained about 30 mg of protein/ml. Total protein recovered was about 900 mg/placenta.

Enzyme activity assay

The microsomal aromatase activity was measured by a modification of the 1 β -³H-release assay described by Tochigi *et al.* (1986) and Thompson & Siiteri (1974*a,b*). The [1 β -³H]androstenedione (27 Ci/mmol; 75%-1 β -³H) was purchased from New England Nuclear Corp. The 0.5 ml reaction mixture contained 10 mM-potassium phosphate buffer, pH 7.4, 5 mM-dithiothreitol, 1 mM-EDTA, 100 mM-KCl, 125 μ M-NADPH, 1 μ M-[1 β -³H]androstenedione (3 Ci/mmol) and the reaction was initiated with about 0.05 mg of microsomal protein at 37 °C in a shaking water bath. Quenching of 0.4 ml aliquots with 5 ml of chloroform was carried out at 5, 10, 15, and 20 min time points, with activity being essentially linear over this period (after subtracting for background). The aqueous and organic phases were separated by low-speed centrifugation (10 min) and a 0.2 ml aliquot of the aqueous phase was counted for radioactivity in 10 ml of ACS scintillant. Batch-1 had an aromatase specific activity of 191 pmol of oestrogens formed/min per mg and Batch-2 had 172 pmol/min per mg.

N.m.r. assignments of 19-oxoandrostenedione (3b) and 19-oxotestosterone (3a)

Standard one-dimensional n.m.r. decoupling experiments were done to assign the protons in the 1.5–3 p.p.m. region. In addition, difference nuclear Overhauser enhancement (n.O.e.) was performed in the standard way on the Varian XL-400 instrument with 19-oxoandrostenedione (3b) by irradiating H-19 and H-1 α . A HOMO2DJ spectrum of 19-oxoandrostenedione (3b) was acquired on the Varian XL-400 instrument by using the standard method, which completely resolved the H-2 β coupling pattern.

Finally a 600 MHz ¹H-n.m.r. spectrum of 19-oxoandrostenedione (3b) resolved essentially all the peaks of interest and helped confirm the assignments.

Transformation of 17-O-THP(tetrahydropyranyl)-19-oxotestosterone (7, Fig. 2 below) into 19-oxotestosterone (3a)

The THP derivative (7) (Cole & Robinson, 1988) (40 mg, 0.104 mmol) was dissolved in 4 ml of dichloromethane/methanol (1:1, v/v) under N₂ with magnetic stirring at room temperature and was subsequently treated with PPTS (pyridinium *p*-toluenesulphonate) (32 mg, 0.127 mmol). After 18 h the reaction was still not complete (by t.l.c.), so more PPTS (32 mg, 0.127 mmol) was added. After 24 h more, the reaction appeared complete and the mixture was partitioned between 50 ml of dichloromethane and 50 ml of water. The separated aqueous phase was extracted twice more with dichloromethane (50 ml each) and the combined organic phases were dried (over anhydrous Na₂SO₄) and concentrated *in vacuo*. The crude oil was flash-chromatographed [3 g of SiO₂; 75% ethyl acetate in hexanes (isomer mixture); 3 ml fractions] giving the pure (by h.p.l.c. and t.l.c.) 17 β -ol (3a) (Rao & Axelrod, 1962) (27 mg, 0.089 mmol, 86% yield). Crystallization from ethyl acetate/hexanes gave 3a as white crystals; m.p. 127–129 °C [literature value (Rao & Axelrod, 1962) 124–126 °C]; h.p.l.c. (56% ethyl acetate in hexane) retention time 18 min; i.r. (chloroform) 3600, 2920, 1715, 1670, 1610 cm⁻¹; u.v. (methanol) 244 nm (ϵ 11600 M⁻¹·cm⁻¹); ¹H n.m.r. (400 MHz) see Table 2 below; high-resolution mass spectrum (h.r.m.s.) calcd. for C₁₉H₂₆O₃, *m/z* 302.1882; found, *m/z* 302.1887.

Transformation of 3-O-t-butyldimethylsilyl-17-O-THP-19-oxoandrost-2,4-diene-3,17 β -diol (6) into 19-oxo[2 β -³H]-testosterone (5a)

PPTS (6.0 mg, 0.024 mmol) was dissolved in 2 ml of [²H₄]methanol/dichloromethane (1:1, v/v) (0.888 g of [²H₄]methanol, 24.7 mmol) and left for 3 min at room temperature. This solution was added to the dienol ether (6) (29 mg, 0.058 mmol) and gently swirled until the white solid dissolved, and the solution was left under N₂ at room temperature. After 1 day, more PPTS (10 mg, 0.040 mmol) was added and, after a second day, transformation to the enone (5a) was shown (by t.l.c.) to be complete. The mixture was partitioned between 40 ml of dichloromethane and 60 ml of water. The separated aqueous phase was extracted twice with dichloromethane (30 ml each) and the combined organic layers were dried (over anhydrous Na₂SO₄) and concentrated *in vacuo* to afford 20 mg of a crude yellow oil. The oil was purified by flash chromatography (2 g of SiO₂, 75% ethyl acetate in hexanes, 3 ml fractions) to afford pure (by h.p.l.c. and t.l.c.) enone (5a) (15 mg, 0.050 mmol, 86% yield). Crystallization from ethyl acetate/hexanes gave 5a as white crystals [t.l.c., m.p., i.r., u.v., h.p.l.c. data were very similar to those for the unlabelled material (3a) above]; ¹H n.m.r. (400 and 600 MHz), same as unlabelled material, except, δ 2.75 (dd, *J* 13.3, 3.0 Hz, 1H, 1 β), 2.37 (bs, 87.3% H, 2 α), 2.31 (bd, *J* 13.3 Hz, 12.7% H, 2 β), 1.76 (dd, *J* 13.3, 6.0 Hz, 1H, 1 α); electron-impact mass spectrometry (e.i.m.s.): *d*₀, 6.7%; *d*₁, 91%; *d*₂, 2.3% (based on an average of ten scans); h.r.m.s. calcd. for C₁₉H₂₅O₃³H, *m/z* 303.1945; found, *m/z* 303.1949.

