# A time-dependent inactivation of aromatase by 19-substituted androst-4-ene-3,6,17-triones

**Mitsuteru Numazawa, Ayako Mutsumi, Naomi Asano, and Yuri Ito**

*Tohoku College of Pharmacy, Aobaku, Sendai, Japan*

*Diastereomeric (19S)- and (19R)-19-ethynyl-19-acetoxy derivatives of androst-4-ene-3,6,17-trione (AT) (**9** and **10**) and 19,19-difluoro AT (**12**) were synthesized. The 19,19-difluoro compound (**12**) was an effective competitive inhibitor of human placental aromatase with an inhibition constant ($k_i$) of 1.8 μM but the acetylenic **9** and **10** were poor inhibitors of the enzyme with $k_i$s of 75 and 67 μM, respectively. Inhibitor **12** caused a time-dependent, biphasic loss of aromatase activity in the presence of reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) in air, whereas the other two caused a time-dependent, pseudo-first-order inactivation of the activity with rate constants for inactivation of 0.250, 0.077, and 0.065 $min^{-1}$ for steroids **12**, **9**, and **10**. NADPH was required for the time-dependent inactivation, and the substrate androst-4-ene-3,17-dione prevented it.* L-*Cysteine did not protect aromatase from the inactivation.* (*Steroids* **58**:40–46, 1993)

**Keywords:** aromatase; suicide substrate; chemical synthesis; 19-ethynyl-19-acetoxyandrost-4-ene-3,6,17-trione; 19,19-difluoroandrost-4-ene-3,6,17-trione; time-dependent inactivation

## Introduction

Aromatase, a unique cytochrome P450 enzyme complex, catalyzes the conversion of androst-4-ene-3,17-dione (androstenedione) and testosterone to estrone and estradiol, respectively.[1–4] The aromatization process is thought to proceed via three sequential oxygenations of the androgen. The initial steps involve two sequential hydroxylations at carbon 19 to yield 19-hydroxyandrostenedione and 19,19-dihydroxyandrostenedione, which may dehydrate to the intermediate 19-oxo derivative.[5–7] The details of the third step remain obscure. Nevertheless, after the final oxygenation, the 19-oxo derivative aromatizes with expulsion of the 19-methyl group as formic acid.[8–10] A selective inhibitor of aromatase might be effective in the treatment of estrogen-dependent breast cancer.[11–15] For this reason, a wide variety of suicide substrates of aromatase, which primarily has made use of the oxygenation of the 19-methyl group in the inactivation process, have been developed. 19-Acetylenic alcohol[16] and 19,19-difluoro derivatives[17] of the natural substrate androstenedione are typical ones among them.

Address reprint requests to Dr. Mitsuteru Numazawa, Tohoku College of Pharmacy, 4-1 Komatsushima-4-chome, Sendai 981, Japan.
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Androst-4-ene-3,6,17-trione (AT) inactivates aromatase in a mechanism-based manner.[18,19] Covey and Hood[18] have postulated that enzymatic oxygenations at C-19 may be involved in the inactivation of aromatase caused by AT. We[20] recently have synthesized 19-hydroxy- and 19-oxo-AT as the possible intermediates in the inactivation and have shown that they also inactivate the enzyme in a time-dependent manner.

As a continuing study of the mechanism of aromatase inactivation by AT, we have further investigated whether the 19-oxygenation is involved in the inactivation process. The studies described in this article focus on the synthesis and biochemical evaluation of 19-acetylenic (**9** and **10**) and 19,19-difluoro (**12**) derivatives of AT. The steroids **9**, **10**, and **12** caused a mechanism-based inactivation of aromatase.

## Experimental

### *Materials and general methods*

[1β-$^3$H]Androstenedione (27.5 Ci/mmol; $^3$H-distribution, 74–79% at 1β) was purchased from New England Nuclear (Boston, MA, USA) and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) from Kohjin Co., Ltd. (Tokyo, Japan).

Melting points were measured on a Yanagimoto melting point apparatus (Kyoto, Japan) and are uncorrected. Infrared (IR) spectra were recorded in KBr pellet on a Perkin Elmer FT-IR

1725X spectrophotometer (Norwalk, CT, USA) and ultraviolet (UV) spectra on a Hitachi 150-20 spectrophotometer (Tokyo, Japan). Proton nuclear magnetic resonance ($^1$H NMR) spectra were obtained in $CDCl_3$ solution with a JEOL GX 400 (400 MHz) spectrometer (Tokyo, Japan) using tetramethylsilane ($\delta$ = 0.00) or $C\underline{H}Cl_3$ ($\delta$ = 7.26) for *tert*-butyldimethylsilyl (TBDMS) derivatives as an internal standard, and mass spectra (MS) with a JEOL JMS-DX 303 spectrometer. Optical rotations were done on a JASCO DIP-360 polarimeter. Analytical thin-layer chromatography (TLC) was performed with E. Merck precoated TLC plates (Rahway, NJ, USA) (silica gel 60F-254, layer thickness 0.25 mm). Silica gel column chromatography was conducted with E. Merck Kieselgel 60 (70-230 mesh).

