Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 32-39

ANDROST-5-ENE-7,17-DIONE: A NOVEL CLASS OF SUICIDE SUBSTRATE OF AROMATASE

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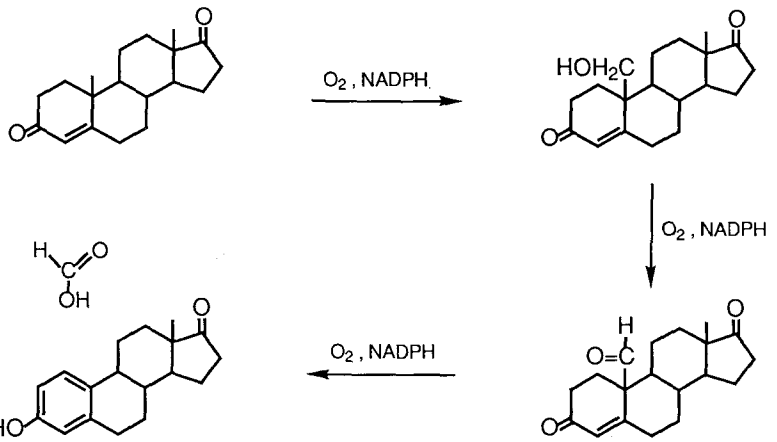
Received May 26, 1992

SUMMARY: 5-En-7-one steroid 1 was found to be a potent inhibitor of aromatase. This along with its 19-hydroxy derivative 7 was characterized as suicide substrate of human placental aromatase (k_{inact}'s of 0.069 and 0.058 min^{-1} and K_I's of 143 nM and 11.1 μM, respectively, for steroids 1 and 7). The results suggest that the 19-oxygenation would be involved in the irreversible inactivation of aromatase by the 5-en-7-one steroids.

Aromatase catalyzes the conversion of 4-en-3-one androgens, androst-4-ene-3,17-dione (androstenedione) and testosterone, to estrogens, estrone and estradiol. The conversions is cytochrome P-450 dependent (1-3) and involves sequential hydroxylations at C-19 as shown in Scheme 1 (4-6). The role of estrogens in promoting some forms of neoplasm, particularly breast cancer, is well documented (7-10). In fact, the estrogen receptor antagonist tamoxifen is used widely in the treatment of breast cancer (11). An alternative approach to blockade of estrogenic activities, which continues to receive attention, involves the lowering of estrogen levels by aromatase inhibition.

A number of potent aromatase inhibitors, analogs of the substrate androstenedione, have been described, including 4-hydroxy (9), 19-ethynyl (12), or 1-methyl-1-ene (13) derivative of androstenedione which is now under clinical evaluation. We have recently developed 3-deoxy C_{19} steroids having a unique 4-ene structure as potent aromatase inhibitors, and demonstrated that a carbonyl function at C-3 of androstenedione is not essential for steroid binding to the active site of aromatase (14-17). As a continuing study of the 3-deoxy steroids as aromatase inhibitors, we have investigated further structure requirements necessary for aromatase inhibition by 5-en-7-one steroids. We report here the synthesis and biochemical evaluation of androst-5-ene-7,17-dione (1) and its 19-hydroxy derivative 7. The 7-one steroids inactivated aromatase in a suicide manner.

0006-291X/92 $4.00



Scheme 1. Proposed mechanism of androstenedione aromatization.

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, washed with 0.5 mM dithiothreitol) were obtained essentially as described by Ryan (18). Androst-5-ene-7,17-dione (1) was synthesized according to the method (19) previously reported.

3β-Acetoxy-19-(tert-butyldimethylsiloxy)androst-5-ene-7,17-dione (3). To a stirred mixture of 3β-acetoxy-19-(tert-butyldimethylsiloxy)androst-5-en-17-one (2) (1.37 g, 2.97 mmol) in benzene (50 ml) and Celite (4.82 g) was added pyridinium dichromate (6.62 g, 17.60 mmol) followed by the addition of 70% tert-butyl hydroperoxide (4.4 g, 48.12 mmol) at 10°C (20). After 15 min at 10°C, the reaction mixture was stirred at room temperature for 24 h under dark. AcOEt (200 ml) was added, and the mixture was filtered through a pad of Celite and washed with 50 ml portions of AcOEt. Combined filtrate was washed with 10% $Na_2S_2O_3$ solution and saturated NaCl solution and dried (Na_2SO_4). After evaporation of the solvent, the residue was recrystallized from acetone-hexane to give 3 (1.04 g, 74%) as colorless plates: mp 145-146°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.04 and 0.07 (3H each, s, 19-OSiMe_2), 0.86 (9H, s, 19-OSi(Me_2)CMe_3), 0.92 (3H, s, 18-Me), 2.06 (3H, s, 3-OCOMe), 3.85 (1H, d, J=10.7 Hz, 19-Ha), 3.94 (1H, d, J=10.7 Hz, 19-Hb), 4.75 (1H, m, 3α-H), 5.93 (1H, d, J=1.5 Hz, 6-H). IR (KBr): 1730, 1700, and 1650 (C=O) cm^{-1}. UV λ_{max} (95% EtOH): 234 nm (ε=1.40 x 10^4). Anal. Calcd for $C_{27}H_{42}O_5Si$: C, 68.31; H, 8.92. Found: C, 68.37; H, 8.86.

