Steroids 75 (2010) 330–337

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Probing the binding pocket of the active site of aromatase with 2-phenylaliphatic androsta-1,4-diene-3,17-dione steroids

Madoka Takahashi, Kouwa Yamashita, Mitsuteru Numazawa*

Tohoku Pharmaceutical University, 4-1 Komatsushima-4-chome, Aobaku, Sendai 981-8558, Japan

ARTICLE INFO

Article history:
Received 18 November 2009
Received in revised form 7 January 2010
Accepted 14 January 2010
Available online 22 January 2010

Keywords:
Aromatase
Competitive inhibitor
Androsta-1,4-diene-3,17-dione
2-Phenylaliphatic substituent
Suicide substrate
Breast cancer

ABSTRACT

A series of 2-phenylaliphatic-substituted androsta-1,4-diene-3,17-diones (**6**) as well as their androstenedione derivatives (**5**) were synthesized as aromatase inhibitors to gain insights of structure–activity relationships of varying the alkyl moiety (C_1 to C_4) of the 2-phenylaliphatic substituents as well as introducing a methyl- or trifluoromethyl function to *p*-position of a phenethyl moiety to the inhibitory activity. The inhibitors examined showed a competitive type inhibition. The 2-phenpropylandrosta-1,4-diene **6c** was the most powerful inhibitor (K_i: 16.1 nM) among them. Compounds **6c** along with the phenethyl derivative **6b** caused a time-dependent inactivation of aromatase (k_{inact}: 0.0293 and 0.0454 min^{-1} for **6b** and **6c**, respectively). The inactivation was prevented by the substrate androstenedione, and no significant effect of L-cysteine on the inactivation was observed in each case. Molecular docking of the phenpropyl compound **6c** to aromatase was conducted to demonstrate that the phenpropyl group orients to a hydrophobic binding pocket in the active site to result in the formation of thermodynamically stable enzyme–inhibitor complex.

1. Introduction

Aromatase is a cytochrome P-450 enzyme that catalyzes the conversion of the androgens, androstenedione (AD) and testosterone, to the estrogens, estrone and estradiol, respectively [1–3]. The aromatase reaction is thought to proceed through three sequential oxygenations at C-19 of the androgens [4–7] (Fig. 1). Aromatase is a potential therapeutic target for the lowering of estrogen levels in patients with advanced estrogen-dependent breast cancer [8–11]. The specific blockade of estrogen biosynthesis has been pursued intensely with the goal of developing practical clinical drugs. For this reason, a number of powerful aromatase inhibitors, which are analogs of the natural substrate AD, have been described by various laboratories.

Androsta-1,4-diene-3,17-dione (Δ^1-AD, **1**), which is the 1-ene derivative of AD, is one of the prototypical mechanism-based inhibitors (suicide substrates); the C(1)═C(2) double bond is the structural feature responsible for aromatase inactivation, although the inactivation mechanism is currently unknown [5,12–21]. Several analogs of compound **1**, having a D-ring lactone (testolactone) [22,23], 1-methyl (atamestane) [24] or 6-methylene (exemestane) [25,26] structure, are clinically evaluated as orally active suicide substrates for aromatase (Fig. 2). We have studied the structure–activity relationships of 6-substituted (alkyl, phenylaliphatic, alkoxy, and ester) Δ^1-AD analogs [27–29] as well as 19-substituted ones [30] as aromatase inhibitors, some of which are among the most potent competitive inhibitors and mechanism-based inhibitors reported so far. The length (bulkiness) and the stereochemistry of the 6-substituents as well as the electronic effects of the 19-substituents play a critical role not only in the binding of inhibitors to the active site of aromatase, but also in the cause of the mechanism-based inactivation of aromatase by the 1,4-dien-3-one steroid **1**. Recently, we have also reported the structure–activity relationships of the 2-alkyl or alkoxy substituted Δ^1-ADs as aromatase inhibitors [31]. Among the 2-substituted derivatives, the 2-hexyl compound is the most powerful competitive inhibitor and inactivates aromatase with the lowest K_I value in a time-dependent manner.

In the cause of our studies on aromatase inhibition by Δ^1-AD analog, we were interest in C-2 substitution of the Δ^1-AD **1** by a phenylaliphatic group. Thus, we synthesized 2-substituted ($C_6H_5CH_n$, $n = 1–4$) Δ^1-AD derivatives **6a**–**6d** along with *p*-methyl and *p*-trifluoromethylphenethyl analogs **6e** and **6f**, and tested their ability to inhibit aromatase activity as well as their ability to inactivate aromatase in a suicide manner. Furthermore, molecular docking studies of the phenpropyl derivative **6c**, the most powerful inhibitor among them, with human placental aromatase protein were performed.

* Corresponding author. Tel.: +81 22 727 0138; fax: +81 22 727 0137.
E-mail address: numazawa@tohoku-pharm.ac.jp (M. Numazawa).

0039-128X/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2010.01.008

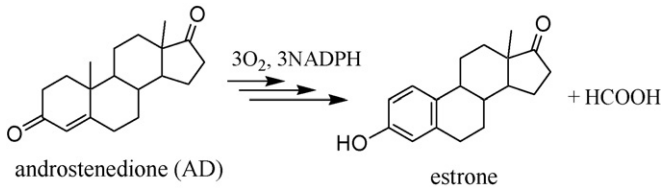

Fig. 1. Aromatization sequence of AD with human placental aromatase.

2. Experimental

2.1. Materials and general methods

17β-*tert*-Butyldimethylsiloxyandrost-4-en-3-one (**2**) [32] was synthesized according to the previous method. [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 Yanagimoto melting point apparatus (Kyoto, Japan) and are uncorrected. Infra red (IR) spectra were recorded on a Perkin-Elmer 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.

2.2. 2α-Phenalkyl-17β-tert-butyldimethylsiloxyandrost-4-en-3-ones (3)

Phenbutyl bromide, *p*-methylphenethyl bromide and *p*-trifluoromethyl bromide (Sigma–Aldrich. Co.) (5.84 mmol) was refluxed in acetone (15.6 ml) containing NaI (2.3 g) for 6 h [33], and the solvent removed in a vacuum. Hexane was added, and the resulting solutions washed with water, dilute H_2SO_4 and water, and dried with Na_2SO_4. Evaporation of the solvent gave phenbutyl iodide, *p*-methylphenethyl iodide and *p*-trifluoromethyl iodide.

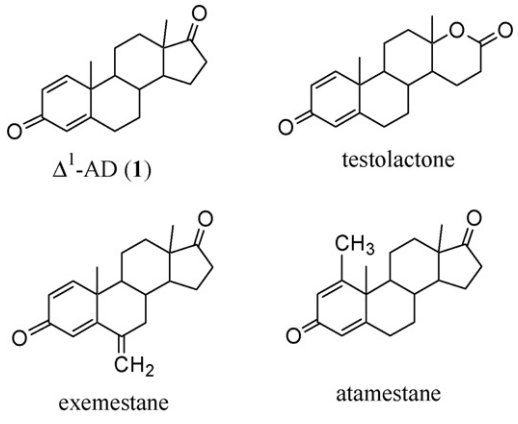

Fig. 2. Structures of suicide substrates of aromatase having a 1,4-dien-3-one structure.

(A) To a stirring solution of 160 μl (1.13 mmol) of diisopropylamine in 1.6 ml of THF, cooled to 0 °C, was added BuLi (690 μL of 1.6 M in hexane). After 20 min, the solution was cooled to −20 °C and 200 mg (0.50 mmol) of compound **2** in 1.6 ml THF was added via cannula to the solution. After an additional 15 min, 170 μl (0.98 mmol) of hexamethylphosphoric triamide (HMPA) was added all at once and the mixture was allowed to stir for another 20 min, and then 2.02 mmol of benzyl bromide or phenbutyl iodide was added dropwise to the mixture [34]. After 1 h at −20 °C reaction, the reaction mixture was quenched with 1 M aqueous HCl and diluted with EtOAc. The organic phase was washed with brine and dried with Na_2SO_4. Evaporation of the solvent gave an oil which was purified by silica gel (20 g) column chromatography with hexane–EtOAc (60:1) to yield an oily substance. Then, the oil was subjected to preparative TLC (hexane–EtOAc, 8:1, multiple developments) to give 2α-benzyl steroid **3a** (64%) or 2α-phenbutyl steroid **3d** (22%).