### *5α-Bromo-3β-(*tert*-butyldimethylsiloxy)-17,17-(ethylenedioxy)androstan-6β,19-oxide (2)*

3β-Hydroxy steroid (**1**) (2.50 g, 5.85 mmol), synthesized according to known method,[21] and imidazole (1.60 g, 23.4 mmol) were dissolved in 40 ml of dimethylformamide (DMF), and TBDMS/Cl (3.50 g, 23.4 mmol) was added to this solution portionwise. The mixture was stirred at room temperature and poured into saturated $NH_4Cl$ solution. The precipitates were collected by filtration, dried under vacuum, and recrystallized from acetone to give **2** (2.90 g, 91%) as colorless needles: mp 204.5–205.5 C; $^1$H NMR $\delta$ 0.070 and 0.073 (3H each, s, 3-OSi$Me_2$), 0.87 (3H, s, 18-Me), 0.88 (9H, s, 3-OSi$Me_2$C$\underline{Me}_3$), 3.68–3.98 (6H, m, 17,17-$OCH_2CH_2O$- and 19-$CH_2$), 4.04 (1H, d, J = 4.8 Hz, 6α-H), 4.09 (1H, m, 3α-H). Analysis calculated for $C_{27}H_{45}O_4SiBr$: C, 59.87; H, 8.37. Found: C, 59.67; H, 8.40.

### *3β-(*tert*-Butyldimethylsiloxy)-17,17-(ethylenedioxy)androst-5-en-19-ol (3)*

Zinc dust (11.5 g) was added to a solution of **2** (3.81 g, 7.05 mmol) in 95% EtOH (480 ml) and the mixture was heated under reflux with stirring for 6 hours. The suspension was filtered and the residue was washed with 95% EtOH. The combined filtrates were condensed to ~30 ml, diluted with AcOEt (500 ml), washed with saturated $NaHCO_3$ solution and water, and dried ($Na_2SO_4$). A solid product, obtained by evaporation of the solvent, was purified by recrystallization from acetone to afford **3** (3.04 g, 93%) as colorless needles: mp 151–153 C; $^1$H NMR $\delta$ 0.03 and 0.04 (3H each, s, 3-OSi$Me_2$), 0.85 (3H, s, 18-Me), 0.86 (9H, s, 3-OSi$Me_2$C$\underline{Me}_3$), 3.51 (2H, m, 3α-H and 19-Ha), 3.72–3.92 (5H, m, 17,17-$OCH_2CH_2O$- and 19-Hb), 5.68 (1H, m, 6-H); IR 3,387 (OH) cm$^{-1}$. Analysis calculated for $C_{27}H_{46}O_4Si$: C, 70.08; H, 10.02. Found: C, 70.00; H, 10.22.

### *3β-(*tert*-Butyldimethylsiloxy)-17,17-(ethylenedioxy)androst-5-en-19-one (4)*

Pyridinium dichromate (1.23 g, 3.27 mmol) was added to a solution of **3** (1.01 g, 2.18 mmol) in $CH_2Cl_2$ (20 ml) and the mixture was stirred at room temperature for 9.5 hours. After this time, the reaction mixture was subjected to column chromatography (silica gel, 70 g; $CH_2Cl_2$) and the product eluted was recrystallized from acetone to give **4** (760 mg, 75%) as colorless plates: mp 130–132 C; $^1$H NMR $\delta$ 0.01 and 0.02 (3H each, s, 3-OSi$Me_2$), 0.77 (3H, s, 18-Me), 0.84 (9H, s, 3-OSiC$Me_2$C$\underline{Me}_3$), 3.46 (1H, m, 3α-H), 3.80–3.91 (4H, m, 17,17-$OCH_2CH_2O$–), 5.78 (1H, d, J = 5.5 Hz, 6-H), 9.63 (1H, s, 19-H); IR 1,718 (C=O) cm$^{-1}$. Analysis calculated for $C_{27}H_{44}O_4Si$: C, 70.39; H, 9.63. Found: C, 70.51, H, 9.54.

### *(19R/S)-19-Ethynyl-3β-(*tert*-butyldimethylsiloxy)-17,17-(ethylenedioxy)-androst-5-en-19-ol (5)*

Acetylene (passed through a −60 C trap and $H_2SO_4$) was bubbled at room temperature into tetrahydrofuran (THF) (145 ml, freshly distilled from $LiAlH_4$) for 10 minutes before and during the dropwise addition of 3 M ethylmagnesium bromide in ether (7 ml, 21 mmol), all at room temperature. Acetylene was bubbled into the clear solution for another hour. $N_2$ was passed over the ethynylmagnesium bromide solution, and 19-oxo steroid (**4**; 1.88 g, 4.07 mmol) in dry THF (15 ml) was added dropwise, followed by rinse with THF (5 ml). After vigorous stirring for 5 hours, the reaction mixture was poured into 500 ml each of AcOEt and saturated $NH_4Cl$ solution, acidified with 10% HCl, shaken, and separated. The organic layer was washed successively with saturated $NaHCO_3$ and NaCl solutions, dried ($Na_2SO_4$), and evaporated to give a solid. The crude product was purified by column chromatography on silica gel (120 g, hexane/AcOEt, 9 : 1) and recrystallized from acetone to afford **5** (1.39 g, 70%) as colorless needles: mp 167–170 C; $^1$H NMR $\delta$ 0.003 and 0.000 (3H each, s, 3-OSi$Me_2$), 0.821 (3H, s, 18-Me), 0.824 (9H, s, 3-OSi$Me_2$C$\underline{Me}_3$), 2.55 (0.8H, d, J = 2.6 Hz, 19-C≡CH), 2.58 (0.2H, d, J = 2.6 Hz, 19-C≡CH), 3.49 (1H, m, 3α-H), 3.79–3.90 (4H, m, 17,17-$OCH_2CH_2O$–), 4.94 (0.8H, d, J = 2.6 Hz, 19-H), 4.96 (0.2H, d, J = 2.6 Hz, 19-H), 5.65 (0.8H, m, 6-H), 5.68 (0.2H, m, 6-H); $[\alpha]_D^{24}$ = +28.3° (c = 0.01). Analysis calculated for $C_{29}H_{46}O_4Si$: C, 71.55; H, 9.53. Found: C, 71.32; H, 9.65.

