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A TIME-DEPENDENT INACTIVATION OF AROMATASE BY 19-OXYGENATED ANDROST-4-ENE-3,6,17-TRIONES

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Summary—19-Hydroxyandrost-4-ene-3,6,17-trione (19-OHAT), its 19-oxo derivative (19-oxo AT) and 4β,5β-epoxyandrostane-3,6,17-trione (**5**) were synthesized as possible intermediates involved in a mechanism-based inactivation of aromatase caused by androst-4-ene-3,6,17-trione (AT). These compounds inhibited the enzyme in a competitive manner with K_i's of 0.61, 7.5 and 5.1 μM for 19-OHAT, 19-oxo AT, and compound **5**. The two 19-oxygenated steroids showed a time-dependent, pseudo-first order rate of inactivation of aromatase with k_{inact}'s of 0.222 and 0.076 min^{-1} for 19-OHAT and 19-oxo AT, respectively, while compound **5** did not. NADPH and oxygen were required for the inactivation. Androstenedione blocked the inactivation, while L-cysteine partially prevented that of 19-OHAT and almost completely that of 19-oxo AT. When the 19-oxygenated steroids were separately subjected to reaction with *N*-acetyl-L-cysteine, these rapidly disappeared from the reaction mixture with $t_{1/2}$ of 25 min (19-OHAT) and 20 s (19-oxo AT). This finding indicates that L-cysteine prevents inactivation by a chemical dependent elimination of the inhibitors from the incubate. These results suggest that the 19-oxygenation rather than the 4,5-epoxidation may be involved in the time-dependent inactivation by AT.

INTRODUCTION

Aromatase, a unique cytochrome *P*-450 monooxygenase complex, catalyzes the synthesis of estrone or estradiol from androst-4-ene-3,17-dione (androstenedione) or testosterone [1–5]. It has been proposed that this aromatization process is achieved via three sequential hydroxylations of the androgen. The first oxygenation occurs on the 19-methyl group of androstenedione to yield 19-hydroxyandrostenedione (19-OHA), followed by the second oxygenation at the same carbon to give the intermediate 19-oxoandrostenedione (19-oxo A) [6–9]. The precise nature of the third step is still not established. Nevertheless, after this final oxygenation, the oxygenated androgen spontaneously aromatizes with expulsion of the 19-methyl group as formic acid [10]. A potent selective inhibitor of aromatase activity might be effective in the treatment of estrogen-dependent breast carcinoma and in the modulation of the reproductive process [10–15]. The specific irreversible blockade of estrogen biosynthesis via mechanism-based inactivation of aromatase has been intensely pursued with the goal of developing practical clinical drugs.

Androst-4-ene-3,6,17-trione (AT) is a mechanism-based inhibitor of aromatase [16, 17]. Its 3α- and 3β-reduced derivatives also inactivate aromatase in a time-dependent manner, although 3-deoxy AT, a very potent competitive inhibitor, does not cause it. These demonstrate that an oxygen function (carbonyl, hydroxyl, ester, or ether group) at the 3-position is essential for a 4-en-6-one steroid to cause the time-dependent inactivation [17–19]. Covey and Hood [16] have postulated without biochemical data that enzymatic oxygenations at C-19 may be involved in the activation by AT. To determine whether the 19-oxygenated or 4,5-epoxy derivatives of AT have an intermediary role in the inactivation by AT, we prepared the possible intermediates and evaluated them as inactivators of aromatase.

EXPERIMENTAL

Materials and methods

[1β-^{3}H]Androstenedione (25.4 Ci/mmol; ^{3}H-distribution, β/α = 74.2/25.8) was purchased from New England Nuclear (Boston, Mass.) and NADPH from Kohjin Co. Ltd (Tokyo,

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Japan). Silica gel thin-layer plates (Kieselgel 60-F_{254}, 0.25 mm thick) were supplied from E. Merck AG (Darmstadt, Fed. Rep. Germany). Androst-4-ene-3,6,17-trione (AT) [17] and 3β-acetoxy-19-hydroxy-androst-5-en-17-one (**1**) [20] were synthesized according to known methods.

Melting points were measured on a Yanagimoto melting point apparatus. i.r. spectra were recorded on a Shimadzu IR-430 spectrophotometer and u.v. spectra on a Hitachi 150-20 spectrophotometer. [^{1}H]NMR spectra were obtained with a JEOL GX 400 (400 MHz) spectrometer using tetramethylsilane (δ = 0.00 ppm) or $CHCl_3$ (δ = 7.26 ppm, for tert-butyldimethylsiloxy compounds) as an internal standard.