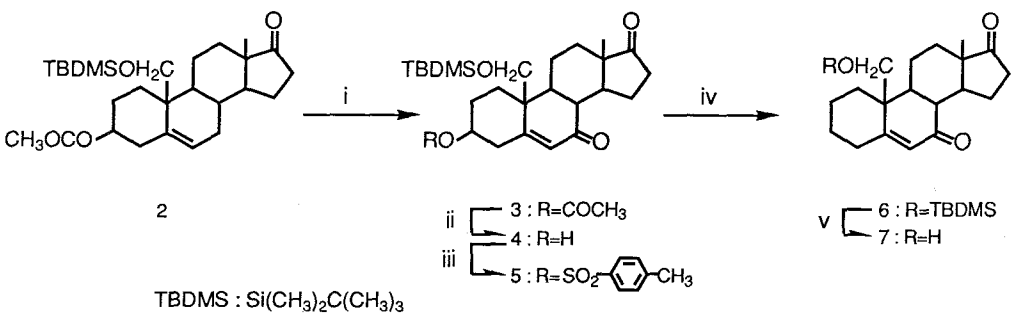
3β-Hydroxy-19-(tert-butyldimethylsiloxy)androst-5-ene-7,17-dione (4). To a solution of compound 3 (1.0 g, 2.11 mmol) in MeOH (50 ml) was added 1 M NaOH solution (5.87 ml). The mixture was stirred at room temperature for 1 h and then poured into saturated NaCl solution (500 ml). The precipitates were collected by filtration, washed with water, dried under vacuum, and recrystallized from acetone-hexane to afford 4 (0.89 g, 98%) as colorless needles: mp 156.5-157°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.03 and 0.06 (3H each, s, 19-OSiMe_2), 0.85 (9H, s, 19-OSi(Me_2)CMe_3), 0.92 (3H, s, 18-Me), 3.72 (1H, m, 3α-H), 3.84 and 3.94 (1H each, d, J=10.7 Hz, 19-H_2), 5.92 (1H, d, J=1.5 Hz, 6-H). IR (KBr): 3350 (OH), 1730 and 1660 (C=O) cm^{-1}. UV λ_{max} (95% EtOH): 239 nm (ε=1.34 x 10^4). Anal. Calcd for $C_{25}H_{40}O_4Si$: C, 69.40; H, 9.32. Found: C, 69.03; H, 9.37.

3β-(p-Toluenesulfonyloxy)-19-(tert-butyldimethylsiloxy)androst-5-ene-7,17-dione (5). p-Toluenesulfonyl chloride (2.0 g, 10.5 mmol) was added to a stirred solution of compound 4 (0.89 g, 2.06 mmol) in pyridine (12 ml) at 0°C. The reaction mixture was stirred at room temperature for 24 h and then poured into chilled water (200 ml). The precipitates were collected by filtration, dried under vacuum, and recrystallized from MeOH to give 5 (1.1 g, 91%) as colorless needles: mp 110-111°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.01 and 0.04 (3H each, s, 19-OSiMe_2), 0.83 (9H, s, 19-OSi(Me_2)CMe_3), 0.89 (3H, s, 18-Me), 3.80 and 3.87 (1H each, d, J=10.8 Hz, 19-H_2), 4.38 (1H, m, 3α-H), 5.82 (1H, s, 6-H), 7.36 and 7.82 (2H each, d, J=7.8 Hz, aromatic protons). IR (KBr): 1732 and 1662 (C=O) cm^{-1}. Anal. Calcd for $C_{32}H_{46}O_6SSi$: C, 65.49; H, 7.90. Found: C, 65.00; H, 7.64.