(B) LDA (1.13 mmol) was prepared similar to the above at 0 °C. A solution of 1 g (2.48 mmol) of compound **2** in 8.00 ml THF was added via cannula to the LDA solution at 0 °C. After the same work-up as the above (78 mmol of HMPA), 13.79 mmol of phenethyl iodide, *p*-methylphenethyl iodide or *p*-trifluoromethyl iodide was added dropwise at 0 °C. After 1.5 h reaction, the reaction mixture was quenched with 1 M aqueous HCl and diluted with EtOAc. The organic phase was washed with brine and dried with Na_2SO_4. Evaporation of the solvent gave an oil which was purified by silica gel (100 g) column chromatography with hexane–EtOAc (30:1) to yield an oily substance. Then, the oil was subjected to preparative TLC (hexane–EtOAc, 5:1) to give 2α-phenethyl steroid **3b**, 2α-*p*-methylphenethyl steroid **3e** or 2α-*p*-trifluoromethyl steroid **3f** (11–22%).

(C) A mixture of 17β-*tert*-butyldimethylsiloxyandrost-4-en-3-one (**2**) (700 mg, 1.74 mmol), 1-iodo-3-phenyl propane (3.6 ml, 23.16 mmol), THF (1.5 ml) and HMPA (0.28 ml) was cooled −60 °C and then *tert*-BuOK (390 mg, 3.48 mmol) in THF (1.5 ml)–HMPA (0.28 ml) was added to this solution [35]. The reaction mixture was stirred at −60 °C for 1.5 h and the temperature was gradually elevated to room temperature. The mixture was extracted of with EtOAc, washed with saturated $NaHCO_3$ solution and saturated NaCl, and dried with Na_2SO_4. Evaporation of the solvent gave an oil which was purified by silica gel (70 g) column chromatography with hexane–EtOAc (60:1) to yield an oily substance. Then, the oil was subjected to preparative TLC (hexane–EtOAc, 20:1, multiple developments) to give 2α-phenpropyl steroid **3c** (193 mg, 22%).

The purities of the oily substances were determined by analytical HPLC (CH_3CN:H_2O = 100:0, v/v; flow rate, 1.0 ml/min) to be more than 98%.

3a: oil; t_R = 19.4 min; IR (KBr): 1672 cm^{-1} (C=O); UV λ_{max} 242 nm (ε = 14,700); ^{1}H NMR δ: 0.01 (6H, s, Si–$(CH_3)_2$), 0.71 (3H, s, 18-Me), 0.88 (9H, s, Si–C$(CH_3)_3$), 1.10 (3H, s, 19-Me), 2.45 (1H, dd, J = 9.2 and 13.9 Hz, phenyl-CH_a), 3.48 (1H, dd, J = 3.8 and 14.0 Hz, phenyl-CH_b), 3.55 (1H, t, J = 8.2 Hz, 17α-H), 5.76 (1H d, J = 1.5 Hz, 4-H), 7.18–7.31 (5H, m, aromatic protons); MS *m/z*: 492 (M^+, 14), 435 (100), 359 (12), 91 (16). HR-MS for $C_{32}H_{48}O_2Si$ (M^+): calcd 492.3424, found 492.3436.

3b: oil; t_R = 24.9 min; IR (KBr): 1672 cm^{-1} (C=O); UV λ_{max} 242 nm (ε = 14,400); ^{1}H NMR δ: 0.01 (6H, s, Si–$(CH_3)_2$), 0.75 (3H, s, 18-Me), 0.88 (9H, s, Si–C$(CH_3)_3$), 1.16 (3H, s, 19-Me), 2.68 (2H, m, phenyl-CH_2), 3.56 (1H, m, 17α-H), 5.69 (1H, d, J = 1.2 Hz, 4-H), 7.17–7.29 (5H, m, aromatic protons); MS *m/z*: 506 (M^+, 1), 499 (68),

402 (100), 373 (9). HR-MS for $C_{33}H_{50}O_2Si$ (M^+): calcd 506.3580, found 506.3576.

3c: oil; t_R = 24.2 min; IR (neat): 1669 cm^{-1} (C=O); UV λ_{max} 242 nm (ε = 8200); ^1H NMR δ: 0.01 (6H, s, Si–$(CH_3)_2$), 0.74 (3H, s, 18-Me), 0.88 (9H, s, Si–C$(CH_3)_3$), 1.17 (3H, s, 19-Me), 2.63 (2H, m, phenyl-*CH_2*), 3.54 (1H, t, *J* = 8.3 Hz, 17α-H), 5.69 (1H, d, *J* = 1.5 Hz, 4-H), 7.16–7.32 (5H, m, aromatic protons); MS *m/z*: 520 (M^+, 86), 463 (100), 402 (20), 387 (14). HR-MS for $C_{34}H_{52}O_2Si$ (M^+): calcd 520.3737, found 520.3748.

3d: oil; t_R = 24.1 min; IR (KBr): 1675 cm^{-1} (C=O); UV λ_{max} 242 nm (ε = 13,200); ^1H NMR δ: 0.01 (6H, s, Si–$(CH_3)_2$), 0.74 (3H, s, 18-Me), 0.88 (9H, s, Si–C$(CH_3)_3$), 1.18 (3H, s, 19-Me), 2.62 (2H, m, phenyl-*CH_2*), 3.55 (1H, t, *J* = 8.4 Hz, 17α-H), 5.69 (1H, s, 4-H), 7.16–7.29 (5H, m, aromatic protons); MS *m/z*: 534 (M^+, 48), 477 (100), 402 (35), 75 (41). HR-MS for $C_{35}H_{54}O_2Si$ (M^+): calcd 534.3893, found 534.3885.

3e: oil; t_R = 25.3 min; IR (neat): 1675 cm^{-1} (C=O); UV λ_{max} 242 nm (ε = 11,000); ^1H NMR δ: 0.01 (6H, s, Si–$(CH_3)_2$), 0.75 (3H, s, 18-Me), 0.88 (9H, s, Si–C$(CH_3)_3$), 1.16 (3H, s, 19-Me), 2.31 (3H, m, *p-Me*-phenethyl), 2.63 (2H, m, *p*-Me-phenyl-*CH_2*), 3.55 (1H, t, *J* = 8.3 Hz, 17α-H), 5.69 (1H, d, *J* = 1.5 Hz, 4-H), 7.05–7.11 (4H, m, aromatic protons); MS *m/z*: 520 (M^+, 2), 463 (18), 402 (100), 345 (32). HR-MS for $C_{34}H_{52}O_2Si$ (M^+): calcd 520.3737, found 520.3732.

3f: oil; t_R = 22.4 min; IR (neat): 1672 cm^{-1} (C=O); UV λ_{max} 242 nm (ε = 10,700); ^1H NMR δ: 0.01 (6H, s, Si–$(CH_3)_2$), 0.75 (3H, s, 18-Me), 0.88 (9H, s, Si–C$(CH_3)_3$), 1.17 (3H, s, 19-Me), 2.75 (2H, t, *J* = 7.8 Hz, *p*-CF_3-phenyl-*CH_2*), 3.55 (1H, m, 17α-H), 5.70 (1H, d, *J* = 1.2 Hz, 4-H), 7.32 and 7.53 (2H each, d, *J* = 8.1 Hz, aromatic protons); MS *m/z*: 574 (M^+, 1), 517 (18), 161 (54), 153 (100). HR-MS for $C_{34}H_{49}F_3O_2Si$ (M^+): calcd 574.3453, found 574.3435.

