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6-Phenylaliphatic-substituted Androst-4-ene-3,17-diones as Aromatase Inhibitors: Structure-activity Relationships

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Two series of 6α- and 6β-phenylaliphatic-substituted androst-4-ene-3,17-diones (3 and 5) were synthesized as aromatase inhibitors to gain insights of structure-activity relationships of varying the *n*-alkyl moiety (C_2 to C_5) of the 6-phenylaliphatic substituents to the inhibitory activity. All of the inhibitors synthesized inhibited human placental aromatase in a competitive manner with apparent K_i values ranging from 16 to 115 nM. The 6α-phenethyl analog 3a and the 6β-phenbutyl analog 5c (K_i=16 nM for the two inhibitors, respectively) were the most potent inhibitors in each series. The inhibitory activities of the 6β-substituted steroids 5 except for the phenethyl compound 5a were more powerful than those of the corresponding 6α-isomers 3. Elongation of the alkyl moiety of the 6-substituent of the 6α-phenethyl steroid 3a up to five methylene units decreased affinity to aromatase in all cases, whereas the addition of two more methylene units to the 6-side chain of the 6β-phenethyl analog 5a increased the affinity in relation to carbon number of the 6-substituent. These results along with molecular modelling with the PM3 method, would give a new information about the formation of thermodynamically stable enzyme-inhibitor complex in a hydrophobic binding pocket in the active site of aromatase. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Placental aromatase is a cytochrome P-450 enzyme that catalyzes the conversion of androgens, androst-4-ene-3,17-dione (androstenedione) and testosterone, to estrogens, estrone and estradiol [1–3]. Aromatase is a potential therapeutic target for the selective lowering of estrogen levels in patients with estrogen-dependent tumors, including breast cancer [4–8]. The specific blockade of estrogen biosynthesis has been pursued intensely with the goal of developing practical clinical drugs.

We previously synthesized 6-*n*-alkyl-substituted (alkyl: methyl to *n*-octyl) androstenediones [9, 10] and their Δ^1-, Δ^6-, and $\Delta^{1,6}$-analogs [11, 12] as aromatase inhibitors, some of which are among the most potent competitive inhibitors reported so far. The structure-activity relationships of these 6-alkyl-substituted steroids to aromatase inhibition activity have revealed that aromatase has a hydrophobic binding pocket with a limited accessible volume in the region of the C-6 position of the natural substrate androstenedione. The 6-phenyl- and 6-benzyl-substituted androstenediones have also been synthesized to act as good competitive inhibitors of aromatase [9]. In connection with work in our laboratory on the spatial aspects of binding of the aromatase inhibitors to the hydrophobic pocket, we were interested in 6-phenylaliphatic-substituted androstenediones with more than two methylene units between the steroid and the phenyl group. Thus, we describe here the preparation and biochemical evaluation of 6α- and 6β-phenylaliphatic androstenediones (3 and 5), having two to five methylene units, as aromatase inhibitors. 6α-Phenethyl- and 6β-phenbutyl-androstenediones were the most potent competitive inhibitors among the synthesized compounds. The inhibition experiments as well as the conformational analysis with the PM3 calculations revealed new information concerning the tolerance of the binding pocket.

MATERIALS AND METHODS

3,3:17,17-Bis(ethylenedioxy)androstane 5α,6α-epoxide (1) was synthesized using the method [9] pre-

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viously reported. [1β-^{3}H]Androstenedione (27.5 Ci/mmol; ^{3}H-distribution, 74–79% at 1β) was obtained from New England Nuclear Corp. (Boston, MA) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Kohjin Co. (Tokyo).

Melting points were measured on a Yanagimoto melting point apparatus (Kyoto) and are uncorrected. Infrared (IR) spectra were recorded on a Perkin Elmer FT-IR 1725X spectrophotometer (Norwalk, CT), and ultraviolet (UV) spectra were determined in 95% ethanol on a Hitachi 150-20 UV spectrophotometer (Tokyo). ^{1}H NMR spectra were obtained in $CDCl_3$ solutions with a JEOL EX 270 (270 MHz) spectrometer (Tokyo) using tetramethylsilane as an internal standard. High resolution mass spectra (HRMS) were determined with a JEOL JMS-DX 303 spectrometer. Thin-layer chromatography (TLC) was performed on Merck precoated silica gel plates (Darmstadt). Column chromatography was conducted with silica gel (Merck, 70–230 mesh). High-performance liquid chromatography (HPLC) was carried out using a Waters 510 pump (Milford, MA), YMC-D-ODS-5 column (250 × 20 mm i.d.; Kyoto) and a UV detector (240 nm).

