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Mechanism for Aromatase Inactivation by a Suicide Substrate, Androst-4-ene-3,6,17-trione

THE 4β,5β-EPOXY-19-OXO DERIVATIVE AS A REACTIVE ELECTROPHILE IRREVERSIBLY BINDING TO THE ACTIVE SITE

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ABSTRACT. Aromatase is a cytochrome P450 enzyme complex that catalyzes the conversion of androst-4-ene-3,17-dione to estrone through three sequential oxygenations of the 19-methyl group. Androst-4-ene-3,6,17-trione **(1)** is a suicide substrate of aromatase. The inactivation mechanism for steroid **1** has been studied to show that the inactivation reaction proceeds through the 19-oxo intermediate **3.** To further clarify the mechanism, 4β,5β-epoxyandrosta-3,6,17,19-tetraone **(6)** was synthesized as a candidate for a reactive electrophile involved in irreversible binding to the active site of aromatase, upon treatment of compound **3** with hydrogen peroxide in the presence of $NaHCO_3$. The epoxide **6** inhibited human placental aromatase in a competitive manner (K_i = 30 μM); moreover, it inactivated the enzyme in an active-site-directed manner in the absence of NADPH (K_I = 88 μM, k_{inact} = 0.071 min^{-1}). NADPH and BSA both stimulated the inactivation rate without a significant change of the K_I in either case (k_{inact}: 0.133 or 0.091 min^{-1}, in the presence of NADPH or BSA, respectively). The substrate androst-4-ene-3,17-dione protected the inactivation, but a nucleophile, L-cysteine, did not. When both the epoxide **6** and its 19-methyl analog **4** were subjected separately to reaction with *N*-acetyl-L-cysteine in the presence of $NaHCO_3$, the 19-oxo steroid **6** disappeared from the reaction mixture more rapidly ($T_{1/2}$ = 40 sec) than the 19-methyl analog **4** ($T_{1/2}$ = 3.0 min). The results clearly indicate that the 4β,5β-epoxy-19-oxo compound **6,** which is possibly produced from 19-oxo-4-ene steroid **3** through the 19-hydroxy-19-hydroperoxide intermediate, is a reactive electrophile that irreversibly binds to the active site of aromatase. BIOCHEM PHARMACOL 52;8:1253–1259, 1996.

KEY WORDS. aromatase; suicide substrate; inactivation mechanism; androst-4-ene-3,6,17-trione; reactive electrophile; 4β,5β-epoxy-19-oxo metabolite

Placental aromatase is a cytochrome P450 enzyme complex that catalyzes the conversion of androgens, androst-4-ene-3,17-dione (androstenedione) and testosterone, to estrogens, estrone, and estradiol [1–3]. Three sequential oxygenations are involved in the production of the estrogen [4]. The first two are sequential hydroxylations of the 19-methyl group to produce 19-hydroxy and 19,19-*gem*-diol intermediates, respectively [5–9]. Dehydration of this *gem*-diol leads to the readily isolated 19-oxo intermediate. In the third step, C-19 and the 1β,2β-protons are eliminated as formic acid and water, respectively, to produce the estrogens [7, 10–13]. However, it is now thought to be a substrate-dependent variation in stereochemistry of the 2β-proton loss [14, 15]. Considerable speculation continues as to the mechanism of attack of the third mole 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 [7, 16–18].

Aromatase is a potential therapeutic target for the selective lowering of estrogen levels in patients with estrogen-dependent tumors, including breast cancer [19–22]. The specific irreversible blockade of estrogen biosynthesis via mechanism-based inactivation has been pursued intensely with the goal of developing practical clinical drugs. Androst-4-ene-3,6,17-trione **(1)** is one of the earliest discovered suicide substrates of aromatase [23–25] (Fig. 1). The 14α-hydroxy derivative of steroid **1** is now under clinical trial [26]. Both 19-hydroxy- and 19-oxo-analogs of steroid **1,** compounds **2** and **3,** inactivate aromatase in a suicide manner [27]. This fact and our recent studies [28] using stereo- and/or regiospecifically labeled [^3H, ^{14}C] steroid **1** have indicated that further oxygenation of the 19-oxo steroid **3** produced by the two initial hydroxylations at C-19 of steroid **1** yields not only 6-oxoestrogens but also a reactive electrophile that immediately binds to the active site of aromatase in an irreversible manner, resulting in inactivation of the enzyme. The aromatase-bound metabolite in a 46–69 kDa fraction retains the 1β-proton, the 19-carbon,