Synthesis

3β-Acetoxy-19-(tert-butyldimethylsiloxy)-androst-5-en-17-one (2). tert-Butyldimethylsilyl chloride (7.21 g, 47.8 mmol) and imidazole (3.07 g, 45.1 mmol) were added to a solution of 3β-acetoxy-19-hydroxyandrost-5-en-17-one (**1**) [20] (4.4 g, 12.7 mmol) in dry dimethylformamide (95 ml). The reaction mixture was stirred at room temperature for 16 h, then diluted with ether (600 ml), and washed with 5% NH_4Cl solution (200 ml × 2) and water. The organic layer was dried (Na_2SO_4) and evaporated to give an oil which was purified by silica gel column chromatography (hexane–AcOEt, 9:1) and then recrystallization from acetone to give **2** (4.99 g, 85%) as colorless needles; m.p. 110–111°C. [^{1}H]NMR ($CDCl_3$) δ 0.04 and 0.05 (3H, s, 19-$SiMe_2$-C), 0.88 (9H, s, 19-$SiMe_2CMe_3$), 0.91 (3H, s, 18-Me), 2.05 (3H, s, 3-OCOMe), 3.61 (1H, d, J = 10.7 Hz, 19-Ha), 3.78 (1H, d, J = 10.7 Hz, 19-Hb), 4.63 (1H, m, 3α-H), 5.62 (1H, m, 6-H). i.r. (KBr) 1740 (C = 0) cm^{-1}. Anal. Calcd for $C_{27}H_{44}O_4Si$: C, 70.39; H, 9.63. Found: C, 70.42; H, 9.57.

3β-Hydroxy-(19-tert-butyldimethylsiloxy)-androst-5-en-17-one (3). To a solution of compound **2** (2.0 g, 4.34 mmol) in MeOH (66 ml) was added 1 M NaOH (7.9 ml) and the reaction mixture was stirred at room temperature for 1 h. After this time, the mixture was poured into ice-water (600 ml) and the precipitates were collected by filtration, dried under vacuum, and recrystallized from AcOEt to give **3** (1.75 g, 96%) as colorless amorphous: m.p. 118–119°C. [^{1}H]NMR ($CDCl_3$) δ −0.01 and 0.00 (3H, s, 19-$SiMe_2$-C), 0.83 (9H, s, 19-$SiMe_2CMe_3$), 0.87 (3H, s, 18-Me), 3.53 (1H, m, 3α-H), 3.56 and 3.75 (1H, d, J = 10.7 Hz, 19-H), 5.55 (1H, m, 6-H). i.r. (KBr) 3435 (OH) and 1740 (C = O) cm^{-1}. Anal. Calcd for $C_{25}H_{42}O_3Si$: C, 71.71; H, 10.11. Found: C, 71.77; H, 9.98.

19-(tert-Butyldimethylsilyloxy)androst-4-ene-3,6,17-trione (4). Jones reagent (4.7 ml) was added to a solution of compound **3** (1.63 g, 3.89 mmol) in acetone (120 ml) and the mixture was stirred at room temperature for 25 min. After this time, the reaction mixture was poured into ice-water (1 l) and the precipitates were collected by filtration, washed with water, dried under vacuum, and purified by silica gel column chromatography (hexane–AcOEt, 7:1, v/v) and recrystallization from acetone to afford **4** (840 mg, 50%) as pale yellow prisms: m.p. 126–127°C. [^{1}H]NMR ($CDCl_3$) δ 0.00 and 0.03 (3H, s, 19-$SiMe_2$-C), 0.86 (9H, s, 19-$SiMe_2CMe_3$), 0.96 (3H, s, 18-Me), 3.79 (1H, d, J = 10.2 Hz, 19-Ha), 3.92 (1H, d, J = 10.2 Hz, 19-Hb), 6.39 (1H, s, 4-H). i.r. (KBr) 1740, 1690 and 1675 (C = O) cm^{-1}. u.v. (95% EtOH) λ_{max} 248.4 nm ($\epsilon = 8.67 \times 10^3$). Anal. Calcd for $C_{25}H_{38}O_4Si$: C, 69.72; H, 8.89. Found: C, 69.50; H, 9.10.

