Pergamon

Biochemical Pharmacology, Vol. 47, No. 4, pp. 717–726, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0029-7

METABOLIC ASPECTS OF THE 1β-PROTON AND THE 19-METHYL GROUP OF ANDROST-4-ENE-3,6,17-TRIONE DURING AROMATIZATION BY PLACENTAL MICROSOMES AND INACTIVATION OF AROMATASE*

MITSUTERU NUMAZAWA,† KOJI MIDZUHASHI and MASAO NAGAOKA
Tohoku College of Pharmacy, Sendai 981, Japan

(*Received* 3 *June* 1993; *accepted* 10 *September* 1993)

Abstract—Aromatase catalyzes the conversion of androst-4-ene-3,17-dione to estrogen through sequential oxygenations at the 19-methyl group. Androst-4-ene-3,6,17-trione (AT) is a suicide substrate of aromatase, and the mechanism of inactivation of aromatase has been postulated to involve enzymatic oxygenation at the 19-position. [1β-^{3}H,4-^{14}C]-, [19-$^3H_3$,4-^{14}C]-, and [1β-^{3}H,19-^{14}C]ATs, with high specific activities, were synthesized to study metabolic aspects and the inactivation mechanism. Incubation of the labeled AT with human placental microsomes yielded the 19-oxygenated derivatives, 19-hydroxy-AT and 19-oxo-AT, as well as the aromatization products, 6-oxoestrone and 6-oxoestradiol. A stereospecific 1β-proton elimination occurred during the aromatization of [1β-^{3}H,4-^{14}C]AT, and a marked tritium isotope effect was observed in the first hydroxylation at C-19 of [19-$^3H_3$,4-^{14}C]AT. After incubation of the three double-labeled ATs, the solubilized proteins were subjected to SDS–PAGE and the ^{3}H/^{14}C ratio of the aromatase-bound metabolite in a 46–69 kDa fraction was analyzed. A marked decrease of the ^{3}H/^{14}C ratio of the metabolite was observed in the experiment using [19-$^3H_3$,4-^{14}C]AT, compared with that of the labeled AT used, but there were no significant changes in the other experiments, indicating that the adduct retains the 1β-proton, the 19-carbon, and one of three 19-methyl protons of AT. Thus, we conclude that further oxygenation of 19-oxo-AT produced by the two initial hydroxylations of AT at C-19 yields not only 6-oxoestrogen (by a mechanism similar to that involved in the aromatization of the natural substrate) but also a reactive electrophile that immediately binds to the active site in an irreversible manner, resulting in inactivation of aromatase.

Key words: androst-4-ene-3,6,17-trione; aromatase inhibitor; 19-oxygenated metabolite; 6-oxoestrogen; aromatase-bound metabolite; inactivation mechanism

The conversion of androgens to estrogens is catalyzed by aromatase, a unique P450 enzyme complex [1–3], whose activity results in aromatization of the A-ring of androgens to form the phenolic A-ring characteristic of estrogens, with concomitant loss of the C-19 angular methyl group. Several mechanistic features of the aromatase reaction have been determined. Three oxygenative steps are involved, each apparently requiring 1 mol of oxygen and 1 mol of NADPH, for a total of 3 mol of each [4]. The first two are sequential, stereospecific hydroxylations at the C-19 methyl group. The 19-hydroxy steroid is initially formed [5], and then the second hydroxylation involves the stereospecific displacement of the 19-pro-R hydrogen atom of the 19-ol to give the 19,19-gem-diol [6–8]. Dehydration of this gem-diol leads to the readily isolated 19-aldehyde intermediate [9]. A tritium isotope effect in the first but not in the second hydroxylation at C-19 is observed in the aromatization of [19-$^3H_3$,4-^{14}C]androstenedione [10]. In the case of androstenedione, following the third oxygenative step, C-19 [11] and 1β,2β-protons [12, 13] are eliminated as formic acid and water, respectively, to produce estrone. It is now thought that a substrate-dependent variation occurs in the stereospecificity of the elimination of the C-2 proton [14]. Considerable speculation continues as to the site and mechanism of attack of the third mol of oxygen. A leading theory for the third step proposes nucleophilic attack of the heme ferric peroxide species on the 19-aldehyde intermediate to produce a 19-hydroxy-19-ferric peroxide intermediate [15–17]. 2β-Hydroxylation of the 19-aldehyde is thought to be one of the alternate biosynthetic routes [18, 19].

The production of estrogen through the aromatase pathway is important in the growth of established estrogen-dependent breast cancer [20–22]. For this reason, various substrate analogs have been tested in a number of laboratories as inhibitors of aromatase. These include competitive as well as irreversible inhibitors. Among the latter, suicide substrate (mechanism-based inhibitors) are of special interest due to their high selectivity. However, to our knowledge, the inactivation process of almost all the inhibitors, except for 19,19-difluoroandrost-4-ene-3,17-dione [23, 24], remains to be solved, principally because of unavailability of their radioactive forms.

* Preliminary accounts of this work were presented at the Third International Conference of Aromatase, Bologna, Italy, June 1992.

† Corresponding author: Dr. Mitsuteru Numazawa, Tohoku College of Pharmacy, 4-1 Komatsushima-4-chome, Aobaku, Sendai 981, Japan. Tel. (22) 234-4181; FAX (22) 275-2013.

AT* is one of the earliest discovered suicide substrates of aromatase [25, 26]. Its 3α- and 3β-reduced derivatives [26–28] and its 19-nor derivative [22] also inactivate aromatase in a time-dependent manner, although the 3-deoxy derivative of AT, a very potent competitive inhibitor, does not [27]. The previous findings have shown that an oxygen function at C-3 is essential for the time-dependent inactivation caused by a 4-en-6-one steroid. Since both 19-hydroxy- and 19-oxo-ATs inactivate the enzyme in a suicide manner [22, 29], it has been suggested that the 19-oxygenation is involved in the aromatase inactivation by AT. However, there is no direct evidence for the production of the 19-oxygenated ATs. On the other hand, we have reported that when [4-^{14}C]AT is incubated with microsomes in the presence of NADPH in air, the AT irreversibly binds to placental aromatase, being activated by the enzyme [26]. To further elucidate the aromatase inactivation process by AT, we synthesized [1β-^{3}H,4-^{14}C]-, [19-$^{3}H_3$,4-^{14}C]-, and [1β-^{3}H,19-^{14}C]ATs and incubated them separately with placental microsomes under various conditions. AT was converted into the 19-oxygenated steroids, 19-hydroxy-AT and 19-oxo-AT, as well as the aromatized products, 6-oxoestrone and 6-oxoestradiol. The aromatization was accompanied by a stereospecific elimination of the 1β-proton and a marked tritium isotope effect in the first hydroxylation at C-19. Two of the three protons of the 19-methyl group and the 1β-proton were retained in an aromatase-bound metabolite.

