Aromatase Inactivation by a Suicide Substrate, Androst-5-ene-4,7,17-trione: The 5β,6β-Epoxy-19-oxo Derivative, as a Possible Reactive Electrophile Irreversibly Binding to the Active Site

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In order to understand the mechanism involved in the aromatase inactivation by androst-5-ene-4,7,17-trione (4), a suicide substrate of aromatase, 5β,6β-epoxyandrost-4,7,17,19-tetraone (6) was synthesized as a candidate for a reactive electrophile involved in irreversible binding to the active site of aromatase upon treatment of 19-oxo-5-ene steroid 5 with hydrogen peroxide in the presence of NaHCO₃. The epoxide 6 was a competitive inhibitor of human placental aromatase (Kᵢ = 34 μM); moreover, it inactivated the enzyme in an active-site-directed manner in the absence of NADPH (Kᵢ = 36 μM, a rate constant for inactivation (kₐ) = 0.027 min⁻¹). NADPH stimulated the inactivation rate, but the substrate androst-4-ene-3,17-dione blocked the inactivation. A nucleophile, l-cysteine, did not cause a significant change in the inactivation. When both the epoxide 6 and its 19-methyl analog 7 were subjected separately to a reaction with N-acetyl-l-cysteine in the presence of NaHCO₃, the 19-oxo compound 6 disappeared from the reaction mixture more rapidly (t₁/₂ = 6.0 min) than the 19-methyl analog 7 (t₁/₂ = 16 min). On the basis of these results, it is suggested that the 5β,6β-epoxy-19-oxo steroid 6 may be the reactive electrophile that alkylates a nucleophilic residue of the amino acid of the active site.

Key words aromatase; suicide substrate; inactivation mechanism; androst-5-ene-4,7,17-trione; reactive electrophile; 5β,6β-epoxy-19-oxo metabolite

Aromatase, a unique cytochrome P-450 enzyme complex, catalyzes the conversion of androst-4-ene-3,17-dione (androstenedione) and testosterone to estrone and estradiol. ¹,² The aromatization process is thought to proceed with three sequential oxidations at C-19 of the androgens.³⁻⁶ Inhibitors of aromatase may be valuable as therapeutic agents in the treatment of estrogen-dependent breast cancer.⁷⁻¹⁰ For this reason, the specific, irreversible blockade of estrogen biosynthesis via a mechanism-based (suicide) inactivation has been pursued with the goal of developing such an agent.

The known suicide substrates primarily involve the oxygenation of the 19-angular methyl of a 4-en-3-one steroid in the inactivation process. We previously reported that a C₁₇₄ steroid having a unique x,β-unsaturated ketone, 4-en-6-one,¹¹ 5-en-4-one,¹² and 5-en-7-one,¹³,¹⁴ instead of the 4-en-3-one, efficiently inhibits the aromatase activity in a competitive manner. Only the 5-en-7-one steroid, among these enones, inactivates aromatase in a suicide manner.¹³,¹⁴ Moreover, the 4-oxo derivative of this 5-en-7-one steroid, androst-5-ene-4,7,17-trione (4), is also a suicide substrate of aromatase.¹² Both 19-hydroxy and 19-oxo derivatives of the 5-en-4,7-dione ⁴¹² inactivate aromatase in a suicide manner, suggesting that the 19-oxygenation would be involved in the aromatase inactivation process by the 5-en-7-one and its 4-oxo derivative 4, respectively. On the other hand, the 6-oxo derivative of androstenedione, androst-4-ene-3,6,17-trione (1), a regioisomer of compound 4, is one of the earliest discussed suicide substrates of aromatase.¹⁵⁻¹⁷ Recently, we definitely established the mechanism involved in the aromatase inactivation by inhibitor 1: the two initial hydroxylations at C-19 of inhibitor 1 produce the 19-oxo compound 2, of which further oxygenation yields the 4β,5β-epoxy-19-oxo steroid 3 through rearrangement of the 19-hydroxy-19-hydroperoxide initially produced, and this epoxide 3 irreversibly binds to the active site of aromatase.¹⁸⁻²⁰ On the basis of the structural similarity between inhibitors 1 and 4, as well as our previous findings, we focused on 5β,6β-epoxyandrost-4,7,17,19-tetraone (6) as a reactive electrophile involved in the aromatase inactivation by compound 4. In this paper, we report the preparation and biochemical and chemical evaluation of the 5β,6β-epoxy-19-oxo steroid 6.

