Aromatase Reaction of 3-Deoxyandrogens: Steric Mode of the C-19 Oxygenation and Cleavage of the $C_{10}-C_{19}$ Bond by Human Placental Aromatase†

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ABSTRACT: Aromatase is a cytochrome P-450 enzyme complex that catalyzes the conversion of androst-4-ene-3,17-dione (AD) to estrone and formic acid through three sequential oxygenations of the 19-methyl group. To gain insight into the catalytic function of aromatase as well as the mechanism of the hitherto uncertain third oxygenation step, we focused on the aromatase-catalyzed 19-oxygenation of 3-deoxyandrogens: 3-deoxy-AD (**1**), which is a very powerful competitive inhibitor but poor substrate of aromatase, and its 5-ene isomer **4**, which is a good competitive inhibitor and effective substrate of the enzyme. In incubations of their 19*S*-^{3}H-labeled 19-hydroxy derivatives **2** and **5** and the corresponding 19*R*-^{3}H isomers with human placental microsomes in the presence of NADPH under air, the radioactivity was liberated in both water and formic acid. The productions of ^3H_2O and ^{3}HCOOH were blocked by the substrate AD or the inhibitor 4-hydroxy-AD, indicating that these productions are due to a catalytic function of aromatase. A comparison of the ^3H_2O production from *S*-^{3}H substrates **2** and **5** with that from the corresponding *R*-^{3}H isomers revealed that the 19-*pro-R* hydrogen atom was stereospecifically (*pro-R*:*pro-S* = 100:0) removed in the conversion of 5-ene substrate **5** into the 19-oxo product **6**, whereas 75:25 stereoselectivity for the loss of the *pro-R* and *pro-S* hydrogen atoms was observed in the oxygenation of the other substrate, **2**. The present results reveal that human placental aromatase catalyzes three sequential oxygenations at C-19 of 3-deoxyandrogens **1** and **4** to cause the cleavage of the $C_{10}-C_{19}$ bond through their 19-hydroxy (**2** and **5**) and 19-oxo (**3** and **6**) intermediates, respectively, where there is a difference in the stereochemistry between the two androgens in the second 19-hydroxylation. It is implied that the aromatase-catalyzed 19-oxygenation of 5-ene steroid **4** but not the 4-ene isomer **1** would proceed in the same steric mechanism as that involved in the AD aromatization.

Aromatase is the cytochrome P-450 enzyme that mediates the biosynthesis of estrogens from androgen precursors (*1*−*3*). The aromatase reaction was shown to involve three oxygenation steps, each of which requires 1 mol of O_2 and 1 mol of NADPH (*4*−*8*) (Figure 1). The first 19-methyl oxygenation occurs with retention of configuration (*9*, *10*). The second hydroxylation reaction, which removes stereospecifically the 19-*pro-R*-hydrogen of the 19-alcohol (*11*, *12*), yields a 19,19-*gem*-diol that chemically eliminates water to give the readily isolated 19-aldehyde. A tritium isotope effect in the first but not in the second hydroxylation is observed in the aromatization of [19-^{3}H,4-^{14}C]AD[1] (*9*, *10*, *13*). In the case of AD, following the third oxygenation step, C-19 and 1β,2β-hydrogens are eliminated as formic acid and water, respectively, to produce estrone. The resulting formic acid retains the 19-*pro-S* hydrogen of the parent 19-alcohol. It is now thought that a substrate-dependent variation occurs in the stereospecificity of the elimination of the C-2 hydrogen (*14*). Considerable speculation continues as to the site and mechanism of attack of the third mole of O_2. 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 (*7*, *15*, *16*). It is proposed that enolization of a carbonyl group at C-3 of the intermediate toward the C-2 position would be a prerequisite for the cleavage of the $C_{10}-C_{19}$ bond (*7*, *16*−*20*).

Aromatase has received considerable attention over the last 40 years, with much effort directed at the development of inhibitors to be used in the treatment of estrogen-dependent breast cancer (*21*−*24*). For this reason, various substrate analogues have been tested in a number of laboratories as the inhibitors. We previously reported that androst-4-en-17-one (**1**) (*25*, *26*), the 3-deoxy analogue of AD, and its 5-ene isomer **4** (*27*) are excellent and good competitive inhibitors of human placental aromatase, respectively, although they are lacking a carbonyl group at C-3, which has been thought to be essential for a proper binding of the substrate to the active site of aromatase (*7*, *17*−*20*, *28*) (Figure 2). It has also been reported that 3-deoxysteroids **1** and **4** are oxygenated with aromatase to produce the corresponding 19-alcohols **2** and **5** and 19-aldehydes **3** and **6** where the less powerful inhibitor **4** is a good substrate for the 19-oxygenation whereas the more powerful inhibitor **1** is a poor substrate (*29*). On the other

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[1] Abbreviations: AD, androstenedione (androst-4-ene-3,17-dione); TLC, thin-layer chromatography.

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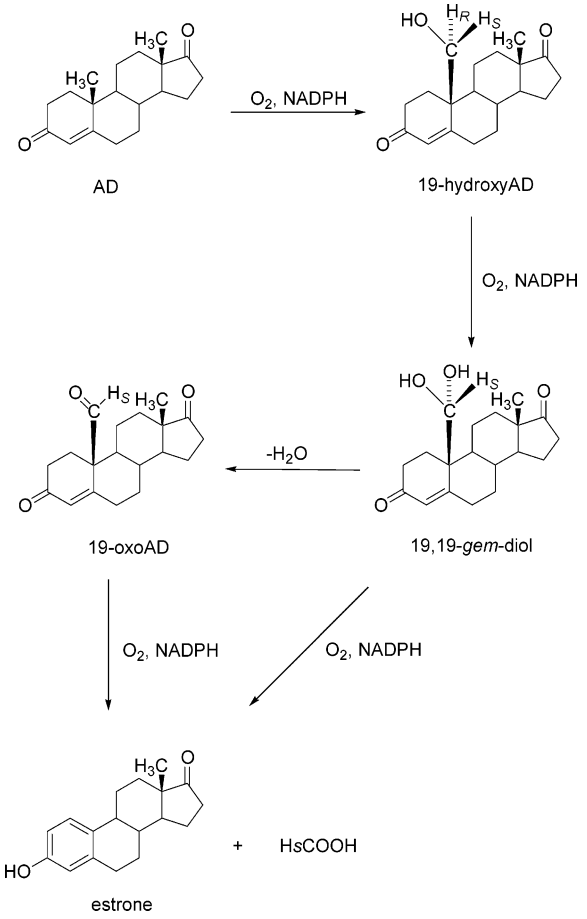

