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Aromatase inactivation by 2-substituted derivatives of the suicide substrate androsta-1,4-diene-3,17-dione

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ABSTRACT

To gain the structure–activity relationship of Δ^1-androstenediones (Δ^1-ADs) as mechanism-based inactivator of aromatase, series of 2-alkyl- and 2-alkoxy-substitiuted Δ^1-ADs (**6** and **9**) as well as 2-bromo-Δ^1-AD (**14**) were synthesized and tested. All of the inhibitors examined blocked aromatase in human placental microsomes in a competitive manner. In a series of 2-alkyl-Δ^1-ADs (**6**), *n*-hexyl compound **6f** was the most powerful inhibitor with an apparent K_i value of 31 nM. The inhibitory activities of 2-alkoxy steroids **9** decreased in relation to length of the alkyl chain up to *n*-hexyloxy group (K_i: 95 nM for methoxy **9a**). All of the alkyl steroids **6** along with the alkoxy steroid **9**, except for the ethyl and *n*-propyl compounds **6b** and **6c**, caused a time-dependent inactivation of aromatase. The inactivation rates (k_{inact}: 0.020–0.084 min^{-1}) were comparable to that of the parent compound Δ^1-AD. The inactivation was prevented by the substrate AD, and no significant effect of L-cysteine on the inactivation was observed in each case. The results indicate that the 2-hexyl compound **6f** act as the most powerful mechanism-based inactivator of aromatase among Δ^1-AD analogs and may be submitted to the preclinical study in estrogen-dependent breast cancer.

1. Introduction

The conversion of androgens to estrogens is catalyzed by aromatase, of which activity results in aromatization of A-ring of androgens to form the phenolic A-ring characteristic of estrogen, with concomitant loss of 19-angular methyl group [1–3] (Fig. 1). Three oxygenation steps are involved, each apparently requiring 1 mol of oxygen and 1 mol of NADPH, for a total of 3 mol of each [4]. The first two are sequential, stereospecific hydroxylation at C-19 methyl group [5–7]. In the third step, C-19 and 1β,2β-hydrogens are eliminated as formic acid and water, respectively, to produce estrogen [8–11]. It is now thought that a substrate-dependent variation occurs in the stereospecificity of the elimination of the C-2 hydrogen [12]. Considerable speculation continue as to the third step and mechanism of attack of the third mol of oxygen. A leading theory for the third step proposes a nucleophilic attack of the heme-ferric peroxide intermediate [8,10,11,13,14].

Inhibitors of aromatase are of interest in the treatment of established breast cancer [15–18]. For this reason, a number of powerful aromatase inhibitors, analogs of natural substrate androstenedione (AD), have been described by various laboratories. Androsta-1,4-diene-3,17-dione (**1**), which can serve as a substrate for aromatase [19–24], is prototypical mechanism-based (suicide) inhibitor of aromatase [9,25–28] (Fig. 2). Several analogs of the 1,4-diene **1**, having a D-ring lactone (Testolactone) [29,30], 1-methyl (Atamestane) [31], or 6-methylene (Exemestane) [32,33] structure, are clinically evaluated as orally active substrate for aromatase. The 1,4-diene-3-one structure is responsible for inactivation, although the inactivation mechanism is currently unknown [9,27]. We have studies the structure–activity relationships of 6α- and 6β-substituted (alkyl, phenylaliphatic, alkoxy, and ester) 1,4-dien-3-ones [34–36] as well as 19-substituted 1,4-dein-3-ones [37] as aromatase inhibitors. Length and/or stereochemistry of the C-6 substituent of compound **1** play a critical role not only in the tight binding to the active site of aromatase but also in a time-dependent inactivation of the enzyme [34–36]. Among the 19-substituted derivatives, powerful competitive inhibitors of aromatase, 19-chloro compound shows the time-dependent inactivation of aromatase probably through steric and electronic reasons [37].

In the course of our study on the structure–activity relationships of the 1,4-dien-3-one **1** as aromatase inhibitors, we were of interest in C-2 substitution of the substrate **1** by an alkyl or alkoxy group. Thus, we synthesized 2-substituted (alkyl and alkoxy) 1,4-dienes and tested their ability to inhibit aromatase activity as well as their ability to inactivate aromatase in a suicidal manner.

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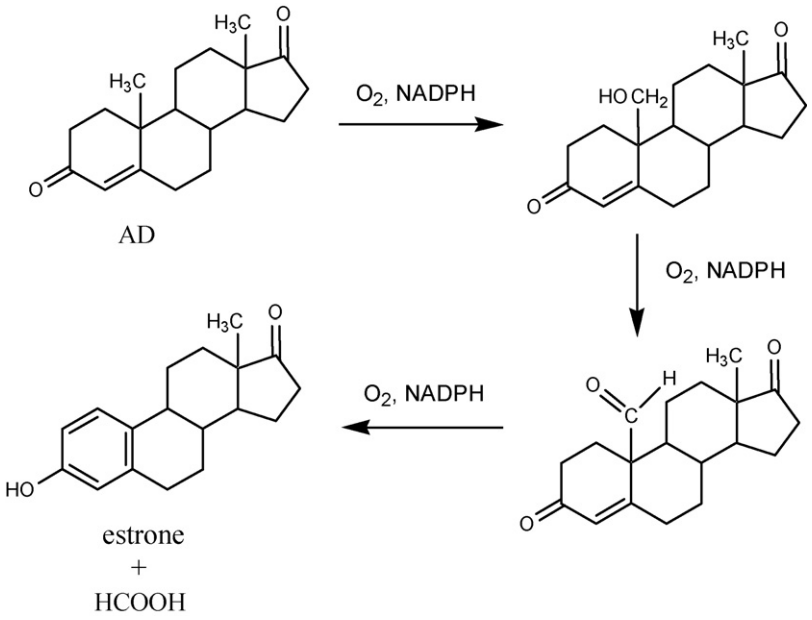

Fig. 1. Aromatization sequence of AD with human placental aromatase.

2. Experimental

2.1. Material and general methods

2-Methyl-Δ^1-AD (**6a**) [38], 2α-hydroxyAD (**7**) [39], 17β-*tert*-butyldimethylsiloxyandrost-4-en-3-one (**2**) [40], 2-bromo-5α-androst-1-en-3-one-17β-ol acetate (**10**) [41], and 2α-ethylandrost-4-ene-3,17-dione (**5b**) [42] were synthesized according to the previous methods. [1β-^{3}H]AD (specific activity 27.5 Ci/mmol; ^{3}H-distribution 74–79%) was obtained from New England Nuclear (Boston, MA, USA). NADPH was purchased from Sigma–Aldrich (St. Louise, MO, USA).

Melting points were measured on a Yanagimato melting point apparatus (Kyoto, Japan) and are uncorrected. Infra red (IR) spectra were recorded on a PerkinElmer FT-IR 1725X spectrophotometer in a KBr pellet or nujol form, and ultra violet (UV) spectra were determined in 95% EtOH on a Hitachi 150-20 spectrophotometer (Tokyo, Japan). ^{1}H-nuclear magnetic resonance (NMR) spectra were obtained in $CDCl_3$ solution with a JEOL LA 400 (400 MHz) and JEOL LA 600 (600 MHz) spectrometers (Tokyo, Japan) using tetramethylsilane as an internal standard, and mass (MS) spectra (electron impact, EI mode) and high resolution (HR)-MS with a JEOL JMS-DX 303 spectrometer. Thin-layer chromatography (TLC) was performed on E. Merck precoated silica gel plates (silica gel 60F-254, Darmstadt, Germany). Column chromatography was conducted with silica gel 60, 70–230 mesh (E. Merck). High-performance liquid chromatography (HPLC) was carried out using a Waters 600 pump and a UV detector (240 nm) (Milford, MA, USA) where Symmetry column (300 mm × 7.8 mm i.d., Waters) was employed for preparative use and Puresil column (150 mm × 4.6 mm i.d., Waters) for purity determination of compounds.