General procedure for Grignard reaction of the 5α,6α-epoxide **1**

A solution of phenylaliphatic bromide (phenylaliphatic: phenethyl, phenpropyl, phenbutyl, and phenpentyl) (103 mmol) in anhydrous THF (20 mL) was added dropwise to a stirred suspension of Mg metal (2.50 g, 103 mmol) in anhydrous THF under N_2 atmosphere, and the mixture was stirred to become a clear solution. A solution of compound **1** (2 g, 5.13 mmol) in anhydrous THF (20 mL) was slowly added to these Grignard reagent solutions, separately, and the mixture was refluxed for 2 h under N_2 atmosphere. After the solution was cooled, saturated NH_4Cl solution (100 mL) was added, and the resulting product was extracted with ethyl acetate (200 mL × 2). The combined organic layer was washed to neutrality with water, dried with Na_2SO_4, and evaporated to dryness. The residue was purified by column chromatography (hexane/ethyl acetate) and/or recrystallization to give 6β-substituted 3,3:17,17-bis(ethylenedioxy)androstan-5α-ols (**2**).

6β-Phenethyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (**2a**). Yield. 82%: mp 158–159°C (from acetone). [^{1}H] NMR δ 0.85 (3H, s, 18-Me), 0.96 (3H, s, 19-Me), 3.82–4.00 (8H, m, OCH_2CH_2O × 2), 7.15–7.30 (5H, m, aromatic protons); FT-IR (KBr) 3494 (OH) cm^{-1}; *Anal.* calculated for $C_{31}H_{44}O_5$: C, 74.96; H, 8.93. Found: C, 75.04; H, 8.98.

6β-Phenpropyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (**2b**). Yield. 94%: mp 177–178°C (from acetone-H_2O). [^{1}H] NMR δ 0.84 (3H, s, 18-Me), 0.95 (3H, s, 19-Me), 3.81–4.02 (8H, m, OCH_2CH_2O × 2), 7.14–7.30 (5H, m, aromatic protons); FT-IR (KBr) 3513 (OH) cm^{-1}. *Anal.* calculated for $C_{32}H_{46}O_5$: C, 75.26; H, 9.08. Found: C, 75.34; H, 9.19.

6β-Phenbutyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (**2c**). Yield. 96%: mp 46–48°C. [^{1}H] NMR δ 0.86 (3H, s, 18-Me), 0.96 (3H, s, 19-Me), 3.82–4.02 (8H, m, OCH_2CH_2O × 2), 7.14–7.30 (5H, m, aromatic protons); FT-IR (KBr) 3505 (OH) cm^{-1}. *Anal.* calculated for $C_{33}H_{48}O_5$: C, 75.53; H, 9.22. Found: C, 75.24; H, 9.52.

6β-Phenpentyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (**2d**). Yield. 99% (oil). [^{1}H] NMR δ 0.85 (3H, s, 18-Me), 0.96 (3H, s, 19-Me), 3.81–4.01 (8H, m, OCH_2CH_2O × 2), 7.14–7.30 (5H, m, aromatic protons); FT-IR (neat) 3513 (OH) cm^{-1}; HRMS (EI) calculated for $C_{34}H_{50}O_5$ 538.3658, found, 538.3658.

Conversion of the bis(ethylenedioxy)steroids **2** *to 6α-phenylaliphatic androst-4-ene-3,17-diones* (**3**)

Hydrochloric acid (1 M, 2.1 mL) was added to a solution of steroids **2** (0.84 mmol) in 95% EtOH (21 mL), and the mixture was refluxed for 8 h. After evaporation of the solvent, the residue was dissolved in ethyl acetate (400 mL), washed with 5% $NaHCO_3$ solution and water, dried with Na_2SO_4, and evaporated to dryness. The residue was purified with column chromatography (hexane/ethyl acetate) followed by HPLC to yield the corresponding 6α-phenylaliphatic steroids **3** along with their 6β-isomers as minor products.

6α-Phenethylandrost-4-ene-3,17-dione (**3a**). Yield. 67%: mp 48–50°C. HPLC, retention time (t_R), 53.3 min (MeCN–H_2O, 70:30, 5 mL/min, t_R=48.2 min for the 6β-isomer **5a** under the conditions); [^{1}H] NMR δ 0.92 (3H, s, 18-Me), 1.18 (3H, s, 19-Me), 5.87 (1H, d, J = 1.5 Hz, 4-H), 7.16–7.33 (5H, m, aromatic protons); FT-IR (KBr) 1737 and 1673 (C=O) cm^{-1}; UV λ_{max} 242 nm (ε = 14,700). *Anal.* calculated for $C_{27}H_{34}O_2$: C, 83.03; H, 8.78. Found: C, 82.86; H, 8.81.

6α-Phenpropylandrost-4-ene-3,17-dione (**3b**). Yield. 48%: mp 127–127.5°C. HPLC, t_R, 74.1 min (MeCN–H_2O, 65:35, 8 mL/min, t_R=63.4 min for the 6β-isomer **5b** under the conditions); [^{1}H] NMR δ 0.91 (3H, s, 18-Me), 1.18 (3H, s, 19-Me), 5.78 (1H, d, J = 1.5 Hz, 4-H), 7.17–7.32 (5H, m, aromatic protons); FT-IR (KBr) 1742 and 1672 (C=O) cm^{-1}; UV λ_{max} 242 nm (ε = 14,800). *Anal.* calculated for $C_{28}H_{36}O_2$: C, 83.12; H, 8.97. Found: C, 83.40; H, 9.23.