FIGURE 1: Aromatization sequence of AD with human placental aromatase.

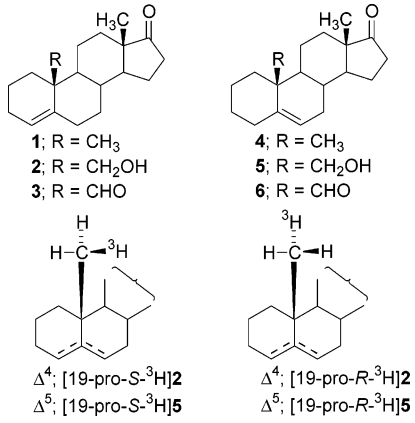

FIGURE 2: Structures of 3-deoxyandrogens and stereochemistry of [19-^{3}H]-19-alcohols **2** and **5**.

hand, the introduction of a hydroxy group at C-19 of 4-ene steroid **1** enhances the affinity to the active site of aromatase (*25*). In contrast, the same molecular modification of 5-ene steroid **4** markedly decreases the affinity (*27*), as evident in the AD series (*2*, *3*). The structure−activity relationships of the 3-deoxysteroid analogue with these previous findings reveal that there is a difference in the binding manner in the active site between the 4-ene and 5-ene series of 3-deoxysteroids.

Taken together, 3-deoxyandrogens seem to play an important role as probes in elucidating not only the spatial aspects of the interactions of the inhibitors (substrate) to the binding (active) site but also the mechanisms of the catalytic function of aromatase. This paper describes the determination of the stereochemistry of the hydroxylation at C-19 of 19-alcohols **2** and **5** as well as the identification of the cleavage reaction of the $C_{10}-C_{19}$ bond by further oxygenation, using human placental microsomes as a source of aromatase and 19*S*-^{3}H-labeled 19-alcohols **2** and **5** as well as their 19*R*-^{3}H isomers as the substrates. 19-*pro*-*R*-Hydrogen was removed stereospecifically in the case of the 5-ene substrate **5** or stereoselectively (*pro*-*R*:*pro*-*S* = 75:25) in the other case. The $C_{10}-C_{19}$ bond cleavage reaction, yielding formic acid, was identified in the aromatase reaction of both substrates.

EXPERIMENTAL PROCEDURES

Materials. The 19*S*-^{3}H-labeled 4-ene- and 5-ene-19-alcohols **2** (0.61 mCi/mmol) and **5** (0.39 mCi/mmol) (19*S*-^{3}H:19*R*-^{3}H = 90:10 for **2** and 70:30 for **5**) and their 19*R*-^{3}H isomers (0.55 mCi/mmol for **2** and 0.21 mCi/mmol for **5**) (19*R*-^{3}H:19*S*-^{3}H = 90:10 for **2** and 70:30 for **5**) were synthesized using a previously reported method (*30*). The ^3H_2O (18 μCi/mmol) was obtained from New England Nuclear Corp. (Boston, MA). The 9-diazomethylanthracene and 9-hydroxymethylanthracene formate were prepared according to previous methods (*31*, *32*). NADPH was purchased from Sigma-Aldrich Co. (St. Louis, MO). 4-Hydroxy-AD was synthesized according to a previous method (*33*).

Enzyme Preparation. Human term placental microsomes (sedimented after 60 min at 105000*g*) were obtained as described by Ryan (*34*). They were washed once with 5 mM dithiothreitol solution, freeze-dried, and stored at −20 °C. There was no significant loss in activity for 2 months.

Incubation Studies. Incubations were carried out using 50 mL Erlenmeyer flasks in a shaking water bath at 37 °C in air. Each flask contained 430 nM [19-^{3}H]-19-alcohol **2** or **5**, 300 mM NADPH, 250 or 500 μg (for the incubation with **5** or **2**) of protein of placental microsomes, 100 μL of methanol, and 67 mM phosphate buffer, pH 7.5, in a total volume of 4 mL. For each experiment, except for those for determining the initial velocity conditions (triplicate incubations), four parallel incubations were performed. The initial velocity conditions were obtained using 125, 250, or 500 μg of protein of placental microsomes and a 5, 10, or 15 min incubation period, respectively. After the incubation period, the incubation flasks were placed in dry ice−ethanol to stop incubation, and 0.3 mL of 10% H_3PO_3 was then added to the incubation mixture to adjust the pH to 3. A 2 mL aliquot of the mixture was subjected to lyophilization. Two portions were taken from the sublimate fraction after lyophilization. One 250 μL fraction was used for counting the radioactivity of ^{3}H. The second portion (1 mL) was adjusted to pH 11 with 0.3 mL of 1 M NaOH solution and lyophilized again. A portion (250 μL) of this sublimate fraction was subjected to radioactivity counting for ^3H_2O, and the residue fraction was dissolved in 1 mL of water of which a 250 μL portion was used for counting for ^{3}HCOOH.