MATERIALS AND METHODS

Materials. [1β-^{3}H]Androst-4-ene-3,17-dione (androstenedione) (27.5 Ci/mmol; ^{3}H-distribution: 1β = 74–79%), [19-^{3}H]androstenedione (79.3 Ci/mmol), [19-^{14}C]androstenedione (60 mCi/mmol), and [4-^{14}C]androstenedione (51.4 mCi/mmol) were purchased from Du Pont–New England Nuclear (Boston, MA); NADPH was obtained from Kohjin Co. Ltd. (Tokyo, Japan). Silica gel coated thin-layer plates (Kiesel-gel 60-F_{254}, 10 × 10 cm, 0.25 mm thick) for TLC and HPTLC were purchased from E. Merck AG (Darmstadt, Germany), Sephadex G-50 was obtained from Pharmacia (Uppsala, Sweden), and molecular weight standards (Rainbow Marker) were from Amersham (Buckinghamshire, UK). Supplies for SDS–PAGE [mini-Protein-Ready gel, 12% (w/w) gel, 0.375 M Tris–HCl] were obtained from Bio-Rad (Richmond, CA).

AT, 19-hydroxy-AT, 19-oxo-AT, 3-hydroxyestra-1,3,5(10)-triene-6,17-dione (6-oxoestrone), and 3,17β-dihydroxyestra-1,3,5(10)-trien-6-one (6-oxoestradiol) were synthesized according to known methods [29, 30]. [4-^{14}C]AT (51 mCi/mmol) was prepared from [4-^{14}C]3β-hydroxyandrost-5-en-17-one according to the method described in a previous report [26].

High-performance liquid chromatography. The HPLC system consisted of a Waters model 510 pump (Tokyo, Japan), a Waters U6K injector, and a JASCO model 100-III UV detector (Tokyo, Japan) set at 240 nm. The analytical columns used were μ-Bondasphere C_{18} [(5 μm, 10 cm long, 3.9 mm i.d., Waters) and ERC-ODS-1161 (5 μm, 10 cm long, 6 mm i.d., Erma CR (Kanagawa, Japan)] stainless steel columns packed with reversed-phase C_{18} material or a μ-Porasil (10 μm, 30 cm long, 3.9 mm i.d., Waters) stainless steel column with normal phase silica. A mixture of acetonitrile and water served as the mobile phase for the reversed-phase HPLC and a mixture of cyclohexane and 1,4-dioxane for the normal phase. Retention time was expressed as t_R.

Preparation of placental microsomes. Human full-term placental microsomes (particles sedimenting at 105,000 *g* for 60 min) were obtained as described by Ryan [31]. They were washed twice with 0.5 mM dithiothreitol solution, lyophilized, and stored at −20°. No loss of aromatase activity occurred over the period of the study.

Preparation of ^{3}H,^{14}C-labeled AT. The double-labeled AT was synthesized essentially according to a method reported previously [32] (Fig. 1). ^{3}H- and ^{14}C-Labeled androstenediones were mixed and used for the AT synthesis without dilution with non-labeled steroid. To a solution of [1β-^{3}H,4-^{14}C]-, [19-$^{3}H_3$,4-^{14}C]-, or [1β-^{3}H,19-^{14}C]androstenedione (45–51 μg, 150–170 μmol; $^{3}H/^{14}C$ = 4.3 to 9.7, specific activities 50.5 to 59.4 mCi/mmol of ^{14}C and 253–489 mCi/mmol of ^{3}H) in *tert*-butanol (50 μL) was added a solution of potassium *tert*-butoxide (0.43 mg, 3.8 μmol) in *tert*-butanol (50 μL). The mixture was heated at 40° for 20 min in air, poured into water (500 μL) and extracted with ethyl acetate (3 mL × 2). The organic layer was washed with water (500 μL) and evaporated to dryness under a stream of nitrogen. The residue was purified by TLC (cyclohexane–ether, 1:2, v/v). The area corresponding to AT (R_f = 0.40) was scraped and eluted with ethyl acetate to yield the crude AT, which was purified further by HPLC (column: μ-Bondasphere; mobile phase: acetonitrile–water, 40:60, v/v; flow rate, 1 mL/min). The fraction corresponding to AT (t_R = 6.0 min) was collected. The radiochemical yield of the labeled AT was about 10%, and the radiochemical purity was determined to be more than 98% by both HPLC (column: ERC-ODS-1161; mobile phase: acetonitrile–water, 45:55, v/v; flow rate, 1 mL/min) and by a reverse isotope dilution method.

Incubation of labeled AT with placental microsomes. Incubations were carried out in air under two conditions. (A) To a test tube containing NADPH (600 μM) and microsomal protein (1 mg) in potassium phosphate buffer (67 mM, pH 7.5) (2 mL), a solution of the appropriate substrate in methanol (25 μL) was added. The mixture was incubated at 37° with reciprocal shaking. Each sample was incubated for 15, 30, 60 and 120 min. The substrates employed were as follows: 0.44 μM [1β-^{3}H,4-^{14}C]AT (^{3}H, 1×10^6 dpm; ^{14}C, 1.03×10^5 dpm), 0.44 μM [1β-^{3}H,19-^{14}C]AT (^{3}H, 5×10^5 dpm; ^{14}C, 1.17×10^5), and 0.54 μM [19-$^{3}H_3$,4-^{14}C]AT (^{3}H, 1×10^6 dpm; ^{14}C, 1.34×10^5 dpm). (B) [4-^{14}C]AT (0.42 μM, 3×10^5 dpm) was incubated with 3 mg of microsomal

* Abbreviations: AT, androst-4-ene-3,6,17-trione; 19-hydroxy-AT, 19-hydroxyandrost-4-ene-3,6,17-trione; 19-oxo-AT, androst-4-ene-3,6,17,19-tetraone; and HPTLC, high-performance TLC.

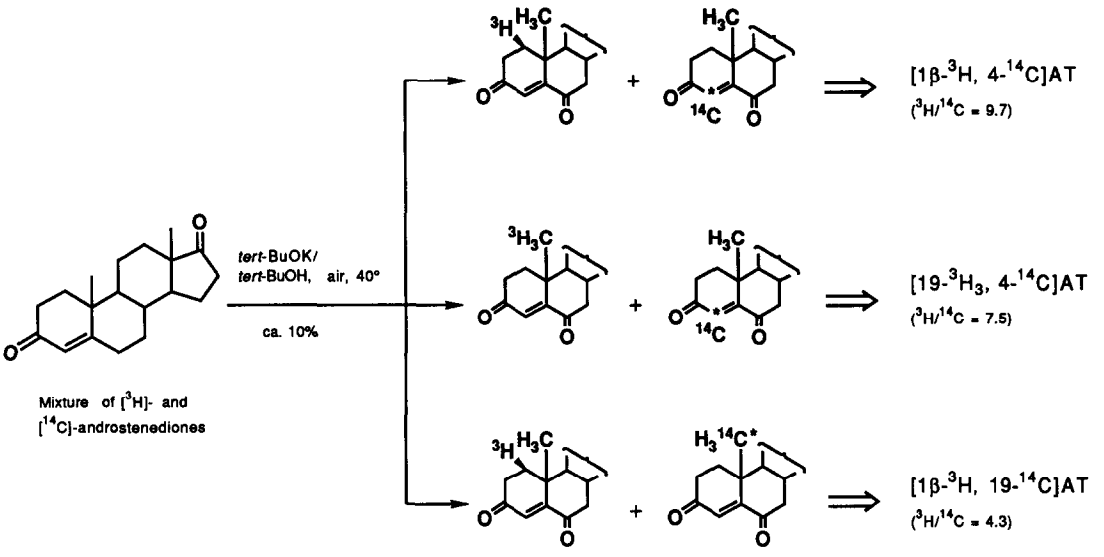

Fig. 1. Synthesis of double-labeled ATs.

protein, 600 μM NADPH, and 25 μL of methanol in 67 mM potassium phosphate buffer, pH 7.5, in a total volume of 6 mL in a 50-mL Erlenmeyer flask at 37° for 30 min.