19-(tert-Butyldimethylsiloxy)androst-5-ene-7,17-dione (6). Zinc powder (0.86 g, 13.15 mmol), NaI (1.0 g, 6.67 mmol), and water (0.86 ml) was added to a solution of compound 5 (1.0 g, 1.70 mmol) and the reaction mixture was heated under reflux for 4 h (2). After this time, the mixture was diluted with AcOEt, washed with saturated NaCl solution, and dried (Na_2SO_4). Evaporation of the solvent yielded an oil which was purified by silica gel column chromatography (hexane/AcOEt) followed by recrystallization from MeOH gave 6 (378 mg, 53%) as colorless needles: mp 114-115°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.02 and 0.05 (3H each, s, 19-OSiMe_2), 0.85 (9H, s, 19-OSi(Me_2)-CMe_3), 0.92 (1H, s, 18-Me), 3.83 and 3.79 (1H each, d, J=10.7 Hz, 19-H_2), 5.87 (1H, s, 6-H). IR (KBr): 1737 and 1666 (C=O) cm^{-1}. UV λ_{max} (95% EtOH): 240 nm (ε=1.20 x 10^4). Anal. Calcd for $C_{25}H_{40}O_3Si$: C,71.24; H, 9.96. Found: C, 71.16; H, 9.61.

19-Hydroxyandrost-5-ene-7,17-dione (7). To a solution of compound 6 (134 mg, 0.32 mmol) in THF (1.5 ml) was added tetra-n-butylammonium fluoride (245 mg, 0.96 mmol). The reaction mixture was allowed to stand at room temperature for 16 h, poured into water (100 ml), and extracted with AcOEt (100 ml). The organic layer was washed with 5% $NaHCO_3$ solution and saturated NaCl solution, dried (Na_2SO_4), and evaporated to give a solid. Recrystallization of the product from acetone yielded 7 (85 mg, 86%) as colorless prisms: mp 190-190.5°C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.94 (1H, s, 18-Me), 3.88 (1H, d, J=11.2 Hz, 19-Ha), 4.09 (1H, d, J=11.2 Hz, 19-Hb), 5.94 (1H, d, J=1.0 Hz, 6-H). IR (KBr): 3350 (OH), 1740 and 1660 (C=O) cm^{-1}. UV λ_{max} (95% EtOH): 242 nm (ε=1.17 x 10^4). Anal. Calcd for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67. Found: C, 75.49; H, 8.54.

Studies of Competitive Inhibition and Time-Dependent Inactivation of Aromatase in Human Placental Microsomes. Aromatase activity was measured by release of tritiated water from [1β-^{3}H]androstenedione during aromatization



Reagents : (i) pyridinium dichromate , tert-BuOH , Celite , benzene ; (ii) NaOH , MeOH
(iii) p-TsCl , pyridine ; (iv) NaI , Zn , $(CH_2OCH_3)_2$; (v) $(n\text{-}Bu)_4NF$, THF

Scheme 2. Synthesis of compound 7.

principally as described by Thompson and Siiteri (1). The inhibition and inactivation studies with compounds 1 and 7 were carried out essentially according to the previous method (16) (Scheme 2).

RESULTS AND DISCUSSION

5-En-7-one steroid 1 was a potent inhibitor of aromatase in human placental microsomes while its 19-hydroxy derivative 7 was a moderately good one (Table 1). 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 (59 $\pm$ 5 nM) for androstenedione as well as an apparent K_i for each steroid (K_i: 300 nM or 15 μM for 1 or 7) was obtained. Compound 1, which is a geometrical isomer of the substrate androstenedione, was a potent competitive inhibitor of aromatase while an introduction of a hydroxy group at C-19 of it provides lowered affinity for the active site. The 19-hydroxy derivative of androstenedione, an intermediate of estrone biosynthesis, has a lower binding affinity for the active site than the parent steroid (2,3). This along with the present results suggests that the 7-one steroids may approach the active site in a similar way to the natural substrate and intermediate, in which hydrogen bonding through the carbonyl oxygen at C-7, instead of that through the C-3 carbony oxygen in a series of androstenedione (22,23), may be involved in their bindings to aromatase.

Two steroids were then tested for their abilities to cause a time-dependent irreversible inactivation of aromatase. Time-dependent, pseudo-first order inactivation was observed when both compounds were separately incubated in the presence of NADPH in air (Figure 2). With increasing inhibitor concentrations, increasing apparent rate constants for inactivation (k_{obsd}'s) were obtained for both compounds. Double-reciprocal plots of k_{obsd} versus inhibitor concentration (24) was linear and gave the apparent K_I's of 143 nM and 11.1 μM and overall rate constants for inactivation (k_{inact}'s) of 0.069 and 0.058 min^{-1}, respectively, for compounds 1 and 7 (Figure 2B). The similarities of the apparent K_I's with the apparent K_i's

