Competitive Inhibition and Suicide Inactivation of Human Placental Aromatase by Androst-4-ene-3,6-dione Derivatives and 3α-Methoxyandrost-4-ene-6,17-dione

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Androst-4-ene-3,6-dione derivatives 2—4 and 3α-methoxy-4-en-6-one steroid 7 were prepared and tested for their ability to inhibit aromatase in human placental microsomes. The 16α-bromide 2, the 16α-alcohol 3, and the 3α-methoxide 7 of this series were effective competitive inhibitors of aromatase with apparent K_i's of 150 nM, 1.18 μM, and 700 nM. Compound 2 caused a time-dependent, biphasic loss of aromatase activity in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) while compound 7 caused a time-dependent, pseudo-first order inactivation of the activity, with k_{inact}'s of 0.417 and 0.036 min^{-1} for compounds 2 and 7. NADPH and oxygen were required for the time-dependent inactivation and the substrate, androst-4-ene-3,17-dione, prevented it in each case.

Keywords 16α-bromoandrost-4-ene-3,6,17-trione; 3α-methoxyandrost-4-ene-6,17-dione; human placental microsome; aromatase; competitive inhibition; suicide inactivation; NADPH requirement; oxygen requirement; substrate protection

Aromatase, a unique cytochrome (cyt.) P-450 monooxygenase complex, catalyzes the synthesis of estrone and estradiol from 4-en-3-one androgens, androst-4-ene-3,17-dione (androstenedione) and testosterone.[1] Estrogens are involved in reproductive processes and are also implicated in estrogen-dependent disease states such as endometrial[2] and breast cancers.[3] Thus, inhibitors of aromatase may be useful in controlling these physiological processes and disease states. For this reason, the specific, irreversible blockade of estrogen biosynthesis through suicide (mechanism-based) inactivation of aromatase has been intensely pursued with the goal of developing practical clinical drugs.[4]

Androst-4-ene-3,6,17-trione (**1**) is a prototypical suicide inhibitor of aromatase.[5] Various derivatives at C-3 of 3β-hydroxy-4-en-6-one steroid **5** also inactivate aromatase in a mechanism-based manner, while the 3-deoxy derivative is a very potent competitive inhibitor but does not cause a time-dependent loss of aromatase activity.[6] On the other hand, effects of a substituent at the 16α-position of a 4-en-3,6-dione steroid and effects of stereochemistry of a substituent at C-3 of a 4-en-6,17-dione steroid on competitive inhibition and suicide inactivation of aromatase have not been studied. Thus, we report here the synthesis and evaluation of the 16α-derivatives of compound **1** and 3α-methoxy steroid **7** as aromatase inhibitors to elucidate the structure–activity relationships of 4-en-6-one steroid and their interaction with the catalytic site(s) of aromatase.

Experimental

Materials and General Methods [1,2-^{3}H]Androstenedione (52 Ci/mmol) (^{3}H-distribution: 1β+2β/1α+2α=71/29) was purchased from New England Nuclear Corp. (Boston, U.S.A.). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was obtained from Kohjin Co., Ltd. (Tokyo, Japan). The 4-ene-3,6-dione steroids **1**—**3** were synthesized according to the methods reported previously.[6,7]

Melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained with JEOL PMX 60 (60 MHz) and JEOL GX (400 MHz) spectrometers using tetramethylsilane as an internal standard, and mass spectrum (MS) by a JEOL JMS-DX 303 spectrometer. Ultraviolet (UV) spectra were determined on a Hitachi UV 150-20 spectrophotometer, and infrared (IR) spectra on a Shimadzu IR-430 spectrophotometer.

