6-Alkyl- and 6-Arylandrost-4-ene-3,17-diones as Aromatase Inhibitors. Synthesis and Structure-Activity Relationships†

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Two series of 6β- and 6α-substituted androst-4-ene-3,17-diones (**5** and **6**) were synthesized as aromatase inhibitors to gain insights of structure-activity relationships of varying substituents (methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, phenyl, benzyl, vinyl, and ethynyl) to the inhibitory activity. All of the inhibitors synthesized prevented human placental aromatase in a competitive manner. The inhibition activities of all the 6-*n*-alkylated steroids **5a-d** and **6a-d** (K_i = 1.4-12 nM) as well as the 6β-vinyl (**5h**), 6α-benzyl (**6g**), and 6-methylene (**10**) compounds (K_i = 5.1, 10, and 4.9 nM, respectively) were very powerful whereas those of the 6-isopropyl (**5e** and **6e**), 6-phenyl (**5f** and **6f**), 6β-benzyl (**5g**), and 6β-ethynyl (**5i**) steroids, having a bulky or polar substituent, were relatively weak. The 6β-ethyl derivative **5b** was the most potent inhibitor among those synthesized. Inhibitors **5a**, **5f**, **5h**, **5i**, **6b**, and **10** did not cause a time-dependent inactivation of aromatase. The 6β-alkyl steroids essentially had higher affinity for the enzyme than the corresponding 6α-isomers, whereas the opposite relation was observed in a series of the aryl steroids. These results along with molecular modeling with the PM3 method clearly indicate that aromatase has a hydrophobic binding pocket with a limited accessible volume in the active site in the region corresponding to the β-side rather than the α-side of the C-6 position of the substrate.

Introduction

Aromatase is the enzyme responsible for catalyzing the conversion of 4-en-3-one androgens, androst-4-ene-3,17-dione (androstenedione) and testosterone, to estrogens, estrone and estradiol, respectively. It is a cytochrome P-450 enzyme,[1] classified as cytochrome P-450XIXA1.[2] The aromatization process is thought to proceed via three sequential oxygenations at C-19 of the androgen.[3] There appears to be a major clinical role for methods of controlling estrogen levels, not least in the treatment of established estrogen-dependent breast cancer. The inhibitors of aromatase is one route to such control.[4]

A number of potent aromatase inhibitors, analogs of the substrate androstenedione, have been described,[4d,f] including as 4-hydroxy,[4b] 19-ethynyl,[5] or 1-methylene[4c] derivatives of androstenedione, which have been the subjects of clinical trials. Several 6-substituted substrate analogs have been synthesized to act as probes of the aromatase active site. 6α-Bromoandrostenedione is an excellent competitive inhibitor (K_i = 3.4 nM), whereas the 6β-bromo isomer appears to be a mechanism-based inhibitor.[6] The 6β-fluoro steroid is also a good inhibitor.[7] The 6α-bromoacetoxy derivative inactivates aromatase in an affinity-labeling manner but the 6β-bromoacetoxy isomer in a mechanism-based manner.[8] Both the 6α- and 6β-hydroperoxy derivatives are not only substrates but also affinity labels for the enzyme.[9] Thus, to our knowledge, there is little literature focusing on structure-activity relationships of 6-substituted androstenediones to aromatase inhibition activity. We describe here the preparation and biochemical evaluation of 6β-alkyl- or -aryl- and 6α-alkyl- or -arylandrostenediones (**5** and **6**). The 6-*n*-alkyl steroids **5a-d** and **6a-d** along with the 6β-vinyl- and 6-methylene derivatives **5h** and **10** were very powerful competitive inhibitors, and the 6β-ethyl compound **5b** was the most potent inhibitor among them. The inhibition experiments as well as the conformational analysis with the PM3 calculations demonstrate for the first time the accessible volume of a binding pocket in the active site of aromatase in the region of the C-6 position of androstenedione.