### *Acetylation of alcohol (5) with acetic anhydride/pyridine and subsequent hydrolysis with hydrochloric acid*

A solution of alcohol (**5**; 530 mg, 1.09 mmol) in acetic anhydride (2.7 ml)/pyridine (6 ml) was heated at 60 C for 2.5 hours. After this time, the reaction mixture was poured into ice-water (150 ml) and extracted with AcOEt (200 ml). The organic phase was washed successively with 5% HCl, saturated $NaHCO_3$ solution and water, dried ($Na_2SO_4$), and evaporated to give a crude product (**6**). To a solution of the crude acetate (**6**; 550 mg) in THF (7 ml) and propan-2-ol (22 ml) was added 3 M HCl (7 ml), and the reaction mixture was stirred at room temperature overnight, condensed to about 10 ml under reduced pressure, and diluted with AcOEt (300 ml). The organic phase was washed with saturated $NaHCO_3$ solution and water, dried ($Na_2SO_4$), and evaporated to give a solid, which was purified by column chromatography (silica gel, 40 g). Elution with hexane/AcOEt (3 : 1 and 5 : 2) yielded three fractions: the first fraction was assigned to be (19S)-19-ethynyl-19-acetoxy-3β-hydroxyandrost-5-en-17-one (**7**) (225 mg, 57% from **5**): mp 203–206 C (colorless plates from acetone); $^1$H NMR $\delta$ 0.99 (3H, s, 18-Me), 2.08 (3H, s, 19-OCOMe), 2.60 (1H, d, J = 2.6 Hz, 19-C≡CH), 3.57 (1H, m, 3α-H), 5.66 (1H, d, J = 4.8 Hz, 6-H), 6.09 (1H, d, J = 2.2 Hz, 19-H); IR 3,305 (OH), 2,117 (C≡CH), 1,735 (C=O) cm$^{-1}$; $[\alpha]_D^{24}$ = +11.86° (c = 0.01). Analysis calculated for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16. Found: C, 74.79; H, 8.02.

The second fraction was shown to be about 1 : 1 mixture of **7** and its stereoisomer (**8**; 30 mg), analyzed by $^1$H NMR spectrometry (19-C≡CH, $\delta$ 2.60 for **7** and 2.56 for **8**) and TLC (Rf, 0.39 for **7** and 0.35 for **8**; hexane/AcOEt, 1 : 1).

The third fraction was (19R)-19-ethynyl-19-acetoxy-3β-hydroxy-androst-5-en-17-one (**8**) (60 mg, 15% from **5**), mp 177.5–179 C (colorless plates from acetone): $^1$H NMR $\delta$ 0.99 (3H, s, 18-Me), 2.13 (3H, s, 19-OCOMe), 2.56 (1H, d, J = 2.6 Hz, 19-C≡CH), 3.55 (1H, m, 3α-H), 5.72 (1H, d, J = 4.6 Hz, 6-H), 5.88 (1H, d, J = 2.2 Hz, 19-H); IR 3,270 (OH), 2,119 (C≡CH), 1,731 (C=O) cm$^{-1}$; EI-MS m/z 328 ($M^+$-42), 255 (base peak); $[\alpha]_D^{24}$ = −1,087° (c = 0.005). Analysis calculated for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16. Found: C, 74.83; H, 8.15.

### *(19S)-19-ethynyl-19-acetoxyandrost-4-ene-3,6,17-trione (9)*

Jones reagent (0.5 ml) was added to a solution of **7** (181 mg, 0.49 mmol) in acetone (20 ml) and the mixture was stirred at 0 C for

1 hour. After this time, the mixture was poured into saturated NaCl solution (250 ml) and extracted with AcOEt (250 ml × 2). The combined organic phase was washed successively with saturated $NaHCO_3$ and NaCl solutions, dried ($Na_2SO_4$), and evaporated to yield a solid, which was purified by column chromatography on silica gel (15 g) (hexane/AcOEt, 1 : 1) and recrystallized from acetone/hexane to give **9** (129 mg, 69%) as colorless needles: mp 173–176 C; $^1$H NMR δ 0.99 (3H, s, 18-Me), 2.06 (3H, s, 19-OCOMe), 2.68 (1H, d, J = 2.2 Hz, 19-C≡CH), 5.76 (1H, d, J = 2.6 Hz, 19-H), 6.80 (1H, s, 4-H); IR 2123 (C≡CH), 1,741 and 1,690 (C=O) cm$^{-1}$; UV $\lambda_{max}^{95\%EtOH}$ (ε): 246 nm (9.4 × 10$^3$); EI-MS m/z 382 (M$^+$); $[\alpha]_D^{24}$ = +131° (c = 0.01). Analysis calculated for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85. Found: C, 72.33; H, 7.71.