19-Hydroxyandrost-4-ene-3,6,17-trione (19-OHAT). To a solution of compound **4** (680 mg, 1.58 mmol) in a mixture of propan-2-ol (18 ml) and tetrahydrofuran (12 ml) was added 3 M HCl (10 ml) and the mixture was stirred at room temperature for 22 h, then diluted with AcOEt (300 ml), washed with saturated $NaHCO_3$ solution and water, and dried (Na_2SO_4). Evaporation of the solvent yielded a solid product which was recrystallized from acetone to give 19-OHAT (450 mg, 90%) as pale yellow plates: m.p. 224–225°C (decomp.). [^{1}H]NMR ($CDCl_3$) δ 0.98 (3H, s, 18-Me), 3.93 and 3.99 (1H, d, J = 10.7 Hz, 19-H), 6.39 (1H, s, 4-H). i.r. (KBr) 3460 (OH) and 1730, 1680 and 1660 (C = O) cm^{-1}. u.v. (95% EtOH) λ_{max} 241.4 nm ($\epsilon = 8.8 \times 10^3$). Anal. Calcd for $C_{19}H_{24}O_4$: C, 72.13; H, 7.65. Found: C, 72.00; H, 7.94.

Androst-4-ene-3,6,17,19-tetraone (19-oxo AT). A solution containing 19-OHAT (32 mg, 0.1 mol) and pyridinium dichromate (57 mg, 0.15 mmol) in dichloromethane (1.5 ml) was stirred at room temperature for 5 h. After this time, the reaction mixture was diluted with AcOEt (50 ml), washed with 5% $NaHCO_3$ solution and saturated NaCl solution, and dried (Na_2SO_4). Evaporation of the solvent gave a solid product which was recrystallized from acetone to give 19-oxo AT (15 mg, 48%) as pale yellow plates: m.p. 205–206.5°C. [^{1}H]NMR ($CDCl_3$) δ 0.89 (3H, s, 18-Me), 6.55 (1H, s, 4-H),

9.93 (1H, s, 19-H). i.r. (KBr) 1730 and 1690 (C = O) cm^{-1}. u.v. (95% EtOH) λ_{max} 238 nm ($\epsilon = 8.9 \times 10^3$). Anal. Calcd for $C_{19}H_{22}O_4$: C, 72.59; H, 7.06. Found: C, 72.76; H, 7.00.

4β,5β-Epoxyandrostane-3,6,17-trione (5). To a solution of AT (1 g, 3.36 mmol) in a mixture of MeOH (100 ml) and 10% NaOH solution (2.0 ml) was added 30% H_2O_2 solution (7.55 ml) dropwise with stirring under ice-cooling and the resulting mixture was allowed to stand at 4°C overnight. The reaction mixture was poured into a chilled saturated NaCl solution (500 ml) and extracted with AcOEt (300 ml × 3). The organic phase was washed with saturated $NaHCO_3$ solution and water and dried (Na_2SO_4). After evaporation of the solvent, the resulting solid was recrystallized from hexane–acetone to afford **5** (258 mg, 25%) as colorless plates: m.p. 158–160.5°C. [^{1}H]NMR ($CDCl_3$) δ 0.93 (3H, s, 18-Me), 1.05 (3H, s, 19-Me), 3.81 (1H, s, 4α-H). i.r. (KBr) 1730 (broad, C = O) cm^{-1}. MS *m/z* 316 (M^+). Anal. Calcd for $C_{19}H_{24}O_4$: C, 72.13; H, 7.65. Found: C, 71.88; H, 7.67.

Reaction of AT, 19-OHAT, 19-oxo AT with N-acetyl-L-cysteine

A solution of steroid (0.01 mmol), *N*-acetyl-L-cysteine (0.08 mmol), $NaHCO_3$ (0.24 mmol) in H_2O (0.32 ml) and MeOH (1.28 ml) was allowed to stand at 37°C. An aliquot (0.1 ml) of the reaction mixture was removed at an appropriate time and diluted with 0.25–0.40 ml of MeCN and then subjected to high-performance liquid chromatography (HPLC). HPLC conditions: pump, Waters 510 pump; solvent, $CH_3CN:H_2O = 45:55$ (for AT) or 30:70 (for 19-OHAT and 19-oxo AT), 1 ml/min; column, ERC-ODS-1161 column (Erma Optical Works Ltd, Tokyo, Japan) (10 cm × 6 mm i.d.); detector, ERC-7211 UV detector (Erma) at 240 nm. t_R: 4.9 min for AT, 4.6 min for 19-OHAT, and 8.1 min for 19-oxo AT.

The reaction mixture was analyzed by thin-layer chromatography (TLC) at 1-min (19-oxo AT) or 30-min (19-OHAT) reaction time. TLC conditions: solvent 1, hexane/AcOEt = 1:2, v/v; solvent 2, chloroform/methanol/formic acid = 10:0.5:0.3. R_f values of 19-oxo AT and 19-OHAT are 0.48 and 0.38 (solvent 1) or 0.36 and 0.33 (solvent 2), respectively.