[^{3}H]*Water and* [^{3}H]*formic acid assay.* [^{3}H]Water released into the incubation medium from the 1β-^{3}H-labeled AT was determined essentially according to the original procedure of Siiteri and Thompson [33]. Chloroform (3 mL) was added to the incubation mixture obtained under condition A (30-min incubation time) and the mixture was vortexed vigorously for 40 sec and centrifuged at 700 *g* for 10 min. To aliquots (1.5 mL) of the aqueous layer were added 500 μL of 24% (w/w) trichloroacetic acid solution and 500 μL of 5% (w/w) Norit A suspension, and the resulting mixture was incubated at 37° with shaking for 30 min. After removal of Norit A by filtration, 100-μL aliquots of the filtrate were mixed with 10 mL of scintillation fluid, and the radioactivity of tritium was counted by a Beckman LS 7800 liquid scintillation counter (Irvine, CA).

[^{3}H]Water and [^{3}H]formic acid released into the incubation mixture (30-min or 1-hr incubation time) from [19-^{3}H$_3$,4-^{14}C]AT were determined essentially according to the method reported by Miyairi and Fishman [10]. Briefly, the incubation mixture was acidified with 1 M phosphoric acid, frozen, and lyophilized. Radioactivity of an aliquot of the first distillate, which consisted of both the tritiated water and formic acid, was determined. Another aliquot was alkalized with 1 M sodium hydroxide solution and re-sublimed. The second distillate contained [^{3}H]water and the residue [^{3}H]sodium formate. The radioactivity of the first distillate was recovered completely in the tritiated water and formic acid fractions of the second lyophilization.

Identification procedure of free steroid metabolites. Steroid metabolites produced by the incubation of [1β-^{3}H,4-^{14}C]AT and [19-^{3}H$_3$,4-^{14}C]AT (condition A), or by incubation of [4-^{14}C]AT (condition B) were extracted with ethyl acetate (2 or 6 mL × 3) and washed with water (2 or 6 mL), respectively. The combined organic layers obtained from three (the double-label experiment) or eight (the ^{14}C-label experiment) incubations were concentrated under reduced pressure to about 200 μL and then subjected to two-dimensional HPTLC (solvent: 1st, chloroform–ethyl acetate, 1:1, v/v; 2nd, hexane–ethyl acetate, 3:4, v/v). Spots corresponding to 19-hydroxy-AT, 19-oxo-AT, 6-oxoestrone, and 6-oxoestradiol (detected by UV 245 nm irradiation) were scraped, and the steroid were eluted with ethyl acetate. After evaporation of the solvent, the residue was further purified by reversed-phase HPLC [column: μ-Bondasphere eluted at 1 mL/min with the indicated mobile phase; (a) acetonitrile–water, 40:60, v/v, for 19-hydroxy-AT (t_R 3.0 min); (b) 45:55, v/v, for 19-oxo-AT (t_R 3.6 min); (c) 30:70, v/v, for 6-oxoestrone (t_R 11.0 min) and 6-oxoestradiol (t_R 5.9 min)]. The isolated radioactive 19-hydroxy-AT, 6-oxoestrone, and 6-oxoestradiol were mixed with about 30 mg of the corresponding authentic samples and recrystallized to a constant specific activity. The 19-oxo-AT was analyzed further by normal phase HPLC (column: μ-Porasil; mobile phase: cyclohexane–1,4-dioxane, 45:55, v/v; flow rate, 1 mL/min; t_R, 4.2 min).

Gel chromatography of protein-bound metabolite. To the incubation mixture obtained under condition A (2-hr incubation time) was added 0.5 mL of 6% (w/w) sodium cholate solution in potassium phosphate buffer, pH 7.25 (final concentration of the detergent, 1.2%) [34]. The resulting mixture was shaken at 4° for 1 hr and centrifuged at 100,000 *g* for 1 hr. An aliquot (1 mL) of the supernatant was applied to a Sephadex G-50 column (void volume, 20 mL, 60 cm long × 1.1 cm i.d.) pre-equilibrated with 100 mM potassium phosphate buffer, pH 7.25, containing 1.2% (w/w) sodium cholate and eluted with the buffer used for the pre-equilibration. Aliquots (750 μL) of each fraction (1 mL) were counted.

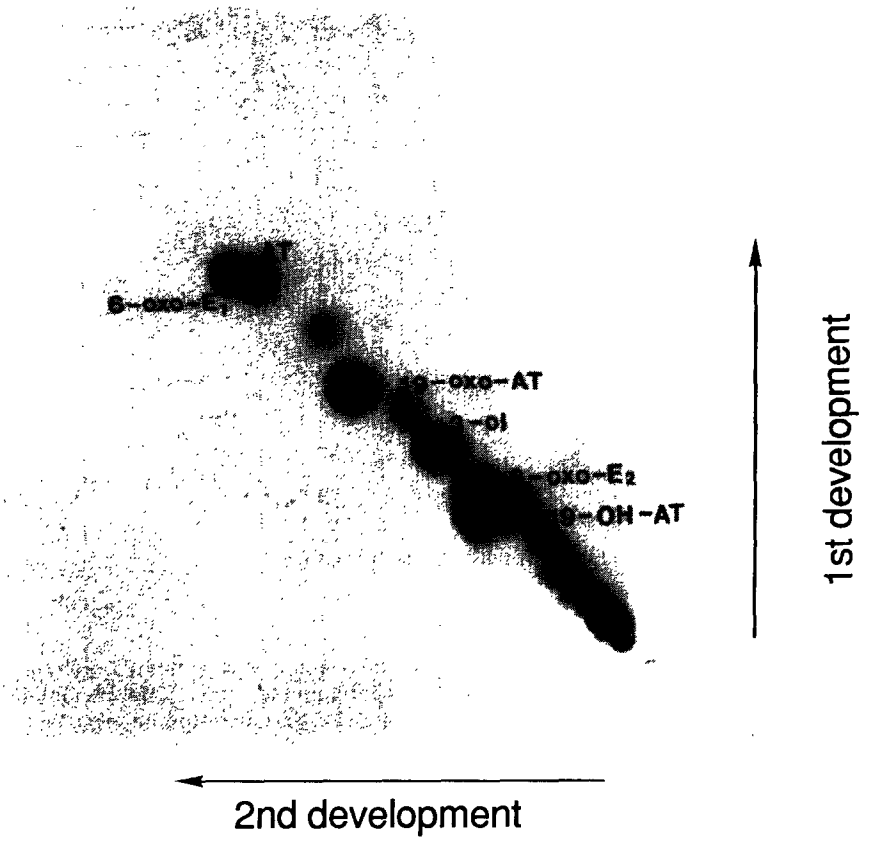

Fig. 2. Metabolites of AT by human placental microsomes. Shown is a two-dimensional high-performance TLC pattern following exposure to radioautography of the metabolites of [4-^{14}C]AT, obtained by ethyl acetate extraction of the incubated obtained under condition B. Solvent system: 1st development, chloroform–ethyl acetate, 1:1, v/v; 2nd development, hexane–ethyl acetate, 3:4, v/v.