## *(19R)-19-ethynyl-19-acetoxyandrost-4-ene-3,6,17-trione (10)*

Compound **8** (130 mg, 0.35 mmol) was oxidized with Jones reagent (0.4 ml) similarly as described for the synthesis of **9** (acetone, 14 ml; reaction time, 1 hour). After essentially the same workup as above, the solid product was subjected to column chromatography (silica gel, 13 g). Elution with hexane/AcOEt (2 : 1) gave the crude product, which was recrystallized from MeOH to afford **10** (81 mg, 62%) as colorless plates: mp 166–169 C; $^1$H NMR δ 0.97 (3H, s, 18-Me), 2.00 (3H, s, 19-OCOMe), 2.69 (1H, d, J = 2.2 Hz, 19-C≡CH), 5.85 (1H, d, J = 2.2 Hz, 19-H), 6.47 (1H, d, J = 1.1 Hz, 4-H); UV $\lambda_{max}^{95\%\ EtOH}$ (ε): 246 nm (9.4 × 10$^3$); IR: 2,124 (C≡CH), 1,741 and 1,687 (C=O) cm$^{-1}$; MS m/z 382 (M$^+$); $[\alpha]_D^{23}$ = +55° (c = 0.01). Analysis calculated for $C_{23}H_{26}O_5$: C, 72.23; H, 6.85. Found: C, 72.51; H, 6.68.

## *19,19-Difluoroandrost-4-ene-3,6,17-trione (12)*

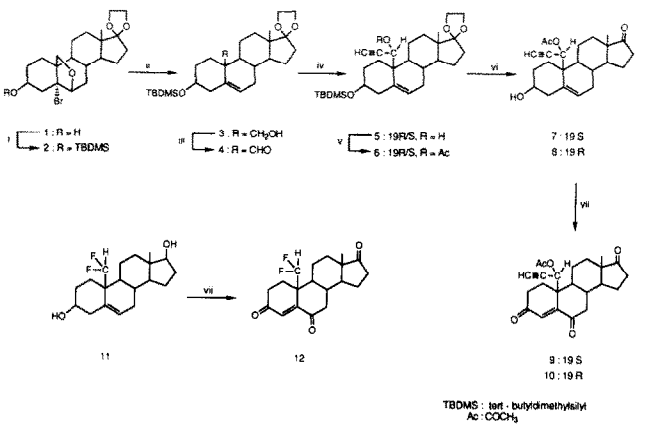
19,19-Difluoroandrost-5-ene-3β,17β-diol (**11**) (272 mg, 0.83 mmol), synthesized according to the reported method,[22] was dissolved in acetone (25 ml). To this solution was added Jones reagent (1.1 ml) and the reaction mixture was stirred at room temperature for 1.2 hours, poured into chilled water (200 ml) saturated with NaCl, extracted with AcOEt (200 ml × 2), washed with saturated NaCl solution and water, and dried ($Na_2SO_4$). Evaporation of the solvent gave a crude product, which was purified by column chromatography (silica gel, 14 g; hexane/AcOEt, 3 : 1) and recrystallized from acetone to give **12** (90 mg, 31%) as pale yellow prisms: mp 259–260 C (decomposition); $^1$H NMR δ 0.97 (3H, s, 18-Me), 6.05 (1H, t, J = 55.1 Hz, 19-H), 6.54 (1H, s, 4-H); IR: 1,733, 1,697, and 1,682 (C=O) cm$^{-1}$; UV $\lambda_{max}^{95\%\ EtOH}$ (ε): 242 nm (1.0 × 10$^4$). Analysis calculated for $C_{19}H_{22}O_3F_2$: C, 67.84; H, 6.59. Found: C, 68.11; H, 6.60.

## *Enzyme preparation*

Human term placental microsomes (particles sedimenting at 105,000 × *g* for 60 min) were obtained as described by Ryan.[23] They were washed twice with 0.5 mM dithiothreitol solution, lyophylized, and stored at −20 C. No loss of activity occurred over the period of the study.

## *Screening assay procedure*

Aromatase activity was measured according to the original procedure of Thompson and Siiteri.[24] All enzymatic studies were conducted in 67 mM phosphate buffer, pH 7.5, at a final incubation of volume of 0.5 ml. The incubation mixture contained 180 μM NADPH, 1 μM [1β-$^3$H]androstenedione (3 × 10$^5$ dpm), 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 minutes in air and terminated by addition




**Scheme 1** Synthesis of 19-substituted androst-4-ene-3,6,17-trione (**9**, **10**, and **12**). Reagents: (i) TBDMS-Cl, imidazole, DMF; (ii) zinc powder, EtOH; (iii) pyridinium dichromate; (iv) HC ≡ CMgBr, THF; (v) acetic anhydride, pyridine; (vi) diluted HCl, THF, propan-2-ol; (vii) Jones reagent, acetone.

of 3 ml of $CHCl_3$, followed by vortexing for 40 seconds. After centrifugation at 700 × *g* for 5 minutes, 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 inhibitors **9**, **10**, and **12** were incubated with or without androstenedione, NADPH (600 μM), and L-cysteine at 37 C with the placental microsomes (1 mg of protein), and MeOH (50 μl) in 67 mM phosphate buffer, pH 7.5, in a total volume of 1 ml in air. Aliquots (50 μl), in duplicate, were removed at various time periods (0–12 minutes) and added to a solution of [1β-$^3$H]androstenedione (1 μM, 2.0 × 10$^5$ dpm) and NADPH (180 μM) in 67 mM phosphate buffer, pH 7.5 (total volume, 0.5 ml), and the mixture incubated at 37 C for 20 minutes. $^3H_2O$ release was determined as described above.