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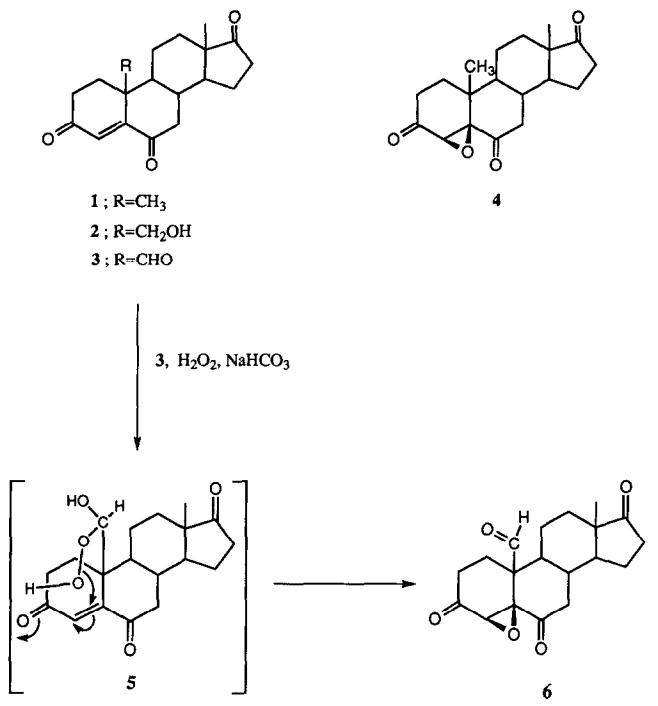

FIG. 1. Structures and synthesis of androst-4-ene-3,6,17-trione derivatives.

and one of the three 19-methyl protons of the parent compound **1**, but the structure of the reactive electrophile is still uncertain. Mechanistic studies by Robinson and coworker [16, 29, 30], on a placental aromatase model reaction have shown that treatment of 2,4-diene analogs of 19-oxo steroids with hydrogen peroxide in the presence of $NaHCO_3$ produces the aromatization product; in contrast, the same reaction of their 4-en-3-one analogs results in the formation of the 4β,5β-epoxide through an intramolecular epoxidation reaction. On the basis of this model reaction as well as our previous findings, we focussed on 4β,5β-epoxyandrosta-3,6,17,19-tetraone **(6)** as the reactive electrophile involved in the aromatase inactivation by compound **1.** In this study, we report the preparation and biochemical and chemical evaluation of the 4β,5β-epoxy-19-oxo steroid **6.** The epoxide **6** inactivated aromatase in human placental microsomes in an affinity labeling manner, and was more reactive towards a nucleophile, L-cysteine, than its 19-methyl analog **(4).**

MATERIALS AND METHODS

Materials

[1β-^{3}H]Androstenedione (27.5 Ci/mmol; ^{3}H-distribution: 1β = 74–79%) was purchased from Du Pont-New England Nuclear (Boston, MA, U.S.A.); NADPH was obtained from the Kohjin Co. Ltd. (Tokyo, Japan). Silica gel (Kieselgel 60, 70–230 mesh) for column chromatography and silica gel thin-layer plates (Kieselgel 60-F_{254}, 0.25 mm thick) were supplied from E. Merck AG (Darmstadt, Germany). BSA (fraction V) was obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Androst-4-ene-3,6,17-trione **(1)** and its 19-hydroxy- **(2)** and 19-oxo- **(3)** derivatives, and 4β,5β-epoxyandrosta-3,6,17-trione **(4)** were synthesized according to known methods [25, 27].

