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# 6α,7α-CYCLOPROPANE DERIVATIVES OF ANDROST-4-ENE: A NOVEL CLASS OF COMPETITIVE AROMATASE INHIBITORS

Mitsuteru Numazawa and Ayako Mutsumi

Tohoku College of Pharmacy, 4-1 Komatsushima-4-chome,
Sendai 981, Japan

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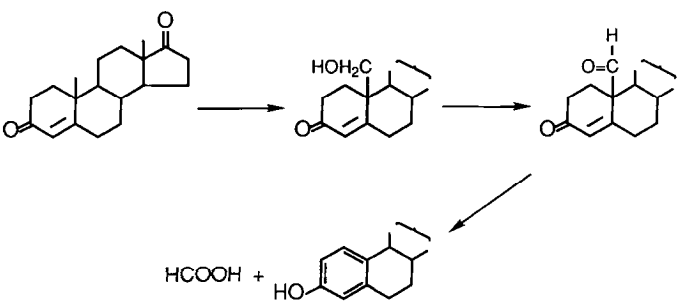
SUMMARY: 6α,7α-Cyclopropane steroids were found to be potent competitive inhibitors of human placental aromatase. 6β,7β-Dihydro-3'H-cyclopropa[6,7]-androst-4-en-17-one (4) has extremely high affinity for aromatase ($K_i$=5 nM) but does not cause a time-dependent inactivation of the enzyme. 

Aromatase is a unique cytochrome P-450 enzyme complex which catalyzes the synthesis of estrone and estradiol from 4-en-3-oxo androgens, androst-4-ene-3,17-dione (androstenedione) and testosterone (1-3). The process involves two successive hydroxylations at C-19 methyl group of the substrate, followed by a third oxygenation step which leads to formation of product with release of the 19-methyl group as formate (Scheme 1) (3-5). Estrogens have been implicated in the development or maintenance of endometrial and mammary carcinoma (6-10). Particularly important may be the effect of periferal extraglandular estrogen production on metastatic carcinoma in postmenopausal women (11,12). Because of this association of estrogens with various disease states, interest has focused on the synthesis of agents which will completely inhibit the enzyme aromatase.

A number of potents aromatase inhibitors, analogs of the substrate androstenedione, have been described, including 4-hydroxy (7), 19-ethynyl




Scheme 1. Proposed mechanism of androstenedione aromatization.

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(13), or 1-methyl-1-ene (8) derivative of androstenedione which are now under clinical evaluation. We have recently developed 3-deoxy $C_{19}$-steroids as potent reversible aromatas .nhibitors, and demonstrated that a carbonyl function at C-3 of androstenedione is not essential for steroid binding to the active site (14-16). As a continuing study of the 3-deoxy steroids as aromatase inhibitors, we have investigated further structure requirement necessary for their interactions with the catalytic (active) site. The studies in this paper focus on the preparation and biochemical evaluation of 6α,7α-cyclopropane derivatives of 3-deoxyandrostenedione.