Results

Chemistry. The synthesis of the 6-substituted androstenediones was carried out principally according to the synthetic sequence previously reported for the synthesis of 6-substituted 4-en-3-one steroids[10] (Scheme 1). The important synthetic intermediate, 3,3:17,17-bis(ethylenedioxy)androstane 5α,6α-epoxide (**2**), was prepared by epoxidation of 5-ene steroid **1** with *m*-chloroperbenzoic acid. The epoxidation produced the 5β,6β-epoxy isomer along with compound **2**, which were successfully separated each other by silica gel column chromatography. The configuration of the α-epoxy ring was determined based on the ^{1}H NMR spectroscopy [6β-H at δ 2.81 ppm (d, J = 4.0 Hz) for **2**, 6α-H at δ 3.07 ppm (d, J = 2.3 Hz) for the β-epoxide].[11] Reactions of the epoxide **2** with alkyl, aryl, vinyl, and ethynyl Grignard reagents in THF on heating under reflux gave the corresponding 6β-substituted bis(ethylenedioxy) 5α-ols **3** (86-98%). Treatment of the 5α-ols **3** with 3 M perchloric acid in THF yielded the hydrolyzed products, 5α-hydroxy 3,17-diones **4**, in excellent yields. The yields of the isopropyl derivatives **3e** and **4e** in these reactions were lower than the others. Thionyl chloride dehydration of compounds **4** in pyrdine then led to the desired 6β-substituted 4-en-3-one steroids **5** in fair yields (Table 1).

The conversion of the 6β-methyl compound **5a** to its 6α-equatorial isomer **6a**, which is thermodynamically more stable than the 6β-axial isomer, was attempted under alkaline conditions (KOH, MeOH) according to the methods[10] previously reported for isomerization of 6β-

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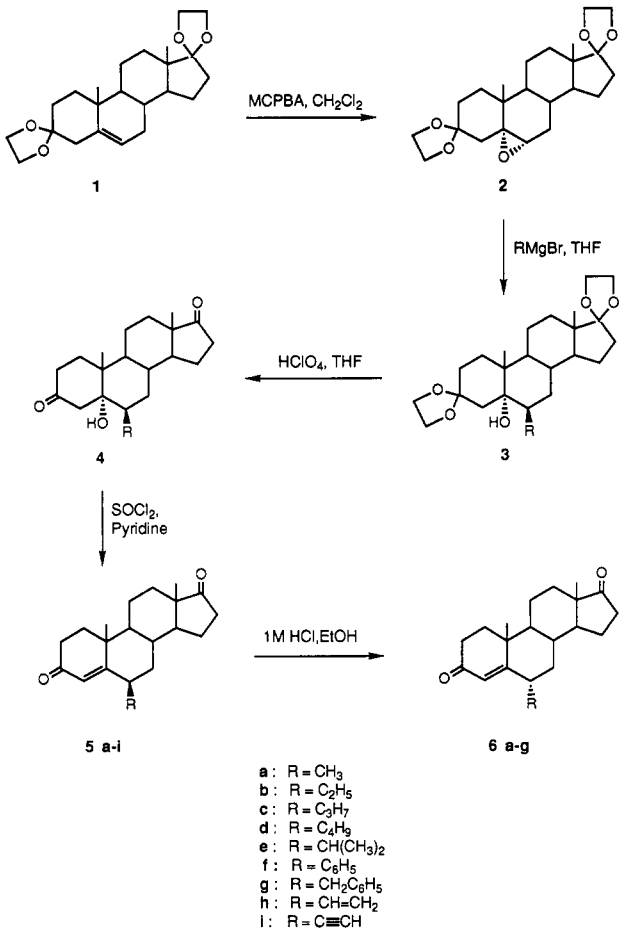
Scheme 1



methyl and 6β-phenyl 4-en-3-one steroids. However, in our hands, the isomerization did not occur at all to result in the recoveries of the starting material. Moreover, when the 6β-phenyl steroid **5f** was subjected to the isomerization reaction, a *ca.* 1:1 mixture of 6α-hydroxy-6β-phenyl 4-ene-3-one **8a** and its 6β-hydroxy isomer **8b** was obtained, instead of the isomerized product, in fair yield. Compounds **8a** and **8b** could be separated by silica gel column chromatography. The configurations of the phenyl group were determined on the basis of the ^1H NMR spectroscopy. The 19-methyl protons of the 6β-phenyl derivative **8a** are shielded by the ring-current effect of benzene ring to appear in the higher field (δ 0.59 ppm), compared to that (δ 1.57 ppm) of the 6α-isomer. The similar shielding effect was also observed in the case of the 6β-phenyl steroids **5f** (δ 0.68 ppm). An attack of hydroxide ion from both the α- and β-faces at C-6 of the 5-ene intermediate **7**, produced under the strong alkaline condition, would occur to yield compounds **8** (Scheme 2). Then, we employed an alternate isomerization condition using acid.[10] Reactions of compounds **5**, except 6β-vinyl- and 6β-ethynyl steroids **5h** and **5i**, with 1 M hydrochrolic acid in EtOH on heating under reflux afforded the desired 6α-substituted derivatives **6a–g** in 16-56% yields, respectively (Table 1).