# Results

## *Chemistry*

Reaction of 5α-bromo-6β,19-epoxy-17,17-(ethylenedioxy)androstan-3β-ol (**1**) with TBDMS chloride in the presence of imidazole in DMF gave 3β-silyl ether (**2**), which was then treated with zinc dust in EtOH under reflux to yield 19-alcohol (**3**) (Scheme 1). Pyridinium dichromate oxidation of the alcohol followed by Grignard reaction with ethynylmagnesium bromide in THF at room temperature afforded (19R/S)-19-acetylenic alcohol (**5**) (53% from steroid **3**). $^1$H NMR analysis showed **5** to be a 4 : 1 mixture of diastereomers with S and R configuration at C-19 [δ 2.55 (0.8H) and 2.58 (0.2H) for 19-C≡CH, 4.94 (0.8H) and 4.96 (0.2H) for 19-H]. Assuming that the addition of ethynylmagnesium bromide to the 19-aldehyde follows the same steric course as the addition of methyl lithium to 3β,17β-diacetoxyandrost-5-en-19-one,[25,26] one could expect the major product to have S configuration and the minor one to have R configuration. Acetylation of **5** with acetic anhydride/pyridine followed by hydrolysis of the TBDMS group at C-3 with diluted HCl gave 19-acet-

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

| Inhibitor | $IC_{50}$ ($\mu$M) | $k_i$ ($\mu$M) |
|---|---|---|
| **9** | >250 (41% inhibition at 250 $\mu$M)[b] | 75 |
| **10** | >250 (47% inhibition at 250 $\mu$M)[b] | 67 |
| **12** | 21[c] | 1.8 |
| AT | 0.96[c] | 0.058 |

[a] Human placental microsomes: 40 $\mu$g protein.
[b] Substrate: 0.3 $\mu$M [1$\beta$ = $^3$H]androstenedione.
[c] Substrate: 1 $\mu$M [1$\beta$-$^3$H]androstenedione.

oxy-3$\beta$-hydroxy-19-ethynyl compounds **7** and **8**. The diastereomeric mixture showed two spots on silica gel TLC ($R_f$, 0.39 and 0.35, hexane/AcOEt, 1 : 1) and could efficiently be separated by silica gel column chromatography: steroid **7** (57% from **5**) [$\delta$ 2.08 (s, 19-OCOMe), 6.09 (d, J = 2.2 Hz, 19-H)]; steroid **8** (15% from **5**) [$\delta$ 2.13 (s, 19-OCOMe), 5.88 (d, J = 2.2 Hz, 19-H)]. Oxidation of the alcohols **7** and **8** with a large excess of Jones reagent[27] at 0 C produced the corresponding 4-ene-3,6,17-triones **9** and **10**.

19,19-Difluoro AT (**12**) was similarly obtained, in 31% yield, by Jones oxidation of 5-ene-3$\beta$,17$\beta$-diol **11**, synthesized according to the method[22] previously reported by Robinson's group.

### *Biochemical properties*

(19S)-19-Acetylenic steroid **9** and its 19R-diastereomer **10** were very weak inhibitors of aromatase in human placental microsomes, whereas 19,19-difluoride **12** was a moderately good inhibitor (Table 1). Aromatase activity in the placental microsomes was determined by the radiometric method in which tritiated water was released from [1$\beta$-$^3$H]androstenedione into the incubation medium during aromatization.[24] 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 Figure 1. All of these inhibitors prevented aromatase activity in a competitive manner with apparent inhibition constants ($k_i$), obtained by analysis of Dixon plots, of 75, 67, and 1.8 $\mu$M for inhibitors **9**, **10**, and **12**, in which $k_m$ for androstenedione is about 58 ± 4 nM and $k_i$ for AT is 58 nM (Table 1).

Time-dependent inactivation was observed when the three inhibitors **9**, **10**, and **12** were incubated with aromatase in the presence of NADPH in air. Pseudo–first-order kinetics were obtained during the first 12 minutes of the incubation of the former two (Figure 2). On the other hand, biphasic inactivation of the enzyme activity was caused by the latter, and the slopes of the rates of inactivation are linear only at the initial time period (i.e., 4 minutes) (Figure 3); these earlier times were used to obtain $k_{obsd}$s of varying concentrations of the inhibitor. With increasing inhibitor concentrations, increasing $k_{obsd}$s were obtained for the

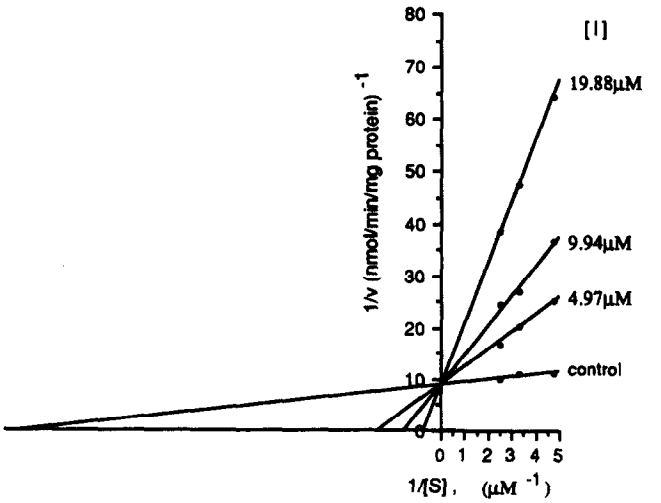



**Figure 1** Lineweaver-Burk plot of inhibition of human placental aromatase by 19,19-difluoride (**12**) with androstenedione as a substrate. Each point represents the mean of two determinations. The inhibition experiments with **9** and **10** gave plots similar to that for **12** (data not shown).

three inhibitors. Double-reciprocal plots of $k_{obsd}$ versus inhibitor concentration[28] yielded $k_i$s of 61, 53, and 1.8 $\mu$M and rate constants for inactivation ($k_{inact}$s) of 0.077, 0.065, and 0.250 min$^{-1}$, respectively, for steroids **9**, **10**, and **12** (Figures 2B, 2D, and 3C).