Table 1. In vitro aromatase inhibitory activity

Compound	IC_{50}, μM[a]	K_i, nM	Inhibition
1	8.1	300	competitive
7	135	15000	competitive
androstenedione	1.0	59 (K_m)	

[a] Substrate: 1 μM [1β-^{3}H]androstenedione

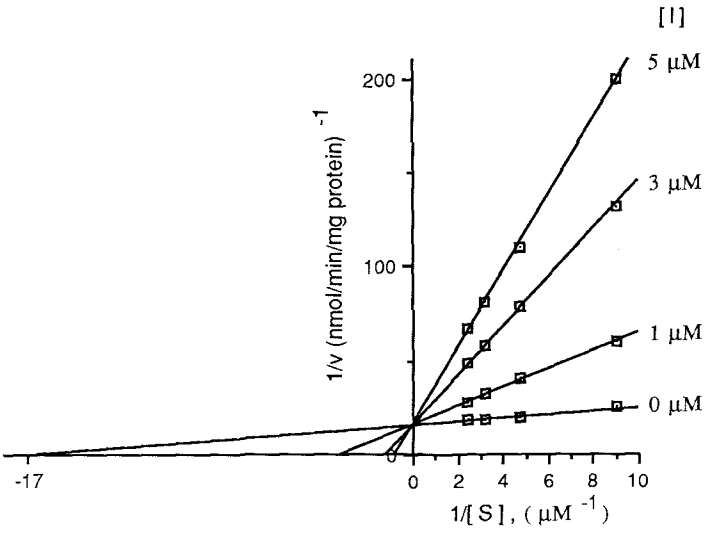


Figure 1. Lineweaver-Burk plot of inhibition of human placental aromatase by compound 1 with androstenedione as a substrate. Each point represents the mean of duplicate determinations. The inhibition experiment with compound 7 gave an essentially similar plot to Figure 1, showing that this is also a competitive inhibitor of aromatase (data not shown).

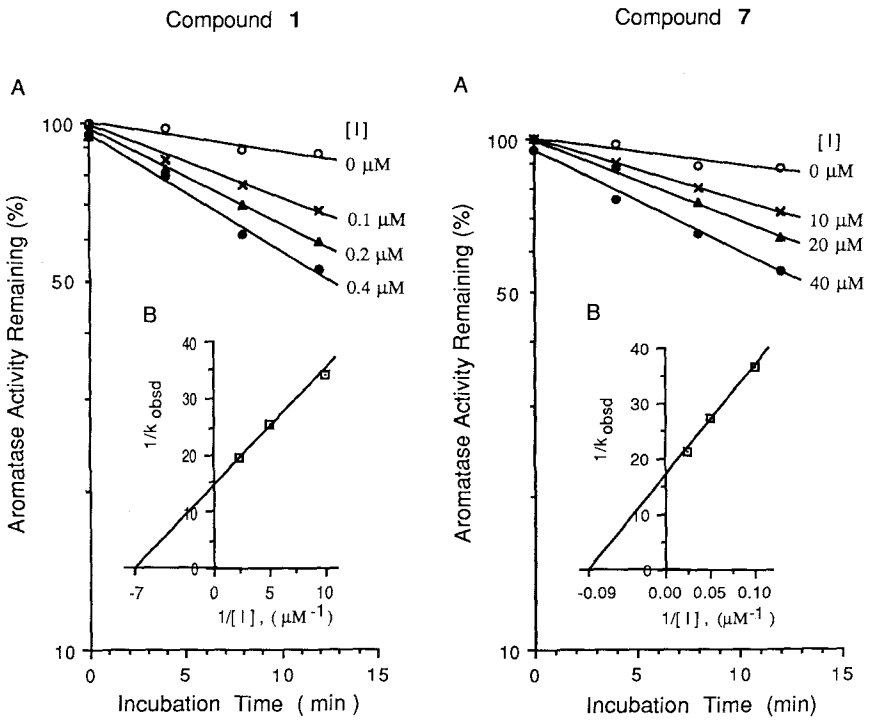


Figure 2. Time- and concentration-dependent inactivation of human placental aromatase by compound 1 or 7 in the presence of NADPH in air (A), and double-reciprocal plot analysis of the kinetics data from A (B).