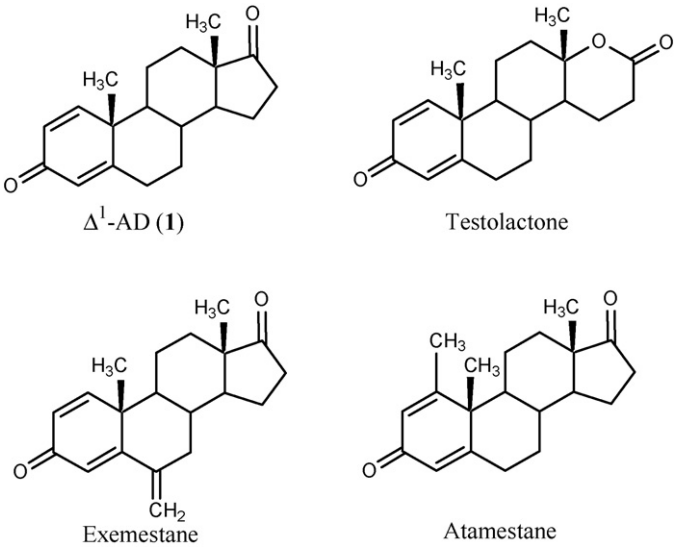

Fig. 2. Structures of suicide substrates of aromatase having a 1,4-dien-3-one structure.

2.2. 2-Ethylandrosta-1,4-diene-3,17-dione (*6b*)

Dichlorodicyanobenzoquinone (DDQ) (272 mg, 1.2 mmol) was added to a solution of 2α-ethyl-AD (**5b**) (314 mg, 0.99 mmol) [42] in dioxane (21 ml) and the reaction mixture was heated under reflux for 30 h. After this time, they mixture was filtrated and extracted with EtOAc (200 ml). The organic layer was washed with 1% NaOH solution and water, and evaporated under reduced pressure to yield an oil which was passed through Al_2O_3 (3 g) column to remove the reagent and then silica gel (20 g) column chromatography (hexane–EtOAc = 5:1) to give a solid. Recrystallization from acetone afford compound **6b** (30 mg, 10%), mp 160–163 °C. IR (KBr): 1623 cm^{-1} (C=C), 1662 and 1736 cm^{-1} (C=O); UV λ_{max}: 249 nm (ε = 15,700); ^{1}H NMR δ: 0.95 (3H, s, 18-Me), 1.08 (3H, t, J = 7.3 Hz, 2-CH_2CH_3), 1.23 (3H, s, 19-Me), 2.33 (2H, m, 2-CH_2CH_3), 6.08 (1H, s, 4-H), 6.75 (1H, s, 1-H); MS *m/z*: 312 (M^+, 57), 295 (26), 150 (100), 136 (18). HR-MS for $C_{21}H_{28}O_2$ (M^+): clad 312.2089, found 312.2086. Anal. calcd for $C_{21}H_{28}O_2$: C, 80.73; H, 9.03. Found C, 80.88; H, 9.00.

2.3. 2α-Alkyl-17β-tert-butyldimethylsiloxyandrost-4-en-3-ones (*3*)

A mixture of 17β-*tert*-butyldimethylsiloxyandrost-4-en-3-one (**2**) (400 mg, 0.99 mmol), *n*-alkyl iodide (1.3 ml), THF (1 ml), hexamethylphosphoric triamide (HMPA) (0.14 ml) was cooled −60 °C and

then *tert*-BuOK (223 g, 1.99 mmol) in THF (0.8 ml)–HMPA (0.14 ml) was added to this solution [43]. The reaction mixture was stirred at −60 °C for 2 h and the temperature was gradually elevated to room temperature. The mixture was extracted of with EtOAc (50 ml ×2), washed with 5% $NaHCO_3$ solution and saturated NaCl, and dried with Na_2SO_4. Evaporation of the solvent gave an oil which was purified by silica gel (40 g) column chromatography with hexane–EtOAc (40:1) to yield an oily substance (0.17–0.25 mmol, 18–25%). The ^{1}H NMR spectrum of the oil substance showed that the alkyl group at C-2 of the compound was about 75–85% of the α-configuration based on the 19-methyl signal (δ: 1.20 and 1.16 ppm for the 2α- and 2β-alkyl). Then, the oil was subjected to preparative TLC (hexane–EtOAc, 20:1, multiple developments) to give 2α-alkyl steroids **3**.

3c: semi-crystalline; IR (KBr): 1671 cm^{-1} (C=O); UV λ_{max} 241 nm (ε = 15,200); ^{1}H NMR δ: 0.003 (6H, s, Si-$(CH_3)_2$), 0.74 (3H, s, 18-Me), 0.88 (9H, s, Si–C$(CH_3)_3$), 0.92 (3H, t, *J* = 7.3 Hz, 2-$(CH_2)_2CH_3$), 1.20 (3H, s, 19-Me), 3.55 (1H, t, *J* = 8.2 Hz, 17α-H), 5.69 (1H, d, *J* = 1.5 Hz, 4-H). MS *m*/*z*: 444 (M^+, 14), 401 (52), 387 (100), 311 (13). HR-MS for $C_{28}H_{48}O_2Si$ (M^+): calcd 444.3424, found 444.3436.

3d: semi-crystalline; IR (KBr): 1714 cm^{-1} (C=O); UV λ_{max} 241 nm (ε = 13,000); ^{1}H NMR δ: 0.002 (3H, s, (Si-$CH_3)_a$), 0.007 (3H, s, Si-$(CH_3)_b$), 0.75 (3H, s, 18-Me), 0.90 (12H, m, Si-C$(CH_3)_3$ and 2-$(CH_2)_3CH_3$), 1.20 (3H, s, 19-Me), 3.55 (1H, t, *J* = 8.2 Hz, 17α-H), 5.70 (1H, s, 4-H); MS *m*/*z*: 458 (M^+, 3), 401 (100), 325 (10), 227 (7). HR-MS for $C_{29}H_{50}O_2Si$ (M^+): calcd 458.3580, found 458.3590.

3e: oil; IR (neat): 1673 cm^{-1} (C=O); UV λ_{max} 241 nm (ε = 9700); ^{1}H NMR δ: 0.001 (3H, s, (Si-$CH_3)_a$), 0.006 (3H, s, Si-$(CH_3)_b$), 0.75 (3H, s, 18-Me), 0.89 (12H, m, Si-C$(CH_3)_3$ and 2-$(CH_2)_4CH_3$), 1.20 (3H, s, 19-Me), 3.55 (1H, t, *J* = 8.4 Hz, 17α-H), 5.69 (1H, s, 4-H). MS *m*/*z*: 472 (M^+, 3), 415 (100), 402 (38), 345 (21). HR-MS for $C_{30}H_{52}O_2Si$ (M^+): calcd 472.3737, found 472.3743.

3f: oil; IR (neat): 1673 cm^{-1} (C=O); UV λ_{max} 240 nm (ε = 9100); ^{1}H NMR δ: 0.004 (6H, s, Si-$(CH_3)_2$), 0.75 (3H, s, 18-Me), 0.88 (12H, m, Si-C$(CH_3)_3$ and 2-$(CH_2)_5CH_3$), 1.20 (3H, s, 19-Me), 3.55 (1H, t, *J* = 8.3 Hz, 17α-H), 5.69 (1H, d, *J* = 1.5 Hz, 4-H). MS *m*/*z*: 486 (M^+, 5), 445 (25), 429 (100), 402 (36). HR-MS for $C_{31}H_{54}O_2Si$ (M^+): calcd 486.3893, found 486.3901.

3g: oil; IR (neat): 1673 cm^{-1} (C=O); UV λ_{max} 241 nm (ε = 10,100); ^{1}H NMR δ: 0.002 (3H, s, Si-$(CH_3)_a$), 0.008 (3H, s, Si-$(CH_3)_b$), 0.75 (3H, s, 18-Me), 0.89 (12H, m, Si-C$(CH_3)_3$ and 2-$(CH_2)_6CH_3$), 1.20 (3H, s, 19-Me), 3.55 (1H, t, *J* = 8.2 Hz, 17α-H), 5.69 (1H, d, *J* = 1.5 Hz, 4-H). MS *m*/*z*: 500 (M^+, 3), 443 (100), 402 (34), 367 (8). HR-MS for $C_{32}H_{56}O_2Si$ (M^+): calcd 500.4050, found 500.4059.