NADPH was essential for the activity loss (Figure 4A) and the substrate androstenedione completely blocked the inactivation (Figure 4B). A nucleophile, L-cysteine, did not protect aromatase from inactivation by **9**, **10**, and **12** (Figure 4C).

## Discussion

We have synthesized diastereomeric 19-acetylene derivatives of AT (**9** and **10**) and 19,19-difluoro AT (**12**), and have established their structures as indicated above except for the configurations at C-19 of the acetylene compounds. The configurations have tentatively been assigned to be 19S for **9** and 19R for **10** according to the previous reports.[25,26] Attempted deprotection of the 19-acetyl of steroids **9** and **10** with various alkaline (NaOH, $Na_2CO_3$, $NaHCO_3$, or $NH_4OH$; MeOH, acetone, or pyridine[29]) and acid (HCl or $H_2SO_4$; MeOH or dioxane[30]) conditions did not yield the desired 19-acetylenic alcohol derivatives of AT, but did yield a complex mixture of the products in every case.

The first series of experiments showed that incorporation of two fluorine atoms or acetylenic acetoxyl group into the 19-position of AT significantly lowered the binding ability for aromatase compared with the parent compound AT, similarly as previously reported in the experiments with androstenedione[16,22] and its 3-deoxy steroid[31] series. In the latter, the large C-19 acetoxyl group may very likely cause a drastic drop of the ability because of its steric hindrance in the interaction with the binding site.

A second series of experiments was performed to

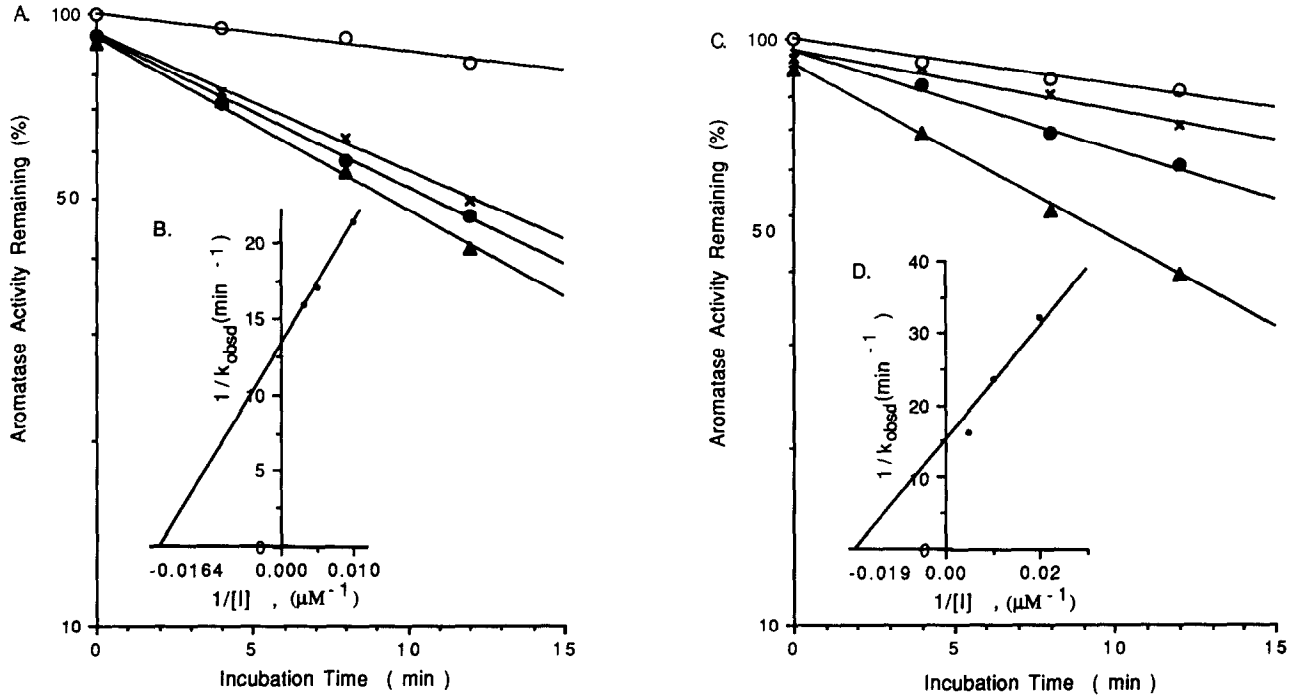



**Figure 2** Time-dependent inactivation and concentration-dependent inactivation of human placental aromatase by 19-acetylenic compounds **9** (A and B) and **10** (C and D) in the presence of NADPH in air. Concentrations of the inhibitors: (A and B for **9**) control (0 $\mu$M), **O**; 100 $\mu$M ×; 200 $\mu$M, ●; 300 $\mu$M, ▲; (C and D for **10**) control (0 $\mu$M), **O**; 50 $\mu$M, ×; 100 $\mu$M, ●; 200 $\mu$M, ▲. Each point represents the mean of two determinations.