Transformation of 19-oxotestosterone (3a) into 19-oxoandrostenedione (3b)

The 17 β -ol (3a) (6.5 mg, 0.0215 mmol) was dissolved in 1 ml of dry dichloromethane, pyridinium chlorochromate (20 mg, 0.093 mmol) was added, and the resultant solution was kept under N₂ with magnetic stirring at room temperature for 45 min,

the colour of the mixture turning from orange to brown. T.l.c. indicated that the reaction was complete, and the mixture was directly flash-chromatographed (0.5 g of SiO_2 , 3 ml of dichloromethane, followed by 10 ml of 75% ethyl acetate in hexanes) to afford clean 17-ketone (**3b**) (Rao & Axelrod, 1962) as a colourless oil (6.0 mg, 0.02 mmol, 93% yield), which appeared pure (by h.p.l.c. and t.l.c.); h.p.l.c. (56% ethyl acetate in hexanes) retention time = 11 min; i.r. (chloroform) 2910, 1735, 1715, 1670, 1610 cm^{-1} ; u.v. (methanol) 244 nm (ϵ 10000 $\text{M}^{-1}\cdot\text{cm}^{-1}$); ^1H n.m.r. (400 and 600 MHz), see Table 2, e.i.m.s. (M^+) m/z 300.

Transformation of 19-oxo[2 β - ^3H]testosterone (**5a**) into 19-oxo[2 β - ^3H]androstenedione (**5b**)

The 17 β -ol (**5a**) (2.7 mg, 0.00891 mmol) was dissolved in 1 ml of dry dichloromethane and pyridinium chlorochromate (15 mg, 0.070 mmol) was added and the reaction was allowed to proceed and worked up as described above to afford 17-ketone (**5b**) (2.48 mg on the basis of u.v., 0.0824 mmol, 92% yield), which appeared pure (by h.p.l.c. and t.l.c.). T.l.c. R_f and h.p.l.c. retention time were identical with those of the unlabelled compound. ^1H n.m.r. (400 MHz), same as unlabelled material, except: δ 2.76 (dd, J 13.9, 3.0 Hz, 1H, 1 β), 2.40 (bs, 87.3% H, 2 α), 2.35 (bd, J 13.9 Hz, 12.7% H, 2 β), 1.81 (dd, J 13.9, 5.4 Hz, 1H, 1 α); e.i.m.s.: d_0 , 3.5%; d_1 , 91.0%; d_2 , 5.2%; d_3 , 0.4% on the basis of an average of ten scans.

Enzymic transformations of labelled androgens

Reactions were allowed to proceed in open 25 ml Erlenmeyer flasks, and reaction volumes were 10 ml total (pH 7.4; 5 mM-dithiothreitol, 100 mM-KCl, 1 mM-EDTA, 10 mM-potassium phosphate, 0.0015 mmol of androgen substrate in 0.2 ml of ethanol; ~60 mg of microsomal protein was used to initiate) (Morand *et al.*, 1975; Caspi *et al.*, 1986). Those reaction mixtures with an NADPH-regenerating system contained 0.04 mmol of NADPH, 56 units of glucose 6-phosphate dehydrogenase and 0.4 mmol of glucose 6-phosphate. The Batch-2 reactions were 4 mM in NADP and 1 mM in NADPH, and the glucose 6-phosphate dehydrogenase as well as the glucose 6-phosphate were omitted. Reactions were allowed to proceed for 60 min at 37 °C before the reaction mixtures were transferred to separation funnels containing 100 ml of water and 200 ml of dichloromethane and shaken. The organic phases were dried (over anhydrous Na_2SO_4) and concentrated *in vacuo*. T.l.c. at this stage revealed complete disappearance of starting material in those reaction mixtures that contained NADPH and the absence of oestrogen product in those reaction mixtures that lacked NADPH. Purifications were done using flash chromatography [0.5 g of SiO_2 , 33% ethyl acetate in hexanes to recover labelled oestradiol (**8a**); 75% ethyl acetate in hexanes to recover labelled 19-oxotestosterone (**5a**); 50% ethyl acetate in hexanes to recover labelled 19-oxoandrostenedione (**5b**); and 25% ethyl acetate in hexanes to recover labelled oestrone (**8b**)]. Yields were determined by u.v. and reported in Table 3 (below). Both ^1H n.m.r. (400 MHz) and e.i.m.s. were used to measure the ^3H levels in the products (see Table 3 below). The n.m.r. ^3H levels represent specific quantities at C-2 compared with the proton signal integration at C-1 and C-4. Reaction mixtures containing NADPH were incubated in duplicate and the results were averaged.

Enzyme transformations on unlabelled androgens

These transformations were performed as described above, except on half the scale. In addition, products were quantified by analytical h.p.l.c. after filtration through silica. Oestrogens were best separated and observed by using 25% ethyl acetate in hexane with u.v. detection at 280 nm [flow rate 1 ml/min;

retention times were about 17 min for oestradiol (**4a**) and 9 min for oestrone (**4b**)]. 19-Oxoandrogens were best separated and observed by using 60% ethyl acetate in hexanes with u.v. detection at 254 nm [flow rate 1 ml/min; retention times were about 9 min for 19-oxoandrostenedione (**3b**) and 16 min for 19-oxotestosterone (**3a**)]. Any changes in the reaction parameters from the standard labelled reactions are noted in the Tables (see Tables 4 and 5 below).