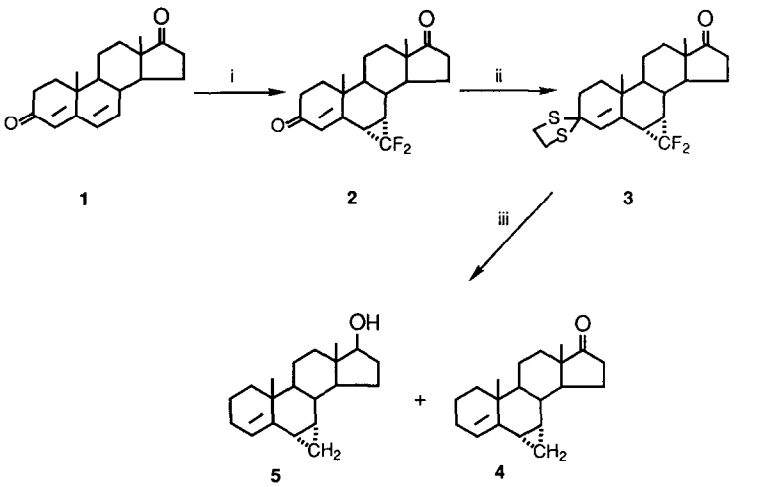
## MATERIALS AND METHODS

[1β-$^3$H]Androstenedione (25.4 Ci/mmol) ($^3$H-distribution: β/α=74.2/25.8) was purchased from New England Nuclear (Boston, MA) and NADPH from Kohjin Co., Ltd. (Tokyo, Japan). Human placental microsomes (particles sedimenting at 105,000 x g for 60 min) were obtained principally as described by Ryan (17). Androsta-4,6-diene-3,17-dione was prepared according to the method previously reported (18).

6β,7β-Dihydro-3'H-3',3'-difluorocyclopropa[6,7]androst-4-ene-3,17-dione (2). Sodium chlorodifluoroacetate (31 g, 203 mmol) in 55 ml of diglyme was added dropwise during 20 min to a boiling solution of androsta-4,6-diene-3,17-dione (1) (2.35 g, 8.27 mmol) in 40 ml of diglyme and the mixture was heated under reflux for 5 h under dark (19). After this time, the reaction mixture was filtered and the filtrate was diluted with AcOEt (500 ml), washed with saturated $NaHCO_3$ solution and water, and dried ($Na_2SO_4$). After evaporation of the solvent, the residue was purified by silica gel column chromatography (hexane/AcOEt) and recrystallization from MeOH to give 2 (1.57 g, 57%) as colorless plates: mp 192.5-193.5°C. $^1$H-NMR (400 MHz, $CDCl_3$): δ 0.94 (3H, s, 18-Me), 1.16 (3H, s, 19-Me), 5.99 (1H, s, 4-H). IR (KBr): 1730 and 1670 (C=O) cm$^{-1}$. UV $\lambda_{max}$ (95% EtOH): 247 nm (ε =2.06 x 10$^4$). EI-MS: m/z 334 ($M^+$). Anal. Calcd for $C_{20}H_{24}O_2F_2$: C, 71.83; H, 7.23. Found: C, 71.77; H, 7.43.

6β,7β-Dihydro-3'H-3',3'-difluorocyclopropa[6,7]-3,3-ethylenedithio-androst-4-en-17-one (3). A solution of ethanedithiol (145 mg, 1.54 mmol) in AcOH (0.5 ml) and p-toluenesulfonic acid (140 mg, 0.74 mmol) were added to a solution of 2 (500 mg, 1.5 mmol) in AcOH (10 ml), and the resulting mixture was stirred overnight at room temperature, poured into water (200 ml), and extracted with AcOEt (200 ml x 2). The organic phase was washed with saturated $NaHCO_3$ solution and water, and dried ($Na_2SO_4$). After usual processing, the product was purified by silica gel column chromatography (hexane/AcOEt) and recrystallization from acetone-water to yield 3 (472 mg, 77%): mp 164-165°C. $^1$H-NMR (60 MHz, $CDCl_3$): δ 0.90 (3H, s, 18-Me), 1.00 (3H, s, 19-Me), 3.33 (4H, br s, $SCH_2CH_2S$), 5.83 (1H, s, 4-H). IR (KBr): 1740 (C=O) cm$^{-1}$. Anal. Calcd for $C_{22}H_{28}S_2OF_2$: C, 64.36; H, 6.87. Found: C, 64.57; H, 6.86.

6β,7β-Dihydro-3'H-cyclopropa[6,7]androst-4-en-17-one (4) and Its 17β-Hydroxy Derivative (5). To a solution of sodium metal (0.81 g) in 60 ml of liquid ammonia was added a solution of 3 (415 mg, 1.01 mmol) in dry tetrahydrofuran (9 ml). The reaction mixture was stirred for 30 min. After removing most of the ammonia and carefully adding of saturated $NH_4Cl$ solution, the mixture was acidified with 5% HCl and extracted with AcOEt (300 ml), and the organic phase was washed with 5% $NaHCO_3$ solution and water, and dried ($Na_2SO_4$). After the usual workup, the resulting oil was subjected to silica gel column chromatography (hexane/AcOEt), in which the less polar




i = $ClF_2CCOONa$, diglyme ; ii = 1,2-ethanedithiol, p-TsOH ; iii = Na-Liq.$NH_3$