2.3. 2α-Phenalkyl-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 17β-hydroxy product which was purified by silica gel (10 g) column chromatography (hexane–EtOAc = 5:1) followed by recrystallization from acetone or trituration from ether to yield 17-hydroxy compounds **4a**–**4d** (35–68%). The purities of the oily substances (**4e** and **4f**) were determined by analytical HPLC (CH_3CN:H_2O = 80:20, v/v; flow rate, 1.0 ml/min) to be more than 98%.

4a: mp 252–254 °C. IR (KBr): 1672 cm^{-1} (C=O); UV λ_{max}: 242 nm (ε = 10,300); ^1H NMR δ: 0.74 (3H, s, 18-Me), 1.10 (3H, s, 19-Me), 2.43 (1H, dd, *J* = 9.3 and 14.1 Hz, phenyl-*CH_a*), 3.47 (1H, dd, *J* = 3.8 and 14.0 Hz, phenyl-*CH_b*), 3.63 (1H, m, 17α-H), 5.75 (1H, d, *J* = 1.5 Hz, 4-H), 7.16–7.30 (5H, m, aromatic protons); MS *m/z*: 378 (M^+, 100), 363 (5), 121 (31), 91 (50). Anal. Calcd for $C_{26}H_{34}O_2$: C, 82.49; H, 9.05. Found C, 82.60; H, 9.27.

4b: mp 143–145 °C. IR (KBr): 1668 cm^{-1} (C=O); UV λ_{max}: 242 nm (ε = 11,200); ^1H NMR δ: 0.80 (3H, s, 18-Me), 1.17 (3H, s, 19-Me), 2.69 (2H, m, phenyl-*CH_2*), 3.65 (1H, m, 17α-H), 5.70 (1H, d, *J* = 1.5 Hz, 4-H), 7.16–7.29 (5H, m, aromatic protons); MS *m/z*: 392 (M^+, 2), 288 (100), 273 (8), 122 (25). Anal. Calcd for $C_{27}H_{36}O_2$: C, 82.61; H, 9.24. Found C, 82.70; H, 9.37.

4c: mp 199–201 °C. IR (KBr): 1667 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 9000); ^1H NMR δ: 0.79 (3H, s, 18-Me), 1.18 (3H, s, 19-Me), 2.63 (2H, m, phenyl-*CH_2*), 3.64 (1H, m, 17α-H), 5.69 (1H, d, *J* = 1.4 Hz, 4-H), 7.15–7.29 (5H, m, aromatic protons); MS *m/z*: 406 (M^+, 100), 301 (30), 288 (53), 273 (6). Anal. Calcd for $C_{28}H_{38}O_2$: C, 82.71; H, 9.42. Found C, 82.55; H, 9.54.

4d: semi-crystalline; mp 87–89 °C. IR (KBr): 1669 cm^{-1} (C=O); UV λ_{max}: 242 nm (ε = 15,300); ^1H NMR δ: 0.80 (3H, s, 18-Me), 1.19 (3H, s, 19-Me), 2.63 (2H, m, phenyl-*CH_2*), 3.65 (1H, m, 17α-H), 5.70 (1H, d, *J* = 1.5 Hz, 4-H), 7.15–7.29 (5H, m, aromatic protons); MS *m/z*: 420 (M^+, 100), 301 (38), 288 (98), 91 (55). HR-MS for $C_{29}H_{40}O_2$: (M^+): calcd 420.3028, found 420.3044.

4e: oil; t_R = 7.0 min; IR (KBr): 1668 cm^{-1} (C=O); UV λ_{max}: 242 nm (ε = 10,600); ^1H NMR δ: 0.80 (3H, s, 18-Me), 1.17 (3H, s, 19-Me), 2.31 (3H, s, *p-Me*-phenethyl), 2.63 (2H, m, *p*-Me-phenyl-*CH_2*), 3.66 (1H, m, 17α-H), 5.69 (1H, d, *J* = 1.5 Hz, 4-H), 7.07–7.11 (4H, m, aromatic protons); MS *m/z*: 406 (M^+, 2), 288 (100), 273 (13), 122 (28). HR-MS for $C_{28}H_{38}O_2$ (M^+): calcd 406.2872, found 406.2883.

4f: oil; t_R = 6.8 min; IR (KBr): 1665 cm^{-1} (C=O); UV λ_{max}: 242 nm (ε = 9800); ^1H NMR δ: 0.80 (3H, s, 18-Me), 1.22 (3H, s, 19-Me), 2.75 (2H, t, *J* = 8.0 Hz, *p*-CF_3-phenyl-*CH_2*), 3.64 (1H, m, 17α-H), 5.71 (1H, d, *J* = 1.5 Hz, 4-H), 7.32 and 7.53 (2H each, d, *J* = 8.1 Hz, aromatic protons); MS *m/z*: 460 (M^+, 1), 345 (7), 288 (100), 273 (11). HR-MS for $C_{28}H_{35}F_3O_2$ (M^+): calcd 460.2589, found 460.2596.

2.4. 2α-phenalkylandrost-4-ene-3,17-diones (*5*)

Jones reagent (11 drops) was added to a solution of the 17β-hydroxide **4** (0.13mmol) 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 silica gel (5 g) column chromatography (hexane–EtOAc = 7:1) to yield an oily substance. Then, the oily substance was subjected to preparative TLC (hexane–EtOAc, 4:1, multiple developments) or preparative HPLC (CH_3CN:H_2O = 60:40, v/v; flow rate 5 ml/min) to obtained compound **5a**–**5f** (49–61%). The purities of oil substances were obtained by analytical HPLC as described for those of compounds **4** were more than 97%.

5a: mp 164–166 °C. IR (KBr): 1664 and 1733 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 13,300); ^1H NMR δ: 0.87 (3H, s, 18-Me), 1.11 (3H, s, 19-Me), 2.45 (1H, m, phenyl-*CH_a*), 3.48 (1H, dd, *J* = 3.8 and 14.1 Hz, phenyl-*CH_b*), 5.78 (1H, d, *J* = 1.8 Hz, 4-H), 7.17–7.30 (5H, m, aromatic protons); MS *m/z*: 376 (M^+, 100), 361 (7), 121 (40), 91 (78). Anal. Calcd for $C_{26}H_{32}O_2$: C, 82.94; H, 8.57. Found C, 83.01; H, 8.80.

5b: mp 129–131 °C. IR (KBr): 1670 and 1742 cm^{-1} (C=O); UV λ_{max}: 240 nm (ε = 15,200); ^1H NMR δ: 0.93 (3H, s, 18-Me), 1.18 (3H, s, 19-Me), 2.70 (2H, m, phenyl-*CH_2*), 5.72 (1H, d, *J* = 1.5 Hz, 4-H), 7.17–7.29 (5H, m, aromatic protons); ^{13}C NMR δ: 13.7, 17.4, 20.2, 21.7, 30.4, 30.7, 31.3, 32.1, 33.0, 35.0, 35.7, 39.2, 41.1, 42.0, 47.5, 50.8, 54.2, 124.0, 125.8, 128.3, 128.4, 142.0, 168.6, 200.8, 220.4; MS *m/z*: 390 (M^+, 2), 286 (100), 271 (16), 122 (27). Anal. Calcd for $C_{27}H_{34}O_2$: C, 83.03; H, 8.77. Found C, 83.34 H, 8.58.

5c: oil; t_R = 7.2 min; IR (neat): 1671 and 1739 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 15,100); ^1H NMR δ: 0.91 (3H, s, 18-Me), 1.20 (3H, s, 19-Me), 2.63 (2H, m, phenyl-*CH_2*), 5.72 (1H, d, *J* = 1.5 Hz, 4-H), 7.16–7.29 (5H, m, aromatic protons); ^{13}C NMR δ: 13.7, 17.4, 20.2, 21.7, 28.7, 29.0, 30.7, 31.2, 32.1, 35.0, 35.7, 36.3, 39.1, 41.8, 41.9, 47.5, 50.8, 54.2, 124.0, 125.7, 128.3, 128.4, 142.5, 168.7, 200.8, 220.5; MS *m/z*: 404 (M^+, 100), 300 (72), 286 (85), 271 (18). HR-MS for $C_{28}H_{36}O_2$ (M^+): calcd 404.2715, found 404.2715.