RESULTS AND DISCUSSION

In order to investigate enzymic enolization, we undertook the synthesis of 19-oxo[2 β - ^3H]testosterone (**5a**). We envisaged the 2,4-dienol compound (**6**) (Fig. 2) to be a direct precursor of the desired testosterone derivative (**5a**). Furthermore, we had previously prepared the dienol compound (**6**) (Cole & Robinson, 1988) from the conjugated ketone precursor (**7**) by using *t*-butyldimethylsilyl triflate and collidine. It is well known that enols of steroid 3-ketones are preferentially protonated on their axial face (Kirk & Hartshorn, 1968). This preference has been exploited in several preparations of ^3H -labelled steroids in the past, in particular by Brodie *et al.* (1969) in their [2 β - ^3H]androstenedione experiments. Therefore the dienol derivative (**6**) was allowed to react with the weak acid PPTS in [^3H]₄methanol. This treatment not only re-ketonized the enol with ^3H -labelling at C-2, but cleaved the 17-THP ether *in situ* to afford labelled 19-oxotestosterone (**5a**) in 86% yield after flash chromatography. The ^3H -labelled compound (**5a**) contained approx. 1 ^3H atom by m.s. and n.m.r. analysis [Table 1, Entry (1)]. Unlabelled 19-oxotestosterone (**3a**) was prepared in 86% yield by treating 17-O-THP-19-oxotestosterone (**7**) with PPTS in methanol.

It should be mentioned that n.m.r. assignment of the A-ring hydrogen atoms of 19-oxotestosterone (**3a**) [and 19-oxoandrostenedione (**3b**)] proved somewhat challenging (see the Materials and methods section). A combination of decoupling, difference n.O.e., and HOMO2DJ experiments performed on a 400 MHz spectrometer strongly supported the stereochemical assignments. In addition, a 600 MHz ^1H n.m.r. spectrum ultimately confirmed the assignments and completely resolved peak overlap (see Fig. 1). Table 2 shows the assigned protons, their chemical shifts and their coupling constants. The chemical shifts of the 2 α - and 2 β -H atoms are inverted with respect to those in androstenedione (**1b**) (Al-Rawi *et al.*, 1976; Pollack *et al.*, 1989). We attribute this chemical-shift peculiarity to a possible shielding effect of the 19-aldehyde group on the 2 β -H atom. It should be emphasized that the coupling constants and n.O.e. data are consistent with a normal half-chair A-ring structure for both **3a** and **3b**.

Table 1. Steroid ^3H levels in the synthetic 19-oxotestosterone and 19-oxoandrostenedione substrates as determined by n.m.r. and m.s.

Entry	^1H n.m.r.	M.s.
(1) [2 β - ^3H]19-OxoT* (5a)	~ 6.9:1 2 β - ^3H /2 α - ^3H	d_0 , 6.7% d_1 , 91.0% d_2 , 2.3% d_3 , 0.4%
(2) [2 β - ^3H]19-OxoA* (5b)†	~ 6.9:1 2 β - ^3H /2 α - ^3H	d_0 , 3.5% d_1 , 91.0% d_2 , 5.2% d_3 , 0.4%

* 19-OxoT and 19-OxoA refer to 19-oxotestosterone and 19-oxoandrostenedione respectively.

† The slightly higher m.s. ^3H levels in **5b** may stem from the fact that it was prepared from a separate batch of **5a** (not from the **5a** with the data shown).

Fig. 1. 600 MHz ^1H -n.m.r. spectrum of 19-oxoandrostenedione (3b) illustrating the 2α -H (peaks 9–16) and 2β -H (peaks 1–8) atoms

That ^2H was incorporated predominantly into the 2β -position of compound 5a was indicated by the 400 MHz ^1H -n.m.r. spectrum. The $2\beta/2\alpha$ -H region of the spectrum showed a broadened singlet with a chemical shift just upfield ($\Delta\delta$ 0.02–0.03 p.p.m.) of the 2α -H of the unlabelled material, presumably due to the α -shift effect of the *gem*- ^2H atom. There was

also a small unresolved shoulder on the upfield side of the peak. The broad singlet represented the expected 2α -H atom, and the shoulder was residual hydrogen at the 2β position. In addition, the splitting patterns of the 1β -H and 1α -H atoms were consistent with nearly complete 2β - ^2H -labelling. For more precise quantification, a 600 MHz ^1H -n.m.r. spectrum was obtained. The spectrum revealed that the labelling ratio (2β - $^2\text{H}/2\alpha$ - ^2H) was approx. 7:1 and that there was essentially no unlabelled material. This ratio is comparable with that reported by Brodie *et al.* (1969) for the base-catalysed exchange of ^2H into androstenedione.

Enzyme experiments were performed as follows. The labelled material (5a; 0.450 mg) was incubated with placental microsomes (~ 60 mg of protein) in 10 ml of buffer at 37 °C for 60 min. The activity of the microsomes, measured by using a 1β - ^3H -release assay (Thompson & Siiteri, 1974a,b; Tochigi *et al.*, 1986) was such that complete aromatization of the 19-oxotestosterone substrate (3a) could take place. Parallel experiments were run, one with NADPH (plus a glucose-6-phosphate dehydrogenase NADPH-regenerating system) and one without NADPH. In the flask which lacked NADPH, there was good recovery of labelled 19-oxotestosterone (5a) (61 % yield after flash-chromatographic purification) and no oestrogens (detectable by t.l.c.). In the flask with NADPH plus the regenerating system (the experiment was performed in duplicate), no starting material was observed (by t.l.c.) and there was a good yield of labelled oestradiol (8a) (72 ± 4 % after flash chromatography). The ^2H levels were quantified by m.s. and ^1H -n.m.r. analysis [see Table 3, Entries (1) and (2)].