SDS–PAGE of protein-bound metabolite. The solubilized protein-bound metabolite was concentrated to 100 μL by ultrafiltration (700 *g* for 12 hr) using Centricut 10 (Kurabo, Osaka, Japan). Discontinuous electrophoresis with a 1.5-mm thick slab gel was performed according to Laemmli [35]. The sample and protein standards were solubilized in 0.063 M Tris–HCl buffer, pH 6.8, containing 2% (w/w) SDS, and 40 mM mercaptoethanol by heating for 2 min at 95°. The slab gels were stained with Coomassie Blue R-250. The zone corresponding to molecular mass 46–69 kDa was cut off, the gel was solubilized in 500 μL of Solvable (Du Pont-New England Nuclear) at 50° for 3 hr, and the radioactivity was counted.

RESULTS

Identification of free steroid metabolites. A two-dimensional HPTLC pattern of the free steroid fraction, the ethyl acetate extract, is shown in Fig. 2. Recrystallizations of 19-hydroxy-AT, 6-oxoestrone, and 6-oxoestradiol from an appropriate solvent to a constant specific activity are shown in Table 1. 19-Oxo-AT could not be subjected to the reverse dilution method because of its instability under the recrystallization conditions. Therefore,

Table 1. Reverse isotope dilution analysis of ^{14}C-labeled metabolites of [4-^{14}C]AT by human placental microsomes*

Recrystallization		Specific activity (dpm/mg)
No.	From	
19-Hydroxy-AT		
2	Acetone	132
3	Acetone	129
4	MeOH-H_2O	132
6-Oxoestrone		
2	MeOH	2,150
3	MeOH	1,880
4	MeOH	1,900
6-Oxoestradiol		
2	MeOH	5,030
3	MeOH	4,710
4	MeOH-H_2O	4,760

* [4-^{14}C]AT was incubated with placental microsomes in the presence of NADPH at 37° for 30 min in air under condition B. The free metabolite fractions obtained from eight incubations were combined and purified by HPTLC and HPLC. The radioactive metabolite isolated, respectively, was diluted by the corresponding authentic sample (35 mg of 19-hydroxy-AT, 30 mg of 6-oxoestrone, or 33 mg of 6-oxoestradiol), and then repeatedly recrystallized from an appropriate solvent.

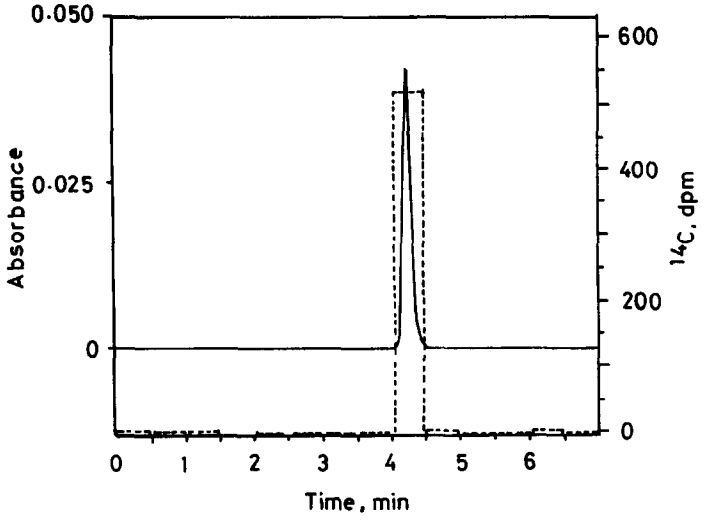

Fig. 3. Identification of [^{14}C]19-oxo-AT by normal phase HPLC. Conditions: column, μ-Porasil; mobile phase, cyclohexane–1,4-dioxane, 45:55, v/v (1 mL/min). Solid line: absorbance at 240 nm; dotted line, radioactivity.

Table 2. ^{3}H/^{14}C Ratios of 6-oxoestrogens and 19-hydroxy-AT produced from [^{3}H,^{14}C]ATs by incubation with human placental microsomes*

Entry	Substrate	Incubation time (min)	Metabolite	^{3}H/^{14}C	Percent of ^{3}H-retention†
A	[1β-^{3}H,4-^{14}C]AT (^{3}H/^{14}C = 9.7)	30	6-Oxoestradiol	2.07	21
			6-Oxoestradiol	2.01	21
			19-Hydroxy-AT	9.6	100
B	[19-^{3}H$_3$,4-^{14}C]AT (^{3}H/^{14}C = 7.5)	15	19-Hydroxy-AT	2.4	32
			Recovered AT	12.2	163
C	[19-^{3}H$_3$,4-^{14}C]AT (^{3}H/^{14}C = 7.5)	30	19-Hydroxy-AT	2.5	33
			Recovered AT	12.0	160

* [^{3}H,^{14}C]AT was incubated with placental microsomes in the presence of NADPH at 37° for 15 min or 30 min in air under condition A. The free metabolite fractions obtained from three incubations were combined, and the isolation of the metabolites and the determination of their specific activities (^{3}H/^{14}C ratios) were carried out in a manner similarly to that described in Table 1.

† Percent of ^{3}H-retention = [(^{3}H/^{14}C ratio of the metabolite)/(^{3}H/^{14}C ratio of the substrate) × 100].

this steroid was further identified by normal phase HPLC, which showed the same t_R as the authentic sample (Fig. 3). Since the recoveries of the metabolites during the isolation procedures, extraction, two-dimensional HPTLC and HPLC, were around 50% (45–56%) for each, their yields can be roughly estimated to be 0.20% for 19-hydroxy-AT, 0.09% for 19-oxo-AT, 2.4% for 6-oxoestrone, and 6.6% for 6-oxoestradiol.

Stereochemistry of the aromatization reaction of AT. [1β-^{3}H,4-^{14}C]AT (^{3}H/^{14}C = 7.4; ^{3}H-distribution, 74–79% at 1β and 26–21% at 1α) was incubated with placental microsomes in the presence of NADPH for 30 min. 6-Oxoestrone and 6-oxoestradiol were isolated similarly as above, and their ^{3}H/^{14}C ratios were obtained by the isotope dilution method (Table 2, entry A). The theoretical tritium percentage in the 6-oxoestrogen is between 21 and 26% of the AT, if the aromatization reaction proceeds through the stereospecific 1β-proton elimination. Our observed retention was 21%, as calculated from the isotope ratios of the 6-oxoestrogens. This change of the ratio is consistent with the stereospecific 1β-proton elimination in the aromatization of AT. On the other hand, the ^{3}H/^{14}C ratio of the 19-hydroxy-AT produced was almost the same as that of the AT.