Synthesis of 4β,5β-Epoxyandrosta-3,6,17,19-tetraone (6)

Aqueous 30% H_2O_2 (0.3 mL, 2.6 mmol) was added to a solution of androst-4-ene-3,6,17,19-tetraone **(3)** (50 mg, 0.16 mmol) in CH_3OH (40 mL) containing anhydrous $NaHCO_3$ (10 mg, 0.12 mmol) [29], and the mixture was allowed to stand at 0° for 40 min. After this time, the reaction mixture was diluted with ethyl acetate (100 mL), washed with $Na_2S_2O_3$ solution and water, and dried (Na_2SO_4). After evaporation of the solvent, an oily product was purified by silica gel column chromatography (hexane–ethyl acetate) followed by recrystallization from acetone to afford pure epoxide **6** (25 mg, 47%): m.p. 246–247° (decomp.); PMR (270 MHz, $CDCl_3$) δ 0.89 (3H, s, 18-CH_3), 3.23 (1H, s, 4-H), 9.82 (1H, d, J = 1.0 Hz, 19-CHO); Fourier transform i.r. (KBr) 1730 cm^{-1}. Anal. calc. for $C_{19}H_{22}O_5$: C, 69.07; H, 6.71. Found: C, 68.78; H, 6.45.

Preparation of Placental Microsomes

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

Aromatase Assay Procedure

Aromatase activity was measured according to the procedure of Siiteri and Thompson [32]. All were carried out in 67 mM phosphate buffer, pH 7.5, at a final incubation volume of 0.5 mL. The incubation mixture for the IC_{50} experiment contained 180 μM NADPH, 1 μM [1β-^{3}H]androstenedione (3×10^5 dpm), 40 μg of protein of the lyophilized microsomes, various concentrations (10, 50, 100, and 150 μM) of inhibitor **6,** and 25 μL of CH_3OH. For kinetic study, various concentrations of inhibitor **6** and [1β-^{3}H]androstenedione and 20 μg of microsomal protein were employed. Incubations were performed at 37° for 20 min in air and terminated by the addition of 3 mL of $CHCl_3$, followed by vortexing for 40 sec. After centrifugation at 700 *g* for 5 min, aliquots (0.25 mL) were removed from the water phase and added to scintillation mixture for determination of ^3H_2O production.

Time-Dependent Inactivation Procedure

Various concentrations (37, 50, 75, and 100 μM) of inhibitor **6** were incubated with or without NADPH (600 μM), androstenedione (0.32 and 3 μM), L-cysteine (0.5 mM), and BSA (0.1%) at 37° with placental microsomes (200 μg

protein) and CH_3OH (25 μL) in 67 mM phosphate buffer, pH 7.5, in a total volume of 500 μL in air. Aliquots (50 μL), in duplicate, were removed at various time periods (0, 4, 8, and 12 min) and added to a solution of [1β-^{3}H]androstenedione (300 nM, 3×10^5 dpm) and NADPH (180 μM) in 67 mM phosphate buffer, pH 7.5 (total volume, 0.5 mL), and the mixture was incubated at 37° for 20 min. ^3H_2O release was determined as described above.

Reaction of the 4β,5β-Epoxides 4 and 6 with N-acetyl-L-cysteine

A solution of the epoxide **4** or **6** (3.2 or 3.3 mg, 10 μmol), N-acetyl-L-cysteine (1.6 mg, 10 μmol), $NaHCO_3$ (1.3 mg, 15 μmol) in H_2O (0.32 mL) and CH_3CN (1.28 mL) was shaken at 37°. An aliquot (100 μL) of the reaction mixture was removed at an appropriate time and diluted with CH_3CN (100 μL). An aliquot (10 μL) of the diluted mixture was then subjected to HPLC. Amounts of the remaining epoxides **4** and **6** were obtained using an absolute calibration method. HPLC conditions: pump, Waters 510 pump; solvent, CH_3CN:H_2O = 50:50 (v/v), 1 mL/min; column, Puresil C_{18} 5 μm 120 A (Waters) (150 mm × 46 mm i.d.); detector, Waters 486 UV detector at 220 nm. Retention time: 5.6 min for compound **4** and 3.0 min for compound **6**. The reaction was also analyzed by TLC. TLC conditions: solvent 1, hexane:ethyl acetate = 1:2 (v/v); solvent 2, $CHCl_3$:CH_3OH:HCOOH = 10:0.5:0.3 (by vol.). The R_f* values of compounds **4** and **6** were 0.77 and 0.54 (solvent 1) or 0.71 and 0.49 (solvent 2), respectively.