There was little ^2H loss as judged by m.s. in the recovered labelled 19-oxotestosterone (5a) [Table 3, Entry (2)]. From the ^1H n.m.r. spectrum, we estimate that there was also less than 5 % epimerization or ^2H loss from C-2. These findings suggest that, if there is an enzyme active-site base positioned near C-2 of 5a (and presumably 3a), it may be shielded from the external environment and thus does not exchange with protons from buffer. This shielding phenomenon has been observed in studies of several enzymes, including oxosteroid isomerase (Kuliopulos

Table 2. ^1H n.m.r. spectral assignments of compounds 3a and 3b

Compound	δ^*	Integral	Assignment	Apparent splitting [J (Hz)]
19-OxoA† (3b)	9.94	1H	19	s
	6.00	1H	4	s
	2.76	1H	1β	ddd (13.9, 5.1, 3.0)
	2.65	1H	6β	tdd (15.0, 5.6, 2.1)
	2.55	1H	6α	ddd (15.0, 4.0, 2.6)
	2.49	1H	16β	dd (19.3, 9.0)
	2.43	1H	2α	ddd (17.1, 5.4, 3.0)
	2.37	1H	2β	ddd (17.1, 13.9, 5.1)
	2.11	1H	16α	dt (19.3, 9.1)
	1.81	1H	1α	td (13.9, 5.4)
	0.90	3H	18	s
19-OxoT† (3a)	9.94	1H	19	s
	5.96	1H	4	s
	3.65	1H	17	t (8.6)
	2.75	1H	1β	ddd (13.3, 5.0, 3.0)
	2.63	1H	6β	td (14.2, 5.2)
	2.50	1H	6α	dt (14.2, 3.2)
	2.40	1H	2α	ddd (17.1, 6.0, 3.0)
	2.34	1H	2β	ddd (17.1, 13.3, 5.0)
	1.76	1H	1α	td (13.3, 6.0)
	0.77	3H	18	s

* Chemical shift (p.p.m.).

† 19-OxoT and 19-OxoA refer to 19-oxotestosterone and 19-oxoandrostenedione respectively.

Fig. 2.

et al., 1989). Alternatively, the oxidation level of the haem iron might need to be adjusted to bring the base into the proximity of H-2. Either case would be very difficult to confirm. Thus the lack of exchange, which may be regarded as weak evidence against an active-site base near H-2, should not be over-interpreted.

We did obtain an unexpected and provocative result, however. The oestradiol (**8a**) that was recovered retained nearly half of the initial ${}^3\text{H}$ content [Table 3, Entry (1)]. Because Brodie, Fishman and co-workers had shown that aromatase specifically removes the $2\beta\text{-H}$ atom in its processing of androstenedione (**1b**) (Brodie *et al.*, 1969; Fishman & Guzik, 1969; Fishman *et al.*, 1969), we were very surprised by such a large ${}^3\text{H}$ retention. We were reasonably sure that our stereochemical assignment was correct. Furthermore, little, if any, epimerization of the starting material presumably took place during the reaction, since in the absence of NADPH the starting material was recovered unchanged (n.m.r., m.s.). During the course of the present study, Fronckowiak & Osawa (1988) presented preliminary data indicating that $[2\beta\text{-}{}^3\text{H}]$ testosterone had shown incomplete stereospecificity with aromatase compared to $[2\beta\text{-}{}^3\text{H}]$ androstenedione. They did not directly analyse the ${}^3\text{H}$ placement by n.m.r., however. Furthermore, these workers extrapolated stereochemical conclusions from a precursor two steps away from the third oxidative step. Consequently, they were not in a position to define rigorously the stereospecificity of the third step. For example, epimerization and/or ${}^3\text{H}$ exchange could occur in the 19-Me (**1**),

19-OH (**2**) and 19-oxo (**3**) intermediates and go undetected. Because of its critical importance to the aromatase mechanism, we decided to explore this issue in more detail.

After demonstrating that unlabelled 19-oxotestosterone (**3a**) could be oxidized in high yield (93 %) to 19-oxoandrostenedione (**3b**) with pyridinium chlorochromate (PCC), we converted the $[2\beta\text{-}{}^3\text{H}]$ 19-oxotestosterone (**5a**) to $[2\beta\text{-}{}^3\text{H}]$ 19-oxoandrostenedione (**5b**) under similar conditions. We chose PCC as an oxidizing agent since it is a weak acid (Corey & Suggs, 1975) and should minimally effect the ${}^3\text{H}$ content at C-2. Indeed, the n.m.r. and m.s. data [Table 1, Entry (2)] were consistent with little ${}^3\text{H}$ exchange or C-2 epimerization in the oxidation. Furthermore, reaction of **5b** with placental microsomes in the absence of NADPH, as described above, led to good recovery of starting material (**5b**) (67 % after flash chromatography), again with negligible ${}^3\text{H}$ loss or epimerization [Table 3, Entry (5)]. In the reaction of **5b** with NADPH (the experiment was performed in duplicate), the major product, surprisingly, was labelled oestradiol (**8a**) (61 ± 4 % yield after flash chromatography), along with a small amount of labelled oestrone (**8b**) (16 ± 1 % yield, percentage of ${}^3\text{H}$ not determined).