We also carried out a series of 15- and 30-min incubations of [19-^{3}H$_3$,4-^{14}C]AT (^{3}H/^{14}C = 7.5) to explore whether a tritium isotope effect operates in the first 19-hydroxylation of AT (Table 2, entries B and C). The loss of one tritium atom from C-19 should, in the absence of the isotope effect, result in 19-hydroxy-AT with a ^{3}H/^{14}C ratio of 5.0 (67%

Table 3. [^{3}H]Water and [^{3}H]formic acid produced from [^{3}H,^{14}C]ATs by incubation with human placental microsomes*

Entry	Substrate	Incubation conditions: Treatment	Incubation conditions: Time (min)	Radioactivity,† dpm (%): [^{3}H]Water	Radioactivity,† dpm (%): [^{3}H]Formate
D	[1β-^{3}H,4-^{14}C]AT	Complete‡	30	78,000 ± 5,910 (7.8)	
		Complete	60	90,900 ± 5,580 (9.1)	
		−NADPH	30	2,600 ± 110 (0.3)	
		+4.4 μM AD§	30	25,400 ± 2,180 (2.5)	
E	[19-^{3}H$_3$,4-^{14}C]AT	Complete	30	16,100 ± 1,330 (1.6)	8,200 ± 610 (0.82)
		Complete	60	25,800 ± 2,440 (2.6)	13,300 ± 1,100 (1.3)
		−NADPH	30	2,500 ± 140 (0.25)	1,100 ± 80 (0.11)
		+5.4 μM AD	30	5,300 ± 220 (0.52)	2,500 ± 170 (0.25)

* [1β-^{3}H,4-^{14}C]- or [19-^{3}H$_3$,4-^{14}C]AT was incubated with placental microsomes in the presence or absence of NADPH or androstenedione.

† Values are means ± SD (N = 4). The mean production rate of [^{3}H]water or [^{3}H]formic acid is expressed as a percentage in parentheses.

‡ Complete: complete incubation system of condition A.

§ AD: androstenedione.

tritium retention). The ratio of 19-hydroxy-AT isolated in each experiment was found to be about 2.5 (33% tritium retention), indicating the presence of a significant tritium isotope effect in the hydroxylation. The existence of this isotope effect was also apparent from the isotope ratio of the recovered AT (^{3}H/^{14}C = *ca.* 12), which was significantly greater than the initial ratio of 7.5.

Determination of [^{3}H]*water and* [^{3}H]*formic acid release from* [1β-^{3}H,4-^{14}C]*AT and* [19-^{3}H$_3$,4-^{14}C]*AT.* The double-labeled ATs were incubated separately with the placental microsomes under the same conditions used for the steroidal metabolite identification experiments. [^{3}H]Water released from [1β-^{3}H,4-^{14}C]AT was determined according to a known method using chloroform extraction [33] (Table 3, entry D). On the other hand, assays of [^{3}H]water and [^{3}H]formic acid produced from [19-^{3}H,4-^{14}C]AT were done by the lyophilization method [10] (Table 3, entry E). Radioactivity of the distillate obtained by the first lyophilization of the incubation mixture under acidic conditions was recovered almost completely in the [^{3}H]water and [^{3}H]formate fractions. The production of radioactive water or the labeled formic acid increased in proportion to the incubation time and required NADPH,

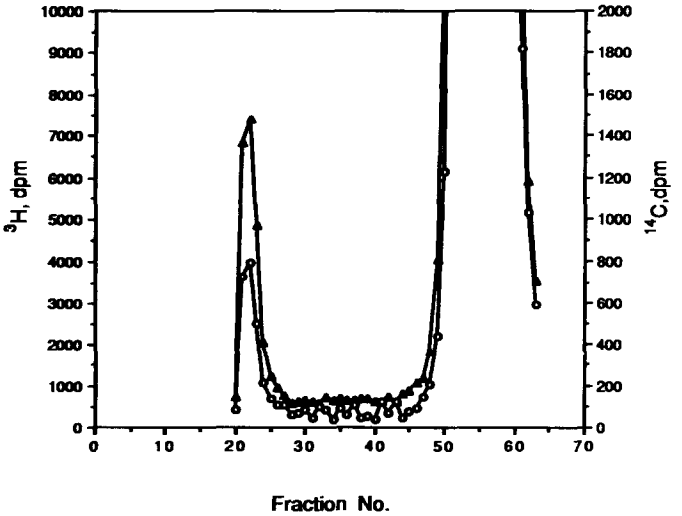

Fig. 4. Sephadex G-50 column chromatography of the solubilized microsomal proteins obtained by incubation with [1β-^{3}H,4-^{14}C]AT under condition A. Key: (▲) ^{3}H, (○) ^{14}C. Essentially the same chromatogram was obtained in the experiment with [19-^{3}H$_3$,4-^{14}C]AT (data not shown).

respectively. The natural substrate, androstenedione, efficiently prevented the production of tritiated water and formate. The [^{3}H]water production from the [1β-^{3}H]AT was comparable to the estrogen production (9.5%) under the same condition, indicating that the 1β-^{3}H elimination observed was dependent primarily on the aromatization reaction. The [^{3}H]water to [^{3}H]formic acid ratio was almost 2. The fact that the production rates of the two labeled substances from the [19-$^3H_3$]AT were less than that of [^{3}H]water from the [1β-^{3}H]AT also demonstrates that a tritium isotope effect was involved in the first 19-oxygenation.

Protein-binding of labeled AT during aromatase inactivation. [1β-^{3}H,4-^{14}C]- or [19-$^3H_3$,4-^{14}C]AT (^{3}H/^{14}C = 9.7 or 7.5) was incubated with placental microsomes in the presence or absence of NADPH for 2 hr, and the microsomal proteins solubilized by sodium cholate were applied to gel filtration using a Sephadex G-50 column. Essentially two radioactive peaks were observed in the fractions of protein-bound metabolite (void volume, fractions 20–24) and steroidal metabolites (fractions 46–65) (Fig. 4). The radiochemical yield of the bound metabolite, based on ^{14}C, was about 2% in the presence of NADPH but 0.5% in the absence of it in each experiment. The ^{3}H/^{14}C ratio of the metabolite changed only in the experiment with [19-$^3H_3$,4-^{14}C]-AT in the presence of NADPH (from 7.5 to 4.3), whereas there was no significant difference of the ratio between the bound metabolite and the inhibitor in the other experiments.