Inhibition Studies with the Substrate AD and the Inhibitor 4-Hydroxy-AD. In the experiments with 4-ene substrate **2** (430 nM), AD at a concentration of 0.5, 1.0, or 10 μM or 4-hydroxy-AD at 1.0 or 10 μM was added to the standard incubation mixture, and in the experiments with 5-ene substrate **5** (430 nM), AD at 25, 50, or 200 nM or 4-hydroxy-AD at 25, 100 or 200 nM was added to the standard

incubation mixture. The incubations were carried out at 37 °C for 15 min. The radioactivity levels of the ^3H_2O and ^{3}-HCOOH fractions released from the ^{3}H substrate **2** or **5** were determined as described above.

Derivatization of ^{3}HCOOH with 9-Diazomethylanthracene. The combined residue fractions obtained from the incubation experiments using 19*S*-^{3}H-labeled substrate **2** or **5** (2.95×10^4 dpm from **2** or 1.72×10^4 dpm from **5**) were separately dissolved in 6 mL of H_2O and HCOONa (4 mg, 59 μmol), and 3 M HCl (2.5 mL) was added to the solution. The mixture was slowly added to a solution of 9-diazomethylanthracene (64 mg, 0.29 mmol) in dry ether (6 mL), stirred at room temperature, and then allowed to stand for 24 h in the dark (*31*, *32*). Subsequently, H_2O (3 mL) was added to the mixture, and then the product was extracted with ether (10 mL × 2). The solvent of the combined organic layer was evaporated under a stream of N_2 gas to yield the residue that was subjected to preparative TLC (silica gel, 0.5 mm thickness, 20 × 20 cm; E. Merck, Darmstadt, Germany; solvent, hexane/EtOAc = 5/1). The 9-hydroxymethylanthranyl formate fraction ($R_f = 0.50$) was collected and extracted with EtOAc. The recovery of HCOOH during the whole procedure was 88% or 90% in the experiment with ^{3}HCOOH from **2** or **5**. After evaporation of the solvent, the obtained ^{3}H-labeled ester (ca. 12 mg) was diluted with nonlabeled ester (22 mg) and recrystallized five times from EtOAc to show a constant specific activity.

RESULTS

Incubation of 19*R*-^{3}H-labeled 19-alcohols **2** and **5** and their 19*S*-labeled isomers with human placental microsomes in the presence of NADPH under aerobic conditions followed by lyophilization under acidic conditions (pH 3) yielded an acid-volatile fraction. This was again lyophilized under alkaline conditions (pH 12) to fractionate the radioactivity of ^{3}H in water and acidic product fractions. When as a control test, ^3H_2O and ^{3}HCOOH were separately added to the incubation mixtures without the labeled substrate, and the resulting mixtures were subjected to the fractionation procedure, the radioactivity of ^3H_2O was recovered in the water fraction, and that of ^{3}HCOOH was done in the acidic fraction, with a rate of more than 97% in each case. The rate of ^{3}H release into the two fractions linearly increased with incubation time (up to 15 min) and with increasing amounts of the microsomal preparation (up to 500 μg of protein) for both substrates (at 430 nM) (data not shown). On the basis of these results, an incubation mixture comprising 430 nM (1.73 nmol) substrate, 500 μg of the microsomal protein for an experiment with 4-ene substrate and 250 μg of the protein for an experiment with 5-ene substrate **5**, and a large excess amount of NADPH (300 mM, 1.3 mmol) at 37 °C for 15 min was chosen as the standard assay conditions. The conversion rates of each substrate to ^3H_2O and ^{3}H acidic product were less than 15% and 5%, respectively, of the used substrate under the standard conditions. A significant amount of the radioactivity of ^{3}H was not observed in the water and acidic product fractions in the absence of NADPH or using the boiled microsomal preparation (95 °C for 10 min).

The initial effort was directed to the identification of ^{3}-HCOOH released in the acidic product fraction. This fraction obtained from the incubations with 19*S*-^{3}H substrate **2** and **5** was diluted with nonlabeled HCOOH and then treated with 9-diazomethylanthracene according to a method previously reported (*31*, *32*), followed by TLC purification, yielding ^{3}H-labeled 9-formyloxymethylanthracene as shown in Figure 3. After addition of nonlabeled 9-formyloxymethylanthracene, the labeled formate ester was subjected to repeated crystallization from ethyl acetate, yielding a constant specific activity level in each case. This shows that ^{3}HCOOH is released by the cleavage of the $C_{10}-C_{19}$ bond of each substrate in the incubation. On the basis of specific activity, it was calculated that approximately 70% or 80% of the radioactivity in the acidic fraction obtained from labeled substrate **2** or **5** was identified as ^{3}HCOOH.

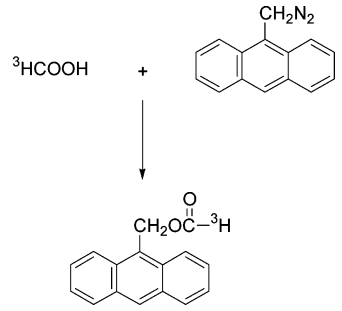

FIGURE 3: Derivatization of ^{3}HCOOH produced from [19-^{3}H]-19-alcohols **2** and **5** into 9-hydroxymethylanthranyl formate.

To clarify whether the release of ^3H_2O and ^{3}HCOOH is due to a catalytic function of aromatase, the natural substrate AD and the inhibitor 4-hydroxy-AD (*35*) were separately added to the standard incubation mixtures, and production of ^3H_2O as well as ^{3}HCOOH was determined. The substrate prevented the production in a dose-dependent manner in all experiments using 19*R*-^{3}H- and 19*S*-^{3}H-labeled substrates **2** and **5** irrespective of the stereochemistry of the labeling; 1 μM AD caused about 90% inhibition of the production in the experiments with substrate **2** whereas 200 nM AD did it in the experiments with the other substrate. The inhibitor also blocked the production from the 19*S*-^{3}H-labeled substrates in a dose-dependent manner (Figure 4).