5d: semi-crystalline; mp 117–119 °C. IR (KBr): 1665 and 1743 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 13,800); ^1H NMR δ: 0.92 (3H, s, 18-Me), 1.21 (3H, s, 19-Me), 2.63 (2H, m, phenyl-*CH_2*), 5.72 (1H, d, *J* = 1.5 Hz, 4-H), 7.15–7.29 (5H, m, aromatic protons); MS *m/z*: 418 (M^+, 100), 299 (30), 286 (85), 271 (13). HR-MS for $C_{29}H_{38}O_2$ (M^+): calcd 418.2872, found 418.2859.

5e: oil; t_R = 7.7 min; IR (KBr): 1671 and 1739 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 12,000); ^1H NMR δ: 0.93 (3H, s, 18-Me), 1.19 (3H, s, 19-Me), 2.31 (3H, s, *p-Me*-phenethyl), 2.65 (2H, m, *p*-Me-phenyl-*CH_2*), 5.72 (1H, d, *J* = 1.5 Hz, 4-H), 7.07–7.11 (4H, m, aromatic protons). ^{13}C NMR δ: 13.7, 17.4, 20.2, 21.0, 21.7, 30.5, 31.3, 32.0, 32.5, 35.0, 35.7, 39.2, 41.1, 42.0, 47.5, 50.8, 54.2, 124.0, 128.3, 129.0,

135.2, 138.9, 168.5, 200.8, 220.4; MS *m/z*: 404 (M^+, 2), 286 (100), 271 (14), 122 (21). HR-MS for $C_{28}H_{36}O_2$ (M^+): calcd 404.2715, found 404.2722.

5f: oil; t_R = 7.4 min; IR (KBr): 1669 and 1739 cm^{-1} (C=O); UV λ_{max}: 241 nm (ε = 11,000); ^1H NMR δ: 0.93 (3H, s, 18-Me), 1.20 (3H, s, 19-Me), 2.75 (2H, t, *J* = 8.0 Hz, *p*-CF_3-phenyl-CH_2), 5.74 (1H, d, *J* = 1.2 Hz, 4-H), 7.32 and 7.53 (2H each, d, *J* = 7.8 Hz, aromatic protons). ^{13}C NMR δ: 13.7, 17.4, 20.2, 21.7, 30.4, 30.7, 31.3, 32.1, 32.9, 35.0, 35.7, 39.3, 41.1, 42.1, 47.5, 50.8, 54.2, 124.0, 125.21, 125.24, 125.3, 128.7, 146.3, 168.8, 200.4, 220.3; MS *m/z*: 458 (M^+, 1), 439 (4), 286 (100), 271 (48). HR-MS for $C_{28}H_{33}F_3O_2$ (M^+): calcd 458.2433, found 458.2430.

2.5. 2-Alkylandrosta-1,4-diene-3,17-diones (**6**)

DDQ (0.15 mmol) was added to a solution of the 4-ene-3,17-diones **5** (0.10 mmol) in dioxane (2 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 purification with preparative TLC (hexane–EtOAc = 4:1, multiple developments) or preparative HPLC (CH_3CN:H_2O = 60:40 (v/v); flow rate 5 ml/min) to obtained compound **6a–6f** (29–49%). The purities of oil substances were obtained by analytical HPLC as described for those of compounds **4** to be more than 98%.

6a: mp 165–168 °C. IR (KBr): 1633 cm^{-1} (C=C), 1662 and 1739 cm^{-1} (C=O); UV λ_{max}: 250 nm (ε = 15,200); ^1H NMR δ: 0.91 (3H, s, 18-Me), 1.19 (3H, s, 19-Me), 3.65 (2H, m, phenyl-CH_2), 6.10 (1H, d, *J* = 1.1 Hz, 4-H), 6.60 (1H, s, 1-H), 7.18–7.30 (5H, m, aromatic protons); ^{13}C NMR δ: 13.8, 18.8, 22.0, 22.2, 31.2, 32.1, 32.4, 35.1, 35.2, 35.7, 43.3, 47.7, 50.4, 52.6, 124.0, 126.2, 128.4, 129.1, 137.5, 139.5, 151.6, 167.7, 185.8, 220.0; MS *m/z*: 374 (M^+, 80), 359 (36), 223 (19), 210 (100). Anal. Calcd for $C_{26}H_{30}O_2$: C, 83.38; H, 8.07. Found C, 83,13; H, 8.28.

6b: mp 197–199°C; IR (KBr): 1629 cm^{-1} (C=C), 1664 and 1739 cm^{-1} (C=O); UV λ_{max}: 250 nm (ε = 14,300); ^1H NMR δ: 0.89 (3H, s, 18-Me), 1.09 (3H, s, 19-Me), 2.77 (2H, m, phenyl-CH_2), 6.08 (1H, d, *J* = 1.0 Hz, 4-H), 6.40 (1H, s, 1-H), 7.08–7.24 (5H, m, aromatic protons); ^{13}C NMR δ: 13.7, 18.6, 21.8, 21.9, 31.1, 31.4, 32.1, 32.4, 34.2, 35.0, 35.6, 43.0, 47.7, 50.4, 52.6, 124.0, 125.7, 128.1, 128.9, 135.8, 141.5, 151.8, 167.6, 186.1, 220.2; MS *m/z*: 388 (M^+, 73), 371 (16), 297 (73), 284 (100). Anal. Calcd for $C_{27}H_{32}O_2$: C, 83.46; H, 8.30. Found: C, 83.43: H, 8.32.

6c: oil; t_R = 5.5 min; IR (neat): 1635 cm^{-1} (C=C), 1665 and 1733 cm^{-1} (C=O); UV λ_{max}: 250 nm (ε = 14,400); ^1H NMR δ: 0.94 (3H, s, 18-Me), 1.21 (3H, s, 19-Me), 2.64 (2H, t, *J* = 7.9 Hz, phenyl-CH_2), 6.08 (1H, d, *J* = 1.5 Hz, 4-H), 6.75 (1H, s, 1-H), 7.16–7.29 (5H, m, aromatic protons); ^{13}C NMR δ: 13.8, 19.0, 21.9, 22.2, 29.0, 30.1, 31.2, 32.1, 32.4, 35.1, 35.7, 43.1, 47.7, 50.5, 52.6, 124.1, 125.7, 128.3, 128.4, 128.6, 137.4, 142.3, 150.3, 167.5, 186.2, 220.1; MS *m/z*: 402 (M^+, 94), 298 (100), 283 (15), 134 (96). HR-MS for $C_{28}H_{34}O_2$ (M^+): calcd 402.2559, found 402.2567.

6d: mp 140–142 °C; IR (KBr): 1626 cm^{-1} (C=C), 1661 and 1742 cm^{-1} (C=O); UV λ_{max}: 249 nm (ε = 14,900); ^1H NMR δ: 0.94 (3H, s, 18-Me), 1.21 (3H, s, 19-Me), 2.62 (2H, t, *J* = 8.0 Hz, phenyl-CH_2), 6.07 (1H, d, *J* = 1.2 Hz, 4-H), 6.73 (1H, s, 1-H), 7.15–7.29 (5H, m, aromatic protons); ^{13}C NMR δ: 13.8, 18.9, 21.9, 22.2, 27.9, 29.0, 31.1, 31.2, 32.1, 32.4, 35.1, 35.6, 35.7, 43.1, 47.7, 50.4, 52.6, 124.1, 125.6, 128.2, 128.4, 137.5, 142.6, 150.2, 167.4, 186.2, 220.0; MS *m/z*: 416 (M^+, 100), 299 (79), 297 (31), 91 (71). Anal. Calcd for $C_{29}H_{36}O_2$: C, 83.61; H, 8.71. Found C, 83.75 H, 8.80.