MATERIALS AND METHODS

[1β,2βH]Androstenedione (27.5 Ci/mmol; ²H-distribution, 1β = 74—79%) was purchased from New England Nuclear (Boston, MA, U.S.A.) and NADPH from Kohjin Co., Ltd. (Tokyo, Japan). Androst-5-ene-4,7,17-trione (4) and androst-5-ene-4,7,17,19-tetraone (5) were synthesized according to the methods¹² previously reported.

Melting points were measured on a Yanagimoto melting point apparatus (Kyoto, Japan) and are uncorrected. IR spectra were recorded in KBr pellet on a Perkin-Elmer FT-IR 1725X spectrophotometer (Norwalk, CT, U.S.A.) and UV spectra in 95% ethanol on a Hitachi 150-20 spectrometer (Tokyo, Japan). ¹H- and ¹³C-NMR spectra were obtained in a CDCl₃ solution with JEOL EX 270 (270 MHz for ¹H) and JEOL GX 400 (100.5 MHz for ¹³C).

![Fig. 1. Structures of Androst-4-ene-3,6,17-trione Derivatives](image_url)

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spectrometers (Tokyo, Japan), respectively, using tetramethylsilane as an internal standard, and MS spectra were obtained with a JEOL JMS-DX 303 spectrometer. TLC was performed with Merck pre-coated TLC plates, Silica gel 60-254, layer thickness 0.25 mm (Darmstadt, Germany). Silica gel column chromatography was conducted with Merck Kiesel gel 60 (70—230 mesh).

5β,6β-Epoxyandrosta-4,7,17,19-tetraene (6) Aqueous 30% hydrogen peroxide (0.4 ml, 3.4 mmol) was added to a solution of the 19-oxo-5-ene compound 5 (42 mg, 0.13 mmol) in methanol (6 ml) containing anhydrous NaHCO₃ (12 mg, 0.14 mmol), and the mixture was stirred at 0°C for 90 min. After this time, the reaction mixture was diluted with ethyl acetate (50 ml), washed with Na₂S₂O₃ solution and water, and dried with Na₂SO₄. After evaporation of the solvent, a crude product was purified by silica gel column chromatography (hexane—ethyl acetate), followed by recrystallization from acetone—hexane to give pure epoxide 6 (18 mg, 42%) as colorless needles; mp 210—212°C. ¹H-NMR δ: 0.88 (3H, s, 18-Me), 3.26 (1H, t, J = 11.5 Hz, 8β-H), 3.43 (1H, s, 6x-H), 10.04 (1H, s, 19-H). FT-IR cm⁻¹: 1718, 1725 and 1741 (C=O). MS m/z (rel. int. %): 330 (M⁺, 50), 301 (12), 283 (13), 273 (32), 255 (17), 227 (18), 199 (16), 189 (20), 161 (25), 153 (45), 131 (35), 105 (36), 91 (71), 79 (67), 67 (43), 55 (85), 41 (100) and 32 (40). Anal. Calcd for C₁₉H₂₂O₄: C, 69.07; H, 6.71. Found: C, 69.37; H, 6.53.

Epoxydation of the 19-Methyl-5-ene Steroid 4 Aqueous 30% hydrogen peroxide (2.0 ml, 17 mmol) was added to a mixture of the 19-methyl-5-ene compound 4 (227 mg, 0.76 mmol), methanol (18 ml), and 4 M NaOH solution (0.3 ml, 1.2 mmol). The resulting mixture was stirred at 0°C for 3.5 h, and then neutralized by adding 10% HCl. After dilution with ethyl acetate (200 ml), the mixture was washed with 5% NaHCO₃ solution and water. The organic layer was dried with Na₂SO₄, and the solvent was evaporated to afford an oil. TLC analysis of the oil revealed that the epoxidation produced a ca. 1:1 mixture of the 5β,6β-epoxy compound 7 and its 5α,6α-isomer, 8. The ca. 1:1 mixture was separated by silica gel column chromatography (hexane—ethyl acetate).

5β,6β-Epoxyandrosta-4,7,17-triene (7) The more polar product obtained by the above epoxidation reaction was recrystallized from acetone to yield the 5β,6β-epoxide 7 (85 mg, 35%) as colorless needles; mp 201—203°C. ¹H-NMR δ: 0.88 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 3.00 (1H, t, J = 11.2 Hz, 8β-H), 3.31 (1H, s, 6x-H). ¹³C-NMR δ: 13.6, 18.5, 19.0, 21.9, 22.2, 30.6, 35.4, 38.2, 38.6, 41.4, 42.4, 43.7, 46.6, 55.2, 62.2, 72.3, 72.8, 203.0, 206.9, 219.3. FT-IR cm⁻¹: 1717 and 1737 (C=O). MS m/z (rel. int. %): 316 (M⁺, 100), 298 (10), 283 (18), 273 (19), 260 (35), 245 (16), 231 (23), 218 (34), 199 (18), 189 (23), 177 (35), 161 (25), 149 (35), 139 (61), 131 (38), 121 (24), 105 (33), 91 (55), 79 (63), 67 (29), 55 (40), 41 (65) and 32 (56). Anal. Calcd for C₁₉H₂₂O₄: C, 72.13; H, 7.65. Found: C, 72.24; H, 7.63.