RESULTS

Synthesis

Reaction of androst-4-ene-3,6,17,19-tetraone (**3**) with hydrogen peroxide in the presence of a weak base, $NaHCO_3$, in CH_3OH gave the 4β,5β-epoxy derivative **6** in a 47% yield (Fig. 1). In contrast, the 19-methyl analog, androst-4-ene-3,6,17-trione (**1**), failed to react with hydrogen peroxide under similar conditions. These results are consistent with the initial reversible formation of 19,19-hydroxy hydroperoxide **5** followed by intramolecular attack of the terminal oxygen of the hydroperoxide on the 4-ene-3,6-dione system, as seen for the hydroxy hydroperoxide of 19-oxoandrostenedione [29]. On the basis of this reaction sequence, it is predicted that compound **6** has the 4β,5β-epoxy ring. However, the stereochemistry of the epoxy ring was determined unambiguously based on its PMR spectrum. Thus, there was no significant NOE of the 19-proton (δ 9.82 ppm) when the 4α-proton (δ 3.23 ppm) was irradiated. A similar NOE result has been reported for the 19-methyl-4β,5β-epoxy analog **4** that was produced by reaction of compound **1** with hydrogen peroxide in the presence of a strong base, NaOH [33]. The spectral data and elemental analysis of compound **6** were consistent with the assigned structure.

Biochemical Properties

Inhibition of aromatase activity in human placental microsomes by epoxide **6** was examined *in vitro* by enzyme kinetics under initial velocity condition. Aromatase activity in placental microsomes was determined using a radiometric assay in which ^3H_2O released from [1β-^{3}H]androstenedione into the incubation medium during aromatization was measured [32]. The inhibitory activity of inhibitor **6** was very weak, and 15% inhibition of the activity was obtained at a 100 μM concentration. To characterize the nature of inhibitor binding to the active site of aromatase, aromatization was measured at several inhibitor and substrate concentrations. The results of this study were plotted on a typical Lineweaver–Burk plot (Fig. 2). The apparent inhibition constant (K_i), which characterizes enzyme affinity, was obtained by a Dixon plot. Inhibitor **6** exhibited clear-cut competitive inhibition with an apparent K_i value of 30 μM in which the apparent K_m value for the substrate androstenedione was found to be 33 nM.

The 4β,5β-epoxy-19-oxo steroid **6** was then tested for its ability to cause a time-dependent inactivation of aromatase. Inhibitor **6** showed a time-dependent inactivation when it was incubated in either the presence or absence of NADPH under aerobic conditions. Pseudo-first-order kinetics were obtained during the first 12 min of the incubation of the inhibitor when the kinetic data were analyzed according to the method of Kitz and Wilson [34] (Fig. 3). Double-reciprocal plots of k_{obs} versus inhibitor concentration gave k_{inact} and K_I values [35], respectively, for the inhibitor (Table 1). Addition of BSA to the incubation mixture did

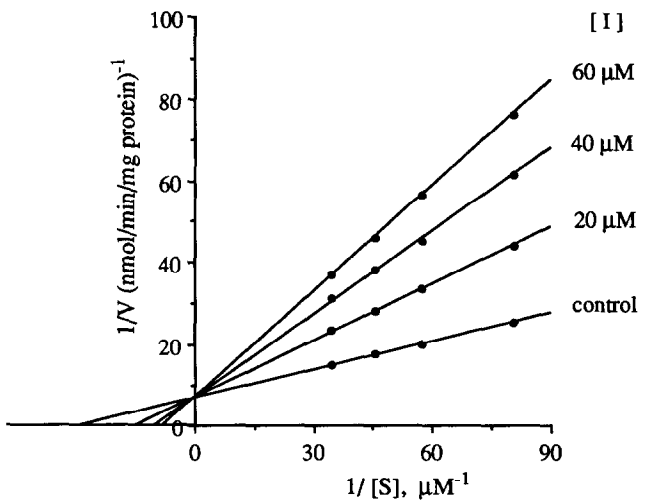

FIG. 2. Lineweaver-Burk plot of the inhibition of human placental aromatase by 4β, 5β-epoxy-19-oxo steroid 6 with androstenedione as a substrate. Each point represents the mean of two determinations which varied by less than 5%.