The configurations of the substituents at C-6 were assigned on the basis of the ^1H NMR spectroscopy. The signal of an olefinic proton at C-4 of the 6β-substituted steroids **5** appears as a singlet at δ 5.74–6.07 ppm whereas that of the 6α-isomers **6** at δ 5.19–5.83 ppm appears as a doublet ($J_{4,6}$ = 1.3 or 1.7 Hz) (Table 1). The similar C-4 proton signals have been reported in series of 6-bromo- and 6-(bromoacetoxy)androstenediones.[8a,12]

The acid-catalyzed isomerization of 6β-vinyl steroid **5h** produced 6-methylene derivative **10**. The UV (λ_{max} 279 nm) and ^1H NMR [two olefinic protons at δ 5.73 (q, J = 12.9 Hz, C=C*H*Me) and 5.84 (s, 4-H)] spectra support the assigned structure. The conformation of an olefinic proton of the methylene function was assigned by the NOE correlation results (20%) between the two olefinic protons to be oriented in the vicinity of the C-4 proton. This conformation corresponds well to the most thermodynamically stable one obtained by the PM3 calculations (Figure 2). Compound **10** would be produced through the 2,4-diene intermediate **9** (Scheme 3).

Biochemical Properties. Reversible inhibition of aromatase activity in human placental microsomes by the 6β- and 6α-substituted androstenediones (**5** and **6a–g**) along with compound **10**, synthesized in this study, was initially studied, and the results are shown in Table 2. Aromatase activity in the placental microsomes was determined by the radiometric method developed by Siiteri and Thompson[13] in which the tritium in [1β-^3H]androstenedione was transferred into water during aromatization. The amount of ^3H_2O released was used as an index of estrogen formation. IC_{50}'s for the inhibitors were first obtained, and then the inhibitors were further studied to characterize the nature of their interactions with the active site under initial velocity conditions with limiting enzyme concentration. Aromatization was measured at several concentrations of the inhibitor in the presence of increasing concentrations of androstenedione. The results of the studies were plotted in a typical Lineweaver–Burk plot. All of the inhibitors exhibited competitive-type inhibition, and the apparent inhibition constants (K_i's), an index of the affinity of the enzyme, were determined by analysis of the Dixon plot. The results for the 6β-ethyl steroid **5b** are shown in the Lineweaver–Burk plot (Figure 1).

The 6-methylene steroid **10** was also identified as a competitive inhibitor of aromatase (IC_{50} = 0.39 μM, K_i = 4.9 nM). In these studies, the apparent K_m for androstenedione was found to be 18 ± 3 nM.

The 6β-methyl (**5a**), 6β-phenyl (**5f**), 6β-vinyl (**5h**), 6β-ethynyl (**5i**), 6α-ethyl (**6b**), and 6-methylene (**10**) inhibitors did not demonstrate a time-dependent inactivation of aromatase.

Molecular Modeling. The minimum-energy conformations of all the inhibitors, assayed in this study, together with androstenedione were determined by the MOPAC package using PM3 Hamiltonian. The steroid backbone of these compounds was excellently superimposed on that of androstenedione in every case. Overlay of inhibitors, the 6-methyl, 6-ethyl, 6-*n*-propyl, 6-*n*-butyl, 6α-benzyl, 6β-vinyl, and 6-methylene steroids, showing the higher affinity for aromatase than androstenedione, is shown in Figure 2. Furthermore, Figure 3 shows the overlay of the 6-isopropyl, 6-phenyl, 6β-benzyl, and 6β-ethynyl compounds which have the lower affinity for the enzyme than the substrate. Comparison of the calculated minimum-energy conformations of the inhibitors in terms of overlap of the steroid nucleus demonstrates that there is some room available in the active site of the enzyme in the β-side, rather than α-side, around the region of C-6, of which the long axis is inclined at an angle of about 62.4° to the C-6 and C-7 edge of the substrate steroid.