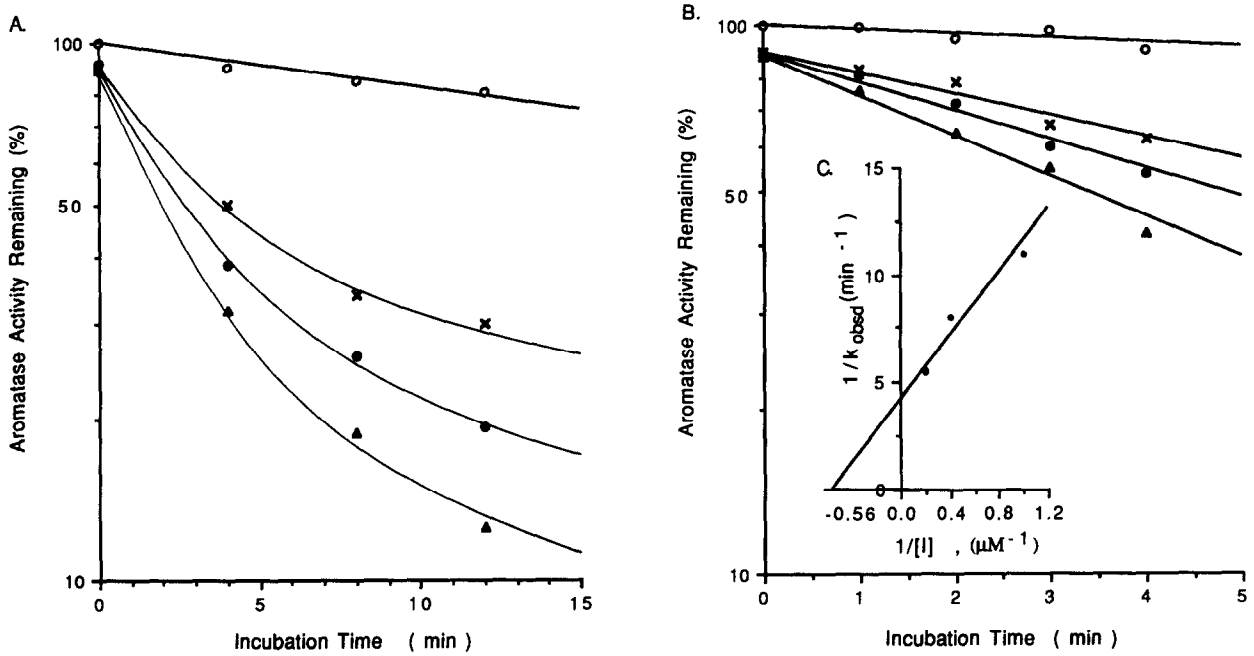



**Figure 3** Time-dependent inactivation (A and B) and concentration-dependent inactivation (C) of human placental aromatase by 19,19-difluoride (**12**) in the presence of NADPH in air. Concentrations of the inhibitor: (A) control (0 $\mu$M), **O**; 2.5 $\mu$M, ×; 5 $\mu$M, ●; 10 $\mu$M, ▲; (B) control (0 $\mu$M), **O**; 1 $\mu$M, ×; 2.5 $\mu$M, ●; 5 $\mu$M, ▲. Each point represents the mean of two determinations.

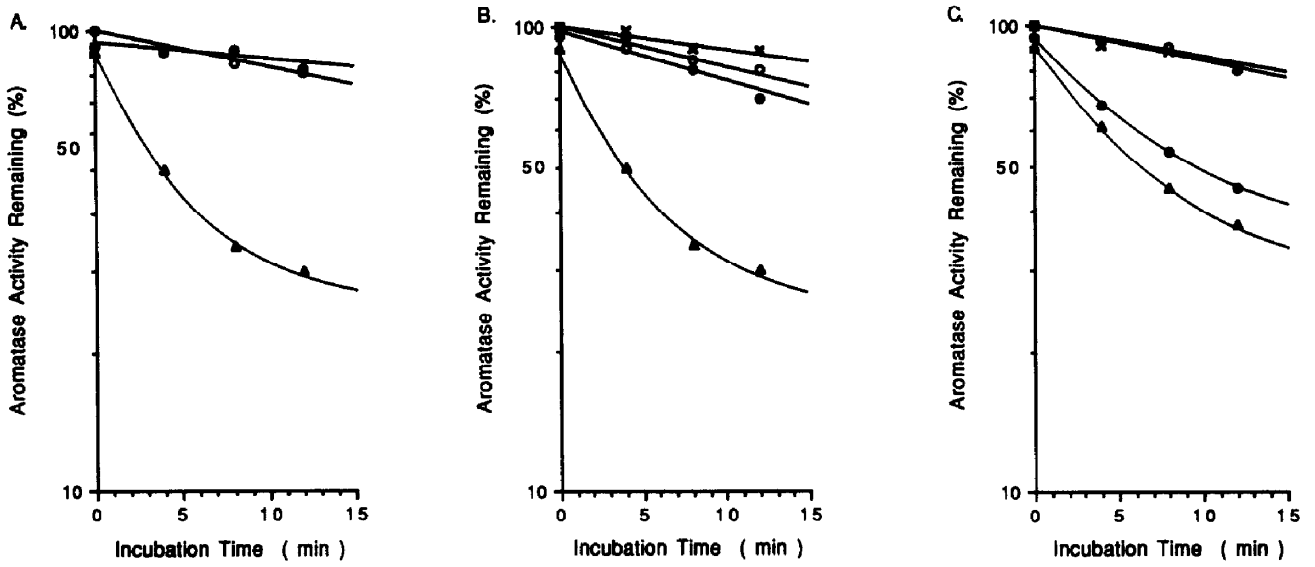
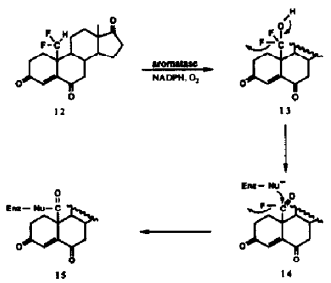