2.4. 2α-Alkyl-17β-hydroxyandrost-4-en-3-ones (*4*)

5% HCl (1.1 ml) was added to a solution of the 17β-siloxy compounds **3** (0.26 mmol) in THF (2 ml) and 2-propanol (2 ml) and the reaction mixture was stand at room temperature for 6 h. After adding $NaHCO_3$, the mixture was extracted with EtOAc (50 ml), washed with 5% $NaHCO_3$ solution and water, and dried with Na_2SO_4. Evaporation of the solvent gave a solid which was purified by silica gel (10 g) column chromatography (hexane–EtOAc = 4:1) followed by recrystallization from acetone to yield 17-hydroxy compounds **4c**–**4f** or purification with preparative TLC (hexane–EtOAc = 4:1, multiple developments) to give **4g** (60–70 mg, 54–63%).

4c: mp 125–128 °C. IR (KBr): 1672 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 13,700); ^{1}H NMR δ: 0.80 (3H, s, 18-Me), 0.92 (3H, t, *J* = 7.3 Hz, 2-$(CH_2)_2CH_3$), 1.21 (3H, s, 19-Me), 3.65 (1H, m, 17α-H), 5.70 (1H, d, *J* = 1.8 Hz, 4-H); MS *m*/*z*: 330 (M+, 24), 288 (100), 273 (16), 122 (39). Anal. calcd for $C_{22}H_{34}O_2$: C, 79.95; H, 10.37. Found C, 79.94; H, 10.65.

4d: mp 199–201 °C. IR (KBr): 1676 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 14,600); ^{1}H NMR δ: 0.80 (3H, s, 18-Me), 0.91 (3H, t, *J* = 6.8 Hz, 2-$(CH_2)_3CH_3$), 1.21 (3H, s, 19-Me), 3.68 (1H, t, *J* = 8.5 Hz, 17α-H), 5.70 (1H, d, *J* = 1.5 Hz, 4-H); MS *m*/*z*: 344 (M+, 7), 288 (100), 135 (12), 122 (33). Anal. calcd for $C_{23}H_{36}O_2$: C, 80.18; H, 10.53. Found C, 80.20; H, 10.45.

4e: mp 140–143 °C. IR (KBr): 1672 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 12,900); ^{1}H NMR δ: 0.80 (3H, s, 18-Me), 0.89 (3H, t, *J* = 7.0 Hz, 2-$(CH_2)_4CH_3$), 1.21 (3H, s, 19-Me), 3.65 (1H, d, *J* = 8.4 Hz, 17α-H), 5.70 (1H, d, *J* = 1.5 Hz, 4-H); MS *m*/*z*: 358 (M^+, 6), 288 (100), 273 (10), 122 (18). Anal. calcd for $C_{24}H_{38}O_2$: C, 80.39; H, 10.68. Found C, 80.40; H, 10.55.

4f: mp 124–128 °C. IR (KBr): 1673 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 12,100); ^{1}H NMR δ: 0.80 (3H, s, 18-Me), 0.88 (3H, t, *J* = 7.2 Hz, 2-$(CH_2)_5CH_3$), 1.21 (3H, s, 19-Me), 3.65 (1H, d, *J* = 8.5 Hz, 17α-H), 5.70 (1H, d, *J* = 1.5 Hz, 4-H); MS *m*/*z*: 372 (M^+, 7), 288 (100), 273 (9), 122 (20). Anal. calcd for $C_{25}H_{40}O_2$: C, 80.59; H, 10.82. Found C, 80.65; H, 10.77.

4g: semi-crystalline; IR (KBr): 1670 cm^{-1} (C=O); UV λ_{max} 241 nm (ε = 11,300); ^{1}H NMR δ: 0.80 (3H, s, 18-Me), 0.88 (3H, t, *J* = 8.3 Hz, 2-$(CH_2)_6CH_3$), 1.21 (3H, s, 19-Me), 3.65 (1H, t, *J* = 8.5 Hz, 17α-H), 5.70 (1H, d, *J* = 1.7 Hz, 4-H); MS *m*/*z*: 386 (M^+, 15), 288 (100), 273 (12), 122 (25). HR-MS for $C_{26}H_{42}O_2$ (M^+): calcd 386.3185, found 386.3180.

2.5. 2α-Alkylandrost-4-ene-3,17-diones (*5*)

Jones reagent (11 drops) was added to a solution of the 17β-hydroxide **4** (0.13–0.15 mmol) in acetone (20 ml) under ice-cooling and the mixture was stirred for 3 min. After this time, MeOH (0.1 ml) was added to the mixture and then extracted with EtOAc (10 ml), washed with 5% $NaHCO_3$ solution and water, dried with Na_2SO_4. Evaporation of the solvent gave an oil which was purified by recrystallization from acetone or by silica gel (5 g) column chromatography (hexane–EtOAc = 7:1) to yield compounds **5** as an oil (32–40 mg, 67–83%). The purities of the oily substances were determined by HPLC (Puresil column, CH_3CN:H_2O = 80:20, v/v; flow rate, 1.0 ml/min) to be more than 98%.

5c: mp 119–122 °C. IR (KBr): 1665 and 1730 cm^{-1} (C=O); UV λ_{max}: 240 nm (ε = 13,200); ^{1}H NMR δ: 0.92 (6H, t, *J* = 7.1 Hz, 18-Me and 2-$(CH_2)_2CH_3$), 1.23 (3H, s, 19-Me), 5.73 (1H, d, *J* = 1.8 Hz, 4-H). MS *m*/*z*: 328 (M^+, 9), 286 (100), 271 (19), 122 (29). Anal. calcd for $C_{22}H_{32}O_2$: C, 80.44; H, 9.82. Found C, 80.58; H, 9.59.

5d: oil; t_R = 6.5 min; IR (neat): 1665 and 1730 cm^{-1} (C=O); UV λ_{max}: 240 nm (ε = 13,200); ^{1}H NMR δ: 0.91 (3H, m, 2-$(CH_2)_3CH_3$), 0.92 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 5.72 (1H, s, 4-H); MS *m*/*z*: 342 (M^+, 5), 286 (100), 122 (36). HR-MS for $C_{23}H_{34}O_2$ (M^+): calcd 342.2559, found 342.2561.

5e: oil; t_R = 8.2 min; IR (neat): 1674 and 1740 cm^{-1} (C=O); UV λ_{max}: 240 nm (ε = 14,700); ^{1}H NMR δ: 0.89 (3H, t, *J* = 7.0 Hz, 2-$(CH_2)_4CH_3$), 0.93 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 5.72 (1H, d, *J* = 7.1 Hz, 4-H); MS *m*/*z*: 356 (M^+, 6), 286 (100), 271 (9), 122 (17). HR-MS for $C_{23}H_{34}O_2$ (M^+): calcd 356.2715, found 356.2726.

5f: oil; t_R = 11.0 min; IR (neat): 1674 and 1740 cm^{-1} (C=O); UV λ_{max}: 240 nm (ε = 12,100); ^{1}H NMR δ: 0.88 (3H, t, *J* = 6.0 Hz, 2-$(CH_2)_5CH_3$), 0.92 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 5.72 (1H, d, *J* = 1.5 Hz, 4-H); MS *m*/*z*: 370 (M^+, 7), 286 (100), 271 (10), 122 (16). HR-MS for $C_{25}H_{38}O_2$ (M^+): calcd 370.2872, found 370.2872.

5g: oil; t_R = 15.0 min; IR (KBr): 1673 and 1739 cm^{-1} (C=O); UV λ_{max}: 240 nm (ε = 10,200); ^{1}H NMR δ: 0.88 (3H, t, *J* = 6.8 Hz, 2-$(CH_2)_6CH_3$), 0.92 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 5.72 (1H, d, *J* = 1.5 Hz, 4-H); MS *m*/*z*: 384 (M^+, 5), 286 (100), 271 (7), 122 (13). HR-MS for $C_{26}H_{46}O_2$ (M^+): calcd 384.3028, found 384.3038.