Discussion

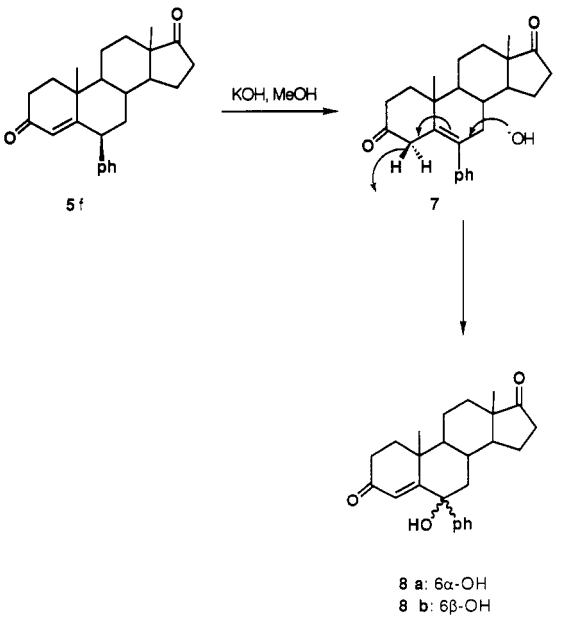
In order to define the effect of varying the C-6 substitution of androstenedione on the activity of aro-

Table 1. Pysicochemical Properties of 6-Substituted Androstenediones **5** and **6**