6α-Phenbutylandrost-4-ene-3,17-dione (**3c**). Yield. 50%: mp 91–93°C. HPLC, t_R, 55.6 min (MeCN–H_2O, 82:18, 5 mL/min, t_R=50.0 min for the 6β-isomer **5c** under the conditions); [^{1}H] NMR δ 0.92 (3H, s, 18-Me), 1.19 (3H, s, 19-Me), 5.80 (1H, d, J = 1.7 Hz, 4-H), 7.16–7.32 (5H, m, aromatic protons); FT-IR (KBr) 1739 and 1674 (C=O) cm^{-1}; UV

λ_{max} 242 nm (ε = 14,500). *Anal.* calculated for $C_{29}H_{38}O_2$: C, 83.21; H, 9.15. Found: C, 82.93; H, 9.40.

6α-Phenpentylandrost-4-ene-3,17-dione (**3d**). Yield. 47% (oil). HPLC, t_R, 58.4 min (MeCN–H_2O, 82:18, 5 mL/min, t_R=51.8 min for the 6β-isomer **5d** under the conditions); [^{1}H] NMR δ 0.92 (3H, s, 18-Me), 1.19 (3H, s, 19-Me), 5.79 (1H, d, J = 1.5 Hz, 4-H), 7.15–7.32 (5H, m, aromatic protons); FT-IR (neat) 1739 and 1669 (C=O) cm^{-1}; UV λ_{max} 242 nm (ε = 14,900); HRMS (EI) calculated for $C_{30}H_{40}O_2$ 432.3028, found 432.3050.

Hydrolysis of bis(ethylenedioxy)steroids **2**

$HClO_4$ (3M, 0.4 mL) was added to a solution of compound **3** (0.46 mmol) in THF (4 mL), and the reaction mixture was stirred at room temperature for 3 h [9, 10]. After this time, the mixture was diluted with ethyl acetate (100 mL), washed with 5% $NaHCO_3$ solution and water, dried (Na_2SO_4), and evaporated to afford the residue of which column chromatography followed by recrystallization yielded the corresponding 3,17-dione **4**.

6β-phenethyl-5α-hydroxyandrostane-3,17-dione (**4a**). Yield. 70%: mp 215–216.5°C (from acetone). [^{1}H] NMR δ 0.91 (3H, s, 18-Me), 1.21 (3H, s, 19-Me), 7.14–7.34 (5H, m, aromatic protons); FT-IR (KBr) 3402 (OH), 1737 and 1703 (C=O) cm^{-1}. *Anal.* calculated for $C_{27}H_{36}O_3$: C, 79.37; H, 8.88. Found: C, 79.50; H, 9.09.

6β-Phenpropyl-5α-hydroxyandrostane-3,17-dione (**4b**). Yield. 76%: mp 173–173.5°C (from acetone). [^{1}H] NMR δ 0.87 (3H, s, 18-Me), 1.18 (3H, s, 19-Me), 7.16–7.33 (5H, m, aromatic protons); FT-IR (KBr) 3402 (OH), 1738 and 1702 (C=O) cm^{-1}. *Anal.* calculated for $C_{27}H_{38}O_3$: C, 79.58; H, 9.06. Found: C, 79.59; H, 9.20.

6β-Phenbutyl-5α-hydroxyandrostane-3,17-dione (**4c**). Yield. 81%: mp 129–130°C (from acetone–hexane). [^{1}H] NMR δ 0.91 (3H, s, 18-Me), 1.20 (3H, s, 19-Me). 7.16–7.32 (5H, m, aromatic protons); FT-IR (KBr) 3409 (OH), 1741 and 1699 (C=O) cm^{-1}. *Anal.* calculated for $C_{29}H_{40}O_3$: C, 79.77; H, 9.23. Found: C, 79.89; H, 9.23.

6β-Phenpentyl-5α-hydroxyandrostane-3,17-dione (**4d**). Yield. 64%: mp 111–113°C (from acetone). [^{1}H] NMR δ 0.90 (3H, s, 18-Me), 1.20 (3H, s, 19-Me), 7.16–7.32 (5H, m, aromatic protons); FT-IR (KBr) 3387(OH), 1741 and 1708 (C=O) cm^{-1}. *Anal.* calculated for $C_{30}H_{42}O_3$: C, 79.95; H, 9.40. Found: C, 79.85; H, 9.35.