The presence of 17β -hydroxysteroid dehydrogenase activity (in oestrone/oestradiol interconversion) in placental microsomes has been previously established (Guller *et al.*, 1986; Blomquist *et al.*, 1987; Fournet-Dulguerov *et al.*, 1987), but the high level of 17-ketone reduction in our case was not expected. Nevertheless,

Table 3. ^2H levels and yields of oestrogens or recovered starting materials from incubations of $2\beta\text{-}[^2\text{H}]19\text{-oxosteroids}$ (**5a** and **5b**) with human placental aromatase

Experiments were performed as described in the Materials and methods section using the substrates described in Table 1. All reactions were done with Batch-1 microsomes, except for Entry (4). The errors listed represent the spans of duplicate runs. The small differences (about 4%) between m.s. and n.m.r. ^2H levels for Entries (1), (3), and (4) probably reflect traces of non-specifically-incorporated ^2H not observed in the n.m.r. spectra.

Entry	M.s.	^1H n.m.r.	Yield (%)
(1) Labelled oestradiol 8a from 5a	$d_0, 52.0 \pm 0.5\%$ $d_1, 47.9 \pm 0.5\%$	$d_0, 56.0 \pm 0.4\%$ $d_1, 44.0 \pm 0.4\%$	$72 \pm 4\%$
(2) 5a recovered from microsomes without NADPH	$d_0, 7.7\%$ $d_1, 87.9\%$ $d_2, 4.4\%$	$\sim 6.9:1\ 2\beta\text{-}^2\text{H}/2\alpha\text{-}^2\text{H}$	61%
(3) Labelled oestradiol 8a from 5b	$d_0, 77.6 \pm 0\%$ $d_1, 22.4 \pm 0\%$	$d_0, 81.3 \pm 0.2\%$ $d_1, 18.7 \pm 0.2\%$	$61 \pm 4\%$ ($16 \pm 1\%$)*
(4) Labelled oestrone 8b , Batch-2 microsomes, 4 mM-NADP ⁺ , 1 mM-NADPH	$d_0, 75.5 \pm 0.3\%$ $d_1, 24.5 \pm 0.3\%$	$d_0, 82.1 \pm 2.6\%$ $d_1, 17.9 \pm 2.6\%$	$68 \pm 5\%$
(5) 5b recovered from microsomes without NADPH	$d_0, 4.7\%$ $d_1, 90.8\%$ $d_2, 4.5\%$	$\sim 6.9:1\ 2\beta\text{-}^2\text{H}/2\alpha\text{-}^2\text{H}$	67%

* Yield of recovered oestrone **8b** (^2H levels undetermined).

the recovered labelled oestradiol (**8a**) showed substantially less ^2H retention, as judged by n.m.r. (18.7%) and m.s. (22.4%) [Table 3, Entry (3)] than was the case for the labelled 19-oxotestosterone (**5a**) experiment [Table 3, Entry (1)]. This 19-oxo[$2\beta\text{-}^2\text{H}$]androstenedione (**5b**) experiment in fact gave results which are consistent with previous studies with labelled androstenedione (Brodie *et al.*, 1969; Fishman & Guzik, 1969; Fishman *et al.*, 1969).

Thus it seemed that there actually was a significant difference between the processing of labelled 19-oxoandrostenedione (**5b**) and 19-oxotestosterone (**5a**) by placental aromatase. The experiment with 19-oxo[$2\beta\text{-}^2\text{H}$]androstenedione (**5b**) showed more ^2H retention than is theoretically possible for a reaction that is 100% stereospecific. We were therefore concerned that 17β -hydroxysteroid dehydrogenase activity might have caused significant 17-ketone reduction *before* aromatization, leading to higher-than-expected ^2H retention at the 2-position. [Unfortunately, the use of purified aromatase to resolve the issue was not technically feasible, owing to the large scale of these aromatase reactions.] Furthermore, we clearly demonstrated the interconversion between 19-oxotestosterone (**3a**) and 19-oxoandrostenedione (**3b**) in placental microsomes. This was accomplished by showing that 19-oxotestosterone (**3a**) was nearly completely oxidized by NADP⁺- (or NAD⁺-) supplemented microsomes to 19-oxoandrostenedione (**3b**) in 60 min [Table 4, Entries (4) and (5)]. All of the abovementioned ^2H -labelled metabolic studies were done with the same batch of placental microsomes (Batch-1).

We then made another unexpected discovery. Preliminary studies with a new batch (Batch-2) of placental microsomes (from a totally separate placenta) indicated that the level of dehydrogenase activity was significantly different. Therefore we decided to compare rigorously the oestradiol (**4a**)/oestrone (**4b**)

Table 4. Reactions of unlabelled 19-oxoandrostenedione (**3b**) and 19-oxotestosterone (**3a**) with placental microsomes under several different conditions

Batch-2 microsomes were used. Percentage yields were determined by h.p.l.c. (see the Materials and methods section). Entries (1), (2) and (3) are control experiments demonstrating that there is negligible endogenous androgen, oestrogen, or nicotinamide cofactors present in the microsomes. Entry (1) contained an NADPH-regenerating (regen.) system.