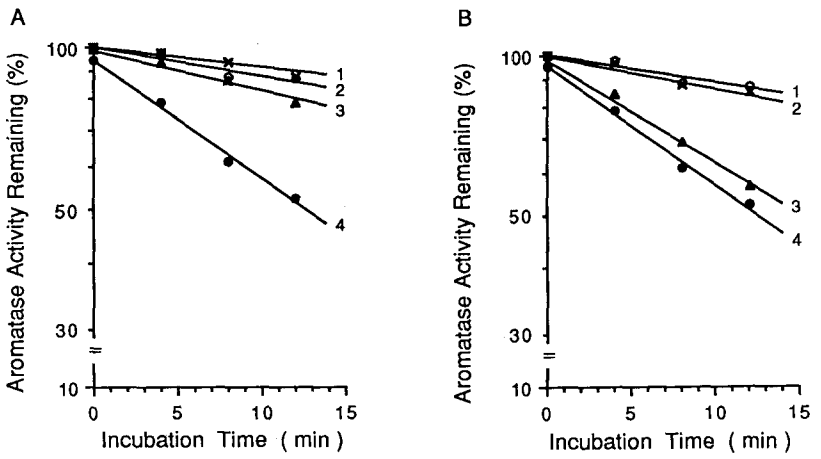


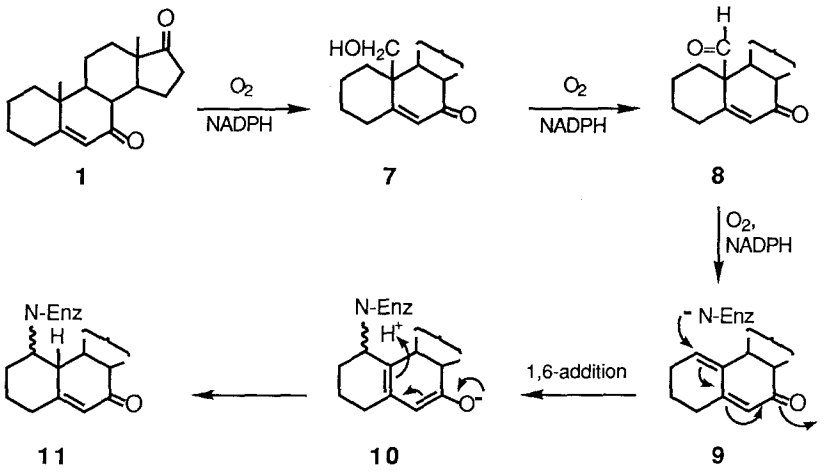
Figure 3. Inactivation of human placental aromatase by compound 1 under various conditions: (A) In the absence of NADPH (line 1) or in nitrogen atmosphere (line 3), the inhibitor (0.4 μM) failed to produce inactivation. Androstenedione at 0 or 1 μM (line 4 or 3) was incubated with aromatase, the inhibitor (0.4 μM), and NADPH in air and protected the enzyme from the inactivation. Control sample (line 2) contained no inhibitor. (B) In the presence (line 3) or absence (line 4) of L-cysteine (0.5 mM), a pseudo-first-order inactivation by the inhibitor (0.4 μM) was observed. Control sample with (line 2) and without (line 1) L-cysteine contained no inhibitor. The inactivation experiments with compound 7 in the absence of NADPH, in nitrogen atmosphere, and in the presence of L-cysteine or androstenedione gave essentially similar results to Figure 3 (data not shown).

obtained from the competitive experiments (Table 1) suggest the initial binding of the inhibitors to aromatase is rate-limiting.

NADPH and oxygen were essential for the activity loss and the substrate androstenedione completely blocked the inactivation (Figure 3A), while a nucleophile, L-cysteine, failed to protect aromatase from the inactivation (Figure 3B). Thus, covalent-bond formation between the enzyme and the reactive intermediate appears to occur rapidly at the active site, therefore, preventing diffusion of the activated inhibitor, a reactive electrophile, in the surrounding media.

The mechanism of aromatase inactivation by compound 1 is presumed to be as follows (Scheme 3); aromatase attacks the 19-carbon of steroid 1 to produce the 19-oxo derivative 8 through 19-alcohol 7, and further oxygenation of compound 8 involves the cleavage of C_{10}-C_{19} bond to generate a reactive electrophile, 1(10),5-dien-7-one 9, which may be attacked by an active site nucleophile in a 1,6-addition manner leading to covalent modification of the enzyme.

The present findings are the first that steroids having a 5-en-7-one system inactivate aromatase in a suicide (mechanism-based) manner, and may have practical applications in drug design of specific inhibitors. At a more fundamental level, presumably the steroids could be play an important



Scheme 3. Proposed mechanism of time-dependent inactivation of aromatase by 5-en-7-one steroids 1 and 7.

role in a understanding of the aromatization mechanism of which details are still a subject of debate.

ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aid for Scientific Research from The Ministry of Education, Science and Culture of Japan.

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