6e: mp 181–182 °C; IR (KBr): 1631 cm^{-1} (C=C), 1664 and 1738 cm^{-1} (C=O); UV λ_{max}: 250 nm (ε = 13,800); ^1H NMR δ: 0.90 (3H, s, 18-Me), 1.10 (3H, s, 19-Me), 2.29 (3H, s, *p*-*Me*-phenethyl), 2.71 (2H, m, *p*-Me-phenyl-CH_2), 6.08 (1H, d, *J* = 1.1 Hz, 4-H), 6.43 (1H, s, 1-H), 6.98–7.04 (4H, m, aromatic protons). MS *m/z*: 402 (M^+, 55), 297 (73), 284 (64), 105 (100). HR-MS for $C_{28}H_{34}O_2$ (M^+): calcd 402.2559, found 402.2556.

6f: oil; t_R = 5.2 min; IR (KBr): 1630 cm^{-1} (C=C), 1664 and 1740 cm^{-1} (C=O); UV λ_{max}: 250 nm (ε = 11,000); ^1H NMR δ: 0.90 (3H, s, 18-Me), 1.08 (3H, s, 19-Me), 2.87 (2H, m, *p*-CF_3-phenyl-CH_2), 6.09 (1H, d, *J* = 1.0 Hz, 4-H), 6.49 (1H, s, 1-H), 7.23 and 7.50 (2H each, d, *J* = 8.1 Hz, aromatic protons). ^{13}C NMR δ: 13.7, 18.7, 21.9, 22.0, 31.1, 31.4, 32.1, 32.4, 34.2, 35.0, 35.6, 43.1, 47.6, 50.4, 52.6, 123.9, 125.00, 125.03, 125.1, 129.1, 135.7 145.8, 151.8, 167.9, 186.0, 219.8; MS *m/z*: 456 (M^+, 62), 438 (6), 297 (57), 284 (100). HR-MS for $C_{28}H_{31}F_3O_2$ (M^+): calcd 456.2276, found 456.2278.

2.6. Enzyme preparation

Human placental microsomes (particles sedimenting at 105,000 × *g* for 60 min) were obtained using the method reported by Ryan [12]. 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).

2.7. Aromatase assay procedure

Aromatase activity was measured according to the procedure of Siiteri and Thompson [36]. 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 [27]. In the time-dependent inactivation experiment, 1/10 of the incubation mixture was used for assays of the remaining aromatase activity.

2.8. Molecular modeling

The crystal structure of human placental aromatase was downloaded from the Protein Data Bank (Accession Code, 3EQM). Molecular modelings, 3D structures, of ligands were built by Spartan'08 software (Wavefunction, Inc., Irvine, CA, USA) using Hartree-Fock 6-31G* calculations. Molecular docking was performed by AutoDock Vina (The Scripps Research Institute, CA, USA) [37]. Docking to aromatase was carried out to propose docked structures with similar calculated affinities (−3.1 to −5.7 kcal/mol). The primary criterion used in choosing best docked structure was the position of the Δ^1-AD derivative relative to the active site aspartate (Asp 309), arginine (Arg 115) and methionine (Met 374), with reference to the bound conformation of AD. The program was able to reproduce the docked conformation of compound **6c** in aromatase with a reasonable rmsd. Graphics were generated with PyMOL software (DeLano Scientific LLC, Palo Alto, CA, USA).

3. Results

3.1. Chemistry

2-Phenyaliphatic-substituted Δ^1-ADs (**6**) were synthesized principally according to reaction sequence of the preparation of 2-alkyl-substituted Δ^1-AD derivatives. Various phenylaliphatic iodide (alkyl: methyl to butyl) and *p*-methyl- and *p*-trifluoromethyl-phenethyl iodide subjected to reaction with testosterone *tert*-butyldimethylsilyl ether (**2**) in presence of *tert*-BuOK or lithium diisopropylamide (LDA) as a base under cooling as a key reaction (Fig. 3). Conditions using *tert*-BuOK at −60 °C was effective only for the formation of 2α-phenpropyl compound

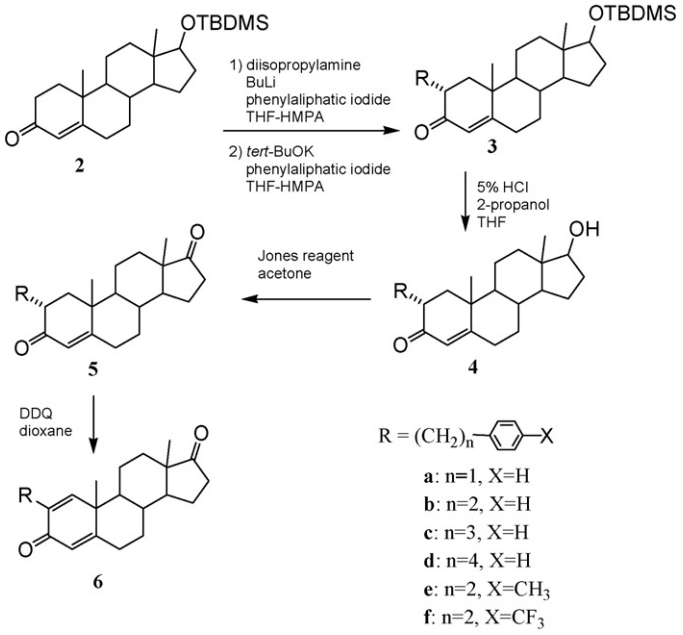

Fig. 3. Synthesis of 2-phenylaliphatic-substituted Δ^1-ADs (**6**).

3c (22% yield). Conditions using LDA at −20 °C were used for the productions of 2α-benzyl- and 2α-phenbutyl- compound **3a** and **3d** (64% for **3a** and 22% for **3d**), whereas 2α-phenethyl- (**3b**), 2α-*p*-methylphenethyl- (**3e**) and 2α-*p*-trifluoromethylphenethyl- (**3f**) compounds were produced under conditions employing LDA at 0 °C (yields: 11–22%). In the reactions, 2α-phenylaliphatic 4-en-3-ones **3** were produced as major products while the 2β-phenyaliphatic isomers, minor products were not isolated. After the purification with silica gel column chromatography or preparative TLC of compounds **3**, these compounds were subjected sequentially to deprotection of the 17β-silyl ethers **3** with diluted HCl in a mixture of THF and 2-propanol and oxidation of the deprotected product 17β-ols **4** with Jones reagent in acetone, giving the 2α-phenylaliphatic 17-keto compounds **5**. Finally, an introduction of a double bond at C-1 of the 17-keto compounds **5**, the AD derivatives with DDQ in dioxane gave 2-phenylaliphatic Δ^1-AD analogs **6**.

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

3.2. Aromatase inhibition

Reversible inhibition of aromatase activity in human placental microsomes by 2-aliphatic-substituted ADs **5** and their Δ^1-AD derivatives **6** was initially studied in vitro by enzyme kinetics. The amount of aromatase activity was measured by determinating the amount of ^3H_2O released from [1β-^{3}H] androstenedione, which is an index of estrogen formation [36]. IC_{50} values were first obtained, and then, to characterize the nature of their binding to the active site of aromatase, aromatization was measured in the presence of several concentrations of the inhibitors and androstenedione. The results of these studies were plotted on typical Lineweaver–Burk plots. All of the steroids examined exhibited clear-cut competitive inhibition. The Lineweaver–Burk plots for the phenpropyl-1,4-dien-3-one **6c** are shown in Fig. 4. The apparent inhibition constants (K_i) were determined by analysis of the Dixon plot and shown in Table 1. In these studies, the apparent K_m and V_{max} for androstenedione was about 40 nM and 120 pmol/min/mg protein, respectively. All the AD derivatives **5** were poor inhibitors of aromatase (IC_{50}: more than 50 μM). In series of the Δ^1-AD steroids, the inhibitory activities of the phenethyl derivative **6b** and its *p*-methyl compound **6e** and the phenpropyl derivative **6c** were relatively high (apparent K_i: 206 nM for **6b**, 16.1 nM for **6c**, and 348 nM for **6e**) whereas the other compounds were poor inhibitors (K_i: more than 2600 nM).