Entry	Yield (%)			
	Oestrone	Oestradiol	3b	3a
(1) 0 mM-Steroid substrate, 2 mM-NADPH, regen.	—	—	x†	x
(2) 3a , 0 mM-NADPH, NADP ⁺	—*	—	—	77
(3) 3b , 0 mM-NADPH, NADP ⁺	—	—	84	—
(4) 3a , 2 mM-NADP ⁺	Not quantified	—	66	†
(5) 3a , 2 mM-NAD ⁺	—	—	57	5.5
(6) 3b ‡, 2 mM-NADPH, regen.	50	29	—	—
(7) 3b , 1 mM-NADP ⁺ , 1 mM-NADPH	71	17	x	x
(8) 3b , 4 mM-NADP ⁺ , 1 mM-NADPH	75	5	x	x
(9) 3b §, 2 mM-NADPH, regen., 4 h	43	27	x	x

* '—' indicates below the limits of detection of h.p.l.c. (about 1%).
† 'x' indicates below the limits of detection of t.l.c.
‡ NADPH-regenerating (regen.) system used; average of two runs.
§ NADPH-regenerating system used, 4 h reaction time.

ratios produced by the two placental preparations using compounds **3a** and **3b**. The results from h.p.l.c. assays are shown in Table 5. There were significant differences for both 19-oxotestosterone (**3a**) and 19-oxoandrostenedione (**3b**) reactions between the two placentas. The most dramatic differences occurred in the experiments with 19-oxoandrostenedione (**3b**). It was initially believed that the Batch-2 19-oxoandrostenedione (**3b**) reaction simply had less dehydrogenase activity and had not reached the same approximate oestradiol/oestrone equilibrium as the others. No significant change in the product ratio was noted, however, even where the 19-oxoandrostenedione (**3b**) Batch-2 reaction was allowed to proceed for a 4-fold longer period [Table 4, Entry (9)]. Furthermore, on the basis of the work of Talalay (1957), the equilibrium in the presence of a 2 mM-NADPH-regenerating system should lie very far toward the $17\beta\text{-ol}$. It was thus difficult to rationalize the formation of 17-ketone from 19-oxotestosterone using a simple equilibrium argument. We recognized that compartmentalization (because of the micellar nature of microsomes) as well as enzyme instability might complicate the outcomes. We thus conceded that the product ratios could not be explained with a simple model.

Nevertheless, we observed that, by adjusting the NADP⁺/NADPH ratio in the Batch-2 reactions (in the absence of an NADPH-regenerating system), we could alter the product ratio of oestrone (**4b**)/oestradiol (**4a**) produced from 19-oxoandrostenedione (**3b**) [see Table 4, Entries (7) and (8)]. At concentrations of 4 mM-NADP⁺ and 1 mM-NADPH we found that the oestrone/oestradiol ratio was about 15:1, with no detectable starting material remaining (as judged by t.l.c.). These conditions were suitable for reliable assessment of the stereospecificity of the

Table 5. Product comparisons from two different batches of microsomes allowed to react with unlabelled 19-oxoandrogens

All reactions mixtures contained 2 mM-NADPH plus an NADPH-regenerating system; errors represent the span of duplicate runs.

Substrate	Product	Yield (%)	
		Batch-1	Batch-2
3a	Oestrone	8 ± 3	20 ± 1
	Oestradiol	69 ± 1	55 ± 2
3b	Oestrone	19 ± 1	50 ± 1
	Oestradiol	56 ± 4	29 ± 7

19-oxo[2 β -³H]androstenedione (**5b**) reaction with aromatase, because contaminating aromatization of 19-oxo[2 β -³H]testosterone (**5a**) was presumably minimal. The results of duplicate experiments are shown in Table 3 [Entry (4)]. The ³H retention in the recovered labelled oestrone (**8b**) was nearly identical with that seen in the labelled oestradiol (**8a**) isolated from the previous experiment involving 19-oxo[2 β -³H]androstenedione (**5b**) and Batch-1 microsomes [Table 3, Entry (3)]. It appears that, at least in these cases, the rate of aromatization is fast compared with 17 β -ol/17-one interconversion. Therefore the incomplete stereospecificity of 19-oxo[2 β -³H]androstenedione (**5b**) aromatization is presumably not a result of prior reduction of **5b** to the 17 β -ol (**5a**).

One can compute the 2 β -H stereoselectivity exhibited by aromatase on the basis of the initial stereochemical purity of the labelled 19-oxoandrogens (**5**), assuming negligible epimerization and kinetic-isotope effect.* The ratio is about 11:1 (β/α) for 19-oxoandrostenedione (**3b**) and about 1.2:1 (β/α) for 19-oxotestosterone (**3a**). It should also be mentioned that the A-ring solution-phase conformations (in [²H]chloroform) of the two 19-oxoandrogens (**3a** and **3b**) are very similar on the basis of the ¹H n.m.r. spectra (Table 2). Calculations using the computer software MACROMODEL (Still, 1986) also predict similar half-chair A-rings for **3a** and **3b**, with approximately similar stability differences [about 11.7 kJ (2.8 kcal)/mol more stable] versus the corresponding 19-oxoandrogens with a half-boat A-ring. In addition, MACROMODEL calculations performed on the half-chair/half-boat forms of compounds **1a** and **1b**, and **9a** and **9b**, suggest that the minimum-energy A-ring conformations should not be affected by 17-ketone versus 17 β -hydroxy functionalities.