* *Abbreviations:* R_f, retardation value; and NOE, nuclear Overhauser enhancement.

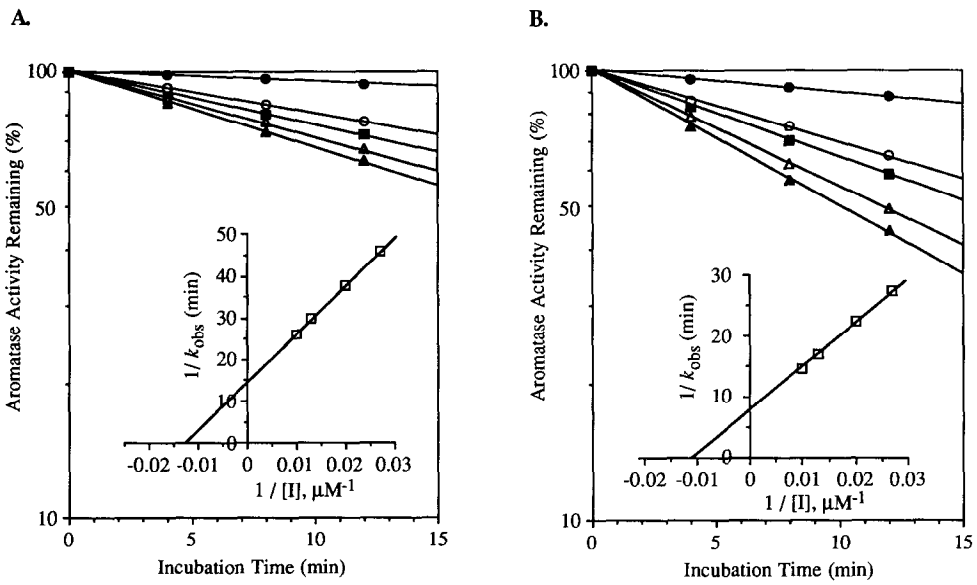

FIG. 3. Time- and concentration-dependent inactivations by 4β,5β-epoxy-19-oxo steroid 6 in the absence (A) or presence (B) of NADPH in air. Concentrations of the inhibitor: control (0 μM) (●); 37 μM (○); 50 μM (■); 75 μM (△); and 100 μM (▲). The aromatase activity remaining under conditions with 0-min preincubation time and no inhibitor equals 100% activity (106 pmol/min/mg protein). Each point represents the mean of two determinations which varied by less than 5%. The time-dependent inactivation experiments with BSA gave essentially similar plots to Fig. 3A and 3B (data not shown), respectively.

not change significantly the K_I values but enhanced the k_{inact} values to about 1.2 to 1.3-fold under the conditions with and without NADPH.

The substrate androstenedione blocked the inactivation caused by inhibitor **6** in either the presence or absence of NADPH (Fig. 4). On the other hand, a nucleophile, L-cysteine, had no significant effect on the inactivation in the absence or presence of NADPH (data not shown).

Reaction of the inhibitors 4 and 6 with N-acetyl-L-cysteine

To determine the chemical reactivity of inhibitor **6** towards a nucleophile, reaction of this compound as well as the 19-methyl-4β,5β-epoxide **4**, with *N*-acetyl-L-cysteine in the presence of $NaHCO_3$ in aqueous CH_3CN was carried out, and disappearance of the inhibitor from the reaction mixture was monitored by HPLC. As shown in Fig. 5, the inhibitors **4** and **6** disappeared in a time-dependent, pseudo-first-order manner with half-lives of 3.0 min for **4** and 40 sec for **6**. TLC analysis of the reaction with the inhibitors at reaction times of 30, 60, or 90 sec for **6** and 2, 3, or 5 min for **4** showed two spots corresponding to the substrate and a polar product [R_f: 0.00 (solvent 1) and 0.24 (solvent 2) for the reaction of **4**, 0.00 (solvent 1) and 0.21 (solvent 2) for the reaction of **6**] in each case.