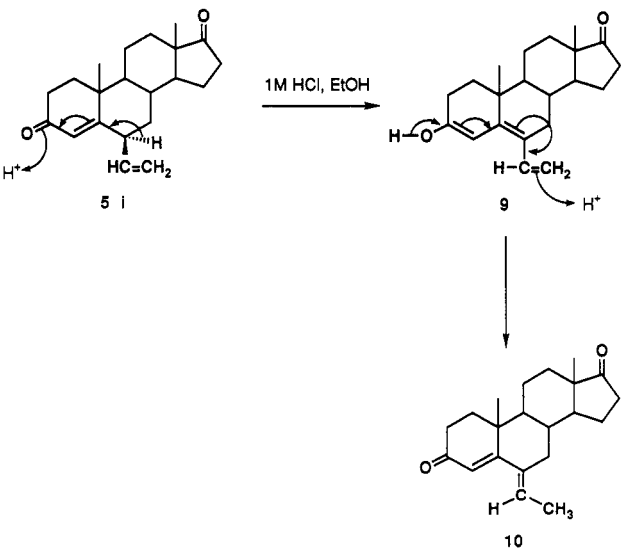
compd	R	yield, %	mp, °C	recryst solvent	formula	anal.[a]	^{1}H NMR ($CDCl_3$), δ: 18-Me	19-Me	4-H ($J_{4,6}$, Hz)	other signals	UV (EtOH), nm (ε)	IR (KBr),[b] cm^{-1}
							6β-Series					
5a	CH_3	59	176–178	acetone	$C_{20}H_{28}O_2$	C, H	0.95	1.30	5.79 (s)	1.27 (d, J = 7.7 Hz)	240.5 (16 300)	1740, 1675
5b	CH_2CH_3	75	116–117	acetone	$C_{21}H_{30}O_2$	C, H	0.90	1.25	5.75 (s)	0.96 (t, J = 9.3 Hz, 6-CH_2*Me*)	241.0 (15 700)	1739, 1676
5c	$(CH_2)_2CH_3$	71	144–145	AcOEt	$C_{22}H_{32}O_2$	C, H	0.94	1.25	5.74 (s)	0.92 (t, J = 8.8 Hz, 6-$(CH_2)_2$*Me*)	241.5 (15 500)	1739, 1676
5d	$(CH_2)_3CH_3$	74	100–102	MeOH	$C_{23}H_{34}O_2$	C, H	0.94	1.25	5.74 (s)	0.90 (t, J = 7.8 Hz, 6-$(CH_2)_3$*Me*)	242.4 (15 400)	1734, 1672
5e	$CH(CH_3)_2$	76	141–142	AcOEt	$C_{22}H_{32}O_2$	C, H	0.93	1.25	5.75 (s)	0.85 and 1.00 (d, J = 5.9 Hz, 6-CH*Me*$_2$)	243.1 (17 900)	1737, 1677
5f	C_6H_5	32	163–166	AcOEt	$C_{25}H_{30}O_2$	C, H	0.91	0.68	6.07 (s)	3.81 (d, J = 5.5 Hz, 6α-H), 7.22–7.34 (aromatic protons)	240.0 (15 100)	1738, 1677
5g	$CH_2C_6H_5$	65	197–199	AcOEt	$C_{26}H_{32}O_2$	C, H	1.00	1.38	5.72 (s)	2.44 (m, 6-C*H*$_2$Ph), 7.12–7.34 (aromatic protons)	244.5 (17 600)	1733, 1672
5h	$CH{=}CH_2$	78	175–177	AcOEt	$C_{21}H_{28}O_2$	C, H	0.93	1.20	5.84 (s)	3.21 (m, 6α-H), 5.10 (m, 6-CH=C*H*$_2$), 5.92 (m, 6-C*H*=CH$_2$)	239.5 (15 800)	1738, 1677
5i	C≡CH	34	202–204	AcOEt	$C_{21}H_{26}O_2$	C, H	0.97	1.48	5.83 (s)	2.20 (s, C≡C*H*), 3.49 (d, J = 2.6 Hz, 6α-H)	237.3 (14 600)	1735, 1685
							6α-Series					
6a	CH_3	44	167–169	AcOEt	$C_{20}H_{28}O_2$	C, H	0.92	1.22	5.81 (d, 1.7)	1.11 (d, J = 6.2 Hz, 6-Me)	239.9 (17 300)	1735, 1667
6b	CH_2CH_3	50	135–137	AcOEt	$C_{21}H_{30}O_2$	C, H	0.93	1.20	5.80 (d, 1.7)	0.96 (t, J = 7.6 Hz, 6-CH_2*Me*)	240.4 (17 100)	1731, 1666
6c	$(CH_2)_2CH_3$	26	oil[c]		$C_{22}H_{32}O_2$	exact MS	0.92	1.20	5.80 (d, 1.7)	0.94 (t, J = 7.0 Hz, 6-$(CH_2)_2$*Me*)	240.3 (16 600)	1743, 1682
6d	$(CH_2)_3CH_3$	46	oil[c]		$C_{23}H_{34}O_2$	exact MS	0.92	1.20	5.81 (d, 1.7)	0.92 (t, J = 6.7 Hz, 6-$(CH_2)_3$*Me*)	239.3 (14 700)	1739, 1675
6e	$CH(CH_3)_2$	59	135–138	AcOEt	$C_{22}H_{34}O_2$	C, H	0.93	1.20	5.83 (d, 1.7)	0.88 and 1.00 (d, J = 6.6 Hz, 6-CH*Me*$_2$)	241.6 (15 300)	1741, 1675
6f	C_6H_5	16	oil[c]		$C_{25}H_{30}O_2$	exact MS	0.96	1.36	5.19 (d, 1.7)	3.56 (m, 6β-H), 7.26–7.37 (aromatic protons)	239.5 (16 000)	1728, 1660
6g	$CH_2C_6H_5$	56	oil[c]		$C_{26}H_{32}O_2$	exact MS	0.86	1.24	5.93 (d, 1.3)	2.44 (m, 6-C*H*$_2$Ph), 7.14–7.34 (aromatic protons)	238.2 (16 600)	1737, 1677

[a] Analytical results obtained for the solid products are within ±0.4% of the theoretical value while the oily products were analyzed by exact mass spectroscopy. [b] IR spectra were obtained in KBr pellets except for the oily compounds of which spectra were obtained in neat forms. [c] Oily compounds were purified by reversed phase HPLC.