**Figure 4** Inactivation of human placental aromatase by 2.5 $\mu$M of 19,19-difluoride (**12**) under various conditions. A: In the absence of NADPH, the inhibitor failed to produce inactivation of the enzyme (●), whereas in the presence of NADPH a biphasic inactivation was observed (▲). Control samples contained no inhibitor (○). B: The inhibitor was incubated with aromatase and NADPH in air in the presence (●) or absence (▲) of androstenedione, and the substrate protected the enzyme from inactivation. Control sample with (×) or without (○) the substrate contained no inhibitor. C: In the presence (●) or absence (▲) of L-cysteine (0.5 mM), a biphasic inactivation of aromatase by the inhibitor was observed. Control sample with (×) and without (○) L-cysteine contained no inhibitor. Each point represents the mean of two determinations. The inactivation experiments with **9** and **10** in the absence of NADPH and in the presence of L-cysteine or androstenedione gave results similar to that for **12** (data not shown).

confirm that the inhibitors are suicide substrates of aromatase. 19,19-Difluoro compound (**12**) inactivated the aromatization of androstenedione in a time-dependent, biphasic manner in the presence of NADPH in air. This biphasic loss is most probably a result of the metabolic-dependent elimination of the inhibitor from the incubate because the fluoro steroid was stable in phosphate buffer at 37 C for up to 1 hour (data not shown). Similar results have previously been reported in the time-dependent inactivation by 19-thioandrostenedione[32] and 16$\alpha$-bromo AT.[27] Recently, Wright et al.[33] showed that 19-thiomethyl- and 19-azido-androstenediones are slow-binding inhibitors of aromatase. From the present results, it is not clear whether the slow-binding inhibition is involved in such biphasic kinetics. Further study should be required to elucidate this. On the other hand, diastereomeric 19-acetylene derivatives **9** and **10** caused a time-dependent, pseudo–first-order loss of aromatase activity in which the apparent $k_i$ and overall $k_{inact}$ values are almost the same, respectively. It has previously been reported that the (19R)-19-acetylenic alcohol derivative of androstenedione has much higher affinity for aromatase than the 19S diastereomer, and only the 19S-isomer inactivates the enzyme in a suicide manner,[16] whereas 6-ene derivatives of both the diastereomers inactivate the enzyme in a mechanism-based manner.[34] The similarities of the apparent $k_i$s for steroids **9**, **10**, and **12** to those obtained from the competition experiment shown in Table 1 (75 versus 61 $\mu$M for **9**, 67 versus 53 $\mu$M for **10**, and 1.8 versus 1.8 $\mu$M for **12**) suggest that initial binding of the inhibitor to the enzyme is a rate-limiting step.

The rate of inactivation decreased when the substrate androstenedione was included in the incubation mixture. In the nucleophilic protection experiments, L-cysteine failed to protect aromatase from inactivation of the inhibitors. Thus, covalent-bond formation between the enzyme and the reactive intermediate appears to occur rapidly at the active site.

The mechanism of inactivation of aromatase by 19,19-difluoroandrostenedione has been confirmed by Robinson's group[17] using isotope-labeled inhibitor; aromatase attacks the 19-carbon of the difluoride to generate an electrophilic acyl fluoride, resulting in covalent modification of the enzyme's active site. Thus, it is presumed that the 19,19-difluoro compound **12** would inactivate aromatase, most likely via a similar mechanism (Scheme 2). For the inactivation by the



**Scheme 2** Proposed mechanisms for the inactivation of aromatase by 19,19-difluoro (**12**).

19-acetylenic alcohol derivative of androstenedione, Covey's group[16] reasoned that an acetylene-containing substituent would be metabolized by aromatase to generate a gem-diol that successively dehydrates to yield a conjugated ketone, an efficient Michael acceptor. Because the gem-diol intermediate seems to be chemically equivalent to its 19-acetoxy-19-hydroxy derivative, the aromatase inactivation by 19-acetoxy-19-acetylenic compounds **9** and **10** may proceed via a mechanism similar to that proposed for the acetylenic alcohol. However, considering both the lack of the stereoselectivity in the inactivations and their poor affinities for the enzyme, involvement of another mechanism cannot be ignored. Further study should be required for the elucidation of the mechanism.

Preliminary experiments have shown that 19-hydroxy- and 19-oxo-AT are produced by incubation of $^{14}$C-labeled AT with the placental microsomes (to be reported elsewhere). This along with the present results suggest that AT would be activated by attack of aromatase at the 19-carbon as part of the inactivation process, although there is no direct evidence. To definitely determine the mechanism, we have now undertaken experiments using radioactive AT that is regiospecifically labeled by $^{3}$H and $^{14}$C.

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