2.6. 2-Alkylandrosta-1,4-diene-3,17-diones (*6*)

DDQ (26–30 mg, 0.10–0.12 mmol) was added to a solution of 4-ene-3,17-diones **5** (0.087–0.11 mmol) in dioxane (2.1 ml) and the mixture was heated under reflux for 19 h. After this time,

the mixture was treated with Al_2O_3 (1 g) column (EtOAc) and the steroidal material was submitted to crystallization from acetone (**6c**) or reversed phase HPLC (Symmetry column; mobile phase, CH_3CN:H_2O = 50:50, v/v; flow rate 5 ml/min) to obtained oily products, compounds **6d**–**6g**, in 35–50% yield. The purities of oil substances were obtained by HPLC as described for those of compounds **5**.

6c: mp 117–120 °C; IR (KBr): 1626 cm^{-1} (C=C), 1664 and 1741 cm^{-1} (C=O); UV λ_{max}: 249 nm (ε = 15,600); ^{1}H NMR δ: 0.92 (3H, t, J = 7.5 Hz, 2-$(CH_2)_2CH_3$), 0.95 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 6.08 (1H, d, J = 1.5 Hz 4-H), 6.76 (1H, s, 1-H), MS m/z: 326 (M^+, 63), 164 (100), 155 (58), 147 (26). Anal. calcd for $C_{22}H_{30}O_2$: C, 80.94; H, 9.26. Found C, 81.10 H, 9.03.

6d: oil; t_R = 4.9 min; IR (neat): 1634 cm^{-1} (C=C), 1668 and 1739 cm^{-1} (C=O); UV λ_{max}: 249 nm (ε = 12,300); ^{1}H NMR δ: 0.91 (3H, t, J = 7.3 Hz, 2-$(CH_2)_3CH_3$), 0.95 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 6.08 (1H, s, 4-H), 6.76 (1H, s, 1-H), MS m/z: 340 (M^+, 94), 178 (100), 159 (61), 134 (35). HR-MS for $C_{23}H_{32}O_2$ (M^+): calcd 340.2402, found 340.2404.

6e: oil; t_R = 5.9 min; IR (neat): 1632 cm^{-1} (C=C), 1666 and 1741 cm^{-1} (C=O); UV λ_{max}: 248 nm (ε = 14,300); ^{1}H NMR δ: 0.88 (3H, t, J = 7.0 Hz, 2-$(CH_2)_4CH_3$), 0.95 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 6.07 (1H, d, J = 1.2 Hz 4-H), 6.75 (1H, s, 1-H), MS m/z: 354 (M^+, 100), 192 (72), 159 (44), 134 (33). HR-MS for $C_{24}H_{34}O_2$ (M^+): calcd 354.2559, found 354.2571.

6f: oil; t_R = 7.7 min; IR (neat): 1633 cm^{-1} (C=C), 1667 and 1739 cm^{-1} (C=O); UV λ_{max}: 249 nm (ε = 17,000); ^{1}H NMR δ: 0.87 (3H, t, J = 7.0 Hz, 2-$(CH_2)_5CH_3$), 0.95 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 6.07 (1H, d, J = 1.4 Hz 4-H), 6.75 (1H, s, 1-H), MS m/z: 368 (M^+, 100), 206 (77), 159 (45), 134 (34). HR-MS for $C_{25}H_{36}O_2$ (M^+): calcd 368.2715, found 368.2711.

6g: oil; t_R = 10.1 min; IR (neat): 1634 cm^{-1} (C=C), 1666 and 1739 cm^{-1} (C=O); UV λ_{max}: 248 nm (ε = 15,100); ^{1}H NMR δ: 0.87 (3H, t, J = 7.0 Hz, 2-$(CH_2)_6CH_3$), 0.95 (3H, s, 18-Me), 1.23 (3H, s, 19-Me), 6.07 (1H, d, J = 1.0 Hz 4-H), 6.75 (1H, s, 1-H), MS m/z: 382 (M^+, 100), 220 (95), 159 (62), 134 (60). HR-MS for $C_{26}H_{38}O_2$ (M^+): calcd 382.2872, found 382.2874.

2.7. 2-Hydroxyandrosta-1,4-diene-3,17-dione (**8**)

Two portions of Bi_2O_3 (124 mg, 0.26 mmol) was added to a solution of 2α-hydroxy-AD (**7**) (100 mg, 0.33 mmol) in acetic acid (0.5 ml), and the mixture was heated for 70 min under reflux. After removing the solid material by filtration, the product was extracted with EtOAc (200 ml), washed 5% $NaHCO_3$ solution and water, dried with Na_2SO_4, and evaporated to afford a solid which was purified by silica gel (10 g) column chromatography (hexane–EtOAc = 4:1) followed by recrystallization from acetone to yield 1,4-diene **8** (77 mg, 78%); mp 149–150 °C. IR (KBr): 1644 and 1735 cm^{-1} (C=O); UV λ_{max}: 253 nm (ε = 13,800); ^{1}H NMR δ: 0.95 (3H, s, 18-Me), 1.27 (3H, s, 19-Me), 6.20 (1H, s, 4-H), 6.31 (1H, s, 1-H); MS m/z: 300 (M^+, 100), 282 (41), 163 (23). Anal. calcd for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05. Found C, 76.05; H, 7.98.

2.8. 2-Alkoxyandrosta-1,4-diene-3,17-diones (**9**)

(A) A mixture of the 2-hydroxy compound **8** (74 mg, 0.24 mmol), *tert*-BuOK (30 mg, 0.27 mmol), and methyl or ethyl iodides (2 ml) was refluxed for 4.5 h [41]. After removing the solid material by filtration, the filtrate was diluted with EtOAc (50 ml) and washed with 5% $NaHCO_3$ solution and water. Organic layer was dried with Na_2SO_4 and evaporated to give an oil which was purified by silica gel (10 g) column chromatography (hexane–EtOAc = 3:1) followed recrystallization to give from MeOH to afford 2-methoxy or 2-ethoxy compound **9a** or **9b** (**9a**; 38 mg, 49%, **9b**; 42 mg, 40%).

9a: mp 240–243 °C. IR (KBr): 1614 and 1638 cm^{-1} (C=C), 1661 and 1736 cm^{-1} (C=O); UV λ_{max}: 253 nm (ε = 15,100); ^{1}H NMR δ: 0.95 (3H, s, 18-Me), 1,28 (3H, s, 19-Me), 3.68 (3H, s, 2-OMe), 5.94 (1H, s, 4-H), 6.13 (1H, s, 1-H); MS m/z: 314 (M^+, 100), 296(13), 164 (28). Anal. calcd for $C_{20}H_{26}O_3$: C, 76.40; H, 8.33. Found C, 76.70; H, 8.13.

9b: mp 173–174 °C. IR (KBr): 1615 and 1641 cm^{-1} (C=C), 1661 and 1740 cm^{-1} (C=O); UV λ_{max}: 253 nm (ε = 14,262); ^{1}H NMR δ: 0.95 (3H, s, 18-Me), 1.27 (3H, s, 19-Me), 1.43 (3H, t, J = 7.0 Hz, 2-OCH_2CH_3), 3.84 (2H, m, 2-OCH_2CH_3), 5.95 (1H, s, 4-H), 6.13 (1H, s, 1-H); MS m/z: 328 (M^+, 100), 165 (45), 150 (26), 137 (26). Anal. calcd for $C_{21}H_{28}O_3$: C, 76.79; H, 8.59. Found C, 76.70; H, 8.88.

(B) A mixture of the 2-hydroxy compound **8** (200 mg, 0.67 mmol), Ag_2O (281 mg, 1.21 mmol), and *n*-butyl or *n*-hexyl iodides (5.3 ml) was refluxed for 2 h [36]. After removing the solid material by filtration, the filtrate was diluted with EtOAc (200 ml) and washed with 5% $NaHCO_3$ solution and water. Organic layer was dried with Na_2SO_4 and evaporated to give a solid or oily product. A solid was recrystallized from acetone to give 2-*n*-butoxy compound **9d** (158 mg, 67%). An oily substance was purified by preparative TLC (hexane–EtOAc = 5:1, multiple developments) to afford 2-*n*-hexyloxy compound **9f** (210 mg, 82%) of which purity was determined by HPLC as described for that of compounds **5**.