The recovery of the radioactivity released from the ^{3}H substrates into ^3H_2O and ^{3}HCOOH under the standard conditions is given in Table 1. The stereochemistry of the 19-hydroxylation of 19-alcohols **2** and **5** was next calculated on the basis of the stereochemistry of the ^{3}H labeling at C-19 of the substrates along with the relative amount of ^3H_2O released from the 19*R*-^{3}H substrate to that of the 19*S*-^{3}H isomer, respectively. The results indicate that the 19-*pro-R* hydrogen atom is stereosepecifically (*pro-R*:*pro-S* = 100:0) removed in the case of 5-ene substrate **5** whereas a 75:25 ratio of removal for *pro-R*:*pro-S* is obtained for 4-ene substrate **2**. Then, the rates of 19-hydroxylation of the 19-alcohols **2** and **5**, giving the corresponding 19-aldehydes **3** and **6**, and the $C_{10}-C_{19}$ bond cleavage, yielding HCOOH, were calculated from the recovery (%) of ^3H_2O and ^{3}HCOOH on the basis of the stereochemistry of the ^{3}H labeling at C-19 of each substrate along with the stereochemistry of the 19-hydroxylation (Table 1). The 19-hydroxylation rate [pmol min^{-1} (mg of protein)$^{-1}$] of 5-ene substrate **5** was about 2-fold that of 4-ene substrate **2** whereas the rate of the $C_{10}-C_{19}$ bond cleavage of substrate **5** was about 4-fold that of the other.

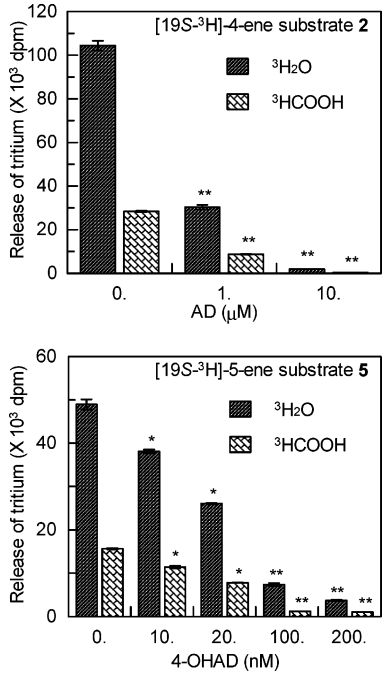

FIGURE 4: Inhibition of production of ^3H_2O and ^3HCOOH from [19*S*-^{3}H]-19-alcohols **2** and **5** by the inhibitor 4-hydroxy-AD. [^{3}H]-19-Alcohols **2** and **5** (0.43 μM) were separately incubated for 15 min with human placental microsomes (500 μg of protein for **2** and 250 μg of protein for **5**), NADPH (300 mM), and 4-hydroxy-AD (1 and 10 μM for experiments with substrate **2** and 10, 20, 100, and 200 nM for those with substrate **5**). The results are presented as the mean ± SD ($n = 4$). (*) $p < 0.01$ and (**) $p < 0.001$, compared with control (0 μM).

DISCUSSION

The intent of this study was to explore the steric mode of the aromatase-catalyzed 19-oxygenation of 19-alcohols **2** and **5** having no oxygen function at C-3, yielding 19-aldehydes **3** and **6**, respectively, as well as whether aromatase catalyzes the cleavage of the $C_{10}-C_{19}$ bond of the aldehydes as observed in the AD aromatization. One of the two hydrogen atoms at C-19 of the alcohols will be transferred to water of the medium by the aromatase-catalyzed 19-oxygenation and the other hydrogen atom released with formic acid by the subsequent 19-oxygenation involving the $C_{10}-C_{19}$ bond cleavage reaction. When 19*R*-^{3}H-labeled 19-alcohols **2** and **5** and their 19*S*-^{3}H-labeled isomers were separately incubated under aerobic conditions with human placental microsomes and NADPH, the radioactivity was recovered in both water and acidic fractions. The production of ^3HCOOH in the acidic fraction was confirmed by the derivatization to [^{3}H]-9-formyloxymethylanthracene. The production of ^3H_2O and ^3HCOOH was efficiently blocked by the natural substrate AD or the typical suicide substrate 4-hydroxy-AD in a dose-dependent manner (Figure 4). There would be scarcely any contamination of the 19-hydroxysteroid dehydrogenase activity in the microsomal preparation as the reason causing extra release of ^3H_2O. The results indicate that the productions of ^3H_2O and ^3HCOOH are principally due to a catalytic function of aromatase. It was previously predicted that 3-deoxyandrogens **1** and **4** are converted into 19-aldehydes **3** and **6** through 19-alcohols **2** and **5** by human placental aromatase in each (*29*). Thus, it is proven that aromatase catalyzes the cleavage of the $C_{10}-C_{19}$ bond of the 19-aldehydes, namely, the third oxygenation of the parent 3-deoxy-19-methylsteroids having no conjugated 4-en-3-one structure.