5α,6α-Epoxyandrosta-4,7,17-triene (8) The less polar epoxidation product obtained above was recrystallized from acetone—hexane to give the 5α,6α-epoxide 8 (78 mg, 32%) as colorless needles; mp 177—179°C. ¹H-NMR δ: 0.88 (3H, s, 18-Me), 1.04 (3H, s, 19-Me), 3.78 (1H, s, 6β-H). ¹³C-NMR δ: 13.9, 14.7, 20.4, 21.0, 23.2, 31.2, 33.5, 35.6, 38.8, 40.2, 42.8, 45.6, 47.3, 48.6, 59.2, 69.3, 203.3, 203.4, 219.3. FT-IR cm⁻¹: 1697, 1719 and 1737 (C=O). MS m/z (rel. int. %): 316 (M⁺, 90), 269 (13), 227 (15), 203 (100), 189 (15), 177 (28), 165 (24), 147 (21), 131 (24), 121 (15), 105 (24), 91 (43), 79 (50), 67 (28), 55 (34), 41 (58) and 32 (15). Anal. Calcd for C₁₉H₂₂O₄: C, 72.13; H, 7.65. Found: C, 72.10; H, 7.65.

Preparation of Placental Microsomes Human term placental microsomes (particles sedimenting at 105000 × g for 60 min) were obtained as described by Ryan. They were washed once with 0.5 mM dithiothreitol solution, lyophilized, and stored at −20°C. No significant 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, in which tritiated water released from 1β,3H-androstenedione into the incubation medium during aromatization was used as an index of the enzyme activity. 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 IC₅₀ experiment contained 180 µM of NADPH, 0.3 µM of the 3H-labeled substrate (3 × 10⁵ dpm), 20 µg of protein of the lyophilized microsomes, various concentrations of each of inhibitors 6 and 7, and 25 µl of methanol, and the entire mixture was incubated at 37°C for 20 min. For kinetic study, various concentrations of each of inhibitors 6 and 7, 14.3, 19.0, 24.0, and 32.2 nm concentrations of the 3H-labeled substrate, and a 5-min incubation time were used. Incubations were terminated by the addition of 3 ml of chloroform, followed by vortexing for 40 s. After centrifugation at 700 × g for 5 min, aliquots (0.25 ml) were removed from the water layer and added to a scintillation mixture for the determination of tritiated water production.

Time-Dependent Inactivation Procedure Various concentrations of each of inhibitors 6 and 7 (40, 80, and 200 µM for 6, 1, 1.5, and 2 µM for 7) were incubated with or without NADPH (600 µM), androstenedione (0.5 µM), and l-cysteine (0.5 mM) at 37°C with placental microsomes (200 µg protein) and methanol (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,3H]androstenedione (300 nM, 3 × 10⁵ 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°C for 20 min. The tritiated water release was determined as described above.

Reaction of the 5β,6β-Epoxides 6 and 7 with N-Acetyl-t-cysteine A solution of the epoxides 6 and 7 (0.66 or 0.63 mg, 2 µmol), N-acetyl-l-cysteine (1.3 mg, 8 µmol), and NaHCO₃ (1.3 mg, 16 µmol) in water (0.1 ml) and methanol (0.4 ml) was shaken at 37°C. Aliquots (50 µl) of the reaction mixture were removed at an appropriate time and diluted with methanol (50 µl). An aliquot (10 µl) of the diluted mixture was then subjected to HPLC. Amounts of the remaining epoxides 6 and 7 were obtained using an absolute calibration method. HPLC conditions: pump, Waters 510 pump; solvent, methanol: water = 60:40 (v/v), 1 ml/min; column, Puresil C₁₈ 5 µm 120A (Waters)
(150 mm × 4.6 mm i.d.); detector, Waters 486 UV detector at 220 nm. Retention time: 3.0 min for 6 and 4.2 min for 7. The reaction was also analyzed by TLC. TLC conditions: solvent 1, hexane : ethyl acetate = 1 : 1 (v/v); solvent 2, CHCl₃ : CH₃OH : HCOOH = 10 : 0.5 : 0.3 (v/v). The Rf values of compounds 6 and 7 were 0.38 and 0.58 (solvent 1) or 0.51 and 0.73 (solvent 2), respectively.