Not only aromatase but also other proteins of the microsomes are involved in the formation of the protein-bound metabolite. The solubilized microsomal proteins obtained by incubations with [1β-^{3}H,19-^{14}C]AT in addition to the two labeled enzymes mentioned above, after concentration by ultrafiltration, were subjected to SDS–PAGE, and the ^{3}H/^{14}C ratio of the zone corresponding to aromatase (46–69 kDa) was analyzed (Table 4). Based on the radioactivity of ^{14}C, aromatase-bound metabolite was produced in about a 0.1% yield in each experiment with NADPH. As expected, its production required NADPH and was prevented by the addition of androstenedione. The ^{3}H/^{14}C ratio of the bound metabolite produced in experiments using [1β-^{3}H,4-^{14}C]AT and [1β-^{3}H,19-^{14}C]AT was almost the same as that of the labeled ATs used, respectively, similarly described as above, showing that the 19-carbon atom and the 1β-proton remain in the steroid-aromatase adduct. Moreover, in the experiment with [19-$^3H_3$,4-^{14}C]AT, the isotope ratio dropped to 2.8 from 7.5 of that of the AT. When non-specific binding observed in the experiment without NADPH was subtracted from the binding with the cofactor, the ratio was 1.7. The loss of two of three tritiums at C-19 through two successive oxygenations, in the absence of an isotope effect, should result in 19-oxo-AT with a ^{3}H/^{14}C ratio of 2.5. Considering the marked isotope effect observed in the first hydroxylation at C-19 of AT, the ratio of 1.7 very likely corresponds to the loss of two tritiums at C-19.

DISCUSSION

We have synthesized [1β-^{3}H,4-^{14}C]-, [19-$^3H_3$,4-^{14}C]-, and [1β-^{3}H,19-^{14}C]ATs with high specific activities, by air oxidation of a mixture of the corresponding ^{3}H- and ^{14}C-labeled androstenediones (Fig. 1). Their structures have been established by chromatographic behavior and a reverse dilution method. There was no significant difference in the ^{3}H/^{14}C ratio between the starting material and the product in each, indicating that no loss of ^{3}H-atoms occurred during the synthetic procedure.

19-Hydroxy-AT, 19-oxo-AT, and the parent inhibitor, AT, inactivate aromatase in human placental microsomes in a time-dependent manner. Therefore, it has been postulated that oxygenation at the C-19 would be involved in the time-dependent inactivation [22, 29]. However, metabolic aspects, except for the conversion into the 3β-reduced derivative of AT, 3β-hydroxy-androst-4-ene-3,17-dione [26], in the placental microsomes, have remained unknown. A detailed analysis (by two-dimensional HPTLC, HPLC and a reverse dilution method) of the metabolites produced from [4-^{14}C] AT by incubation with microsomes in the presence of NADPH unambiguously identified for the first time the aromatized products, 6-oxoestrone and 6-oxoestradiol, along with the 19-oxygenated intermediates, 19-hydroxy-AT and 19-oxo-AT. As shown in Fig. 2, in addition to the four metabolites, the production of other unidentified metabolites was also observed. It is presumed that 17β-reduced derivatives of the 19-oxygenated ATs may also be produced. However, they could not be identified because their authentic samples are unavailable. About 50% of the radioactivity of the substrate was recovered in the unidentified metabolite fractions on TLC.

We then explored the molecular events that occur in the aromatization of AT. The present results (Table 2) show that the AT aromatization process involves the stereospecific elimination of the 1β-proton and proceeds with a marked tritium isotope effect in the first hydroxylation at C-19. The elimination is similar to that in the androstenedione aromatization, although the extent of the isotope effect observed in this study is somewhat smaller [10]. On the other hand, PM3 molecular orbital calculations of the conformational features of the 19-oxygenated ATs suggest that the stereospecific pro-R-proton elimination may occur in the oxygenation of 19-hydroxy-AT to give the 19-oxo derivative [36], in a manner similar to a series of androstenedione derivatives [6, 7]. These facts led us to presume that the same molecular events would occur in the enzymatic aromatization of both AT and androstenedione. Beusen *et al.* [9] have also reported that the aromatization of an alternate substrate, androsta-4,6-diene-3,17-dione, proceeds through the same mechanism as that of androstenedione. Furthermore, Osawa *et al.* [37] recently demonstrated metabolic switching for the first 19-hydroxylation of androstenedione; as much as 20–30% of [19-$^3H_3$]androstenedione is diverted to non-estrogenic 1β- and 2β-hydroxylated products, whereas only a small percentage of the 4-^{14}C-labeled substrate is diverted. Thus, it is likely that the metabolic switching, as well as the isotope effect,

Table 4. Radioactivity analysis of protein-bound or aromatase-bound metabolite*

[^{3}H,^{14}C]AT	Incubation conditions	Bound metabolite		
		^{3}H (dpm)	^{14}C (dpm)	^{3}H/^{14}C (range)
Gel filtration method (protein-bound metabolite)				
[1β-^{3}H,4-^{14}C]AT	Complete†	21,805	2,255	9.7 (9.2–10.4)
(^{3}H/^{14}C = 9.7)	−NADPH	4,605	536	8.6 (8.2–9.0)
[19-^{3}H$_3$,4-^{14}C]AT	Complete	7,007	1,570	4.5 (3.9–5.0)
(^{3}H/^{14}C = 7.5)	−NADPH	3,500	498	7.0 (6.7–7.5)
	Complete minus −NADPH	3,507	1,072	3.2‡
SDS–PAGE method (aromatase-bound metabolite)				
[1β-^{3}H,4-^{14}C]AT	Complete	955	96	10.0 (9.3–10.8)
(^{3}H/^{14}C = 9.7)	−NADPH	153	15	10.0 (9.6–10.4)
	+4.4 μM AD§	344	39	8.8 (8.4–9.6)
[19-^{3}H$_3$,4-^{14}C]AT	Complete	270	95	2.8 (2.5–3.1)
(^{3}H/^{14}C = 7.5)	−NADPH	140	19	7.4 (7.0–7.9)
	+5.4 μM AD	165	30	5.5 (4.8–6.0)
	Complete minus −NADPH	130	76	1.7
[1β-^{3}H,19-^{14}C]AT	Complete	486	97	5.0 (4.1–5.3)
(^{3}H/^{14}C = 4.3)	−NADPH	81	18	4.5 (4.0–4.9)
	+5.4 μM AD	160	37	4.3 (4.2–4.7)

* The [^{3}H,^{14}C]AT was incubated with placental microsomes in the presence or absence of NADPH or androstenedione. Protein-bound metabolite and aromatase-bound metabolite fractions were obtained by Sephadex G-50 gel chromatography and SDS–PAGE, respectively. Values are expressed as means of three determinations, and the ranges of the ^{3}H/^{14}C ratios of the bound metabolites are indicated in parentheses.

† Complete: complete incubation system of condition A.

‡ The radioactivity observed in the complete system of condition A minus that of the non-specific binding observed without NADPH.

§ AD: androstenedione.

may be operative in the first hydroxylation at C-19 of AT, although there is no direct evidence.