We then studied the stereochemistry of 19-hydrogen removal during the conversion of 19-alcohols **2** and **5** to the corresponding 19-aldehydes **3** and **6**. The stereochemistry of the 19-hydrogen removal was calculated to be 75:25 in the case of 4-ene substrate **2** or 100:0 in the case of 5-ene substrate **5** for 19-*pro-R*:19-*pro-S* (Table 1). The apparent kinetic tritium isotope effect has not been observed for the hydroxylation of 19-hydroxy-AD (*11*−*13*), giving 19-oxo-AD. Moreover, we recently studied the kinetic deuterium isotope effect on the 19-oxygenation of 19-alcohols **2** and **5** with human placental aromatase, using their 19,19-$^2H_2$-labeled analogues as substrates (*36*). Gas chromatography−mass spectrometric analysis of the recovered 19-alcohols and the products, 19-aldehydes **3** and **6**, revealed that there is no apparent kinetic isotope effect on the 19-oxygenation in each case. On the basis of this, it is reasonable to think that the apparent tritium isotope effect is not involved in the hydroxylation of 19-alcohols **2** and **5**, although there is no direct evidence. Thus, the stereochemistry of the hydroxylation is valid. The stereospecific *pro-R* hydrogen loss is consistent with the previous result (*11*, *12*) obtained in the oxygenation of 19-hydroxy-AD in the estrogen production from the natural substrate AD, indicating that a 4-en-3-one system, especially the C-3 carbonyl group of AD, does not essentially play a critical role in the stereospecific *pro-R* hydrogen loss during the aromatization sequence. We previously reported that the 4-ene steroid **1**, an excellent competitive inhibitor of aromatase ($K_i = 37$ nM), is a poor substrate for the 19-oxygenation by the enzyme, while the 5-ene isomer **4**, a relatively weak competitive inhibitor ($K_i = 120$ nM), is a good substrate for the 19-oxygenation [9.7 vs 45 pmol min^{-1} (mg of protein)$^{-1}$ for **1** vs **4**] (*25*−*27*, *29*). Introduction of a hydroxy group at C-19 of the former steroid increases the affinity to aromatase ($K_i = 13$ nM for 19-alcohol **2**) (*25*); in contrast, the same molecular modification of the latter markedly decreases the affinity ($K_i = 1000$ nM for 19-alcohol **5**) (*27*) as seen in a series of ADs (*2*, *3*). Taken together, we proposed that 3-deoxysteroids **1** and **4** would bind to the active site of aromatase in a different manner where the 5-ene steroid **4** would bind to the active site in a manner similar to that involved in the AD binding (*27*, *29*). The present findings concerning the stereochemistry of the 19-oxygenation of 19-alcohols **2** and **5** are consistent with the previous proposal from the viewpoint of the steric mode of aromatase reaction.

Introduction of the 19-hydroxy group to parent steroids **1** and **4** increased the ability to serve as a substrate for the 19-hydroxylation, the ^3H_2O production from [19-^{3}H]-19-alcohols **2** and **5**, respectively [about 34 vs 78 pmol min^{-1} (mg of protein)$^{-1}$ for **2** vs **5**] (Table 1). The ^3HCOOH production (the $C_{10}-C_{19}$ bond cleavage) rate of 19-^{3}H alcohol **5** through 19-aldehyde **6** was about four times that of 4-ene isomer **2** through aldehyde **5** [about 7.5 vs 31 pmol min^{-1} (mg of protein)$^{-1}$ for **2** vs **5**]. It is implied that higher production rates of ^3HCOOH, compared to those above, should be obtained under substrate saturation (initial velocity) conditions. Kinetic constants for aromatization of 19-hydroxy-AD, an intermediate of aromatization of the substrate AD, with aromatase in human placental microsomes have been reported to be 200 pmol min^{-1} (mg of protein)$^{-1}$

Table 1: Recovery of Radioactivity Released from 19-^{3}H-Labeled 19-Alcohols **2** and **5** into ^3H_2O and ^{3}HCOOH Fractions by Human Placental Aromatase and Stereochemistry of 19-Hydrogen Atom Removal by 19-Oxygenation

	recovery, %[a] [production rate, pmol min^{-1} (mg of protein)$^{-1}$][c]		stereochemistry of 19-H removal[b]
substrate	^3H_2O	^{3}HCOOH	*R*:*S* (range)
[19*R*-^{3}H]-19-alcohol **2**	10.32 ± 0.19 (33.9 ± 0.62)	1.15 ± 0.03 (8.8 ± 0.23)	75:25 (74.7:25.3−75.5:24.5)
[19*S*-^{3}H]-19-alcohol **2**	4.39 ± 0.14 (33.9 ± 1.08)	1.88 ± 0.06 (6.2 ± 0.20)	
[19*R*-^{3}H]-19-alcohol **5**	11.81 ± 0.48 (78.0 ± 3.2)	2.31 ± 0.09 (35.5 ± 1.4)	100:0 (99.7:0.3−100.7:−0.7)
[19*S*-^{3}H]-19-alcohol **5**	5.06 ± 0.25 (78.4 ± 3.9)	4.14 ± 0.15 (26.3 ± 1.2)	

[a] [19-^{3}H]-19-Alcohols **2** and **5** were separately incubated with human placental microsomes under the respective standard conditions (n = 4, mean ± SD). The radioactivity of the ^3H_2O and ^{3}HCOOH fractions obtained by two successive lyophilizations was counted as described and expressed as percent of the radioactivity of the used substrate. [b] The stereochemistry was calculated on the basis of the equation: (recovery rate of ^3H_2O from [19*R*-^{3}H] substrate, %)/(recovery rate of ^3H_2O from [19*S*-^{3}H] substrate, %) = (X × R_R + (1 − X) × R_S)/(X × S_R + (1 − X) × S_S). X = the ratio of removal of the 19-*pro*-*R*-hydrogen atom to that of the total 19-hydrogen atom. R_R or S_R = the stereochemistry of *pro*-*R* labeling at C-19 of [19*R* or *S*-^{3}H] substrate used. R_S or S_S = the stereochemistry of *pro*-*S* labeling at C-19 of [19*R* or *S*-^{3}H] substrate used. (R_R and S_S = 0.90 each for **2** and 0.70 each for **5**; S_R and R_S = 0.10 each for **2** and 0.30 each for **5**). [c] The production rate [pmol min^{-1} (mg of protein)$^{-1}$] was calculated on the basis of the recovery (%), incubation period (15 min), amounts of microsomal protein (500 μg for **2**, 250 μg for **5**) and substrate (1.73 nmol), and the stereochemistry of ^{3}H labeling of the used substrate.