Scheme 2. Synthesis of compounds 4 and 5.

fraction gave 4 (113 mg, 40%) and the more polar fraction gave 5 (43 mg, 15%). 4: mp 135.5-136°C (colorless needles, from acetone); $^1$H-NMR (400 MHz, $CDCl_3$): δ 0.08 (1H, m, cyclopronane-ring proton), 0.58 (2H, m, cyclopropane-ring protons), 0.93 (3H, s, 18-Me), 0.99 (3H, s, 19-Me), 5.52 (1H, br s, 4-H); IR (KBr): 1740 (C=O) $cm^{-1}$; EI-MS: m/z 284 ($M^+$). Anal. Calcd for $C_{20}H_{28}O$: C, 84.45; H, 9.92. Found: C, 84.41; H, 10.12. 5: mp 157-158°C (colorless needles, from benzene); $^1$H-NMR (400 MHz, $CDCl_3$): δ 0.04 (1H, m, cycropropane-ring proton), 0.48 (2H, m, cyclopropane-ring protons), 0.80 (3H, s, 18-Me), 0.98 (3H, s, 19-Me), 3.65 (1H, m, 17α-H), 5.49 (1H, br s, 4-H); EI-MS: m/z 286 ($M^+$). Anal. Calcd for $C_{20}H_{30}O$: C, 83.86; H, 10.56. Found: C, 83.58; H, 10.75 (Scheme 2).

Screening Assay Procedure. Aromatase activity was measured according to the radiometric method originally developed by Thompson and Siiteri (1). 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 (3 x $10^5$ dpm), 40 μg of protein of the lyophilized placental microsomes, various concentrations of inhibitors, and 25 μl of MeOH. Incubations were performed at 37°C for 20 min in air and terminated by addition of 3 ml of $CHCl_3$, followed by vortexing for 40 seconds. After centrifugation at 700 x g for 5 min, aliquots (0.25 ml) were removed from the water phase and added to sacintillation mixture for determination of $^3H_2O$ production.

Time-Dependent Inactivation Procedure. Various concentrations of inhibitor 2, 4, or 5 were incubated with the placental microsomes (1 mg of protein), 600 μM NADPH, 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, 4, 8, and 12 min) and added to a solution of [1β-$^3$H]androstenedione (1 μM, 3 x $10^5$ 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 AND DISCUSSION

Reversible inhibition of aromatase activity in human placental microsomes by 6α,7α-cyclopropane derivatives (2, 4, and 5), synthesized in this

Table 1. Aromatase inhibition by cyclopropane-substituted androst-4-ene

| Compound | $IC_{50}$, μM[a] | Ki, nM | Inhibition |
|---|---|---|---|
| 2 | 1.15 | 50 | competitive |
| 4 | 0.15 | 5.0 | competitive |
| 5 | 2.70 | 120 | competitive |
| for comparison | | | |
| 4-Hydroxyandrostenedione | 0.41 | | |
| 3-Deoxyandrostenedione | 0.53 | 37 | competitive |
| Androstenedione | 1.0 | 55 (Km) | |

a. Substrate: 1 μM [1β-$^3$H]androstenedione.

study, was initially studied and the results are shown in Table 1. In addition to the above compounds, 3-deoxyandrostnedione (15) and 4-hydroxyandrostenedione (7), potent aromatase inhibitors, are listed for comparison. Aromatase activity in the placental microsomes was determined by the radiometric method in which tritiated water release from [1β-$^3$H]androstenedione into the incubation medium during aromatization (1). $IC_{50}$'s for the cyclopropane-substituted steroids showed that they are potent aromatase inhibitors and the inhibitors were further studied in order to characterize the nature of their interactions with the catalytic site of aromatase. Lineweaver-Burk and Dixon plots were linear and showed that the inhibition was competitive (Figure 1). In each set of competition experiments an apparent $K_m$ (55 ± 5 nM) for androstenedione as well as an apparent $K_i$ for each cyclopropane-substituted steroid ($K_i$: 50, 5.0, or 120 nM for 2, 4, or 5) was determined. Compound 4 bound surprisingly with seven times the affinity of the parent steroid, 3-deoxyandrostenedione, for the enzyme, while an intro-