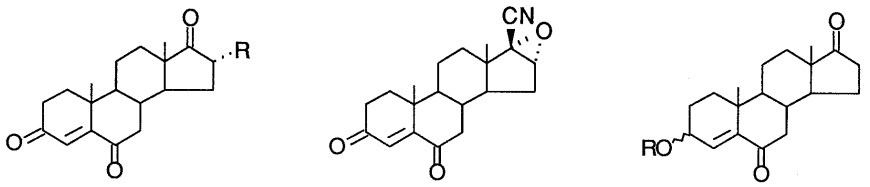
17β-Cyano-16α,17α-epoxyandrost-4-ene-3,6-dione (4) Jones reagent (1.11 ml) was added to a solution of 17β-cyano-16α,17α-epoxyandrost-5-en-3β-ol (300 mg, 0.96 mmol), obtained according to a previous paper,[8] in acetone (30 ml) at room temperature and the resulting mixture was stirred at room temperature for 30 min. The reaction mixture was then poured into water (300 ml) and extracted with AcOEt (200 ml × 2). The organic layer was washed with 5% $NaHCO_3$ solution and water and dried (Na_2SO_4). Evaporation of the solvent gave a solid residue, which was recrystallized from MeOH to afford **4** (155 mg, 50%) as pale yellow plates: mp 221.5—223 °C. ^{1}H-NMR (60 MHz, $CDCl_3$) δ: 1.03 (3H, s, 18-Me), 1.20 (3H, s, 19-Me), 3.93 (1H, s, 16β-H), 6.26 (1H, s, 4-H). IR (KBr): 2250 (CN), 1705 and 1680 (C=O) cm^{-1}. UV $\lambda_{max}^{95\%\,EtOH}$ nm (ε): 250.1 (1.14×10^4). *Anal.* Calcd for $C_{20}H_{23}NO_3$: C, 73.82; H, 7.12, N, 4.31. Found: C, 73.56; H, 7.18; N, 4.25.

3α-Methoxyandrost-4-ene-6,17-dione (7) *p*-Toluenesulfonyl chloride (1.8 g, 9.44 mmol) was added to a solution of 3β-hydroxyandrost-4-ene-6,17-dione (**5**) (1.0 g, 3.31 mmol) in pyridine (34 ml) under ice-cooling and the mixture was first stirred under ice-cooling for 20 min and then stirred at room temperature for 20 h. The reaction mixture was poured into water (350 ml) and extracted with AcOEt (300 ml × 2). The organic layer was washed with 5% HCl, saturated $NaHCO_3$ solution and water, and dried (Na_2SO_4). After evaporation of the solvent, an oily residue obtained was subjected to silica gel column chromatography (silica gel 80 g, hexane–AcOEt) to give the crude 3β-tosylate **6** (350 mg), of which methanolic solution (10 ml) was heated at 80 °C for 30 min. Evaporation of the solvent afforded a solid, which was recrystallized from MeOH to give **7** (120 mg, 11% from **5**) as colorless needles: mp 141—142 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.910 (3H, s, 18-Me), 0.973 (3H, s, 19-Me), 3.384 (3H, s, 3α-OMe), 3.715 (1H, m, 3β-H), 6.381 (1H, dd, J=1.5, 4.9 Hz, 4-H). IR (KBr): 1740 and 1690 (C=O) cm^{-1}. UV $\lambda_{max}^{95\%\,EtOH}$ nm (ε): 235.5 (6.02×10^3). EI-MS *m/z*: 316 (M^+). *Anal.* Calcd for $C_{20}H_{28}O_3$; C, 75.91; H, 8.92. Found: C, 75.64; H, 8.96.

Enzyme Preparation Human term placentas were obtained from Imaizumi Hospital (Sendai, Japan) and were used within a few hours of delivery. Placental microsomes (particles sedimenting at 105000 × *g* for 60 min) were obtained as described by Ryan.[9] They were washed twice with 0.5 mM dithiothreitol solution, lyophilized, and stored at −20 °C. No loss of activity occurred during the period of the study.

Screening Assay Procedure Aromatase activity was measured according to the original procedure of Thompson and Siiteri.[1a] This assay quantitates the production of ^3H_2O release from [1,2-^{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 [1,2-^{3}H]androstenedione (3.0×10^5 dpm), 40 μg of placental microsomal protein, various concentrations of inhibitors, and 25 μl of MeOH. Incubations were performed at 37 °C for 20 min in air and terminated by the addition of 3 ml of $CHCl_3$, followed by vortexing for 40 s. After centrifugation at 700 × *g* for 5 min, aliquots (0.3 ml) were removed from the water phase and added to a scintillation mixture for the determination of tritiated water production.