TABLE 1. Kinetic analysis of time-dependent inactivation of aromatase caused by the 4β,5β-epoxy-19-oxo steroid 6 under various conditions*

Condition†			
NADPH	BSA	K_I (μM)	k_{inact} (min^{-1})
No	No	88	0.071
No	Yes	90	0.091
Yes	No	94	0.133
Yes	Yes	80	0.159

* Apparent K_I and k_{inact} were obtained by a Kitz-Wilson plot. Each result represents the mean of two determinations which varied by less than 10%.

† NADPH and BSA were added to the preincubation mixture at concentrations of 600 μM and 0.1%, respectively.

DISCUSSION

We synthesized and evaluated the 4β,5β-epoxy-19-oxo steroid **6** as a chemically reactive electrophile involved in the aromatase inactivation caused by the suicide substrate androst-4-ene-3,6-dione steroid **1**. The epoxide **6** inhibited aromatase activity in a competitive manner with a much greater apparent K_i value than those [27] of the 19-methyl-4β,5β-epoxide **4** and the 19-oxo steroid **3** (K_i = 5.1 and 7.5 μM, respectively). It has been reported previously that the epoxy steroid **4** does not inactivate aromatase in a mechanism-based (with NADPH) or affinity-labeling (without NADPH) manner, whereas the 19-oxo compound **3** does it only in a mechanism-based manner [27]. In contrast, inhibitor **6** having both 4β,5β-epoxy and 19-oxo structures, inactivated aromatase in a time-dependent manner in the absence of NADPH. Inhibitor **6** is competing for the same site as the natural substrate of aromatase; the presence of

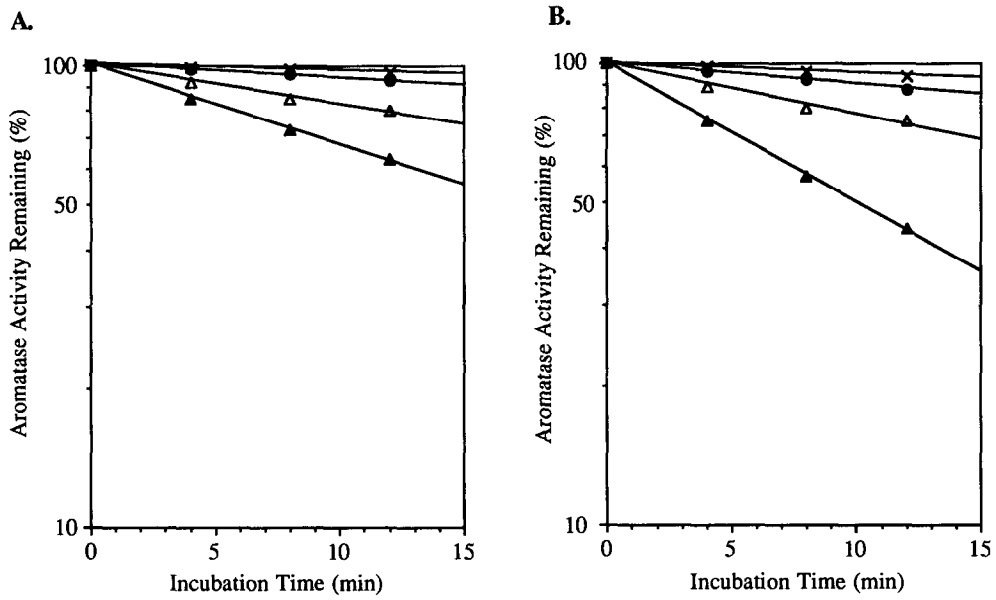

FIG. 4. Effect of androstenedione on time-dependent inactivation by 4β,5β-epoxy-19-oxo steroid 6 in the absence (A) or presence (B) of NADPH in air. Key: control (0 μM inhibitor) (●); control with androstenedione (0.32 μM) or the inhibitor (100 μM) with androstenedione (3 μM) (X); the inhibitor (100 μM) with androstenedione (0.32 μM) (△); the inhibitor (100 μM) only (▲). Each point represents the mean of two determinations which varied by less than 5%.

androstenedione blocked the time-dependent inactivation by the inhibitor. A nucleophile, L-cysteine, had no significant effect on the inactivation. These results indicate that the 4β,5β-epoxy-19-oxo compound **6** is an active-site-directed irreversible inhibitor, affinity-labeling agent, of aromatase.