9d: mp 105–107 °C; IR (KBr): 1611 and 1635 cm^{-1} (C=C), 1659 and 1733 cm^{-1} (C=O); UV λ_{max}: 254 nm (ε = 13,600); ^{1}H NMR δ: 0.96 (6H, m, 18-Me and 2-$OCH_2(CH_2)_2CH_3$), 1.27 (3H, s, 19-Me), 3.75 (2H, m, 2-$OCH_2(CH_2)_2CH_3$), 5.94 (1H, s, 4-H), 6.13 (1H, d, J = 1.5 Hz, 1-H). MS m/z: 356 (M^+, 100), 300 (10), 193 (22), 137 (49). Anal. calcd for $C_{23}H_{32}O_3$: C, 77.49; H, 9.05. Found C, 77.51; H, 9.28.

9f: oil; t_R = 5.1 min; IR (KBr): 1613 and 1638 cm^{-1} (C=C), 1663 and 1739 cm^{-1} (C=O); UV λ_{max}: 254 nm (ε = 13,500); ^{1}H NMR δ: 0.89 (3H, t, J = 7.0 Hz, 2-$OCH_2(CH_2)_4CH_3$), 0.95 (3H, s, 18-Me), 1.27 (3H, s, 19-Me), 3.74 (2H, m, 2-$OCH_2(CH_2)_2CH_3$), 5.94 (1H, s, 4-H), 6.13 (1H, d, J = 1.1 Hz, 1-H). MS m/z: 384 (M^+, 100), 150 (42), 137 (63), 43 (34). HR-MS for $C_{25}H_{36}O_3$ (M^+): calcd 384.2664, found 384.2646.

2.9. 2,4α-Dibromo-17β-acetoxy-5α-androst-1-en-3-one (**11**)

One drop of 30% HBr in CH_3COOH and saturated Br_2 in CH_3COOH (6.4%, 0.78 ml) were added to 2-bromide **10** (130 mg, 0.31 mmol) in CH_3COOH (6.5 ml) and the reaction mixture was stirred at room temperature over night [44]. After this time, reaction mixture was extracted with EtOAc (100 ml), washed with 5% $NaHCO_3$ solution and water, and dried with Na_2SO_4. Evaporation of the solvent gave an oil which was purified by silica gel (13 g) chromatography (hexane–EtOAc = 15:1) followed by recrystallization from acetone yielded 2,4α-dibromide **11** (105 mg, 69%), mp 205–208 °C. IR (KBr): 1702 and 1728 cm^{-1} (C=O); UV λ_{max}: 262 nm (ε = 6500); ^{1}H NMR δ: 0.83 (3H, s, 18-Me), 1.11 (3H, s, 19-Me), 2.05 (3H, s, 17-$OCOCH_3$), 4.61 (1H, dd, J = 7.7 and 9.2 Hz, 17α-H), 4.72 (1H, d, J = 13 Hz, 4-H), 7.63 (1H, s, 1-H); MS m/z: 488 (M^+, 10), 486 and 490 (M^+, 5), 446 (14), 347 (83). HR-MS for $C_{21}H_{28}O_3Br_2$ (M^+): calcd 486.0405, 488.0385 and 490.0364; found 486.0408, 488.0363 and 490.0359. Anal. calcd for $C_{21}H_{28}O_3Br_2$: C, 51.66; H, 5.78. Found C, 51.89; H, 5.50.

2.10. 2-Bromo-17β-acetoxyandrosta-1,4-dien-3-one (**12**)

A solution of 2,4-α-dibromide **11** (93 mg, 0.19 mmol) in collidine (0.54 ml) was heated under reflux, and then the mixture was filtrated and the filtrate was diluted with EtOAc (30 ml). The organic layer was washed with 5% HCl, saturated $NaHCO_3$ solution, and water, sequentially, and dried with Na_2SO_4. Evaporation of the solvent afforded an oil which was purified by silica gel (10 g) column chromatography (hexane–EtOAc = 10:1) and recrystallization from EtOAc to give 1,4-diene **12** (55 mg, 71%), mp 205–207 °C. IR (KBr): 1600 cm^{-1} (C=C), 1658 and 1730 cm^{-1} (C=O); UV λ_{max}: 254 nm

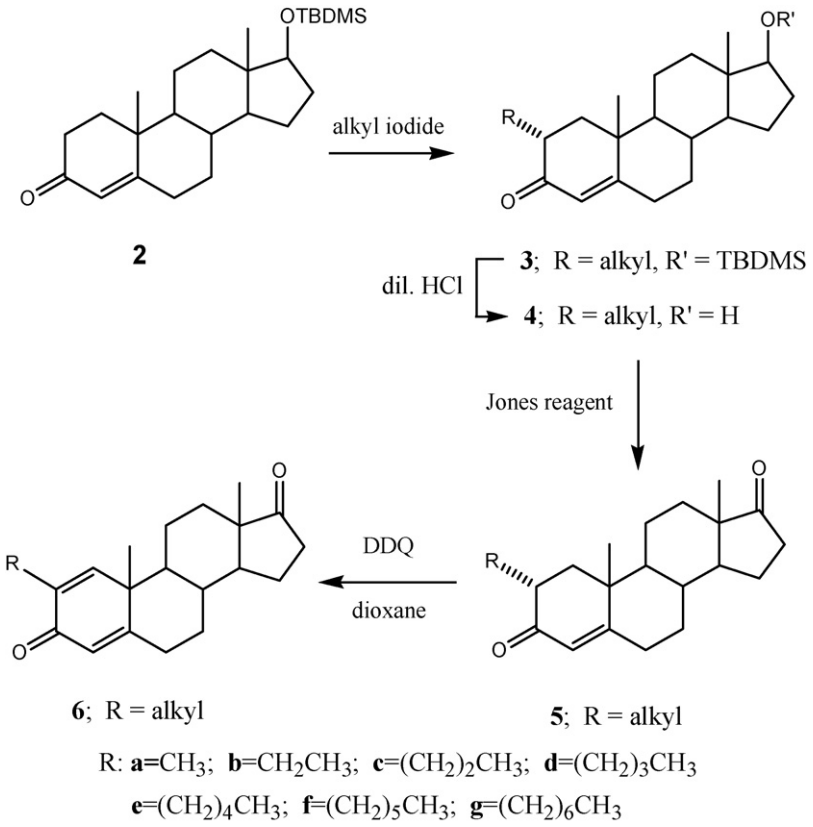

Fig. 3. Synthesis of 2-alkyl-substituted Δ^1-ADs (**6**).

(ε = 13,800); ^{1}H NMR δ: 0.87 (3H, s, 18-Me), 1.28 (3H, s, 19-Me), 2.05 (3H, s, 17β-$OCOCH_3$), 4.58 (1H, dd, J = 7.7 and 9.2 Hz, 17α-H), 6.17 (1H, s, 4-H), 7.48 (1H, s, 1-H); MS m/z: 406 and 408 (M^+, 19), 346 (20), 200 (100). HR-MS for $C_{21}H_{27}O_3Br$ (M^+): calcd 406.1144 and 408.1123. Found 406.1147 and 408.1129. Anal. calcd for $C_{21}H_{27}O_3Br$: C, 61.92; H, 6.68. Found C, 70.10; H, 6.51.