Time-Dependent Inactivation Procedure Various concentrations of inhibitors **2** and **7** 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. Aliquots (50 μl), in duplicate, were removed at various time periods (0—12 min) and added to a solution of



1 : R=H
2 : R=Br
3 : R=OH

4

5 : β ,R=H
6 : β ,R=*p*-MeC$_6$H$_4$SO$_2$-
7 : α ,R=Me
8 : β ,R=Me

Chart 1

TABLE I. *In Vitro* Aromatase Inhibitory Activity[a]

Inhibitor	IC_{50}[b] (μM)	K_i[c] (μM)
4-Ene-3,6-dione steroid		
1 17-one	0.48	0.045
2 16α-bromo-17-one	3.2	0.150
3 16α-hydroxy-17-one	31	1.18
4 17β-cyano-16α,17α-epoxide	>100	
4-Ene-6,17-dione steroid		
7 3α-methoxide	8.2	0.700
8 3β-methoxide	>100	

a) Substrate: 1 μM [1,2-^{3}H]androstenedione. *b*) The concentration of inhibitor required to give 50% inhibition of aromatase activity. *c*) K_i was obtained by analysis of Dixon plots (see Figs. 1B and 2B), in which K_m for the natural substrate, androstenedione, was about 60 nM.

[1,2-^{3}H]androstenedione (1 μM, 3×10^5 dpm), NADPH (180 μM) in 67 mM phosphate buffer, pH 7.5 (total volume, 0.5 ml); the mixture was then incubated at 37 °C for 20 min. Tritiated water released was determined as described above.

Results and Discussion

Chemistry Oxidation of 17β-cyano-16α,17α-epoxyandost-5-en-3β-ol,[8] obtained from the corresponding 16α-bromo-17-keto steroid by reaction with potassium cyanide under controlled conditions (aqueous pyridine,[10] room temperature), with a large excess of Jones reagent at room temperature produced the 4-ene-3,6,17-trione **4**[11] (50%). Reaction of 3β-hydroxy-4-ene-3,17-dione **5** with *p*-toluenesulfonyl chloride in pyridine gave the unstable 3-tosylate **6**, which, without purification, was treated with MeOH under heating to gave the 3α-methoxide **7** (11%). The stereochemistry at the C-3 position was unambiguously determined by the spectral data along with a comparison with the 3β-methoxy isomer **8**.[6c]

Biochemical Properties Results of studies on the reversible inhibition of aromatase activity in human placental microsomes by the 4-ene-3,6-dione steroids **2**—**4** and the 3α-methoxide **7** are shown in Table I; compound **1** and 3β-methoxide **8** are listed for comparison. Analysis of the data in the table shows that an introduction of a hydroxyl group or a bromine atom to C-16α of compound **1** lowered the inhibitory activity toward aromatase in each case. It is noteworthy that the 16α-bromide **2** which has a more lipophilic substituent than the 16α-alcohol **3**, is a more effective inhibitor, in which IC_{50}, the concentration rquired to give 50% inhibition of the enzyme activity, was 3.2 μM for the bromide **2** and 31 μM for the alcohol **3** (*cf.*, IC_{50} for compound **1**: 0.48 μM). Compound **4** having a cyano group at the C-17 position was a very weak aromatase inhibitor (IC_{50} > 100 μM). The 3α-methoxy steroid **7** with a 4-en-6-one system was also an effective inhibitor of aromatase (IC_{50} = 8.2 μM) and, moreover, blocked the enzyme activity to a much greater extent than the 3β-isomer (IC_{50} > 100 μM).