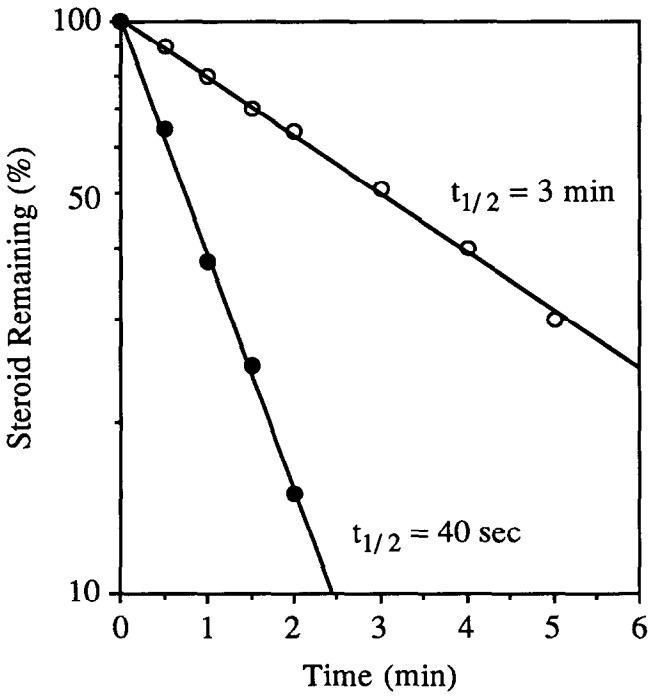

FIG. 5. Time–course for disappearance of 19-methyl-4β,5β-epoxide 4 (○) and 19-oxo-4β,5β-epoxide 6 (●) by reaction with N-acetyl-L-cysteine at 37° in the presence of $NaHCO_3$ in CH_3CN.

The K_I usually is taken as a measure of an affinity-labeling agent for the enzyme [35, 36]. This term is equal to $(k_{-1} + k_2)/k_1$ on the basis of the following equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} E - X$$

The K_I can be greater than the K_i, observed from competitive inhibition kinetics, if k_2 is partially rate-limiting. In this study, the K_I (88 μM) was somewhat greater than the K_i (30 μM), indicating that k_2 is partially rate-limiting. The active-site-directed agent **6** would bind in a favorable way when it undergoes the chemical reaction, alkylation of a nucleophile residue of amino acid of the active site of aromatase. This agent also inactivated aromatase in a time-dependent manner in the presence of NADPH with about 1.9-fold of the inactivation rate of that obtained in the absence of NADPH (k_{inact}: 0.071 vs 0.133 min^{-1}). NADPH did not increase the stability of the microsomal aromatase during the preincubation in the absence of the affinity label; about 12 and 7% loss of the aromatase activity were observed at the 12-min preincubation time with and without NADPH, respectively. The exact reason why NADPH causes an increased inactivation rate is unknown. However, a similar result has been obtained previously from the inactivation of aromatase by the affinity-labeling agent 6β-bromoacetoxyandrostenedione [37, 38]. BSA also stimulated the inactivation rate in either the presence or absence of NADPH (k_{inact} with BSA: 0.159 or 0.091 min^{-1}, respec-

tively). The k_{inact} values obtained under the conditions with NADPH and with NADPH plus BSA were comparable to that (0.145 min^{-1}) of the parent compound **1** [39].

A model reaction of the epoxy-19-oxo steroid **6** with a nucleophile, *N*-acetyl-L-cysteine, showed that this compound was more reactive towards the amino acid in the presence of $NaHCO_3$ than the other epoxide **4.** TLC analysis of the reaction mixtures strongly suggested that the reaction products would be steroid-amino acid adducts in each case, based on the R_f values of the products. Since nucleophilic opening of an epoxide with an SH compound yields stereospecifically a *trans* diaxial thiohydrin, the adducts produced in the model reactions should be the corresponding 5α-alkylthio-4β-hydroxy derivatives, respectively. It is reasonable to imply that a similar reaction should be operative in the alkylation of a nucleophilic residue of the active site of aromatase by steroid **6.** Hydrogen bonding between an oxygen atom of the 4β,5β-epoxy ring and a hydrogen atom of the 19-aldehyde group (see compound **6** of Fig. 6) would accelerate the epoxy-ring opening, namely the formation of the steroid-amino acid or steroid-aromatase adduct.