Dehydration of 5α-hydroxy steroids **4**

Thionyl chloride (0.15 mL) was added to a chilled solution of compound **4** (0.25 mmol) in dry pyridine (1.5 mL) [9, 10]. The mixture was stirred for 3 min at 0°C, poured into 30 mL of ice–water, further stirred for 30 min, and then extracted with ethyl acetate (50 mL × 2). The combined organic layer was washed with water, dried with Na_2SO_4, and evaporated to give the crude product, which was purified by column chromatography followed by HPLC (t_R values were given above in the synthesis of the 6α-isomers **3**) and recrystallization. This yielded the corresponding 4-ene-3,17-dione **5**.

6β-Phenethylandrost-4-ene-3,17-dione (**5a**). Yield. 99%: mp 48–49.5°C. [^{1}H] NMR δ 0.92 (3H, s, 18-Me), 1.31 (3H, s, 19-Me), 5.76 (1H, s, 4-H), 7.15–7.35 (5H, m, aromatic protons); FT-IR (KBr) 1737 and 1673 (C=O) cm^{-1}; UV λ_{max} 244 nm (ε = 15,100). *Anal.* calculated for $C_{27}H_{34}O_2$: C, 83.03; H, 8.73. Found: C, 83.17; H, 8.86.

6β-Phenpropylandrost-4-ene-3,17-dione (**5b**). Yield. 89%: mp 34–34.5°C. [^{1}H] NMR δ 0.91 (3H, s, 18-Me), 1.19 (3H, s, 19-Me), 5.74 (1H, s, 4-H), 7.15–7.32 (5H, m, aromatic protons); FT-IR 1737 and 1673 (C=O) cm^{-1}; UV λ_{max} 243 nm (ε = 16,000). *Anal.* calculated for $C_{28}H_{36}O_2$: C, 83.12; H, 8.97. Found: C, 82.82; H, 9.09.

6β-Phenbutylandrost-4-ene-3,17-dione (**5c**). Yield. 96%: mp 118–119°C (from ethyl acetate–hexane). [^{1}H] NMR δ 0.94 (3H, s, 18-Me), 1.22 (3H, s, 19-Me), 5.73 (1H, s, 4-H), 7.15–7.31 (5H, m, aromatic protons); FT-IR (KBr) 1741 and 1676 (C=O) cm^{-1}; UV λ_{max} 243 nm (ε = 15,200). *Anal.* calculated for $C_{29}H_{38}O_2$: C, 83.21; H, 9.15. Found: C, 83.61; H, 9.45.

6β-Phenpentylandrost-4-ene-3,17-dione (**5d**). Yield. 68%: mp 102–103°C (from acetone). [^{1}H] NMR δ 0.93 (3H, s, 18-Me), 1.21 (3H, s, 19-Me), 5.73 (1H, s, 4-H), 7.16–7.31 (5H, m, aromatic protons); FT-IR (KBr) 1740 and 1676 (C=O) cm^{-1}; UV λ_{max} 243 nm (ε = 11,600). *Anal.* calculated for $C_{30}H_{40}O_2$: C, 83.28; H, 9.32. Found: C, 83.27; H, 9.46.

Enzyme preparation

Human placental microsomes (particles sedimenting at 105,000*g* for 60 min) were obtained using the method reported by Ryan [13]. They were washed once with 0.05 mM dithiothreitol solution, lyophilized, and stored at −20°C. No significant loss of activity occurred during this study (2 months).

Aromatase assay procedures

Aromatase activity was measured according to the procedure of Siiteri and Thompson [14]. The screening assay for determination of IC_{50} value and the kinetic assay were carried out essentially according to the assay methods described in our previous work [15]. Briefly, 20 μg of protein of the lyophilized microsomes and a 20 min incubation time for the screening assay and 20 μg of protein of the microsomes and a 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 (pH 7.5) in the presence of NADPH in air.

Molecular modelling studies

Molecular models were constructed on a Silicon Graphics IRIS 4D workstation starting from data of semiempirical molecular orbital calculations with the PM3 method (MOPAC 93 Revision 2, this was obtained through Japan Chemistry Program Exchange) using the 3D graphic option of the MOL-GRAPH software (Daikin, Tokyo). Each compound discussed in this study was subjected to a systematic conformational analysis to determine all of its minimum-energy conformations. Geometries were considered minimized when the energy change between two subsequent structures was less than 0.001 kcal/mol. The energy barrier for rotation of the aliphatic chains of compounds **3a**, **3b**, **5a** and **5b**, which have two or three methylene units, is more than 32 kcal/mol, whereas that of compounds **3c**, **3d**, **5c** and **5d**, which have four or five methylene units, is more than 5.0 kcal/mol. Low (global minimum)-energy conformations were overlapped within MOL-GRAPH which uses a least-squares fitting algorithm to minimize the displacement between matching atoms in the structures that are superimposed.