Summary and conclusions

We have prepared, characterized and studied [2 β -³H]19-oxoandrogens (**5**) and their metabolism by placental microsome aromatase. Although there appears to be little enzyme-induced solvent exchange or epimerization of hydrogen atoms at C-2 in the absence of NADPH cofactor, we cannot exclude enzyme-catalysed enolization of the 3-ketone. Processing of 19-oxoandrostenedione (**3b**) by aromatase apparently leads to at least 11:1 2 β -H/2 α -H removal, whereas 19-oxotestosterone (**3a**) undergoes about 6:5 2 β -H/2 α -H removal. Although in principle it is possible that placental microsomes contain more than one aromatase, recent molecular-cloning experiments of Simpson

and colleagues (Corbin *et al.*, 1988; Means *et al.*, 1989) argue for one enzyme (see above). Although large differences between apparent 17 β -hydroxysteroid dehydrogenase levels may occur in different microsomal preparations, the dehydrogenase reaction may still be slow compared with the aromatization rate, as it appeared to be in our cases.

We infer from these studies that 2-H loss from the 3-ketone is probably enzyme-mediated for both 19-oxoandrogens (**3a** and **3b**) or some subsequent short-lived intermediates. The fact that the stereoselectivity of 2-H abstraction for compound **3a** is so low may seem surprising, as enzymes are generally considered to be stereospecific. On the other hand, other steroid-enolizing enzymes known to involve active-site general bases have been shown to be non-stereospecific with respect to proton abstraction (Smith & Brooks, 1977; Viger *et al.*, 1981). The experimental solution n.m.r. data for **3**, as well as theoretical studies for compounds **3**, **1** and **9**, suggest similar A-ring conformations for these compounds regardless of 17-ketone versus 17 β -hydroxy functionality. The intrinsic 2-H reactivity of these compounds with buffer would therefore not be expected to show dependence on the presence of a 17-ketone or 17 β -hydroxy group. We believe that the enzyme probably binds the 17-ketone group sufficiently differently from the 17 β -hydroxy group so that the active-site base is located in a different relative position *vis-à-vis* C-2 for the two substrates. The timing of 3-ketone enolization with respect to H-1 β bond cleavage has still not been determined, although we are tempted to favour enolization before, or accompanying, H-1 β cleavage, because of the model reaction studies discussed above (Cole & Robinson, 1988). On the basis of the work of Fronckowiak & Osawa (1988) with 19-methyl substrates, it seems probable that 19-oxoandrogens are processed similarly to 19-methylandrogens with respect to H-2 selectivity. This lends further support to the notion that 19-oxoandrogens are not just aberrant metabolites, as has previously been suggested (Kelly *et al.*, 1977; Bednarski & Nelson, 1989). It should also be mentioned that an interesting aromatase-catalysed metabolic switching phenomenon was observed to be quite different for [19-³H]androstenedione versus [19-³H]testosterone (Osawa *et al.*, 1987). This may also relate to a subtle difference in active-site binding between the two substrates.

Finally, these experiments cast further doubt on the theory that 2 β -hydroxylation is the obligatory aromatase third step (Hahn & Fishman, 1984; Caspi *et al.*, 1984), since much of the 17 β -hydroxy substrate undergoes 2 α -H removal during aromatization. Cytochrome P-450 hydroxylation reactions generally occur with retention of configuration, and the aromatase haem iron is thought to be positioned over the top of the A-ring near C-19 (Kellis & Vickery, 1987b; Kellis *et al.*, 1987). Thus iron-oxo-catalysed 2 α -H removal, which would be required as part of the 2 β -hydroxylation theory, seems improbable.

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* A partial kinetic-isotope effect, or low levels of non-specific epimerization (less than ~ 5%, the estimated detection limit) would make the true stereoselectivity even greater than 11:1 for aromatization of **5b**. No significant $V_{\max}$ or K_m isotope effect was detected for aromatization of **5a** using human placental aromatase (J. T. Kellis, P. A. Cole, L. E. Vickery & C. H. Robinson, unpublished work). The computations were performed by setting the initial 2-³H level equal to 95% (6.9:1 β/α) for **5a** and **5b** and using the n.m.r. data for **8a** and **8b** (see Table 1).