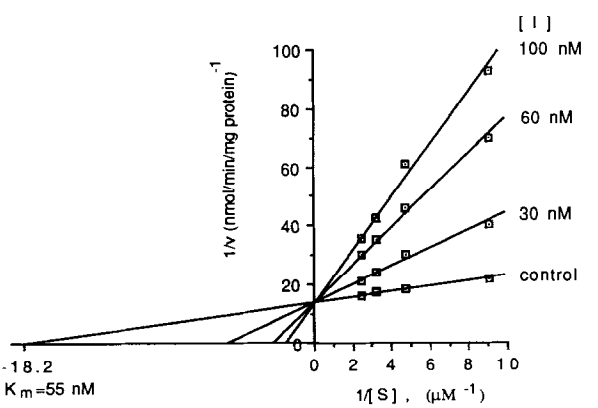



Figure 1. Lineweaver-Burk plot to determine the apparent inhibition constant ($K_i$) for compound 4. Velocity (V) of androstenedione aromatization is expressed as nmol/min/mg protein. Each point represents the mean of duplicate determinations. The inhibition experiments with compounds 2 and 5 gave plots that were essentially the same as those in Figure 1, showing that these steroids are also competitive inhibitors of aromatase (data not shown).

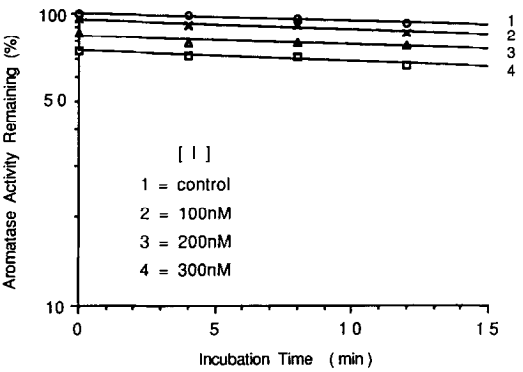



Figure 2. Time course for decrease in aromatase activity by compound 4 in the presence of NADPH and oxygen. Control: does not contain inhibitor 4. The inactivation experiments with compounds 2 and 5 afforded essentially similar results to those in Figure 2; The time-dependent inactivation was not observed in each experiment.

duction of cyclopropane ring at the 6- and 7-positions of androstenedione did not cause a significant effect on the affinity ($K_i$ 50 nM for 2 vs $K_m$ 55 nM for androstenedione).

6α-Bromo- (20) or 7α-(4'-amino)phenylthio- (21) androstenedione has been found to be among the most potent competitive inhibitors produced to date ($K_i/K_m$=approximately 0.35 each). $K_i/K_m$ value of inhibitor 4 is approximately 0.1, indicating that this compound binds to the enzyme 3.5-fold more tightly than the 6α- or 7α-substituted steroid. Substitution of cyclopropane ring for protons at C-6α and C-7α would cause significant structure perturbation. This structural feature may play an important role in the tight binding, although the exact stereochemical aspects of steroid binding is not clear. The 17β-hydroxy derivative 5 had much less affinity for the enzyme compared to the 17-ketone 4, similaly as previously reported for a 3-deoxyandrostenedione series (15). This confirms that a 17-carbonyl function rather than a 3-carbonyl function plays an important role in steroid binding to aromatase.

Three steroids were then tested for its ability to cause a time-dependent irreversible inactivation of aromatase. No time-dependent inactivation was observed after 12 min when each steroid was incubated with the placental microsomes in the presence of NADPH and oxygen (Figure 2). There was no evidence of enzymatic generation of a reactive substance.

The present results encourage further development of the cyclopropane-substituted steroids as potential medicinal agents for the treatment of estrogen-dependent disease states such as breast and endometrial cancer.

ACKNOWLEDGMENT

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