RESULTS

Chemistry

The 6-substituted androstenediones (**3** and **5**), having a phenyl group at the terminal of the 6-alkyl chains, were synthesized principally according to the

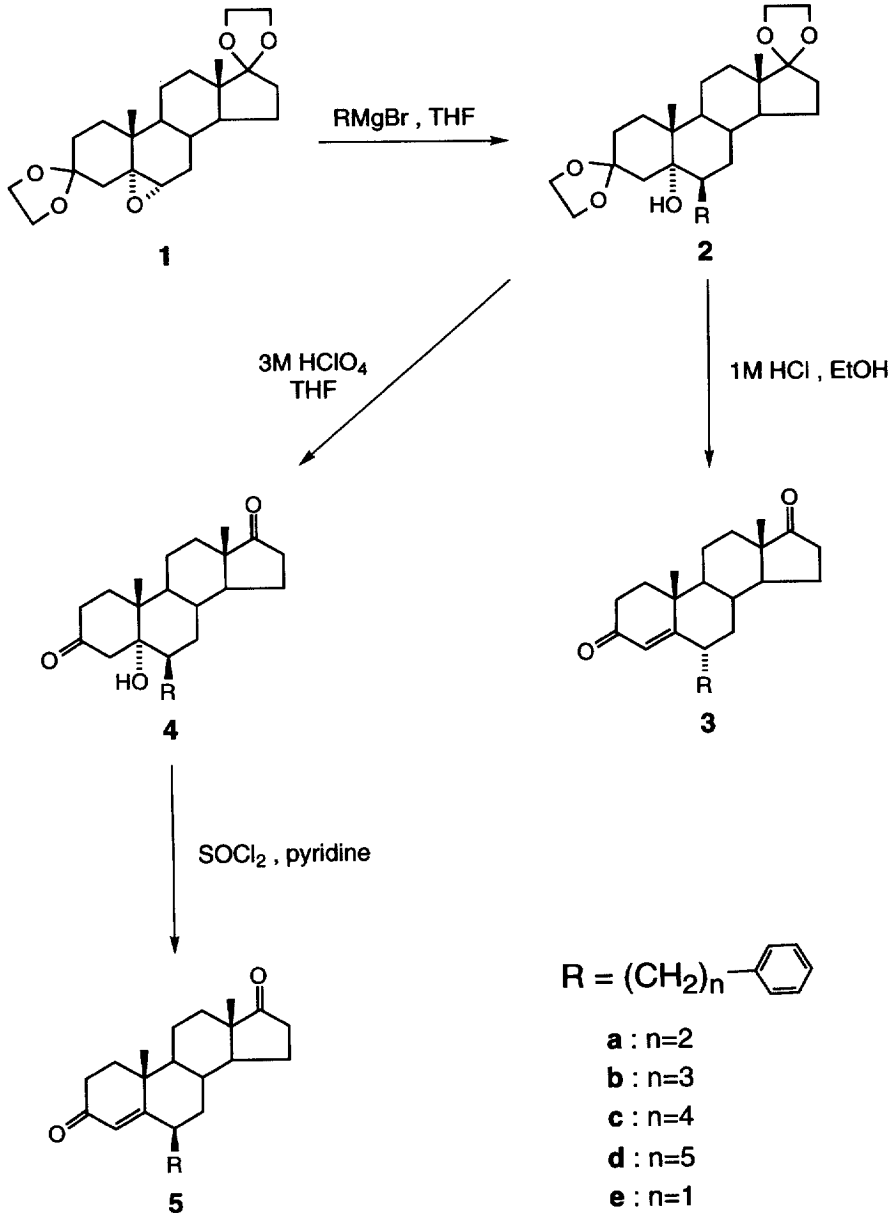

Fig. 1. Synthesis of 6α-and 6β-phenylaliphatic steroids 3 and 5.

methods [9, 10] previously reported. Grignard reaction of 3,3:17,17-bis(ethylenedioxy)androstane-5α,6α-epoxide (**1**) with phenylaliphatic bromides [aliphatic: $(CH_2)_2$, $(CH_2)_3$, $(CH_2)_4$ and $(CH_2)_5$] and Mg metal in THF under reflux gave the corresponding 6β-phenylaliphatic-substituted bis(ethylenedioxy)5α-ols **2** in high yields (Fig. 1). Treatment of compounds **2** with 1 M hydrochloric acid in ethanol under reflux yielded the deprotected, dehydrated, and isomerized products, the desired 6α-substituted 4-ene-3,17-diones **3** (37–66%), along with small amounts of their 6β-isomers **5** (7–15%) in one step. Reverse phase HPLC using a ODS column and acetonitrile-water as the mobile phase was effective for the separation of the 6α-compound and the corresponding 6β-isomer. The 6β-substituted steroids **5**, which were initially produced in the reaction sequence, respectively, should be isomerized to the 6α-equatorial isomers **3**, which are thermodynamically more stable than the corresponding 6β-axial isomers **5**, under the acidic conditions as previously reported [9, 10]. On the other hand, reaction of the ethylenedioxy 5α-ols **2** with 3 M perchloric acid in THF at room temperature afforded the deprotected products, 5α-hydroxy-3,17-diones **4**, in good yields. Dehydration of the 5α-ols **4** with thionyl chloride in pyridine then led to another desired 6β-substituted 4-ene-3,17-diones **5** in good to excellent yields. The configurations of the 6-phenylaliphatic group were assigned on the basis of ^1H NMR spectroscopy [4-H: δ 5.78–5.80 (d, $J_{4,6}$=1.5–1.7 Hz) for the 6α-substituted steroids **3** and δ 5.73–5.76 (s) for the 6β-isomers **5**]. The similar C-4 proton signals have been reported in series of 6-alkyl-, 6-bromo-, and 6-(bromoacetoxy)androstenediones [9, 16, 17].