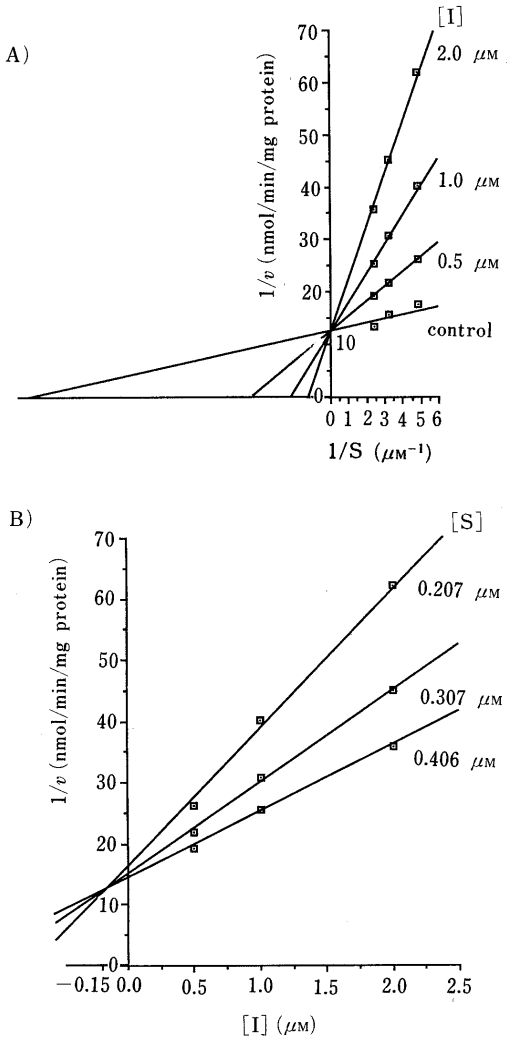


Fig. 1. Lineweaver–Burk Plot (A) and Dixon Plot to Determine the Apparent Inhibition Constant K_i (B) for the 16α-Bromide **2**

Each point represents the mean of duplicate determinations. Inhibition experiments with the 16α-alcohol **3** gave essentially similar plots (data not shown).

The three effective inhibitors, **2**, **3** and **7**, were further studied in order to characterize the nature of their

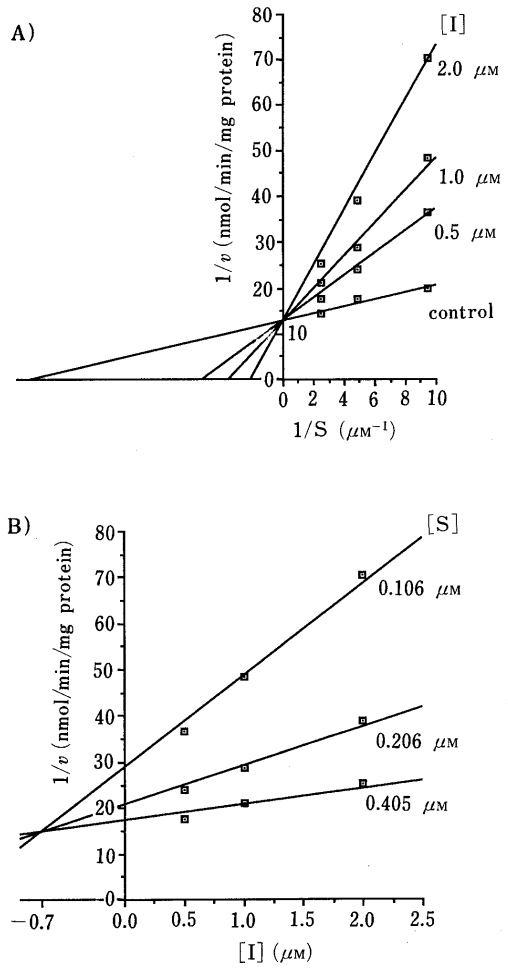


Fig. 2. Lineweaver–Burk Plot (A) and Dixon Plot (B) to Determine the Apparent Inhibition Constants (B) for the 3α-Methoxide **7**

Each point represents the mean of duplicate determinations.

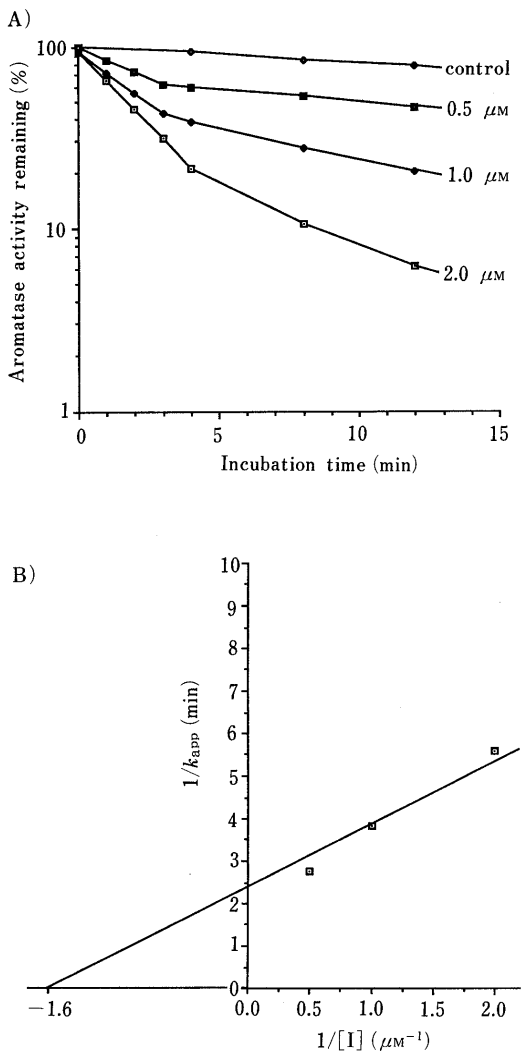


Fig. 3. Time-Dependent Inactivation (A) and Concentration-Dependent Inactivation (B) by the 16α-Bromide **2** of Aromatase Activity