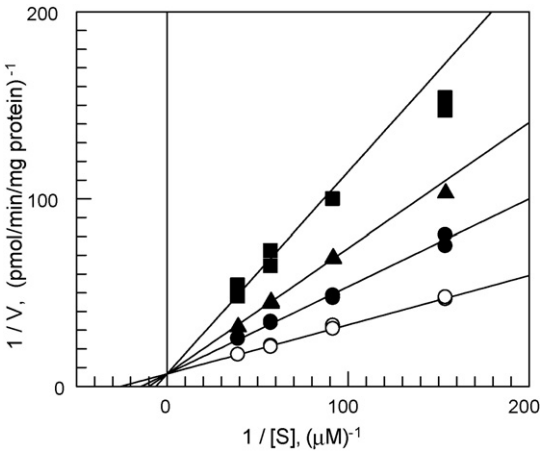

Fig. 4. Lineweaver–Burk plots of inhibition of human placental aromatase by 2-phenpropyl-Δ^1-AD (**6c**) with AD as the substrate. Concentrations of inhibitor: (○) control (0 nM); (●) 12.5 nM; (▲) 25 nM; (■) 50 nM. The inhibition experiments with all the other steroids examined gave essentially similar plots to Fig. 4 (data not shown).

Compounds **6b**, **6c**, and **6e** with the high affinity for aromatase were then tested for their ability to cause a time-dependent inactivation of aromatase. The phenethyl and phenpropyl compounds **6b** and **6c** showed the time-dependent inactivation only in the presence of NADPH under aerobic conditions. Pseudo-first-order kinetics were obtained during the first 12 min of inactivation with the inhibitors, and the kinetic data were analyzed according to the method of Kitz and Wilson [38] (Fig. 5). Double-reciprocal plots of K_{obs} vs. inhibitor concentration gave k_{inact} and K_I values, respectively (Table 2). The K_I and k_{inact} values for compound **6b** and **6c**

Table 1
In vitro aromatase inhibition by 2α-phenylaliphatic-ADs (**5**) and 2-phenylaliphatic-Δ^1-ADs (**6**).

Compound	IC_{50}, μM[a]	Apparent K_i, nM[b]
2α-phenylaliphatic-ADs (**5**)		
5a, benzyl	466	–
5b, phenethyl	126	–
5c, phenpropyl	50.2	5110 ± 260
5d, phenbutyl	222	–
5e, *p*-methylphenethyl	38.8	4095 ± 191
5f, *p*-trifluoromethylphenethyl	46.6	5142 ± 288
2-phenylaliphatic-Δ^1-ADs (**6**)		
6a, benzyl	86.4	4500 ± 290
6b, phenethyl	2.57	206 ± 8.2
6c, phenpropyl	0.145	16.1 ± 0.58
6d, phenbutyl	34.2	2690 ± 190
6e, *p*-methylphenethyl	6.36	348 ± 21.7
6f, *p*-trifluoromethylphenethyl	34.12	4002 ± 354
For comparison		
Δ^1-AD[c]	0.830	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 40 nM and 120 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 [27] and 65 ± 3.5 nM [28].

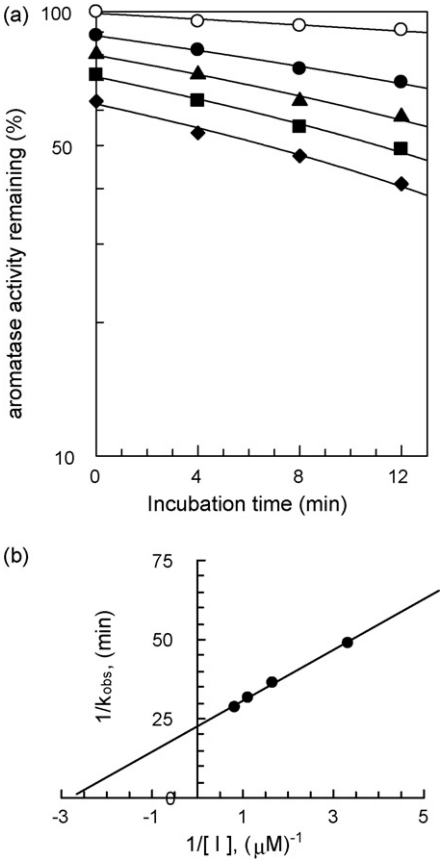

Fig. 5. Time-dependence (a) and concentration-dependence (b) of inactivation of human placental aromatase by 2-phenpropyl-Δ^1-AD (**6c**) in the presence of NADPH in air. Concentrations of the inhibitor: (○) control (0 nM); (●) 300 nM; (▲) 600 nM; (■) 900 nM; (ϑ) 1200 nM. The time-dependent inactivation experiments with 2-phenethyl-Δ^1-AD (**6b**) gave essentially similar plots to Fig. 5 (data not shown).

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

Compound	K_I, nM	k_{inact}, min^{-1}
6b, phenethyl	8124	0.0293
6c, phenpropyl	370	0.0454
6e, *p*-methylphenethyl	NT[b]	–
For comparison		
Δ^1-AD [27]	952	0.059

[a] The time-dependent inactivation of compound **6a**, **6d** and **6f** were not determined because of their poor affinity for aromatase. Apparent K_I and k_{inact} were obtained by Kitz–Wilson plot [38].
[b] The time-dependent inactivation was not observed.

were 8124 nM and 0.0293 min^{-1} for compound **6b** and 370 nM and 0.0454 min^{-1} for compound **6c**. The *p*-methylphenethyl compound **6e** did not cause the time-dependent inactivation.

The substrate AD significantly blocked the inactivation by the 1,4-diene inhibitors, while a nucleophile, L-cysteine, had no significant effect (Fig. 6).

3.3. Molecular docking

Phenpropyl-Δ^1-AD steroid (**6c**) was selected for docking to the active site of human placental aromatase. As shown in Fig. 7, the compound **6c** positions in the active site of the enzyme with hydrogen bondings of the C-3 and C-17 carbonyl groups with polar amino acid residues Asp 309 and Arg 115 or Met 374, respectively, similarly to the natural substrate AD. Interestingly, accessible volume,

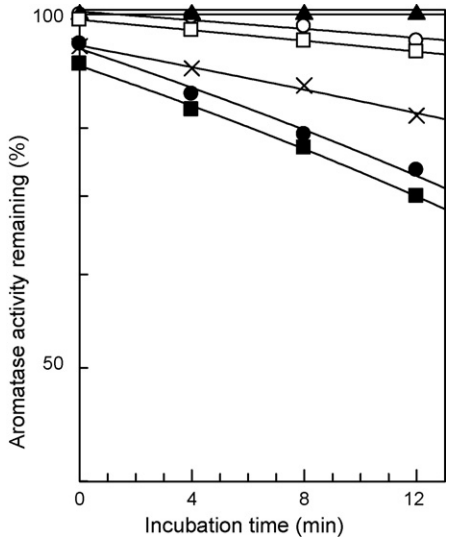

Fig. 6. Inactivation of human placental aromatase by 2-phenpropyl-Δ^1-AD (**6c**) under various conditions: (○) control; (●) inhibitor (600 nM); (▲) control + AD (5.4 μM) or inhibitor (600 nM) + AD (5.4 μM); (□) control + L-cystein (0.5 mM); (■) inhibitor (600 nM) + L-cystein (0.5 mM); (×) inhibitor (600 nM) in the absence NADPH.

hydrophobic binding pocket, extends to towards the C-2 position of compound **6c** and a bulky phenyl group of the phenpropyl group orients to the accessible volume of the binging pocket. The hydrophilic amino acids such as Ile 132, Ile 133, Ile 305, Phe 148, Met 303, and Ala 306 are involved in the binding pocket and may play a critical role in the binding to the active site (Fig. 7).