Biochemical properties

Inhibition of aromatase activity in human placental microsomes by the 6α- and 6β-phenylaliphatic androstenediones (**3** and **5**) synthesized in this study, was examined *in vitro* by enzyme kinetics. The results are shown in Table 1. Aromatase activity was measured using a radiometric assay in which tritiated water released from [1β-^{3}H]androstenedione into the incubation medium during aromatization was measured [14]. IC_{50} values were initially obtained under initial velocity conditions where the conversion rate of the tritiated substrate was less than 10%. In order to characterize the nature of inhibitor binding to the active site of aromatase, aromatization was measured at several inhibitor and substrate concentrations. The results of these results were plotted on typical Lineweaver–Burk plots. All of the inhibitors studied exhibited a clear-cut competitive inhibition. The apparent inhibition constants (K_i) were obtained by analysis of Dixon plot. The Lineweaver–Burk plot of aromatase inhibition by steroid **3a** is shown in Fig. 2. In these studies, the apparent K_m for androstenedione was found to be in the range of 30–35 nM.

Table 1. Aromatase inhibition by 6α- and 6β-phenylalkyl steroids

R	IC_{50} (μM)[a]	K_i (nM)[b]	Relative affinity[c]
	6α-Substituted steroids **3**		
3a, $(CH_2)_2Ph$	140	16	2.06
3b, $(CH_2)_3Ph$	400	44	0.75
3c, $(CH_2)_4Ph$	340	40	0.83
3d, $(CH_2)_5Ph$	560	62	0.53
	6β-Substituted steroids **5**		
3a, $(CH_2)_2Ph$	1050	115	0.29
3b, $(CH_2)_3Ph$	290	34	0.97
3c, $(CH_2)_4Ph$	135	16	2.06
3d, $(CH_2)_5Ph$	450	52	0.63
	For comparison[d]		
3e, 6α-CH_2Ph	–	10	1.83
5e, 6β-CH_2Ph	–	63	0.29

[a][1β-^{3}H]androstenedione (300 nM) and 20 μg of protein from human placental microsomes were used.
[b]Apparent inhibition constants (K_i) were obtained by Dixon plot. All of the inhibitors studied showed a competitive type of inhibition based on Lineweaver–Burk analysis.
[c]Relative affinity was a ratio of K_m (33 nM) for androstenedione to K_i (K_m/K_i).
[d]Ref. [9].

The 6α- and 6β-phenpropylandrostenediones (**3b** and **5b**), at concentrations of 440, 1100, and 2200 nM for **3b** and 340, 850 and 1700 nM for **5b**, did not cause a time-dependent inactivation of aromatase.

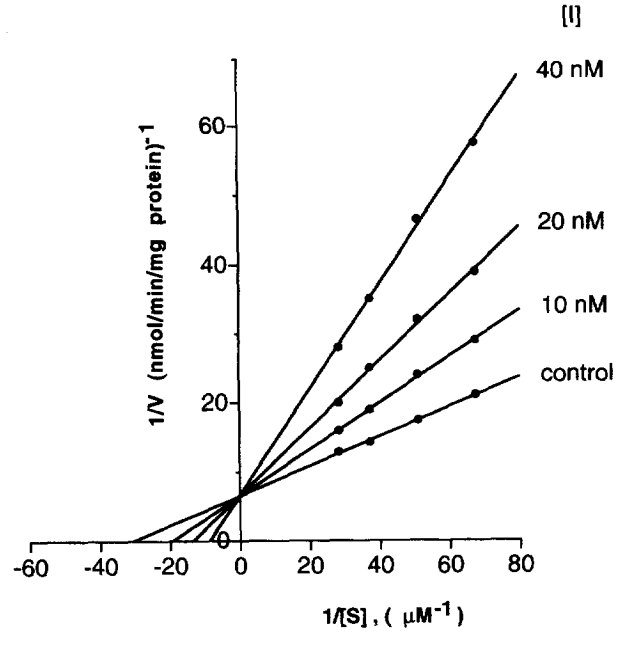

Fig. 2. Lineweaver–Burk plot of 6α-phenethyl androst-4-ene-3,17-dione (3a). Each point represents the mean of two determinations which varied by less than 5% of the mean. The inhibition experiments with all the other steroids examined gave essentially similar plots to Fig. 2 (data not shown).