Each point represents the mean of duplicate determinations. The results obtained by the first 3-min preincubation time in Fig. 3A were employed for the analysis in Fig. 3B. B: $k_{inact}=0.417\,min^{-1}$.

interactions with the catalytic site(s). Aromatization was measured at several concentrations of the inhibitor in the presence of increasing concentrations of the androstenedione substrate. Lineweaver–Burk plots of the results obtained are shown in Figs. 1A and 2A. All three steroids exhibited clear-cut competitive-type inhibition with the apparent inhibition constants (K_i), obtained by analysis of Dixon plots (Figs. 1B and 2B), of 150 nM, 1.18 μM, and 700 nM for **2**, **3**, and **7**, respectively, in which the apparent K_m for androstenedione is about 60 nM (Table I). The 16α-bromide **2** has relatively high affinity ($K_i/K_m=2.5$) for binding to the catalytic site(s) of aromatase. The 16α-alcohol **3** has much higher K_i than that (45 nM) of the parent steroid **1**. This is conceivable from the previous findings on the relative affinities of the natural substrates,[12] androstenedione and 16α-hydroxyandrostenedione, for aromatase (60 nM for the former *vs.* 1.06 μM for the latter). It has previously been reported that steroids having an α-side substituent at C-6[13] and C-7[14] of androstenedione have relatively high affinity for aromatase. The present results obtained with the 3α-methoxide **7** along with the previous ones[13,14] are helpful in understanding the stereochemical properties of aromatase inhibitor.

The 16α-bromide **2**, the most potent 4-en-3,6,17-trione derivative examined in this study and the 3α-methoxide **7** were then tested for their abilities to cause a time-dependent inactivation of aromatase. The two compounds were incubated with aromatase in the presence of NADPH in air and remaining aromatase activity was measured. The latter caused a time-dependent, pseudo-first order inactivation of aromatase during the first 12 min. On the contrary, a time-dependent, biphasic inactivation of aromatase activity was caused by the former, and since the slopes of the rates of inactivation are linear only at the initial time period (*i.e.*, 3 min), these earlier times were used to obtain k_{app} values of varying concentrations of inhibitor. With increasing inhibitor concentrations, increasing k_{app} values were obtained for both compounds (Figs. 3 and 4). Double reciprocal plots of k_{app} *vs.* inhibitor concentration yielded K_i's of 625 and 800 nM and k_{inact}'s of 0.417 and 0.036 min^{-1}, respectively, for compounds **2** and **7** (Figs. 3B and 4B).

NADPH and oxygen were essential for the time-dependent activity loss, and the addition of equimolar androstenedione (1 μM for compound **2** and 6 μM for compound **7**) prevented the inactivation in each case (Table II).

We[6a,c] previously reported that compound **1** and its 3β-reduced steroid derivatives are suicide substrates for