4. Discussion

The 2-phenylaliphatic Δ^1-ADs (**6a**–**6d**), which have various lengths of methylene units from a methyl to a butyl as well as the *p*-methylphenyl- and *p*-trifluoromethylphenylethyl derivatives **6e** and **6f** were synthesized. The effect of introducing the phenylalkyl chain at the 2-position of Δ^1-AD were examined with respect to affinity for aromatase and rate of inactivation of the enzyme. The 2-phenpropyl derivative **6c** was very powerful competitive inhibitor of aromatase in human placental microsomes with apparent K_i value of 16.1 ± 0.58 nM where the K_m/K_i ratio of compound **6c** is about 2.5. This corresponds well to those obtained for 6β-alkyl- and 6β-phenylaliphatic-Δ^1-ADs which are among the most powerful inhibitors of aromatase [27,28]. Compound **6c** inactivated aromatase in a mechanism-based manner. The rate of inactivation decreased when the substrate AD was included in the incubation mixture while L-cysteine, a nucleophile, failed to protect aromatase significantly from the inactivation by the inactivator. Thus, covalent-bound formation between aromatase and the reactive intermediate appears to occur at the active site, therefore, preventing diffusion of the active inhibitor, a reactive electrophile, in the surrounding media [39]. Recently, we suggested that the mechanism-based inactivator Δ^1-AD, a parent compound of the phenpropyl steroid **6c**, may inactivate aromatase through oxygenation of 19-oxo function derived from two sequential oxygenations at the C-19 position of the parent compound [30,40]. The aromatase-bound metabolite may be produce during the cleavage of the 19-oxo group, although the exact mechanism is clear so far. Covey [5] has suggested previously that aromatase inactivates itself because the mechanism-based aromatase inhibitor induces the enzyme to autoxidize itself. Since we have no evidence for covalent modification of aromatase by the compound **6c**, a substrate analog-induced autoxidation mechanism for inactiva-

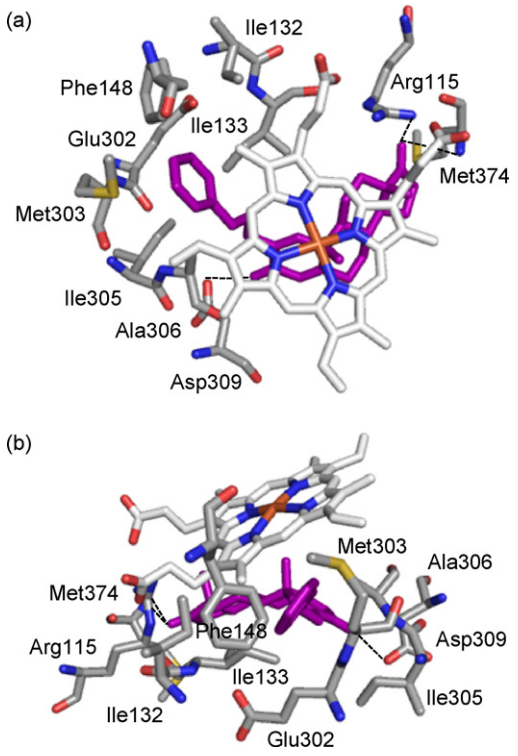

Fig. 7. 2-Phenpropyl-Δ^1-AD (**6c**) is docked into the active site of the human placental aromatase. Views from the heme iron (a) and from the C-2 phenpropyl group (b) are shown in selected active site residues of amino acids which are displayed for gray or white for carbon, blue for nitrogen, red for oxygen and yellow for sulfur. Compound **6c** is colored magenta. The C-3 and C-17 carbonyl groups of compound **6c** positions to hydrogen bonding with Asp 309 and Arg 115 or Met 137, respectively, and the phenpropyl group is oriented in the lipophilic binding pocket of the active site to produce thermodynamically stable enzyme–steroid complex. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

tion may be considered as an alternate explanation for the observed time-dependent inactivation.

The aromatase inhibitory activities of the phenethyl and *p*-methylphenethyl compounds **6b** and **6e** were less powerful than the phenpropyl derivative **6c** but still good inhibitors for the enzyme. The other compounds, the benzyl, phenbutyl, and *p*-trifluoromethylphenethyl-1,4-dienes **6a**, **6d**, and **6f**, showed poor activities of the aromatase inhibition. Only the phenethyl compound **6b** among them caused the time-dependent inactivation of aromatase. Compound **6e** has a *p*-methylphenethyl moiety at the 2-position of Δ^1-AD (**1**), which is similar length of the C-2 substituent to a phenpropyl moiety. This suggests that the terminal phenyl group would play a critical role in the suicide inactivation of aromatase and a steric reason would be operative in the aromatase-catalyzed activation reaction of the phenethyl derivative **6e**. All of the time-dependent activators **6b** and **6c** showed that the K_I values obtained from the inactivation experiments are more than 20 times higher than the corresponding apparent K_i values from the competitive inhibition experiments. The similar tendency has previously been reported in the inactivation experiments using other Δ^1-AD steroid derivatives [27–31]. When the observed inactivation is based on the aromatase reaction *per se*, 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 of the inhibitor becomes rate determining or partial rate determining [39].

All of the AD derivatives **5** were poor inhibitors of aromatase (K_i: more than 4 μM). The previous findings demonstrated that 2-alkyl-substituted ADs have high affinity to the binding site of aromatase; the 2α-methyl- and 2α-ethyl compounds were powerful inhibitors of the enzyme and elongation of the 2α-subsituent up to the hexyl derivative still showed high affinity to the enzyme [31]. The results implied that the binding geometry of the 2-phenylaliphatic steroids **5** in the active site of aromatase would be different from those of the alkyl steroids. The phenyl moiety would prevent the formation of thermodynamically stable inhibitor–enzyme complex.

The crystal structure of human placental aromatase has recently been reported by Ghosh et al. [41]. A molecule of exemestane, one of Δ^1-AD analogs, is build into the active site by using the AD backbone. AD and exemestane superimposed quite well accepts the difference in puckering of the A-ring. Thus exemestane remains tightly bound in the pocket through hydrogen bondings of the C-3 and C-17 carbonyl groups to Asp 309 and Arg 115 or Met 374, respectively, and van der Waals interaction between C-6 methylidene and The 310 is observed. Docking experiment of phenpropyl-Δ^1-AD (**6c**), the most powerful aromatase inhibitor and inactivator, to the crystal structure of the enzyme showed that this compound was bound to the active site in the anchoring effect of hydrogen bondings of the C-3 and C-17 carbonyl groups, as observed in a exemestane molecule, and the lipophilic amino acid residues, Ile 132, Ile 133, Ile 305, Phe 148, Met 303, and Ala 306, in the vicinity of the 2-phenpropyl moiety would also play a critical role in binding of the active site.

5. Conclusion

2-Phenylaliphtic Δ^1-AD (**6**) were synthesized from testosterone 17-*tert*-butyldimethylsilyl ether (**2**) using phenylaliphatic halides in the presence of LDA or $KOC(CH_3)_3$ as a key reaction as the aromatase inhibitors. The phenpropyl compound **6c** was the most powerful inhibitor and this inhibitor inactivated aromatase in a mechanism-based manner. Docking experiments demonstrate that compound **6c** is oriented to the active site of aromatase, binding pocket, though the hydrogen bondings of the 3- and 17-carbonyl groups and the lipophilic interactions of the phenpropyl group to produce thermodynamically stable enzyme–steroid complex.

Acknowledgments

This work was supported in part by a High Technology Research Center Project from the Ministry of Education, Culture, Sports, and Technology of Japan. Human term placenta was kindly donated by Dr. Michihiro Yuki of Yuki Lady's Clinic.