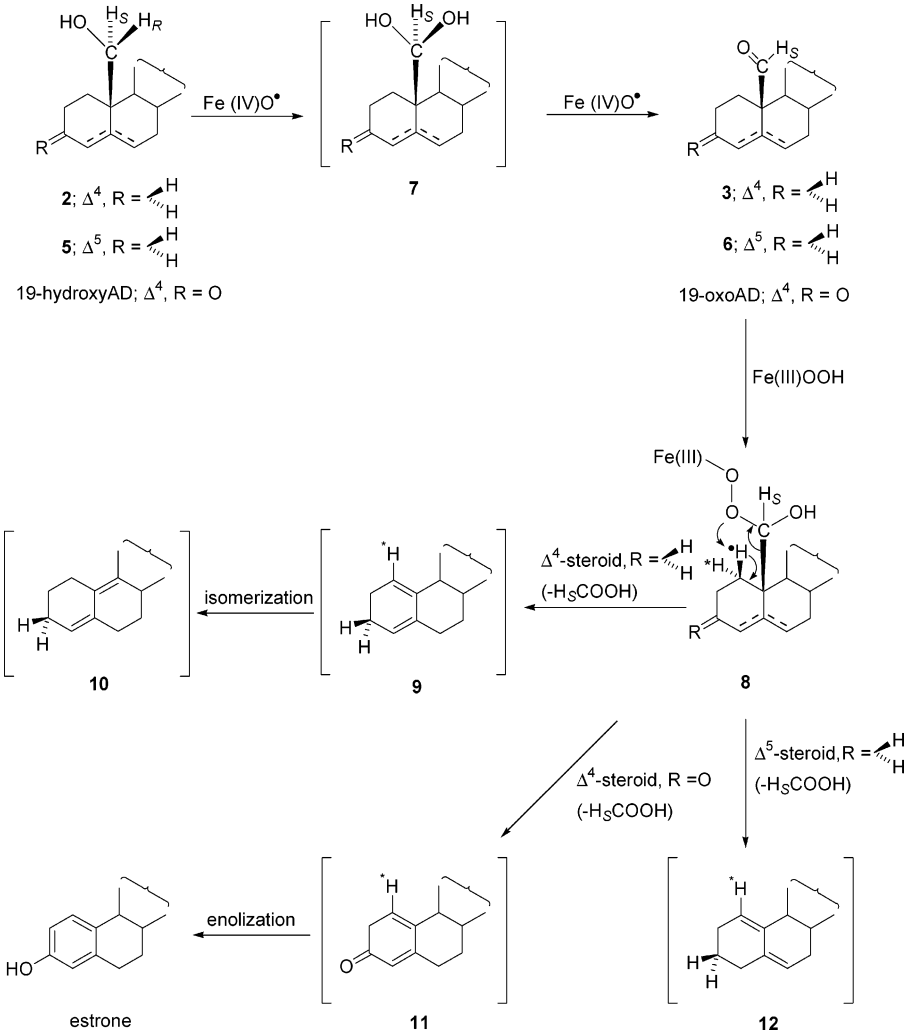

FIGURE 5: Proposed mechanisms for the $C_{10}-C_{19}$ bond cleavage of 3-deoxyandrogens and AD aromatization by placental aromatase.

of the V_{max} value and 46 nM of the K_m value [cf. K_m and V_{max} values for AD aromatization: 14 nM and 96 pmol min^{-1} (mg of protein)$^{-1}$, respectively] (*2*). Thus, it was found that the 5-ene 19-alcohol **5** served as a good substrate for aromatase.

The third step of the overall process, AD → estrone, involves the oxidative $C_{10}-C_{19}$ bond cleavage and aromatization of the steroidal A-ring. The original theory of Akhtar and co-workers shows that the third oxygenation utilizes a ferric peroxy intermediate of the cytochrome P-450 heme in a nucleophilic attack on the aldehyde function of the 19-oxo-AD to yield a peroxy intermediate **8** (Figure 5) (*5*, *37*). The fragmentation of the 19-hydroxy ferric peroxide directly occurs with the release of formic acid and estrogen. Robinson and co-workers indicated, via the use of chemical model and enzymatic studies, the importance of both the C_{19}-aldehyde function and enolization of the C-3 carbonyl group, producing a 3-hydroxy-2,4-diene-19-aldehyde molecular species,

in facilitating aromatization (*7*, *16*, *19*). Semiempirical molecular orbital calculations have also demonstrated that the enolization activates the C_1-position for hydrogen atom abstraction in the aromatization sequence (*18*). These previous findings predicted that the enolization occurs prior to or concomitant with the cleavage of the $C_{10}-C_{19}$ bond in AD aromatization. It was also reported that the 2-ene derivative of 4-ene 19-aldehyde **3**, of which the C_1-position is activated by the C-2 double bond, serves as a substrate for aromatase, with conversion to 3-deoxyestradiol and 3-deoxyestrone (*39*). However, 3-deoxy-19-alcohols **2** and **5** do not have the C-2 double bond which activates their C_1-positions, respectively. Thus, the present findings that the aromatase-catalyzed $C_{10}-C_{19}$ bond cleavage of 19-alcohols **2** and **5** proceeded through their 19-aldehydes **3** and **6**, yielding formic acid, clearly indicate that the activation of the C_1-position is not essential in the bond cleavage reaction. It is likely that the A-ring aromatization sequence proceeds through the $C_{10}-C_{19}$ bond cleavage of 19-oxo-AD, producing the 1(10),4-dien-3-one intermediate **11**, followed by enolization of the 3-carbonyl group to yield estrone as shown in Figure 5.