2.11. 2-Bromo-17β-hydroxyandrosta-1,4-dien-3-one (*13*)

A mixture of 17β-acetate **12** (58 mg, 0.14 mmol), K_2CO_3 (18 mg, 0.13 mmol), and MeOH (2 ml) was heated under reflux under N_2 gas for 1.5 h. After dilution with EtOAc (20 ml), the organic layer was washed with water, dried with Na_2SO_4, and evaporation of the solvent to yield a solid. This was recrystallized from acetone to afford 17β-ol **13** (51 mg, 99%), mp 222–226 °C. IR (KBr): 1597 cm^{-1} (C=C), 1655 cm^{-1} (C=O); UV λ_{max}: 255 nm (ε = 15,300); ^{1}H NMR δ: 0.82 (3H, s, 18-Me), 1.28 (3H, s, 19-Me), 3.56 (1H, m, 17α-H), 6.17 (1H, s, 4-H), 7.50 (1H, s, 1-H); MS m/z: 364 and 366 (M^+, 42), 267 (19), 200 (100). HR-MS for $C_{19}H_{25}O_2Br$ (M^+): calcd 364.1038 and 366.1017. Found 364.1052 and 366.1035. Anal. calcd for $C_{19}H_{25}O_2Br$: C, 62.47; H, 6.90. Found C, 62.30; H, 7.11.

2.12. 2-Bromoandrosta-1,4-dien-3-one (*14*)

Jones reagent was added dropwise to a solution of 17β-ol **13** (48 mg, 0.13 mol) in acetone (3 ml) at 0 °C with stirring until the orange color of the reagent remains, and the mixture was stirred for 3 min. After this time, the mixture was poured into water and the product was extracted with EtOAc (25 ml × 2). The combined organic layer was washed with 5% $NaHCO_3$ solution and water, dried with Na_2SO_4, and evaporated to give a solid which was recrystallized from acetone to yield 17-one **14** (34 mg, 71%), mp 266–268 °C. IR (KBr): 1601 cm^{-1} (C=C), 1655 and 1733 cm^{-1} (C=O); UV λ_{max}: 254 nm (ε = 15,200); ^{1}H NMR δ: 0.95 (3H, s, 18-Me), 1.30 (3H, s, 19-Me), 6.19 (1H, s, 4-H), 7.49 (1H, s, 1-H); MS m/z: 362 and 364 (M^+, 64), 265 (12), 200 (100). HR-MS for $C_{19}H_{23}O_2Br$ (M^+): calcd 362.0881 and 364.0861; found 362.0898 and 364.0867. Anal. calcd for $C_{19}H_{23}O_2Br$: C, 62.82; H, 6.38. Found C, 62.86; H, 6.51.

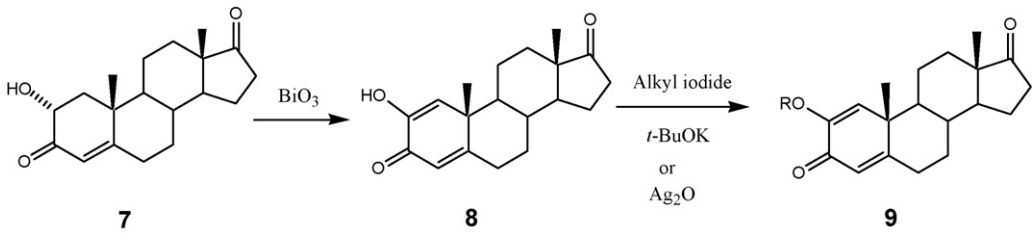

Fig. 4. Synthesis of 2-alkoxy-substituted Δ^1-ADs (**9**).

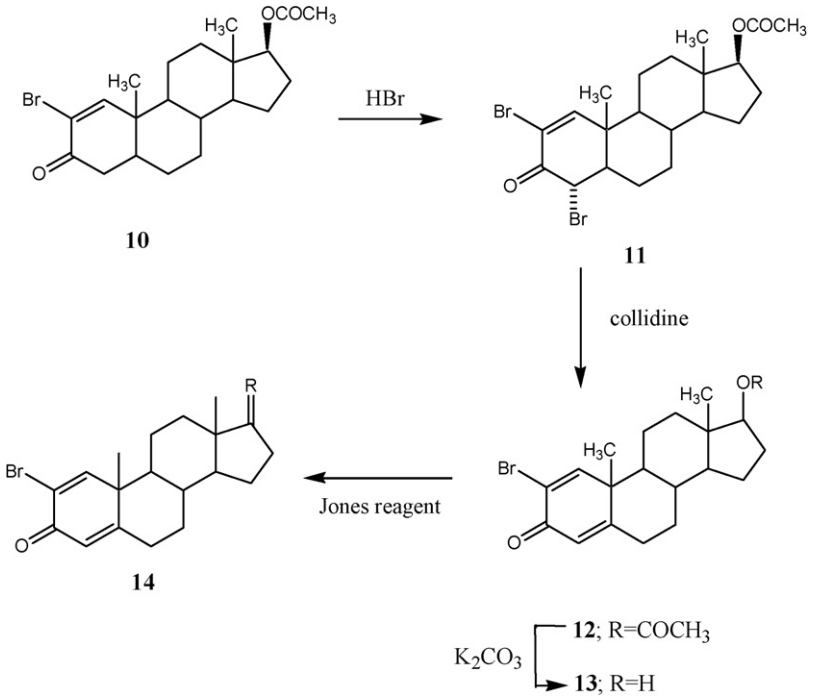

Fig. 5. Synthesis of 2-bromo-1,4-dien-3-one compound **14**.

2.13. Enzyme preparation

Human placental microsomes (particles sedimenting at 105,000 g for 60 min) were obtained using the method reported by Ryan [19]. They were washed once with 0.05 mM dithiothreitol solution, lyophilized, and stored at −80 °C. No significant loss of activity occurred during this study (2 months).

2.14. Aromatase assay procedure

Aromatase activity was measured according to the procedure of Siiteri and Thompson [45]. The screening assay for determination of IC_{50} value, the kinetic assay, and the time-dependent assay were carried out essentially according to the assay methods described in our previous work. Briefly, 20 μg of protein of the lyophilized microsomes and 20-min incubation time for the screening assay, and 20 μg of protein of the microsomes and 5-min incubation time for the kinetic assay, respectively, were employed in this study, and the assays were carried out in 67 mM phosphate buffer in the presence of NADPH in air [34]. In the time-dependent inactivation experiment, 1/10 of the incubation mixture was used for assays of the remaining aromatase activity.

3. Results

3.1. Chemistry

2-Alkylandrosta-1,4-diene-3,17-diones were synthesized by 2α-alkylation of testosterone *t*-butyldimethylsilyl ether (**2**) with *n*-alkyl iodide (alkyl: methyl to *n*-heptyl) and *tert*-BuOK as a key reaction. In the reactions, 2α-alkyl-4-en-3-one steroids **3** were isolated as major products (75–85% yields), whereas the 2β-alkyl isomers were also produced as minor products. After the purification with preparative TLC, compounds **3** was subjected to deprotection of the 17-silyl ether, oxidation of the 17-ol **4**, and dehydrogenation of the 2α-alkyl compounds **5** (Fig. 3). 2-Hydroxy-1,4-dien-3,17-dione **8**, obtained from 2α-hydroxyAD (**7**) by reaction with BiO_3, was alkylated with alkyl iodide and with *tert*-BuOK (alkyl: methyl and ethyl) or Ag_2O (alkyl: *n*-butyl and *n*-hexyl) to afford the 2-alkoxy compounds **9** (Fig. 4). Synthesis of 2-bromo-1,4-diene-3,17-dione **14** was started from 2-bromo-3-one (**10**) [41]. Treatment of compound **10** with hydrogen bromide in acetic acid gave 2,4α-dibromo ketone (**11**) of which dehydrobromination with collidine followed by alkaline hydrolysis with NaOH and oxidation yielded the 2-bromide **14** (Fig. 5).

The structures of the compounds synthesized were confirmed by the spectrometric analysis and HR-MS, elemental analysis, or HPLC analysis.