Enzyme preparation

Human term placental microsomes (particles sedimenting at 105,000 *g* for 60 min) were obtained as described by Ryan [21]. They were washed twice with 0.5 mM dithiothreitol solution, lyophilized, and stored at −20°C. No loss of aromatase activity occurred over the period of the study.

Screening assay procedure

Aromatase activity was measured according to the original procedure of Thompson and Siiteri [22]. This assay quantitates the production of ^3H_2O released from [1β-^{3}H]androstenedione by aromatization. All enzymatic studies were carried out in 67 mM phosphate buffer, pH 7.5, at a final incubation volume of 0.5 ml. The incubation mixture contained 180 μM NADPH, 1 μM [1β-^{3}H]androstenedione, 40 μg of protein of the lyophilized microsomes, various concentrations of inhibitors, and 25 μl of MeOH. Incubations were performed at 37°C for 20 min in air, terminated by addition of 3 ml $CHCl_3$, and vortexed for 40 s. 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.

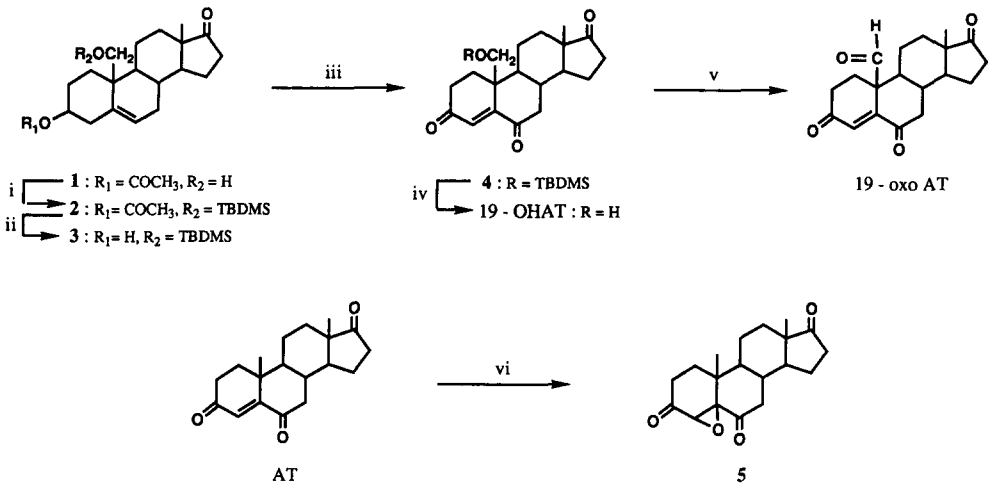
Time-dependent inactivation procedure

Various concentrations of 19-OHAT, 19-oxo AT, and compound **5** were incubated with placental microsomes (1 mg protein), 600 μM NADPH, and MeOH (50 μl) in 67 mM phosphate buffer, pH 7.5, in a total volume of 1 ml at 37°C in air. In experiments with compound **5**, this was also incubated under the above conditions without NADPH. 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 (1 μM, 3.0×10^5 dpm), 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. ^3H_2O release was determined as described above.

RESULTS

Chemistry

Reaction of 3β-acetoxy-19-hydroxyandrost-5-en-17-one (**1**) with tert-butyldimethylsilyl chloride in the presence of imidazole in dimethylformamide gave 19-silyl ether **2**, which was treated with methanolic NaOH solution followed by oxidation with a large excess of Jones reagent at room temperature to yield 19-siloxy-3,6,17-trione steroid **4** (Fig. 1). Deprotection of the 19-silyl ether by treatment with



TBDMS : $Si(CH_3)_2C(CH_3)_3$

Fig. 1. Synthesis of 19-OHAT and 19-oxo AT. Reagents: (i) TBDMS–Cl, imidazole, DMF; (ii) 1 M NaOH, MeOH; (iii) Jones reagent; (iv) 3 M HCl, THF-propan-2-ol; (v) Pyridinium dichromate, CH_2Cl_2; and (vi) H_2O_2, NaOH.

diluted HCl afforded the 19-alcohol derivative (19-OHAT). Oxidation of 19-OHAT with pyridinium dichromate in dichloromethane yielded the 19-oxo derivative (19-oxo AT). 4β,5β-Epoxy steroid **5** was synthesized from AT by treatment with hydrogen peroxide in the presence of NaOH. The configuration of the epoxy ring was assigned based on the previous result [23] concerning the epoxidation of androstenedione with hydrogen peroxide and a chemical shift of 19-methyl protons (δ 1.05 ppm) in the [^{1}H]NMR spectrum. The structures of these steroids were identified by spectral data and elemental analysis.