References

[1] Thompson Jr EA, Siiteri PK. The involvement of human placental microsomal cytochrome P-450 in aromatization. J Biol Chem 1974;249:5373–8.
[2] Kellis Jr J, Vickery LE. Purification and characterization of human placental aromatase cytochrome P-450. J Biol Chem 1987;262:4413–20.
[3] Yoshida N, Osawa Y. Purification of human placental aromatase cytochrome P-450 with monoclonal antibody and its characterization. Biochemistry 1991;30:3003–10.
[4] Thompson Jr EA, Siiteri PK. Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. J Biol Chem 1974;249:5364–72.
[5] Covey DF. Aromatase inhibitors: specific inhibitors of estrogen biosynthesis. In: Berg D, Plempel M, editors. Sterol biosynthesis inhibitors: pharmaceutical and agrochemical aspects. Chichester: Eliss Horwood Ltd.; 1988. p. 534–71.
[6] Oh SS, Robinson CH. Mechanism of human placental aromatase: a new active site model. J Steroid Biochem Mol Biol 1993;44:389–97.
[7] Akhtar M, Lee-Robichaud P, Akhtar ME, Wright JN. The impact of aromatase mechanism on other P450s. J Steroid Biochem Mol Biol 1997;61:127–32.
[8] Bossche HV. Inhibitors of P450-dependent steroid biosynthesis: from research to medical treatment. J Steroid Biochem Mol Biol 1992;43:1003–21.
[9] Brodie AM, Santen RJ. Aromatase and its inhibitors in breast cancer treatment—overview and perspective. Breast Cancer Res Treat 1994;30:1–6.
[10] Johnston JO. Aromatase inhibitors. Crit Rev Biochem Mol Biol 1998;33:375–405.

[11] Harper-Wynne C, Dowsett M. Recent advances in the clinical application of aromatase inhibitors. J Steroid Biochem Mol Biol 2001;76:179–86.
[12] Ryan KJ. Biological aromatization of steroids. J Biol Chem 1959;234:268–72.
[13] Gual C, Morato T, Hayano M, Gut M, Dorfman RI. Biosynthesis of estrogens. Endocrinology 1962;71:920–5.
[14] Meigs RA, Ryan KJ. Enzymatic aromatization of steroids. I. Effects of oxygen and carbon monoxide on the intermediate steps of estrogen biosynthesis. J Biol Chem 1971;246:83–7.
[15] Milewich L, Bradfield DJ, Coe LD, Masters BSS, MacDonald P. Metabolism of 1,4-androstadiene-3,17-dione by human placental microsomes. Enzyme properties and kinetic parameters in the formation of estrogens and 17β-hydroxy-1,4-androstadien-3-one. J Steroid Biochem 1981;14:1115–25.
[16] Numazawa M, Yoshimura A, Oshibe M. Enzymic aromatization of 6-alkyl-substituted androgens, potent competitive and mechanism-based inhibitors of aromatase. Biochem J 1998;329:151–6.
[17] Numazawa M, Yoshimura A. Biological aromatization of $\Delta^{4,6}$- and $\Delta^{1,4,6}$-androgens and their 6-alkyl analogs, potent inhibitors of aromatase. J Steroid Biochem Mol Biol 1999;70:189–96.
[18] Schwarzel WC, Kruggel WG, Brodie HJ. Studies on the mechanism of estrogen biosynthesis. VIII. The development of inhibitors of the enzyme system in human placenta. Endocrinology 1973;92:866–80.
[19] Covey DF, Hood WF. Enzyme-generated intermediates derived from 4-androstene-3,6,17-trione and 1,4,6-androstatriene-3,17-dione cause a time-dependent decrease in human placental aromatase activity. Endocrinology 1981;108:1597–9.
[20] Covey DF, Hood WF. A new hypothesis based on suicide substrate inhibitor studies for the mechanism of action of aromatase. Cancer Res 1982;42(Suppl.):3327s–33s.
[21] Marsh DA, Brodie HJ, Garrett W, Tsai-Morris CH, Brodie AMH. Aromatase inhibitors. Synthesis and biological activity of androstenedione derivatives. J Med Chem 1985;28:788–95.
[22] Segaloff A, Weeth JB, Rongone EL, Murison PJ, Bowers CY. Hormonal therapy in cancer of the breast. XVI. The effect of Δ^1-testololactone on clinical course and hormonal excretion. Cancer 1960;13:1017–20.
[23] Lerner LJ, Bianchi A, Borman A. Δ^1-Testololactone, a nonadrogenic augmentor and inhibitor of androgens. Cancer 1960;13:1201–5.
[24] Henderson D, Norbisrath G, Kerb U. 1-Methyl-1,4-androstadiene-3,17-dione (SH 489): characterization of an irreversible inhibitor of estrogen biosynthesis. J Steroid Biochem 1986;24:303–6.
[25] Salle ED, Briatico G, Giudici D, Ornati G, Zaccheo T. Aromatase inhibition and experimental antitumor activity of FCE 24304, MDL 18962 and SH 489. J Steroid Biochem 1989;34:431–4.
[26] Salle ED, Ornati G, Giudici D, Lassus M, Evans RJ, Coombes RC. Exemestane (FCE 24304) a new steroidal aromatase inhibitor. J Steroid Biochem Mol Biol 1992;43:137–43.
[27] Numazawa M, Oshibe M, Yamaguchi S, Tachibana M. Time-dependent inactivation of aromatase by 6-alkylandrosta-1,4-diene-3,17-diones. Effects of length and configuration of 6-alkyl group. J Med Chem 1996;39:1033–8.
[28] Numazawa M, Yamaguchi S. Synthesis and structure–activity relationships of 6-phenylaliphatic-substituted C_{19} steroids having a 1,4-diene, 4,6-diene, or 1,4,6-triene structure as aromatase inhibitors. Steroids 1999;64:187–96.
[29] Numazawa M, Shelangouski M, Nagasaka M. Probing the binding pocket of the active site of aromatase with 6-ether or 6-ester substituted androst-4-ene-3,17-diones and their diene and triene analogs. Steroids 2000;65:871–82.
[30] Numazawa M, Nagaoka M, Handa W, Ogawa Y, Matsuoka S. Studies directed towards a mechanistic evaluation of inactivation of aromatase by the suicide substrates androta-1,4-diene-3,17-diones and its 6-ene derivatives. Aromatase inactivation by the 19-substituted derivatives and their enzymic aromatization. J Steroid Biochem Mol Biol 2007;107:211–9.
[31] Takahashi M, Handa W, Umeta H, Ishikawa S, Yamashita K, Numazawa M. Aromatase inactivation by 2-substituted derivatives of the suicide substrate androsta-1,4-diene-3,17-dione. J Steroid Biochem Mol Biol 2009;116:191–9.
[32] Hosoda H, Yamashita K, Sagae H, Nambara T, Steroids CI. Dimethyl-*tert*-butylsilyl ethers of steroids. Chem Pharm Bull 1975;23:2118–22.
[33] Abraham EP, Smith JC. Addition of hydrogen chloride and iodide to olefins. Undecenoic acid. J Chem Soc 1936:1605–7.
[34] Roel B, Joannes W, Aede G. A novel route to the marasmane skeleton via a tandem rearrangement-cyclopropanation reaction. Total synthesis of (+)-isovelleral. J Org Chem 2001;66:2350–7.
[35] Nedelec L, Gasc JC, Bucourt R. The kinetically controlled methylation of conjugated polycyclic ketones. Tetrahedron 1974;30:3263–8.
[36] Siiteri PK, Thompson EA. Human placental aromatase. J Steroid Biochem 1975;6:317–22.
[37] Trott A, Olson J. Autodock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. J Comput Chem 2009, available online.
[38] Kitz R, Wilson IB. Effects of methanesulfonic acid as irreversible inhibitors of acetylcholine esterase. J Biol Chem 1962;237:3245–9.
[39] Silverman RB. Mechanism-based enzyme inactivation, vol. 1. Boca Raton, FL: CRC press; 1988. Chapter 1, p. 3–30.
[40] Numazawa M, Yamashita K, Kimura N, Takahashi M. Chemical aromatization of 19-hydroxyandrosta-1,4-diene-3,17-dione with acid or alkaline: elimination of the 19-hydroxy methyl group as formaldehyde. Steroids 2009;74:208–11.
[41] Ghosh D, Griswold J, Erman M, Pangborn W. Structural basis for androgen specificity and oestrogen synthesis in human aromatase. Nature 2009;457:219–23.