The structural features of the steroidal moiety produced by the aromatase-catalyzed $C_{10}-C_{19}$ bond cleavage of the 3-deoxyaldehydes **3** and **6** are currently unknown. On the basis of the mechanism of the $C_{10}-C_{19}$ bond cleavage of 19-oxo-AD, 1(10),4- or 1(10),5-diene steroid **9** or **12** may be produced from 19-aldehydes **3** or **6** where the endocyclic diene **9** may be isomerized to the thermodynamically more stable exocyclic diene **10**. The study of the structural determination is now underway in our laboratory.

In conclusion, human placental aromatase catalyzes the stereoselective or stereospecific 19-*pro-R*-hydrogen atom removal from [19-^{3}H]-19-alcohol **2** or **5**, yielding the corresponding 19-aldehydes **3** and **6** as well as the cleavage of the $C_{10}-C_{19}$ bond of 19-aldehydes **3** and **6**. We reported that the C-17 carbonyl function is essential for tight binding to the active site in series of 3-deoxysteroids (*25*−*27*). Thus, this function would play a critical role in the steric mode of the 19-hydroxylation followed by the cleavage of the $C_{10}-C_{19}$ bond by anchoring the 3-deoxysteroids via hydrogen binding to an active site amino acid. It has been thought that anchoring the substrate AD by hydrogen binding of an active site amino acid of aromatase such as ^{302}Glu (*40*), ^{473}Lys (*41*), ^{310}Thr (*28*), or ^{480}His (*20*) with the C-3 carbonyl function is necessary for the whole process of the aromatization reaction. The molecular structure of the A−B−C−D ring system of AD, or an androst-4-ene system, along with the 17-carbonyl group rather than C-3 carbonyl group would play an important role in the recognition and binding of a substrate (inhibitor) by aromatase. The present results give new insights into not only a catalytic function of aromatase but also the mechanism of the natural substrate aromatization.

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REFERENCES

1. Thompson, E. A., Jr., and Siiteri, P. K. (1974) The involvement of human placental microsomal cytochrome P-450 in aromatization, *J. Biol. Chem. 249*, 5373−5378.
2. Kellis, J. T., Jr., and Vickery, L. E. (1987) Purification and characterization of human placental aromatase cytochrome P-450, *J. Biol. Chem. 262*, 4413−4420.
3. Yoshida, N., and Osawa, Y. (1991) Purification of human placental aromatase cytochrome P-450 with monoclonal antibody and its characterization, *Biochemistry 30*, 3003−3010.
4. Meyer, A. S. (1955) Conversion of 19-hydroxy-D-4-androstene-3,17-dione to estrone by endocrine tissue, *Biochim. Biophys. Acta 17*, 441−442.
5. Akhtar, M., Calder, M. R., Corina, D. L., and Wright, J. N. (1982) Mechanistic studies on C-19 demethylation in oestrogen biosynthesis, *Biochem. J. 201*, 569−580.
6. Cole, P. A., and Robinson, C. H. (1990) Mechanism and inhibition of cytochrome P-450 aromatase, *J. Med. Chem. 33*, 2933−2944.
7. Oh, S. S., and Robinson, C. H. (1993) Mechanism of human placental aromatase: new active site model, *J. Steroid Biochem. Mol. Biol. 44*, 389−397.
8. Akhtar, M., Lee-Robichaud, P., Akhtar, M. E., and Wright, J. N. (1997) The impact of aromatase mechanism on other P450s, *J. Steroid Biochem. Mol. Biol. 61*, 127−132.
9. Caspi, E., Arunachalam, T., and Nelson, P. A. (1983) Biosynthesis of estrogens: the steric mode of the initial C-19 hydroxylation of androgens by human placental aromatase, *J. Am. Chem. Soc. 105*, 6987−6989.
10. Caspi, E., Arunachalam, T., and Nelson, P. A. (1986) Biosynthesis of estrogens: aromatization of (19R)-, (19S)-, and (19RS)-[19-^{3}H,^{2}H,^{1}H]-3β-hydroxyandrost-5-en-17-ones by human placental aromatase, *J. Am. Chem. Soc. 108*, 1847−1852.
11. Osawa, Y., Shibata, K., Rohrer, D., Weeks, C., and Duax, W. L. (1975) Reassignment of the absolute configuration of 19-substituted 19-hydroxysteroids and stereomechanism of estrogen biosynthesis, *J. Am. Chem. Soc. 97*, 4400−4402.
12. Arigoni, D., Battaglia, R., Akhtar, M., and Smith, T. (1975) Stereospecificity of oxidation at C-19 in oestrogen biosynthesis, *J. Chem. Soc., Chem. Commun.*, 185−186.
13. Osawa, Y., Higashiyama, T., Fronckowaik, M., Yoshida, N., and Yarborough, C. (1987) Aromatase, *J. Steroid Biochem. 27*, 781−789.
14. Cole, P. A., and Robinson, C. H. (1990) Conversion of 19-oxo[2 beta-2H]androgens into oestrogens by human placental aromatase. An unexpected stereochemical outcome, *Biochem. J. 268*, 553−561.
15. Akhtar, M., Corina, D., Pratt, J., and Smith, T. (1976) Studies on the removal of C-19 in oestrogen biosynthesis using $^{18}O_2$, *J. Chem. Soc., Chem. Commun.*, 854−856.
16. Cole, P. A., and Robinson, C. H. (1988) Peroxide model reaction for placental aromatase, *J. Am. Chem. Soc. 110*, 1284−1285.
17. Graham-Lorence, S., Khalil, M. W., Lorence, M. C., Mendelson, C. R., and Simpson, E. R. (1991) Structure−function relationships of human aromatase cytochrome P-450 using molecular modeling and site-directed mutagenesis, *J. Biol. Chem. 266*, 11939−11946.
18. Korzekwa, K. R., Trager, W. F., Smith, S. J., Osawa, Y., and Gillette, J. R. (1991) Theoretical studies on the mechanism of conversion of androgens to estrogens by aromatase, *Biochemistry, 30*, 6155−6162.
19. Oh, S. S., and Robinson, C. H. (1994) Synthesis of and chemical model reaction studies with 3-deoxyandrogens: evidence supporting a 2,3-enolization hypothesis in human placental aromatase catalysis, *J. Chem. Soc., Perkin Trans. 1*, 2237−2243.
20. Kao, Y.-C., Korzekwa, K. R., Laughton, C. A., and Chen, S. (2001) Evaluation of the mechanism of aromatase cytochrome P450, *Eur. J. Biochem. 268*, 243−251.
21. Hervey, H. A., Lipton, A., and Santen, R. J. (1982) Aromatase: new perspectives for breast cancer, *Cancer Res. 42* (Suppl.), 3261s−3269s.
22. Brodie, A. M. H. (1994) Aromatase inhibitors in the treatment of breast cancer, *J. Steroid Biochem. Mol. Biol. 49*, 281−287.
23. Harper-wynne, C., and Dowseff, M. (2001) Recent advances in the clinical application of aromatase inhibitors, *J. Steroid Biochem. Mol. Biol. 76*, 179−186.
24. Howell, A., Howell, S. J., Clarke, R., and Anderson, E. (2001) Where do selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs) now fit into breast cancer treatment algorithms?, *J. Steroid Biochem. Mol. Biol. 79*, 227−237.
25. Numazawa, M., Mutsumi, A., Hoshi, K., and Koike, R. (1989) 19-Hydroxy-4-androsten-17-one: potential competitive inhibitor of estrogen biosynthesis, *Biochem. Biophys. Res. Commun. 160*, 1009−1014.