Biochemical properties

Reversible inhibition of aromatase activity in human placental microsomes by 19-OHAT, 19-oxo AT, and the epoxide **5**, compared to the parent inhibitor AT is shown in Table 1. 19-OHAT, 19-oxo AT and compound **5** were effective inhibitors of aromatase, the rank order being AT > 19-OHAT > compound **5** > 19-oxo AT. The three steroids were further studied to characterize the nature of their interactions with the active site. Aromatization was measured at several concentrations of the inhibitor in the presence of increasing concentrations of androstenedione. Lineweaver–Burk plots of the results are shown in Fig. 2. All of these inhibitors exhibited clear cut competitive-type inhibition with apparent inhibition constants (K_i), obtained by analysis of Dixon plots, of 0.61, 7.5 and 5.1 μM for 19-OHAT, 19-oxo AT, and the epoxide **5**, in which K_m for androstenedione is about 60 ± 7 nM and K_i for AT is 60 nM (Table 1).

Time-dependent inactivation was observed when the 19-oxygenated steroids, 19-OHAT and 19-oxo AT, were incubated with aromatase in the presence of NADPH in air (Fig. 3), while compound **5** at the concentrations employed (10, 20 and 30 μM) behaved only as a competi-

Table 1. *In vitro* aromatase inhibitory activity[a]

Inhibitor	IC_{50} (μM)	K_i (μM)[b]	Inhibition
19-OHAT	15	0.61	Competitive
19-oxo AT	84	7.5	Competitive
4β,5β-epoxide **5**	53	5.1	Competitive
AT	0.96	0.060	Competitive

[a]Substrate: 1 μM [1β-^{3}H]androstenedione; human placental microsomes: 40 μg protein.
[b]K_i was obtained by analysis of Dixon plots, in which K_m for the natural substrate, androstenedione, was 60 ± 7 nM.

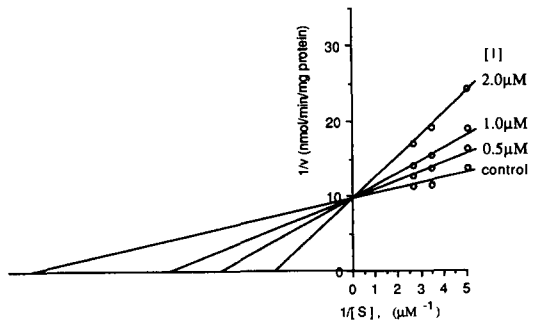


Fig. 2. Lineweaver–Burk plot of inhibition of human placental aromatase by 19-OHAT using androstenedione as the substrate. Each point represents the mean of two determinations. The inhibition experiments with compound **5** and 19-oxo AT gave essentially similar plots of Fig. 2 (data not shown).