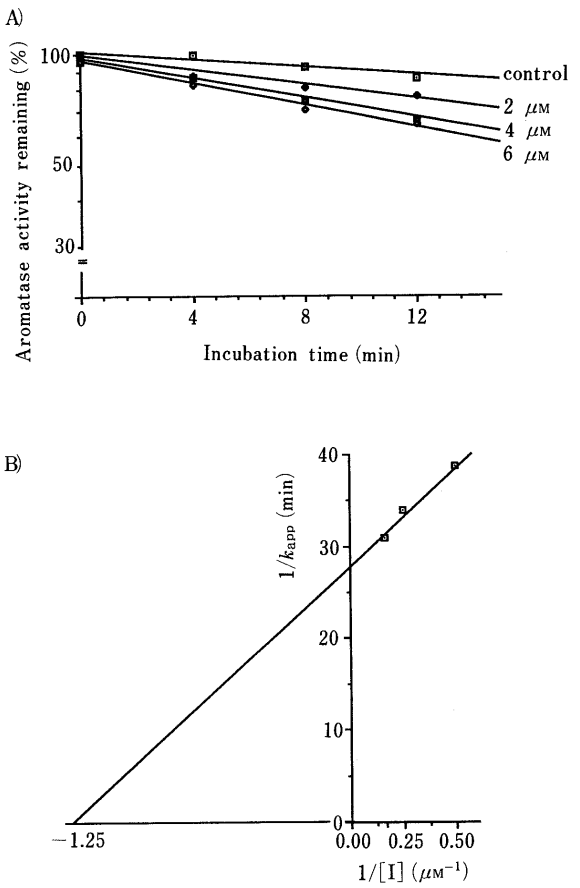


Fig. 4. Time-Dependent Inactivation (A) and Concentration-Dependent Inactivation (B) by the 3α-Methoxide **7** of Aromatase Activity

Each point represents the mean of duplicate determinations.
B: $k_{inact} = 0.036\,min^{-1}$.

TABLE II. NADPH and Oxygen Dependency and Substrate Protection Experiments for Compounds **2** and **7**

Inhibitor and conditions	Activity remaining (%)[a]	Inhibitor and conditions	Activity remaining (%)[a]
2[b]	22	**2**+N_2 atmosphere	93
7[b]	59	**7**+N_2 atmosphere	94
2−NADPH	96	**2**+androstenedione[c]	91
7−NADPH	95	**7**+androstenedione[c]	98

a) Preincubations with inhibitors were carried out for 8 min at 37 °C. The values are the mean of triplicate determinations. *b*) Compounds **2** (1 μM) and **7** (6 μM) were preincubated in the presence of NADPH (600 μM) in air. *c*) Equimolar androstenedione (1 μM for experiment with compound **2** and 6 μM for experiment with compound **7**) was added to the incubate.

aromatase and irreversibly bind to aromatase, probably through a sulfhydryl function, and that the oxygen function (carbonyl, hydroxyl, or ester group) at C-3 of the inhibitors is essential for a mechanism-based inactivation of aromatase activity, although the mechanism itself is unknown. Considering these along with the present results (Figs. 3 and 4 and Table II), it can be concluded that compounds **2** and **7** are suicide substrates for aromatase analogous to compound **1** and its 3β-reduced derivative, respectively, although their irreversible bindings to aromatase have not been unambiguously determined.[15] Furthermore, the 16α-alcohol **4** having a 4-ene-3,6-dione structure may also be a suicide substrate for aromatase. Compound **2** has a chemically reactive α-bromoketone structure in the D-ring.[11] However, this did not cause any significant time-dependent inactivation under conditions without NADPH in an affinity-labeling manner (Fig. 3A), showing that the time-dependent inactivation observed in this study depends on a mechanism-based inactivation.[16] However, compound **2** may interact irreversibly with the microsomal proteins rather than aromatase to result in the disappearance of the steroid from the incubate (chemical dependent elimination). Bednarski *et al.*[17] previously reported that a time-dependent, biphasic loss of aromatase activity caused by suicide substrates containing a 19-thiol group may be responsible for the metabolic dependent elimination from the incubate. Thus, the time-dependent, biphasic loss caused by compound **2** is probably a result of the chemical- or metabolic-dependent elimination of the inhibitor from the incubate, since compound **2** was stable in phosphate buffer at 37 °C for up to 10 h (data not shown).

The 3α-methoxide **7** is the first suicide substrate for aromatase having a 3α-oxygen function in a 4-en-6-oxo system. It is not clear whether or not the 3α-oxygen function plays a similar role to the 3β one in the formation of the reactive intermediate (a potent electrophile) which binds in a covalent manner with nucleophile in the aromatase active site. Human placental microsomes contain non-aromatase cyt. P-450 catalyzing benzphetamine demethylation[18] and hydroxysteroid oxidoreductases.[19] Considering this, it is speculated that the time-dependent inactivation observed with compound **7** would, in part, depend on the 3-oxo steroid **1** produced from it during the preincubation through demethylation of the 3α-methoxyl substituent and subsequent oxidation of the resulting 3α-hydroxyl function. It is believed that compound **7** with its unique structural feature as aromatase inhibitor can facilitate further the elucidation of the spatial relationships of the substrate molecule to the active site of aromatase and of the development of a suicide inhibitor with a new structural feature.

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