We have reported previously that the NADPH-dependent production of the protein-bound metabolite of AT is prevented by anti-aromatase monoclonal antibody, yielding the non-specific binding level observed without NADPH or with androstenedione, and that the non-specific binding would depend mainly on a chemical 1,4-addition reaction of AT with a nucleophile, such as a thiol of the microsomal protein [26]. The fact that there was no significant difference in the ^{3}H/^{14}C ratio between the protein-bound or aromatase-bound metabolite produced by the non-specific binding and the labeled AT used further confirms this (Table 4). Thus, since the 46–69 kDa zone in the SDS–PAGE of the solubilized microsomal proteins contains not only aromatase but also other proteins, the radioactivity of this zone observed in the presence of NADPH should consist of the mechanism-based binding as well as the non-specific binding to other proteins. In the androstenedione addition experiment with [19-^{3}H$_3$,4-^{14}C]AT and NADPH, the isotope ratio (^{3}H/^{14}C = 5.5) of the aromatase-bound metabolite could not be completely recovered to that of the used AT (^{3}H/^{14}C = 7.4). This was probably due to the metabolic elimination of the added steroid during the incubation.

Covey and Hood [38] have previously postulated the mechanism of the aromatase inactivation by AT by comparison with the proposed mechanism of the androstenedione aromatization; the third oxygenation of 19-oxo-AT, produced from AT by two sequential hydroxylations at C-19, occurs at the C-2 position to liberate the 19-methyl as formic acid accompanied with the attachment of aromatase to the C-4 position through its nucleophilic residue, followed by the stereospecific elimination of the 1β-proton. However, the present results showing that the 1β-proton, the 19-carbon atom, and one of three protons at C-19 of AT are retained in the steroid-aromatase adduct clearly indicate that the postulated mechanism is incorrect.

When an enzyme catalyzes the conversion of a suicide substrate to its reactive form, there are two processes that can follow. The altered form can be released as a product or it can react with the enzyme in an irreversible manner to result in inactivation of the enzyme [39]. Thus, mechanisms for the aromatization of AT and for aromatase inactivation by the steroid are proposed (see Fig. 5). The sequential two hydroxylations initially occur at C-19 to give 19-oxo-AT through 19-hydroxy-AT. 19,19-Hydroxyferric peroxide intermediates probably are formed from 19-oxo-AT and/or its enol form, a 3-hydroxy-2,4-dien-6-one, by the third oxygenation [15–17]. Having a 2,4-dien-3-ol structure, the 19-peroxide intermediate would be converted principally into 6-oxoestrone in a fashion similar to that proposed in the aromatization of 19-oxoandrostenedione into

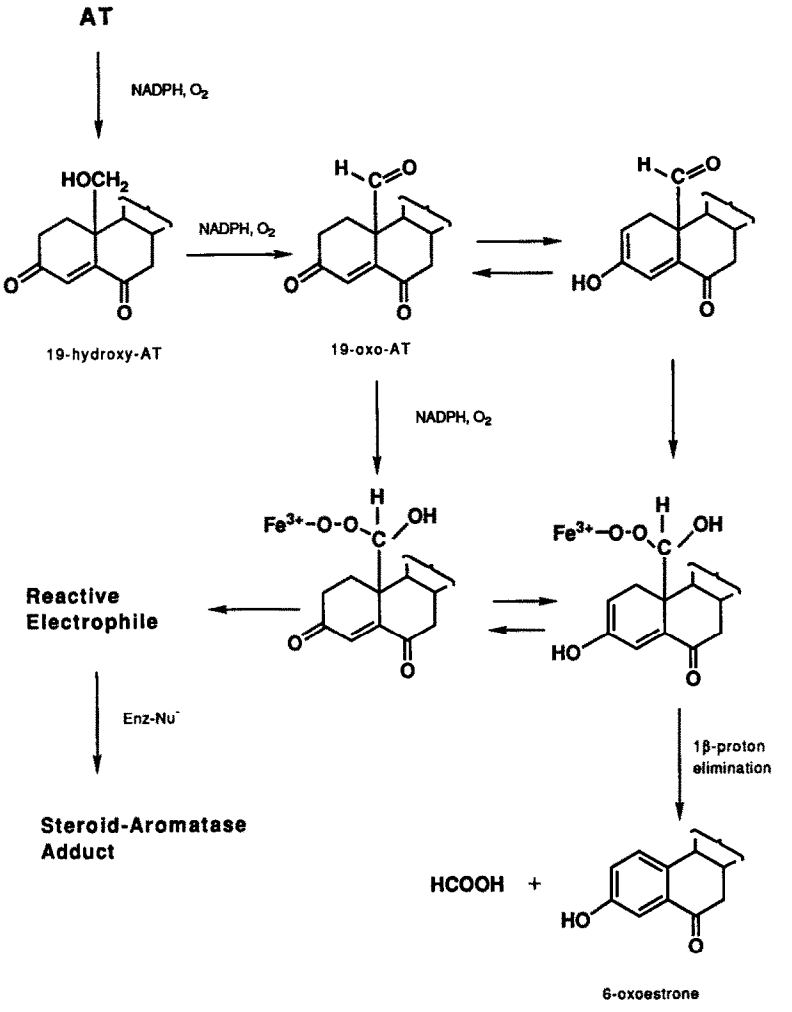

Fig. 5. Proposed mechanisms for the aromatization of AT and for aromatase inactivation by AT.

estrone [14, 40, 41]. Furthermore, the peroxides may be involved, at least in part, in the formation of a reactive electrophile which immediately reacts with the active site of aromatase to result in the irreversible inactivation of the enzyme. Study on the characterization of the electrophile is now underway in our laboratory.

Acknowledgement—This work was supported in part by a Grant-in-Aid for Scientific Research from The Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. Thompson EA Jr and Siiteri PK, The involvement of human placental microsomal cytochrome P-450 in aromatization. *J Biol Chem* **249**: 5373–5378, 1974.
2. Kellis J Jr and Vickery LE, Purification and characterization of human placental aromatase cytochrome P-450. *J Biol Chem* **262**: 4413–4420, 1987.
3. Yoshida N and Osawa Y, Purification of human placental aromatase cytochrome P-450 with monoclonal antibody and its characterization. *Biochemistry* **30**: 3003–3010, 1991.
4. Thompson EA and Siiteri PK, Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. *J Biol Chem* **249**: 5364–5372, 1974.
5. Meyer AS, Conversion of 19-hydroxy-Δ-4-androstene-3,17-dione to estrone by endocrine tissue. *Biochim Biophys Acta* **17**: 441–442, 1955.
6. Arigoni D, Battaglia R, Akhtar M and Smith T, Stereospecificity of oxidation at C-19 in oestrogen biosynthesis. *J Chem Soc, Chem Commun* 185–187, 1975.
7. Osawa Y, Shibata K, Rohrer D, Weeks C and Duax WL, Reassignment of the absolute configuration of 19-substituted 19-hydroxysteroids and stereomechanism of estrogen biosynthesis. *J Am Chem Soc* **97**: 4400–4402, 1975.
8. Caspi E, Arunachalam T and Nelson PA, Biosynthesis of estrogens: Aromatization of (19*R*)-, (19*S*)-, and (19*RS*)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-ones by human placental aromatase. *J Am Chem Soc* **108**: 1847–1852, 1986.
9. Beusen DD, Carrell ML and Covey DF, Metabolism of 19-methyl-substituted steroids by human placental aromatase. *Biochemistry* **26**: 7833–7841, 1987.