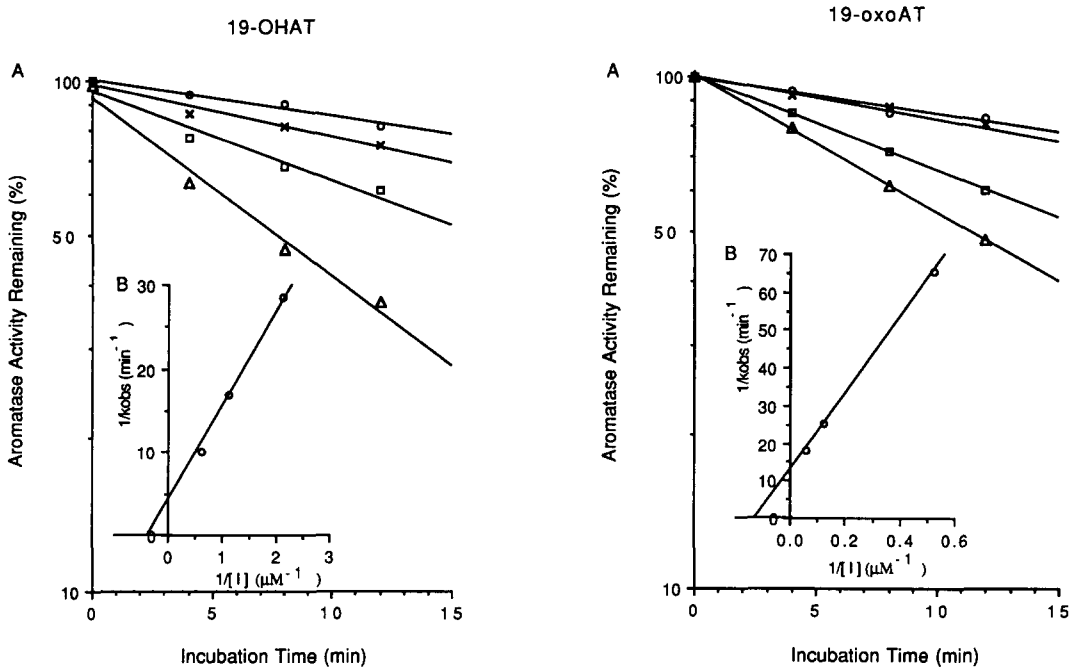


Fig. 3. Time-dependent (A) and concentration-dependent (B) inactivations of human placental aromatase by 19-OHAT and 19-oxo AT in the presence of NADPH in air. Concentrations of 19-OHAT were: control (0 μM), ○; 0.5 μM, ×; 1 μM, □; 2 μM, △. Concentrations of 19-oxo AT were: control (0 μM), ○; 2 μM, ×; 10 μM, □; 30 μM, △. Each point represents the mean of two determinations.

tive inhibitor with no evidence of enzymatic generation of reactive substance in the presence of NADPH (Fig. 4). The inhibitor **5** also did not cause the inactivation of aromatase in a time-dependent manner in the absence of NADPH. Pseudo-first order kinetics were obtained during the first 12 min of the incubation of 19-OHAT

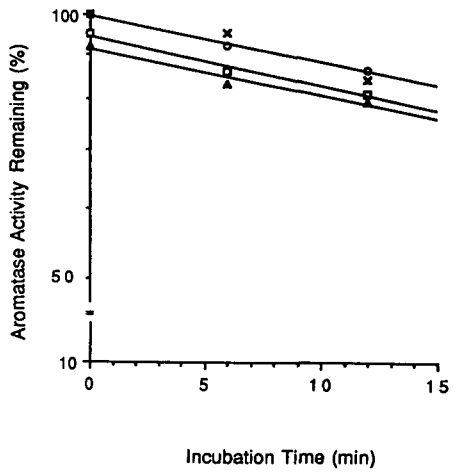


Fig. 4. Time-course for decrease in human placental aromatase activity by compound **5** in the presence of NADPH in air. Concentrations of the inhibitor were: control (0 μM), ○; 10 μM, ×; 20 μM, □; 30 μM, △. Each point represents the mean of two determinations. The inactivation experiments with inhibitor **5** in the absence of NADPH afforded essentially similar plots to Fig. 4 (data not shown).

and 19-oxo AT. With increasing inhibitor concentrations, increasing k_{obsd}'s were obtained for both compounds. Double-reciprocal plots of k_{obsd} versus inhibitor concentration [24] yielded K_i's of 2.5 and 7.7 μM and k_{inact}'s of 0.222 and 0.076 min^{-1}, respectively, for 19-OHAT and 19-oxo AT [Fig. 3(B)]. NADPH and oxygen were essential for the activity loss [Fig. 5(A)] and the substrate androstenedione completely blocked the inactivation [Fig. 5(B)].

A nucleophile, L-cysteine, partially protected the enzyme from inactivation by 19-OHAT [Fig. 5(C)], while, for 19-oxo AT, the nucleophile almost completely protected it (Fig. 6). To know the stability of these inhibitors in the incubation medium containing the thiol, reaction of 19-oxo AT, 19-OHAT, or AT with *N*-acetyl-L-cysteine in the presence of $NaHCO_3$ in aqueous methanol was carried out and disappearance of the inhibitor from the reaction mixture was monitored by HPLC. As shown in Fig. 7, 19-oxo AT and 19-OHAT disappeared in a time-dependent, pseudo-first order manner with half-lives of 20 s and 25 min, respectively, whereas AT was stable under the conditions. TLC analysis of the reaction with 19-oxo AT at 1-min reaction time showed two spots corresponding to the substrate and a polar product [R_f, 0.00 (solvent 1) or 0.15 (solvent 2)] in an