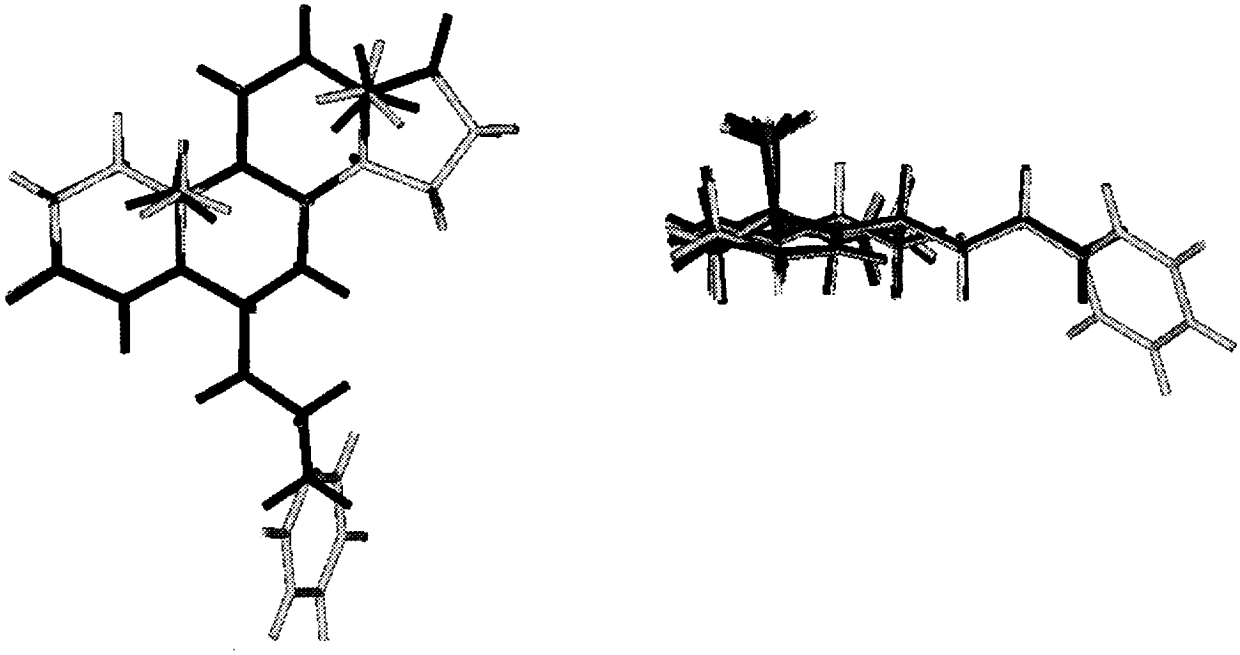

Fig. 3. Overlay of the 6α-phenethyl- steroid 3a (light line) and the 6α-*n*-propyl analog (dark line) having the same methylene unit number each other by superimposing their respective steroid nucleus. Views from the β-side (left) and from the C(2)–C(3)–C(4) (right).

Molecular modelling

The minimum-energy conformations of all the inhibitors assayed in this study were determined by the MOPAC package using the PM3 Hamiltonian. The backbone of these steroids as well as those of 6-phenyl- and 6-benzyl-steroids were excellently superimposed on androstenedione. All of the methylene moieties of the 6-phenylaliphatic side chains were linearly extended in the similar geometry as that previously reported for the 6-*n*-alkyl side chains [9, 10]. Overlays of the inhibitors, the 6β-phenbutyl- (**5c**) and 6α-phenethyl- (**3a**) steroids, showing the highest affinity for aromatase in each series, and the 6β-*n*-pentyl- (K_m/K_i=7.5) and 6α-*n*-propyl- (K_m/K_i=2.7) steroids [9, 10] having the same methylene unit numbers as compounds **5c** and **3a**, respectively, are shown in Figs 3 and 4. Comparison of the calculated minimum-energy conformations of the inhibitors in