10. Miyairi S and Fishman J, Radiometric analysis of oxidative reactions in aromatization by placental microsomes: Presence of differential isotope effects. *J Biol Chem* **260**: 320–325, 1985.
11. Akhtar M, Calder MR, Corina DL and Wright JN, Mechanistic studies on C-19 demethylation in oestrogen biosynthesis. *Biochem J* **201**: 569–580, 1982.
12. Brodie HJ, Kripalani KJ and Possaza G, Studies on the mechanism of estrogen biosynthesis. VI. The stereochemistry of hydrogen elimination of C-2 during aromatization. *J Am Chem Soc* **91**: 1241–1242, 1969.
13. Fishman J, Guzik H and Dixon D, Stereochemistry of estrogen biosynthesis. *Biochemistry* **8**: 4304–4309, 1969.
14. Cole PA and Robinson CH, Conversion of 19-oxo[2β-^{2}H]androgens into oestrogens by human placental aromatase. An unexpected stereochemical outcome. *Biochem J* **268**: 553–561, 1990.
15. Akhtar M, Corina D, Pratt J and Smith T, Studies on the removal of C-19 in oestrogen biosynthesis using $^{18}O_2$. *J Chem Soc, Chem Commun* 854–856, 1976.
16. Cole PA and Robinson CH, A peroxide model reaction for placental aromatase. *J Am Chem Soc* **110**: 1284–1285, 1988.
17. Stevenson DE, Wright JN and Akhtar M, Mechanistic consideration of P-450 dependent enzymic reactions: Studies on oestriol biosynthesis. *J Chem Soc, Perkin Trans I* 2043–2052, 1988.
18. Hahn EF and Fishman J, Immunological probe of estrogen biosynthesis. Evidence for the 2β-hydroxylative pathway in aromatization of androgens. *J Biol Chem* **259**: 1689–1694, 1984.
19. Caspi E, Dharmaratne HRW, Roitman E and Shackleton C, Estrogen biosynthesis: 2β-hydroxy-19-oxoandrost-4-ene-3,17-dione revisited. *J Chem Soc, Perkin Trans I* 1191–1195, 1993.
20. Harvey HA, Lipton A and Santen R, New perspectives for breast cancer. *Cancer Res* (Suppl 42): 3261s–3269s, 1982.
21. Brodie AMH, Coombes RC and Dowsett M, Aromatase inhibitors: Their biochemistry and clinical potential. *J Steroid Biochem* **27**: 899–903, 1987.
22. Covey DF, Aromatase inhibitors: Specific inhibitors of oestrogen biosynthesis. In: *Steroid Biosynthesis Inhibitors: Pharmaceutical and Argochemical Aspects* (Eds. Berg D and Plemel M), pp. 534–571. Ellis Horwood, Chichester, England, 1988.
23. Marcotte PA and Robinson CH, Inhibition and inactivation of estrogen synthetase (aromatase) by fluorinated substrate analogues. *Biochemistry* **21**: 2773–2778, 1982.
24. Furth PS and Robinson CH, Tritium release from [19-^{3}H]19,19-difluoroandrost-4-ene-3,17-dione during inactivation of aromatase. *Biochemistry* **28**: 1254–1259, 1989.
25. Covey DF and Hood WF, Enzyme-generated intermediates derived from 4-androstene-3,6,17-trione and 1,4,6-androstatriene-3,17-dione cause a time-dependent decrease in human placental aromatase activity. *Endocrinology* **108**: 1597–1599, 1981.
26. Numazawa M, Tsuji M and Mutsumi A, Studies on aromatase inhibition with 4-androstene-3,6,17-trione: Its 3β-reduction and time-dependent irreversible binding to aromatase with human placental microsomes. *J Steroid Biochem* **28**: 337–344, 1987.
27. Numazawa M, Mutsumi A and Tsuji M, 3β-Hydroxyandrost-4-en-6-one derivatives as aromatase inhibitors. *Steroids* **54**: 299–311, 1989.
28. Numazawa M, Mutsumi A and Kigawa H, Competitive inhibition and suicide inactivation of human placental aromatase by androst-4-ene-3,6-dione derivatives and 3α-methoxyandrost-4-ene-6,17-dione. *Chem Pharm Bull (Tokyo)* **38**: 3076–3080, 1990.
29. Numazawa M, Mutsumi A, Hoshi K, Kigawa H and Oshibe M, A time-dependent inactivation of aromatase by 19-oxygenated androst-4-ene-3,6,17-triones. *J Steroid Biochem Mol Biol* **39**: 959–966, 1991.
30. Burrows PE, Jones SL and Smith HE, 6α- and 6β-Hydroxyestradiol. Circular dichroism and substantiation of configurational assignments. *J Org Chem* **38**: 3797–3798, 1973.
31. Ryan KJ, Biological aromatization of steroids. *J Biol Chem* **234**: 268–272, 1959.
32. Marsh DA, Brodie HJ, Garrett W, Tsai-Morris C-H and Brodie AMH, Aromatase inhibitors. Synthesis and biological activity of androstenedione derivatives. *J Med Chem* **28**: 788–795, 1985
33. Siiteri PK and Thompson EA, Human placental aromatase. *J Steroid Biochem* **6**: 317–322, 1975.
34. Mendelson CR, Wright EE, Evans CT, Porter JC and Simpson ER, Preparation and characterization of polyclonal and monoclonal antibodies against human aromatase cytochrome P-450 ($P\text{-}450_{AROM}$), and their use in its purification. *Arch Biochem Biophys* **243**: 480–491, 1985.
35. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
36. Numazawa M, Oshibe M and Matsuzaki H, 1,4-Addition reaction with thiols and conformational analysis with PM3 molecular orbital calculations of 19-oxygenated androst-4-ene-3,6,17-triones. *Steroids*, **58**: 423–428, 1993.
37. Osawa Y, Yoshida N, Fronckowiak M and Kitawaki J, Immunoaffinity purification of aromatase cytochrome P-450 from human placental microsomes, metabolic switching from aromatization to 1β and 2β monohydroxylation, and recognition of aromatase isozymes. *Steroids* **50**: 11–28, 1987.
38. Covey DF and Hood WF, A new hypothesis based on suicide substrate inhibitor studies for the mechanism of action of aromatase. *Cancer Res* (Suppl 42): 3327s–3333s, 1982.
39. Silverman RB, Rate constant and dissociation constant terminology. In: *Mechanism-Based Enzyme Inactivator: Chemistry and Enzymology* (Ed. Silverman RB), Vol. 1, pp. 4–5. CRC Press, Boca Raton, 1988.
40. Cole PA and Robinson CH, Synthesis and reactivity studies with 19-peroxide-androstenedione derivatives: Analogues of a proposed aromatase intermediate. *J Chem Soc, Perkin Trans I* 2119–2125, 1990.
41. Cole PA and Robinson CH, Mechanistic studies on a placental aromatase model reaction. *J Am Chem Soc* **113**: 8130–8137, 1991.