26. Numazawa, M., Mutsumi, A., Hoshi, K., Oshibe, M., Ishikawa, E., and Kigawa, H. (1991) Synthesis and biochemical studies of 16- or 19-substituted androst-4-enes as aromatase inhibitors, *J. Med. Chem. 34*, 2496−2504 (1991).
27. Numazawa, M., Mutsumi, A., Tachibana, M., and Hoshi, K. (1994) Synthesis of androst-5-en-7-ones and androsta-3,5-dien-7-ones and their related 7-deoxy analogues as conformational and catalytic probes for the active site of aromatase, *J. Med. Chem. 37*, 2198−2205.
28. Laughton, C. A., Zvelbil, M. J. J. M., and Neidle, S. (1998) A detailed molecular model for human aromatase, *J. Steroid Biochem. Mol. Biol. 44*, 399−407.
29. Numazawa, M., Nagaoka, M., Morio, M., and Kamiyama, T. (1999) 19-oxygenations of 3-deoxy androgens, potent competitive inhibitors of estrogen biosynthesis, with human placental aromatase, *J. Steroid Biochem. Mol. Biol. 71*, 173−179.
30. Numazawa, M., Sohtome, N., and Nagaoka, M. (2004) Stereochemistry of $NaBH_4$ reduction of a 19-carbonyl group of 3-deoxy androgens. Synthesis of [19*S*-^{3}H]- and [19*R*-^{3}H]-labeled aromatase inhibitors having a 19-hydroxy group, *Chem. Pharm. Bull. 52*, 722−726.
31. Baker, S. A., Monti, J. A., Christian, S. T., Benington, F., and Morin, R. D. (1980) *Anal. Biochem. 107*, 116−123.
32. Beusen, D. D., Kalmen, B. L., and Covey, D. F. (1986) Role of substrate conformational features in the stereospecificity of aromatase, *Biochemistry 25*, 662−667.
33. Numazawa M., and Ogata M. (1986) Methoxylation of enolizable steroidal 4-ene-3-ones using hypervalent iodine, *J. Chem. Soc., Chem. Commun.*, 1092−1093.
34. Ryan, K. J. (1959) Biological aromatization of steroids, *J. Biol. Chem. 234*, 268−272.
35. Brodie, A. M. H., Grarrett, W. M., Hendrickson, J. R., Tsai-Morris, C. H., Marcotte, P. A., and Robinson, C. H. (1981) Inactivation of aromatase in vitro by 4-hydroxy-4-androstene-3,17-dione and 4-acetoxy-4-androstene-3,17-dione and sustained effects in vivo, *Steroids 38*, 693−702.
36. Nagaoka, M., and Numazawa, M. (2005) Gas chromatography mass spectrometric analysis of oxidative reactions of [19,19-$^2H_2$]-19-hydroxy-3-deoxy androgens, aromatase inhibitors, by placental aromatase. Absence of deuterium-isotope effect, *Steroids* (in press).
37. Akhtar, M., Vincent, C. O., and Wright, J. N. (1993) Mechanistic studies on aromatase and related C- - -C bond cleaving *P*-450 enzymes, *J. Steroid Biochem. Mol. Biol. 44*, 375−387.
38. Cole, P. A., and Robinson, C. H. (1991) Mechanistic studies on a placental aromatase model reaction, *J. Am. Chem. Soc. 113*, 8130−8137.
39. Cole, P. A., Bean, J. M., and Robinson, C. H. (1990) Conversion of a 3-Desoxysteroid to 3-Desoxyestrogen by Human Placental Aromatase, *Proc. Natl. Acad. Sci. U.S.A. 87*, 2999−3003.
40. Koymans, L. M. H., Moereels, H., and Bossche, H. V. (1995) A molecular model for the interaction between vorozole and other non-steroidal inhibitors and human cytochrome *P*450 19 (*P*450 Aromatase), *J. Steroid Biochem. Mol. Biol. 53*, 191−197.
41. Grahan-Lorence, S., Amarneh B., White, R. E., Peterson, J. A., and Simpson, E. R. (1995) A three-dimensional model of aromatase cytochrome P450, *Protein Sci. 4*, 1065−1080.

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