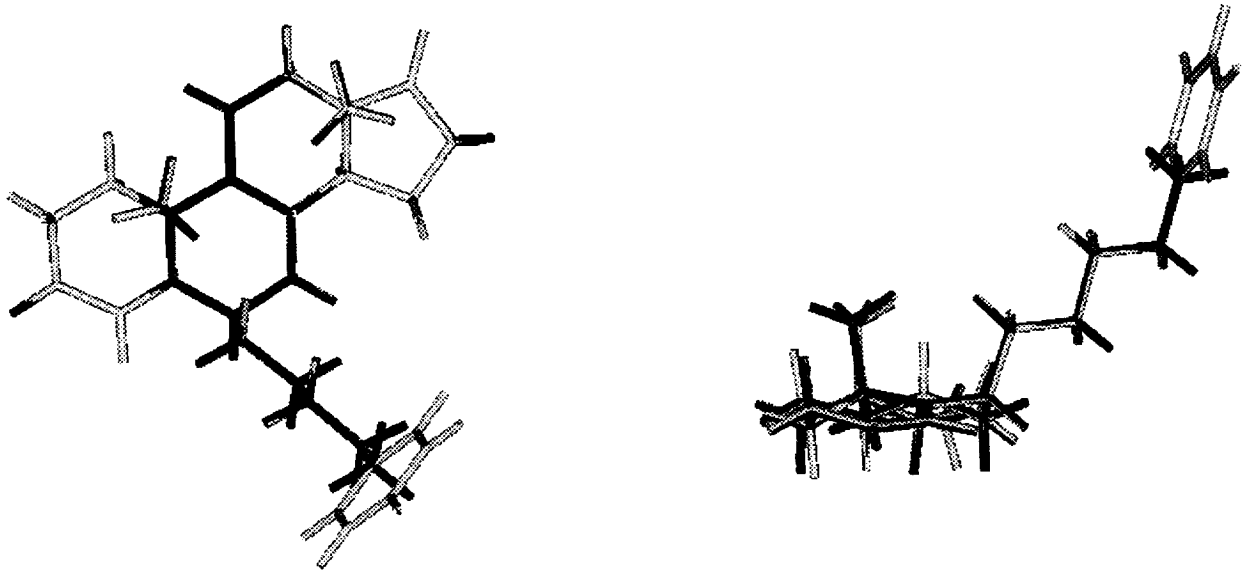

Fig. 4. Overlay of the 6β-phenbutyl steroid 5c (light line) and the 6β-*n*-pentyl analog (dark line) having the same methylene unit number each other by superimposing their respective steroid nucleus. Views from the β-side (left) and from the C(2)–C(3)–C(4) (right).

terms of overlap of the steroid nucleus suggests that an interaction of the phenyl moiety with the amino acid residue of the active site through not only steric reasons but also an electrostatic effect would be operative in the tight bindings of the 6α-phenethyl- and 6α-benzyl-analogs **3a** and **3e** as well as the 6β-phenbutyl analog **5c**.

DISCUSSION

We synthesized two stereoisomeric series of 6-phenylaliphatic-substituted androstenediones (**3** and **5**) having more than two methylene units between the steroid and the phenyl group and carried out a series of experiments with these steroids to determine the structure-activity relationships of aromatase inhibition. The 6α-phenethyl- (**3a**) and 6β-phenbutyl- (**5c**) steroids were very potent competitive inhibitors of human placental aromatase with apparent K_i of 16 nM, respectively. The two inhibitors bound to aromatase with about two times the affinity of the substrate androstenedione (K_m= ~ 33 nM).

Addition of one methylene unit to the methylene moiety of the 6-benzyl group of the 6α-steroid **3e** slightly increased affinity for aromatase (K_m/K_i: 1.83 for the benzyl **3e** vs 2.06 for the phenethyl **3a**). Further elongation of the methylene unit up to C-5 decreased affinity in which the K_m/K_i values ranged between 0.53 and 0.75. In the 6β-series, an introduction of one methylene to the methylene moiety of the 6β-benzyl compound **5e** did not change, to a significant extent, the affinity (K_m/K_i=0.29 for both the 6β-phenethyl **5a** and for the 6β-benzyl **5f**), in contrast, elongation of the methylene side chain of the phenethyl group up to C-4 increased affinity in proportion to carbon number, but further elongation of the side chain decreased it. The results indicated that the inhibitors **3a** and **3e** having the phenethyl and benzyl groups at the 6α-position as well as the 6β-substituted steroids **5b** and **5c** having the phenpropyl and phenbutyl groups at C-6, all of which show more than 1.0 of the K_m/K_i ratio, can be tolerated in the pocket of the active site of aromatase.

We previously reported that an introduction of a *n*-alkyl group containing one to six carbons at C-6α and C-6β positions of androstenedione gives rise to an increased affinity for aromatase. On the basis of the structure-activity relationships, it has previously been shown that the binding pocket is in the active site of aromatase in the region corresponding to the β-side rather than the α-side and can form a stable enzyme-inhibitor complex not only with the 6β-ethylandrostenedione but also with the 6β-*n*-pentyl derivative [9, 10].

Molecular modelling with the PM3 method shows that the 6-methylene moiety between the steroid and phenyl group in the phenylaliphatic steroids **3** and **5** extends in the similar direction to that of the 6-*n*-alkyl chain of the 6-alkylandrostenediones. This suggest that the 6β-phenbutyl steroid may bind tightly to the active site probably in the similar geometry to that involved in the binding of 6β-*n*-pentyl steroid. However, not only the intrinsic steric factor but also an electronic one (cf. π–π interaction) between the phenylaliphatic group of the inhibitors and the amino acid residue of the active site may be involved in the inhibitory activities of the phenylaliphatic steroids **3** and **5**.

In conclusion, a variation of the chain length between the steroid and phenyl group could influence binding to aromatase, giving new aspects of binding of the inhibitors to the binding pocket.

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