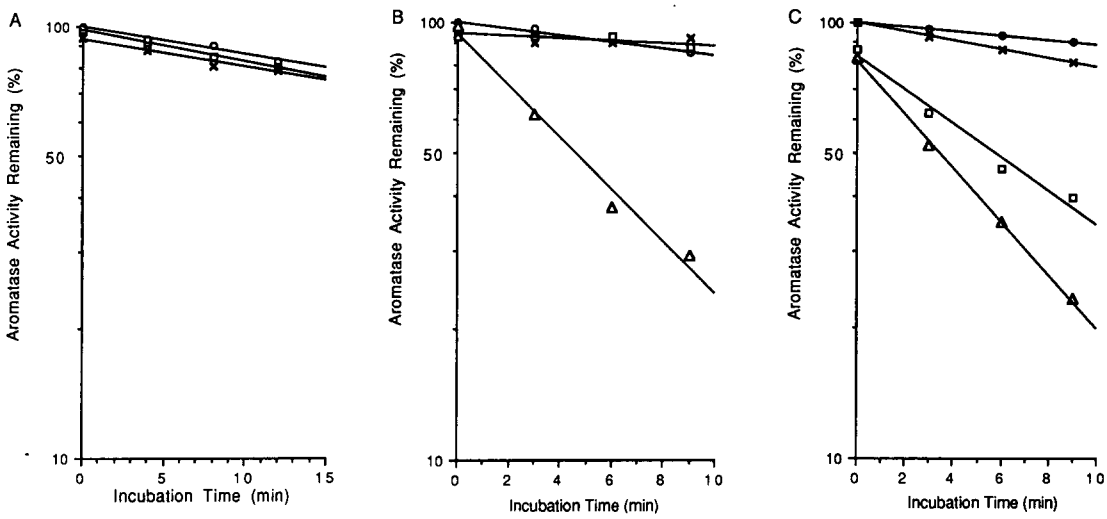


Fig. 5. Inactivation of human placental aromatase by 19-OHAT (2 μM) under various conditions. (A) In the absence of NADPH (×) or in N_2 atmosphere (□), the inhibitor failed to produce the inactivation. Control does not contain the inhibitor (○). (B) Androstenedione at concentrations (0 μM, △; 2 μM, ×) was incubated with aromatase, the inhibitor, and NADPH in air and protected the enzyme from inactivation. Control with (□) or without (○) androstenedione (2 μM) does not contain the inhibitor. (C) In the presence (□) of L-cysteine (0.5 mM), a time-dependent inactivation of aromatase by the inhibitor, observed in the absence of the nucleophile (△), was partially protected. Control with (×) or without (○) the nucleophile contains no inhibitor. Each point represents the mean of two determinations. The inactivation experiments with 19-oxo AT in the absence of NADPH and in N_2 atmosphere gave essentially similar results to Fig. 5A and B (data not shown).

approximate 1:10 ratio. In the experiment with 19-OHAT (reaction time, 30 min), the formation of polar product [R_f, 0.00 (solvent 1) and 0.10 (solvent 2)], as a sole product, along with the substrate (approximate 1:1 ratio) was similarly detected by TLC.

DISCUSSION

4β,5β-Epoxidation [25] as well as Baeyer–Villiger type oxygen insertion [9], 2β-hydroxylation [26], and 19,19-hydroxyperoxidation [27] has been proposed for the last oxygenation step in the aromatase reaction but shown to be unlikely. However, there is no report concerning the epoxidation of AT by the aromatase reaction. Then, we have synthesized 19-hydroxy, 19-oxo, and 4β,5β-epoxy derivatives of AT (19-OHAT, 19-oxo AT, and compound **5**, respectively) and carried out a series of experiments using these steroids to determine whether the 19-oxygenation or the 4β,5β-epoxidation is indeed involved in the suicide inactivation of aromatase by AT. 19-OHAT and 19-oxo AT inactivated aromatase activity in a time-dependent manner in the presence of NADPH and O_2 (Fig. 3) while the epoxide **5**, an effective competitive aromatase inhibitor, did not (Fig. 4). The similarities of the apparent K_i's for 19-OHAT and 19-oxo AT, obtained according to Kitz and Wilson [24], with those obtained from the competition experiments described in Table 1 suggest that the initial binding of the inhibitors to the enzyme is rate-limiting. The inhibitor **5** also did not inactivate the enzyme in an affinity labeling manner in the absence of

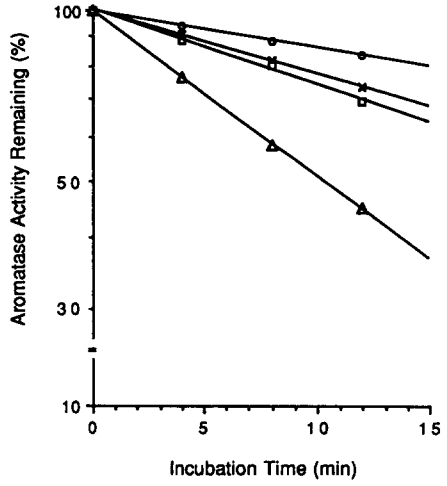


Fig. 6. Complete prevention of the time-dependent inactivation by 19-oxo AT in the presence of L-cysteine. 19-oxo AT (20 μM) was incubated without (△) or with (□) L-cysteine (0.5 mM). Control with (×) or without (○) L-cysteine does not contain the inhibitor. Each point represents the mean of two determinations.