RESULTS

Synthesis Reaction of androst-5-ene-4,7,17,19-tetraone (5) with hydrogen peroxide in the presence of a weak base, NaHCO₃, in methanol gave the 5β,6β-epoxy derivative 6 in a 42% yield (Fig. 2). In contrast, the 19-methyl steroid, androst-5-ene-4,7,17-trione (4), did not react with hydrogen peroxide under similar conditions. The results are consistent with the initial reversible formation of 19-hydroxy-19-hydroperoxide, followed by intramolecular attack of the terminal oxygen of the hydroperoxy function on the 5-ene-4,7-dione system, as seen for the hydroxy hydroperoxide of 19-oxo androstenedione or its 6-oxo analog. On the basis of this reaction sequence, the stereochemistry of the 5,6-epoxy ring was assigned as β. In contrast, the reaction of the 19-methyl compound 4 in the presence of a strong base, NaOH, afforded the 5β,6β-epoxide 7 along with the 5α,6α-isomer 8 as a ca. 1 : 1 mixture. Their structures were determined, based principally on their NMR spectroscopies (H, ¹³C, C–H correlated spectroscopy (COSY), H–H COSY, and NOESY). The β-configuration of the epoxy ring of compound 7 was consistent with a lower field shift of the 19-methyl proton signal (δ = 1.23) and a higher field shift of the epoxy proton at C-6 (δ = 3.31) compared to the α-epoxide 8 (δ = 1.04 for the 19-methyl protons and 3.78 for the epoxy proton at C-6). Furthermore, the 8β-proton of the β-epoxide 7 resonated at a relatively low field, 3.00 ppm. These signals for the 8β-proton and the epoxy proton at C-6 of compound 6 also support the configuration of the epoxy ring of the 5β,6β-epoxy-19-oxo steroid 6 (δ = 3.26 for 8β-H and δ = 3.43 for 6α-H).

Biochemical Properties Reversible inhibition of aromatase activity in human placental microsomes by the 5β,6β-epoxy-19-oxo steroid 6 was initially examined in vitro by enzyme kinetics under an initial velocity condition. The inhibitory activity of inhibitor 6 was very weak, and 12% inhibition of the activity was obtained at 40 μM concentration. To characterize the nature of the inhibitor binding to the active site of aromatase, aromatization was measured at several inhibitor and substrate concentrations. The results of the study were plotted on a typical Lineweaver–Burk plot (Fig. 3). The apparent inhibition constant (Kᵢ), an index for the affinity of the enzyme for the inhibitor, was obtained by a Dixon plot. Inhibitor 6 exhibited clear-cut competitive inhibition with an apparent Kᵢ value of 34 μM, in which the apparent Kᵢ value for the substrate androstenedione was 33 nM.

The 5β,6β-epoxy-19-oxo compound 6 was then tested for its ability to cause a time-dependent inactivation of aromatase. Inhibitor 6 showed time-dependent inactivation when it was incubated in either the presence or absence of NADPH in air. Pseudo-first-order kinetics were obtained during the first 12 min of the incubation of the inhibitor in each experiment when the kinetic data were analyzed according to Kitz and Wilson (Fig. 4). With increasing inhibitor concentration, increasing kᵢobs was obtained. Double-reciprocal plots of kᵢobs versus inhibitor concentration gave a rate constant for inactivation (kᵢinact) of 0.074 min⁻¹ and Kᵢ of 33 μM in the experiment with NADPH and kᵢinact of 0.027 min⁻¹ and Kᵢ of 36 μM in the experiment without NADPH.

The substrate androstenedione blocked the inactivation caused by inhibitor 6 in either the presence or absence of NADPH (Fig. 5). A nucleophile, l-cysteine, had no significant effect on the inactivation in the presence or absence of NADPH (data not shown). Reaction of the Epoxides 6 and 7 with N-Acetyl-l-cysteine The chemical reactivity of the 19-oxo-5β,6β-

![Fig. 3. Lineweaver–Burk Plot of the Inhibition of Human Placental Aromatase by 5β,6β-Epoxide-19-oxo Steroid 6 with Androstenedione as a Substrate](image-url)
epoxy steroid 6 towards a nucleophile was then examined. This inhibitor, as well as the 19-methyl analog 7, were subjected to reaction with N-acetyl-l-cysteine in the presence of NaHCO₃ in aqueous methanol, and the disappearance of the epoxides from the reaction mixture was monitored by HPLC. These epoxides disappeared in a time-dependent manner with half-lives of 6.0 min for the 19-oxo compound 6 and 16 min for the 19-methyl analog 7 (Fig. 6). TLC analysis of the reaction with the epoxides at reaction times of 5, 10, and 20 min for compounds 6 and 7, respectively, showed two spots corresponding to the substrate and a polar product [RF: 0.00 (solvent 1) and 0.22 (solvent 2) for the reactions of 6, 0.00 (solvent 1) and 0.25 (solvent 2) for the reactions of 7] in each case.