3.2. Aromatase inhibition

Inhibition of aromatase activity in human placental microsomes by 2-alkyl- and 2-alkoxy-substituted 1,4-diene-3-one steroids **6** and **9** as well as 2-bromide **14** was examined in vitro enzyme kinetics. The results are given in Table 1. Aromatization activity in placental microsomes was determined using a radiometric assay in which tritiated water released from [1β-^{3}H]AD into the incubation medium during aromatization was measured [45]. To characterize the nature of inhibitor binding to the active site of aromatase, aromatization was measured at several concentrations and substrate concentrations. The results of these studies were plotted on a typical Lineweaver-Burk plot in all cases (Fig. 6). In these studies, the apparent K_m and V_{max} values for AD were about 35 nM and 110 pmol/min/mg protein, respectively. All of the steroids showed clear-cut competitive inhibition. The apparent K_i values were obtained by Dixon plots. The inhibitory activities of 2-methyl (**6a**), 2-*n*-pentyl (**6e**), 2-*n*-hexyl (**6f**) and 2-*n*-heptyl (**6g**) -Δ^1-ADs as well as 2-methoxy analog (**9a**) were high (K_i; less than 95 nM) and they were powerful aromatase inhibitors.

3.3. Time-dependent inactivation of aromatase

The inhibitors having a 1,4-dien-3-one structure was then tested for their ability to cause a time-dependent inactivation of aromatase. All of 2-alkyl-1,4-dien-3-one steroids **6**, except for 2-ethyl and 2-propyl steroids **6b** and **6c**, as well as 2-alkoxy steroids **9**

Table 1
In vitro aromatase inhibition by 2α-alkyl-ADs and 2-alkyl-, 2-alkoxy- and 2-bromo-substituted Δ^1-ADs (**5**, **6**, **9** and **14**).

Compound	IC_{50} (nM)[a]	Apparent, K_i (nM)[b]
2α-Alkyl ADs **5**		
Methyl, **a** [42]	460	45 ± 2.5
Ethyl, **b** [42]	480	45 ± 4.1
n-Propyl, **c**	1,460	152 ± 7.1
n-Butyl, **d**	1,930	289 ± 26
n-Pentyl, **e**	1,280	241 ± 17
n-Hexyl, **f**	10,400	558 ± 48
n-Heptyl, **g**	>30,000	–
2-Alkyl-Δ^1-ADs **6**		
Methyl, **a** [38]	550	46 ± 3.2
Ethyl, **b**	960	116 ± 5.0
n-Propyl, **c**	1,750	134 ± 4.0
n-Butyl, **d**	1,840	146 ± 14
n-Pentyl, **e**	743	66 ± 5.0
n-Hexyl, **f**	298	31 ± 1.9
n-Heptyl, **g**	605	51 ± 4.8
2-Alkoxy-Δ^1-ADs **9**		
Methoxy, **a**	1,100	95 ± 9.1
Ethoxy, **b**	17,600	1360 ± 80
n-Butoxy, **d**	29,600	2870 ± 210
n-Hexyloxy, **f**	>50,000	–
2-Bromo-Δ^1-AD **14**	1,840	170 ± 15
For comparison Δ^1-AD[c]	830	90 ± 6.4

[a] 300 nM of [1β-^{3}H]androstenedione and 20 μg of protein from human placental microsomes were used.

[b] Apparent inhibition constant (K_i) was obtained by Dixon plot. 20 μg of protein from human placental microsomes and 5 min of incubation were employed. All of the inhibitors examined showed a competitive type of inhibition based on analysis of the Lineweaver–Burk plot. The results were means ± S.E. Apparent K_m and V_{max} values for AD were about 35 nM and 110 pmol/min/mg protein, respectively, in this study.

[c] The K_i values of Δ^1-AD were previously obtained to be 43 ± 3.0 nM [34] and 65 ± 3.5 nM [35].

showed the time-dependent inactivation only in the presence of NADPH under aerobic conditions (Figs. 7A and 8A). Pseudo-first order kinetics was obtained during the first 12 min of inactivation of inhibitors and kinetic data were analyzed according to the method of Kitz and Willson [46] (Figs. 7B and 8B). Double-reciprocal plots of k_{obs} versus inhibitor concentration gave K_I and k_{inact} values, respectively. The K_I values were 0.304–2.42 μM for the 2-alkyl compounds **6** and 1.67–25 μM for the 2-alkoxides **9**, and k_{inact} values were 0.020–0.073 min^{-1} for compounds **6** and 0.018–0.084 min^{-1} for compounds **9** (Table 2).

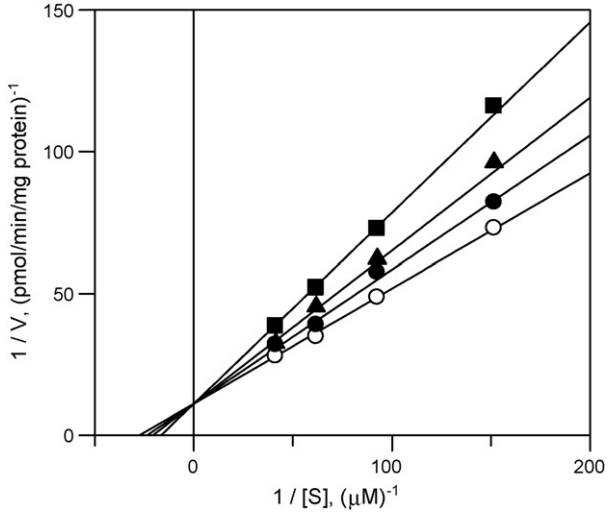

Fig. 6. Lineweaver–Burk plots of inhibition of human placental aromatase by 2-hexyl-Δ^1-AD (**6f**) with AD as the substrate. Concentrations of inhibitor: (○) control (0 nM); (●) 5 nM; (▲) 10 nM; (■) 20 nM. The inhibition experiments with all the other steroids examined gave essentially similar to Fig. 6 (data not shown).

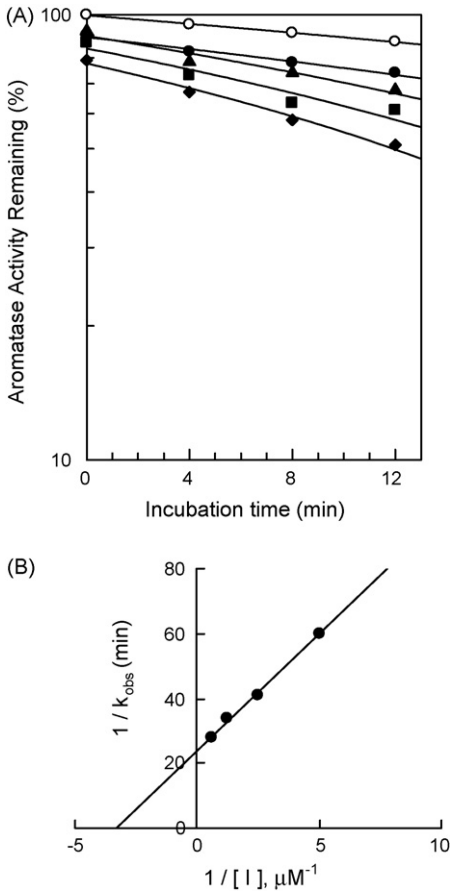

Fig. 7. Time-dependence (A) and concentration-dependence (B) of inactivation of human placental aromatase by 2-hexyl-Δ^1-AD (**6f**) in the presence of NADPH in air. Concentrations of the inhibitor: (○) control (0 nM); (●) 200 nM; (▲) 400 nM; (■) 800 nM; (♦) 1600 nM. The time-dependent inactivation experiments with other the 2-alkyl-Δ^1-AD steroids except for ethyl and *n*-propyl gave essentially similar plots to this figure (data not shown).

The substrate AD significantly blocked inactivation by the inhibitors, whereas a nucleophile, L-cysteine, had no significant effect (data has not been shown). The results indicate that the

Table 2
Kinetic analysis of time-dependent inactivation of aromatase caused by 2-substituted Δ^1-ADs[a] (**6** and **9**).