Robinson's group has reported that the aromatase model reaction of a 2,4-dien-3-ol analog of the 19-aldehyde intermediate with hydrogen peroxide is faithful to the actual aromatase-catalyzed reaction [30]; in contrast, the model reaction of the 19-aldehyde having a 4-en-3-one structure results in the formation of the 4β,5β-epoxy derivatives [29]. Based on these facts along with our previous studies that both the aromatization reaction of the 4-ene-3,6-dione steroid **1** and the aromatase inactivation by this steroid proceed through further oxygenation of the 19-aldehyde intermediate **3,** it is presumed that the 19-hydroxy-19-ferric hydroperoxide intermediate **7** having a 2,4-dien-3-ol structure will be converted into 6-oxoestrone, whereas the similar hydroperoxide intermediate **8** having a 4-en-3-one structure will be rearranged to the 4β,5β-epoxide **6** (Fig. 6). The intermediate **6** immediately alkylates a nucleophilic residue of the active site, without diffusion to the surrounding medium, causing the inactivation. This sequence is consistent with previous results obtained using the [^{3}H, ^{14}C]-compound **1.** The electron-withdrawing effect of a 6-carbonyl group of compound **3** will prevent, in part, the enolization of the other carbonyl at the C-3 position towards the C-2 position. This chemical nature may be suitable for the production of the electrophile **6.** To our knowledge, the structure of a reactive electrophile has been elucidated only for 19,19-difluoroandrostenedione among suicide substrates of aromatase [40, 41]. Thus, the present results are the first to show that a 4β,5β-epoxidation is involved in the inactivation of aromatase by a suicide substrate. This study would be helpful for understanding not only the function of aromatase but also the aromatization mechanism of the natural substrate.

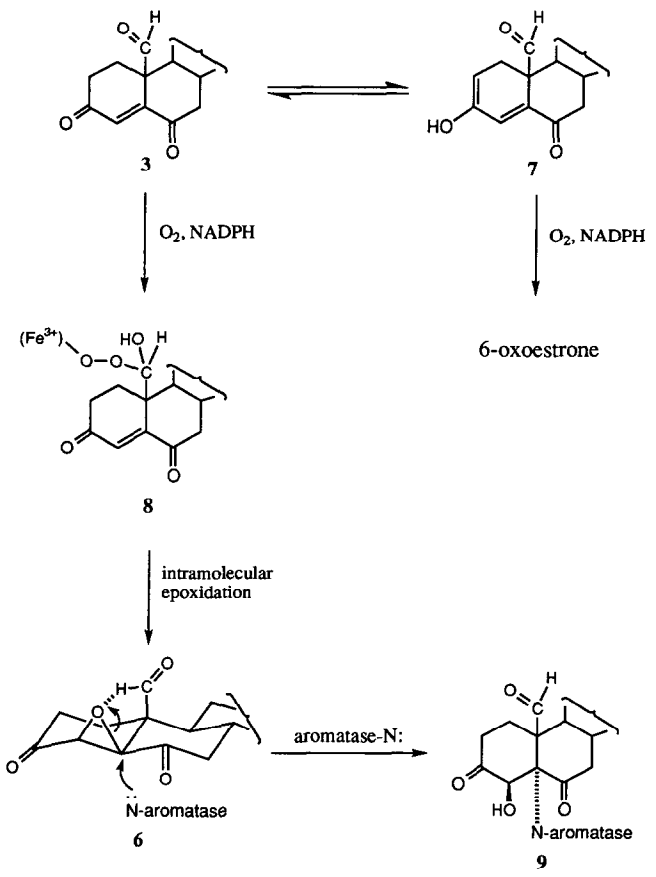

FIG. 6. Proposed mechanism for aromatase inactivation by the suicide substrate androst-4-ene-3,6,17-trione (1).

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