REFERENCES

- Akhtar, M., Corina, D., Pratt, J. & Smith, T. (1976) *J. Chem. Soc. Chem. Commun.* 854–856
- Akhtar, M., Calder, M. R., Corina, D. L. & Wright, J. N. (1981) *J. Chem. Soc. Chem. Commun.* 129–130
- Akhtar, M., Calder, M. R., Corina, D. L. & Wright, J. N. (1982) *Biochem. J.* **201**, 569–580
- Al-Rawi, J. M. A., Bloxidge, J. P., Elvidge, J. A. & Jones, J. R. (1976) *Steroids* **28**, 359–375
- Arigoni, D., Battaglia, R., Akhtar, M. & Smith, T. (1975) *J. Chem. Soc. Chem. Commun.* 185–186
- Bednarski, P. J. & Nelson, S. D. (1989) *J. Steroid Biochem.* **32**, 309–316
- Beusen, D. D., Carrell, H. L. & Covey, D. F. (1987) *Biochemistry* **26**, 7833–7841
- Blomquist, C. H., Lindemann, N. J. & Hakanson, E. Y. (1987) *J. Clin. Endocrinol. Metab.* **65**, 647–652
- Brodie, A. M. H. (1985) *Biochem. Pharmacol.* **34**, 3213–3219
- Brodie, H. J., Kripalani, K. J. & Possanza, G. (1969) *J. Am. Chem. Soc.* **91**, 1241–1242
- Caspi, E., Wicha, J., Arunachalam, T., Nelson, P. & Spiteller, G. (1984) *J. Am. Chem. Soc.* **106**, 7282–7283
- Caspi, E., Arunachalam, T. & Nelson, P. A. (1986) *J. Am. Chem. Soc.* **108**, 1847–1852
- Cole, P. A. & Robinson, C. H. (1986) *J. Chem. Soc. Chem. Commun.* 1651–1653
- Cole, P. A. & Robinson, C. H. (1988) *J. Am. Chem. Soc.* **110**, 1284–1285
- Corbin, C. J., Graham-Lorence, S., McPhaul, M., Mason, J. I., Mendelson, C. R. & Simpson, E. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8948–8952
- Corey, E. J. & Suggs, J. W. (1975) *Tetrahedron Lett.* 2647–2650
- Covey, D. F., Hood, W. F., Beusen, D. D. & Carrell, H. L. (1984) *Biochemistry* **23**, 5398–5406
- Fishman, J. & Guzik, H. (1969) *J. Am. Chem. Soc.* **91**, 2805–2806
- Fishman, J. & Hahn, E. F. (1987) *Steroids* **50**, 339–345
- Fishman, J., Guzik, H. & Dixon, D. (1969) *Biochemistry* **8**, 4304–4309
- Fournet-Dulguerov, N., MacLusky, N. J., Leranthe, C. Z., Todd, R., Mendelson, C. R., Simpson, E. R. & Naftolin, F. (1987) *J. Clin. Endocrinol. Metab.* **65**, 757–764
- Fronckowiak, M. D. & Osawa, Y. (1988) *Endocr. Soc. Meet.* June 8–11, New Orleans, abstr. 430
- Guller, S., Gravanis, A. & Guripide, E. (1986) *J. Steroid Biochem.* **24**, 935–944
- Hahn, E. F. & Fishman, J. (1984) *J. Biol. Chem.* **259**, 1689–1694
- Johnston, J. O. & Metcalf, B. W. (1984) in *Novel Approaches to Cancer Chemotherapy* (Sunkara, P., ed.), pp. 307–328, Academic Press, New York
- Kellis, J. T., Jr. & Vickery, L. E. (1987a) *J. Biol. Chem.* **262**, 4413–4420
- Kellis, J. T., Jr. & Vickery, L. E. (1987b) *J. Biol. Chem.* **262**, 8840–8844
- Kellis, J. T., Jr., Childers, W. E., Robinson, C. H. & Vickery, L. E. (1987) *J. Biol. Chem.* **262**, 4421–4426
- Kelly, W. G., Judd, D. & Stolee, A. (1977) *Biochemistry* **16**, 140–145
- Kirk, D. N. & Hartshorn, M. P. (1968) *Steroid Reaction Mechanisms*, pp. 154–157, Elsevier, Amsterdam
- Kuliopulos, A., Mildvan, A. S., Shortle, D. & Talalay, P. (1989) *Biochemistry* **28**, 149–159
- Mastalerz, H. & Morand, P. (1982) *J. Chem. Soc. Perkin Trans. I* 2611–2615
- Means, G. D., Mahendroo, M. S., Corbin, C. J., Mathis, J. M., Powell, G. E., Mendelson, C. R. & Simpson, E. R. (1989) *J. Biol. Chem.* **264**, 19385–19391
- Meyer, A. S. (1955a) *Biochim. Biophys. Acta* **17**, 441–442
- Meyer, A. S. (1955b) *Experientia* **11**, 99–102
- Morand, P., Williamson, D. G., Layne, D. S., Lompa-Krzymien, L. & Salvador, J. (1975) *Biochemistry* **14**, 635–638
- Ortiz de Montellano, P. R. (ed.) (1986) *Cytochrome P-450: Structure, Mechanism and Biochemistry*, Plenum Press, New York
- Osawa, Y., Shibata, K., Rohrer, D., Weeks, C. & Duax, W. L. (1975) *J. Am. Chem. Soc.* **97**, 4400–4402
- Osawa, Y., Yoshida, N., Fronckowiak, M. & Kitawaki, J. (1987) *Steroids* **50**, 11–27
- Pollack, R. M., Zeng, B., Mack, J. P. G. & Eldin, S. (1989) *J. Am. Chem. Soc.* **111**, 6419–6423
- Rao, P. N. & Axelrod, L. N. (1962) *J. Org. Chem.* **27**, 4694–4696
- Ryan, K. J. (1959) *J. Biol. Chem.* **234**, 268–272
- Siiteri, P. K. & Thompson, E. A. (1975) *J. Steroid Biochem.* **6**, 317–322
- Smith, A. G. & Brooks, C. J. W. (1977) *Biochem. J.* **167**, 121–129
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- Stevenson, D. E., Wright, J. N. & Akhtar, M. (1985) *J. Chem. Soc. Chem. Commun.* 1078–1080
- Stevenson, D. E., Wright, J. N. & Akhtar, M. (1988) *J. Chem. Soc. Perkin Trans. I* 2043–2052
- Still, W. C. (1986) *MACROMODEL*, Columbia University
- Still, W. C., Kahn, M. & Mitra, A. (1978) *J. Org. Chem.* **43**, 2923–2925
- Talalay, P. (1957) *Rec. Chem. Prog.* **18**, 31–49
- Thompson, E. A. & Siiteri, P. K. (1974a) *J. Biol. Chem.* **249**, 5364–5372
- Thompson, E. A. & Siiteri, P. K. (1974b) *J. Biol. Chem.* **249**, 5373–5378
- Tochigi, B., Osawa, Y. & Osawa, Y. (1986) *Endocr. Res.* **12**, 105–113
- Townsend, J. D., Possanza, G. J. & Brodie, H. J. (1966) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **25**, 282 (abstr. 520)
- Viger, A., Coustal, S. & Marquet, A. (1981) *J. Am. Chem. Soc.* **103**, 451–458
- Watanabe, Y. & Ishimura, Y. (1989) *J. Am. Chem. Soc.* **111**, 8047–8049

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