DISCUSSION

The 5β,6β-epoxy-19-oxo steroid 6 was synthesized and evaluated as a chemically reactive electrophile involved in the aromatase inactivation caused by the suicide substrate androst-5-ene-4,7-dione steroid 4. The epoxide 6 inhibited aromatase activity in a competitive manner, with a greater apparent Kᵢ value than that of the 19-oxo-5-ene steroid 5 (Kᵢ = 34 for 6 vs. 6.3 μM) for 5). Furthermore, inhibitor 6 inactivated aromatase in a time-dependent manner in the absence of NADPH. Androstenedione blocked the time-dependent inactivation, in a dose-response manner, by the inhibitor, with an apparent Kᵢ value of 36 μM, indicating that the inhibitor is competing for the same site as the natural substrate of aromatase. A nucleophile, l-cysteine, had no significant effect on the inactivation.
These results show that the 5β,6β-epoxy-19-oxo steroid 6 is an active-site-directed irreversible inhibitor, affinity-labeling agent of aromatase.

The $K_{ii}$, observed from the time-dependent inactivation kinetics, was almost the same to the $K_{ii}$, observed from competitive inhibition kinetics, indicating that reversible binding of inhibitor 6 to the active site of aromatase is rate-limiting for the inactivation by the inhibitor. The active-site-directed agent 6 binds in a favorable way when it undergoes the following chemical reaction: alkylation of a nucleophilic residue of amino acid of the active site. In the presence of NADPH, this agent also inactivated aromatase in a time-dependent manner with about 2.7-fold of the inactivation rate of that obtained in the absence of NADPH ($k_{\text{inact}}$, 0.027 vs. 0.074 min$^{-1}$). About 10 and 6% loss of the aromatase activity were observed at the 12-min preincubation time with and without NADPH, respectively, showing that NADPH did not increase the stability of the microsomal aromatase during the preincubation. Although the exact reason why NADPH causes such an increased inactivation rate is unknown, similar results have been obtained previously from the inactivation of aromatase by the affinity-labeling agents 6β-bromoacetoxysteroid,27,28 and the 4β,5β-epoxy-19-oxo-3 steroid.20)

The 5β,6β-epoxy-19-oxo compound 6 was more reactive than the 19-methyl-5β,6β-epoxide 7 toward a nucleophile, N-acetyl-L-cysteine, in a model reaction with the amino acid in the presence of NaHCO$_3$. The $R_f$ values of the products on TLC strongly suggested that the reaction products would be steroid-amino acid adducts in each case. Furthermore, the adducts should be trans dixial thiolhydrins, 5α-alkylthio-6β-hydroxy derivatives, respectively, based on the chemical nature of nucleophilic opening of an epoxide. 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 the epoxide 6. Hydrogen bonding between an oxygen atom of the 5β,6β-epoxy ring and a hydrogen atom of the 19-aldehyde group, or coordination of a lone pair of an oxygen atom of the epoxy ring to a carbon atom of the 19-aldehyde group, would accelerate the epoxy-ring opening, similarly to the reaction of the other affinity labeling agent, 4β,5β-epoxy-19-oxo steroid 3, with the amino acid.20)

The present results, along with our previous findings, suggest that further oxygenation of the 19-aldehyde intermediate, 5, which produced two sequential oxygenations of the 19-methyl of the parent steroid, 4, will yield the 19-hydroxy-19-ferric hydroperoxide intermediate 9 which is rearranged to the 5β,6β-epoxide 6. (Fig. 7). The reactive electrophile 6 immediately alkylates a nucleophilic residue of the active site, without diffusion to the surrounding medium, causing irreversible inactivation. The structural similarity of inhibitor 4 to the suicide substrate 1, of which the aromatase inactivation mechanism has definitely been determined,20 also support the proposed inactivation sequence described above. Further study is now underway to directly detect the aromatase-catalyzed 19-oxygenation of steroid 4.

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