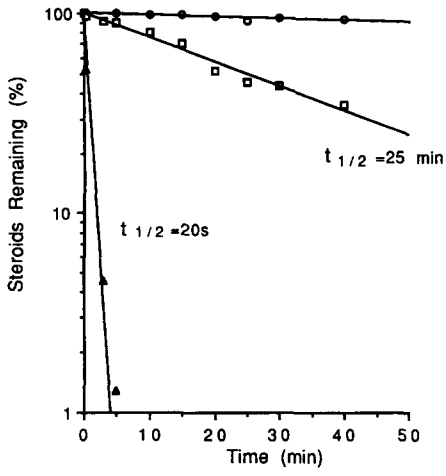


Fig. 7. Time-course for disappearance of AT (○), 19-OHAT (□), and 19-oxo AT (△) by reaction with *N*-acetyl-L-cysteine in the presence of sodium bicarbonate in methanol.

NADPH. The results indicate that 19-oxygenation rather than the epoxidation seems to be involved in a time-dependent inactivation of aromatase caused by AT.

19-OHAT and 19-oxo AT are competing for the same site as the natural substrate of aromatase; the presence of androstenedione blocked the time-dependent inactivation by the two inhibitors [Fig. 5(B)]. A nucleophile, L-cysteine, almost completely prevented the inactivation of aromatase by 19-oxo AT (Fig. 6) and partially by 19-OHAT [Fig. 5(C)]. The 19-oxygenated AT derivatives rapidly disappeared, with half-lives of 20 s (19-oxo AT) and 25 min (19-OHAT), from the reaction mixture containing *N*-acetyl-L-cysteine in a pseudo-first order manner (Fig. 7). This strongly suggests that the partial or complete prevention of the inactivation by L-cysteine would principally be due to a chemical dependent elimination of the inhibitor. We have previously reported that AT reacts with a thiol to yield a 1,4-adduct in which a thiol attaches to the C-4α position of a steroid molecule through a sulfur atom [28]. Moreover, reaction of 19-OHAT with *N*-acetyl-L-cysteine also yields the similar 1,4-adduct (reported elsewhere). Considering these, it is concluded that the 1,4-addition reaction should principally be involved in the chemical dependent elimination of the inhibitors. Although there is no evidence, a conformational transmission of distortion through C-19, C-10, and C-5 or a stereoelectronic effect might be in operation in a marked difference of the reactivity toward a thiol between the AT derivatives.

The affinities of intermediates involved in the androgen aromatization for aromatase decrease in the following order: androstenedione > 19-OHA > 19-oxo A [5] for estrone biosynthesis, and 16α-hydroxyandrostenedione (16α-OHA) > 19-hydroxy-16α-OHA > 19-oxo 16α-OHA [29] for estriol biosynthesis. The overall similarity of the affinities of the AT derivatives to those of the natural substrates and intermediates was observed (AT > 19-OHAT > 19-oxo AT).

The present findings that 19-OHAT and 19-oxo AT are mechanism-based inhibitors of aromatase as well as AT, along with their relative affinities to the enzyme, strongly suggest that AT may inactivate the enzyme through 19-oxo AT which is produced by subsequent two oxygenations at C-19. Oxidative biotransformation of 19-oxo AT may produce a more reactive electrophile (X) which immediately binds to a nucleophile of the active site (path a, Fig. 8) to result in the inactivation of aromatase. However, since 19-oxo AT in itself is a very reactive electrophile, 19-oxo AT may directly bind to a nucleophile of the active site, without further activation process, in a 1,4-addition

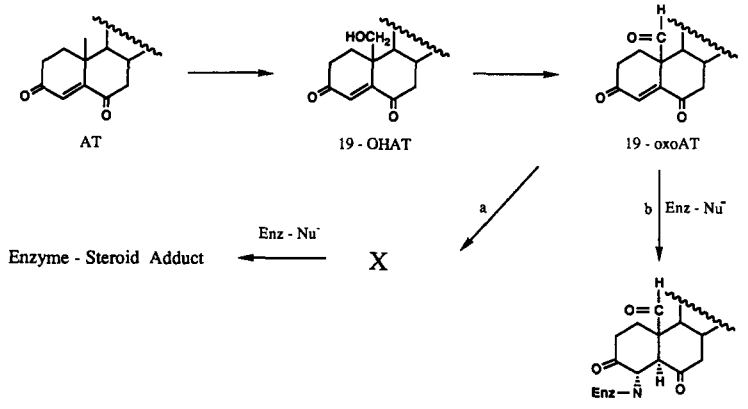


Fig. 8. Proposed mechanisms for the inactivation of aromatase by AT.

manner (path b, Fig. 8). To further clarify the inactivation mechanism, radioactive AT which is regio- or stereo-selectively labeled with ^{3}H or ^{14}C is required and the study is now under way.

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