Compound	K_I (nM)	k_{inact} (min^{-1})
2-Alkyl-Δ^1-ADs **6**		
Methyl, **a** [38]	909	0.035
Ethyl, **b**	NT[b]	–
n-Propyl, **c**	NT[b]	–
n-Butyl, **d**	2,420	0.020
n-Pentyl, **e**	1,170	0.033
n-Hexyl, **f**	304	0.034
n-Heptyl, **g**	2,340	0.073
2-Alkoxy-Δ^1-ADs **9**		
Methoxy, **a**	1,670	0.084
Ethoxy, **b**	25,000	0.018
n-Butoxy, **d**	12,670	0.025
n-Hexyloxy, **f**	23,200	0.056
For comparison Δ^1-AD [34]	952	0.059

[a] Apparent K_I and k_{inact} were obtained by Kitz–Wilson plot [46].

[b] NT: the time-dependent inactivation was not observed.

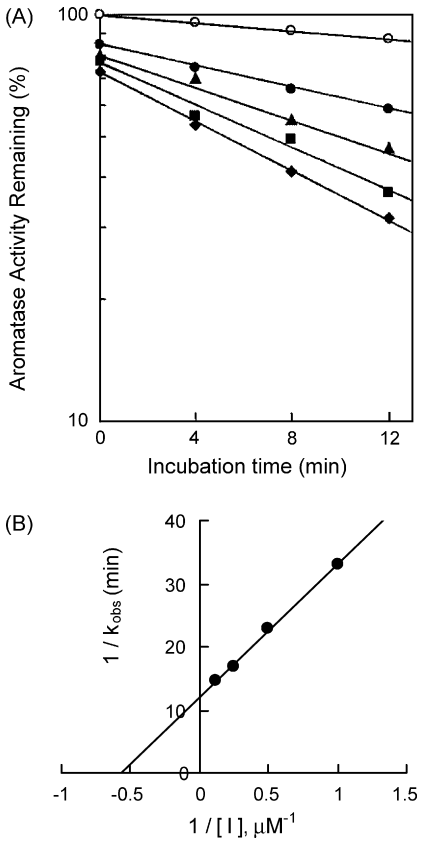

Fig. 8. Time-dependence (A) and concentration-dependence (B) of inactivation of human placental aromatase by 2-methoxy-Δ^1-AD (**9a**) in the presence of NADPH in air. Concentrations of the inhibitor: (○) control (0 μM); (●) 1 μM; (▲) 2 μM; (■) 4 μM; (♦) 8 μM. The time-dependent inactivation experiments with other the 2-alkoxy-Δ^1-AD steroids gave essentially similar plots to this figure (data not shown).

inhibitors examined are mechanism-based inactivators of aromatase.

4. Discussion

In order to define the effect of the C-2 substitution of 1,4-dien-3-one steroid, Δ^1-AD, on the activity of aromatase inhibition in a reversible or irreversible (time-dependent) manner, series of 2-alkyl steroids **6** and 2-alkoxy steroids **9** as well as 2-bomo compound **14** were synthesized and tested. All of the 2-substituted compounds **6** along with the 2-bromide **14** were powerful competitive inhibitors of aromatase in human placental microsomes with apparent K_i's ranging from 46 to 170 nM, whereas the alkoxides **9**, except for 2-methoxide **9a** (K_i: 95 nM), were relatively poor inhibitors (K_i: more than 1360 nM). The hexyl compound **6f** showed the highest affinity for aromatase (K_i: 31 nM), and its affinity was almost same to that of AD (K_m: 35 nM). The affinities of the 2-alkyl steroids **6** as well as the 2-methoxide **9a** and the 2-bromide **14** (K_i: 170 nM) were higher than that of 2-hydroxy compound **8** (K_i: 510 nM), demonstrating that a high degree of bulk tolerance with a lipophilic moiety exists in the region of C-2 of Δ^1-AD steroids.

Affinity of the 2-alkyl steroids **6** for aromatase decreased in accordance with the bulkiness of the alkyl substituent up to C_4, *n*-butyl moiety (K_i: CH_3 and $(CH_2)_3CH_3$, 46 and 146 nM, respectively). Further elongation of the alkyl substituent up to C_6 vice versa increased the affinity (K_i: $(CH_2)_4CH_3$ and $(CH_2)_5CH_3$, 66 and 31 nM, respectively) and the addition of one more methylene unit decreased it (K_i: 51 nM for $(CH_2)_6CH_3$). In contrast, in a series of the 2-alkoxy compounds **9**, the affinity decreases in relation to a carbon number of the C-2 substituent from C_1 to C_6, OCH_3 to $O(CH_2)_5CH_3$. The IC_{50} value of the *n*-hexyloxy compound **9f** was more than 50 μM. Considering the differences of the inhibitory activities between the alkyl and alkoxy compounds **6** and **9**, an electrostatic effect of an oxygen atom but not a carbon atom at the C-2 position on the affinity might be involved in the difference; for example, the hydrogen bonding to the nucleophilic residue of aromatase interferes the tight binding of the alkoxide **9** to the active site, although there is no evidence.

On the other hand, 2α-methyl- and 2α-ethyl-AD analogs (**5a** and **5b**) were powerful inhibitors of aromatase (K_i; 45 nM each) and elongation of the 2α-substituent up to the heptyl derivative **5g** (IC_{50}: more than 30 μM) decreased the inhibitory activity in proportion to the carbon number. The results demonstrate that the binding geometries of Δ^1-AD analogs **6** in the active site of aromatase are different from those of AD analogs **5**.

All of the alkyl compounds **6** as well as the alkoxy compounds **9**, except for the 2-ethyl and 2-butyl steroids **6b** and **6c**, caused a time-dependent inactivation of aromatase with k_{inact} of 0.018–0.084 min^{-1}. The rates of inactivation were corresponding well to those of suicide substrates with a double bond at C-1, reported previously, 0.033 min^{-1} for Testolactone [48] and 0.050 min^{-1} for Exemestane [33] and 0.055 and 0.059 min^{-1} for Δ^1-AD [27,34]. The inactivation rate decreased when the substrate AD was incubated in the incubation mixture while L-cysteine, a nucleophile, failed to protect aromatase from the inactivation in each case. Thus, covalent-bond formation between aromatase and the reactive intermediate appears to occur at the active site, therefore, preventing diffusion of the activated inhibitors, a reactive electrophile, in the surrounding media [47].

The inhibition constants (K_I's) obtained from the inactivation experiments are more than 10–20 times higher than the corresponding apparent K_i's from the competitive inhibition experiments. The similarly tendency has previously been reported in the inactivation experiments with other Δ^1-AD derivatives, K_i and K_I of 242 and 770 nM for Testolactone [48], 4.3 and 26 nM for Exemestane [33], and 43 and 952 nM for Δ^1-AD [34]. This relation of the K_I value to the K_i value suggests that binding of the activated inhibitor to the nucleophilic residue of the active site rather than activation become rate determining or partial rate determining [47].

Two sequences for the inactivation of aromatase have been proposed by a Δ^1-AD steroid with no evidence [9]. The first mechanism is involvement of oxygenation at C-19 and the other is removal of an electron from the C-1 double bond by perferry oxygen. Our previous results [37], a positive relation between the aromatization and the inactivation reaction, support the C-19 oxygenation mechanism. Covey [9] has suggested previously that aromatase inactivates itself because the suicide substrates induce the enzyme to autoxidize itself. We have currently no evidence for covalent modification of aromatase by compounds, however, a substrate analog-induced autoxidation mechanism would be considered as an alternate explanation for the observed time-dependent inactivation.

During the preparation of this manuscript, Ghosh et al. [49] have reported the crystal structure of human placental aromatase and discussed how a mechanism-based steroidal inhibitor, Exemestane, blocks the aromatization process by using the AD backbone. From modeling experiment of Exemestane molecule, it was not clear whether there is an existence of bulk tolerance corresponding to the extending region of the C-2 position. The present results that Δ^1-AD analogs with longer alkyl chains at C-2, *n*-pentyl, *n*-hexyl, and *n*-heptyl groups, were powerful inhibitor of aromatase predict the existence of it.

On the basis of the structure–activity relationship of 2-alkyl- and 2-alkoxy-Δ^1-ADs (**6** and **9**) as aromatase inhibitors, 2-hexyl-

Δ^1-AD (**6f**) was the most powerful mechanism-based inactivator of aromatase which may be submitted for preclinical study in estrogen-dependent breast cancer.

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