Scheme 2



Scheme 3



matase inhibition, two stereoisomeric series of the inhibitors **5** and **6** were tested, different saturated and unsaturated hydrocarbons being substituted at the C-6β and C-6α positions. Compounds **5a–d** and **6a–d**, having an *n*-alkyl group, as well as the 6α-benzyl and 6β-vinyl steroids **6g** and **5h** were very potent competitive inhibitors of aromatase in human placental microsomes with apparent K_i's ranging from 1.4 to 12 nM. On the other hand, the inhibitory activities of the 6-phenyl (**5f** and **6f**), 6β-benzyl (**5g**), and 6-isopropyl (**5e** and **6e**) derivatives, which have a bulky substituent, and the 6β-ethynyl steroid **5i** with a polar function were weaker than the above, however, still good to fair inhibitors (K_i: 37 and 21 nM for **5f** and **6f**, 63 nM for **5g**, 22 and 31 nM for **5e** and **6e**, and 62 nM for **5i**).

The 6-ethyl steroids **5b** and **6b** (K_i: 1.4 nM for **5b** and 4.7 nM for **6b**) were the most potent inhibitors in the respective series. It is surprising that the 6β-ethyl isomer **5b** binds with about 12 times the affinity of the substrate

Table 2. Aromatase Inhibition[a] by 6β- and 6α-Substituted Steroids **5** and **6**

R	IC_{50},[b] μM		K_i,[c] nM		relative K_i	
	5 (6β)	**6** (6α)	**5** (6β)	**6** (6α)	6α/6β	inhibition[c]
a, CH_3	0.72	0.35	11	5.6	0.51	competitive
b, CH_2CH_3	0.14	0.26	1.4	4.7	3.4	competitive
c, $(CH_2)_2CH_3$	0.24	0.39	4.6	6.7	1.5	competitive
d, $(CH_2)_3CH_3$	0.51	0.79	8.8	12	1.4	competitive
e, $CH(CH_3)_2$	1.1	1.4	22	31	1.4	competitive
f, C_6H_5	1.7	1.1	37	21	0.6	competitive
g, $CH_2C_6H_5$	4.0	0.66	63	10	0.16	competitive
h, $CH{=}CH_2$	0.32		5.1			competitive
i, $C{\equiv}CH$	1.9		62			competitive

[a] Inhibition type (all competitive) was determined by Lineweaver–Burk plot. [b] Substrate, [1β-^{3}H] androstenedione (1 μM); enzyme preparation, human placental microsomes (20 μg of protein); incubation time, 20 min. [c] K_i was obtained by Dixon plot in which K_m for androstenedione was 18 ± 3 nM. Human placental microsomes, 10 μg of protein; incubation time, 5 min.

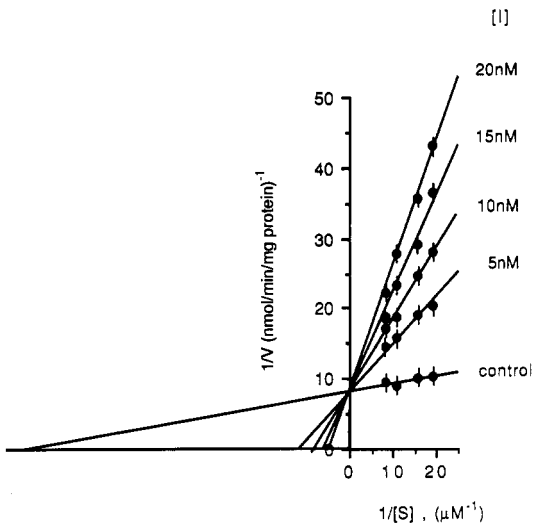


Figure 1. Lineweaver–Burk plot of inhibition of human placental aromatase by the 6β-ethyl compound **5b** with androstenedione as a substrate. Each point represents the mean value of two determinations with the range. The inhibition experiments with the other inhibitors examined in this study gave essentially similar results to Figure 1 (data not shown).

androstenedione to the enzyme (K_m = *ca.* 18 nM). 6α-Bromo-,[6] 7α-[(4′-aminophenyl)thio]-,[14] 2β,19-methylene-,[15] 3-methylene-,[16] and 3-deoxy-6α,7α-cyclopropanoandrostenediones[17] and (19*R*)-10-thiiranylestr-4-ene-3,17-dione[18] have been found to be among the most potent competitive inhibitors synthesized so far (K_i = *ca.* 1–5 nM). The inhibitory activities of the 6β-ethyl derivative **5b** as well as its 6α-isomer **6b** and the 6β-propyl (**5c**), 6β-vinyl (**5h**), and 6-methylene (**10**) compounds are comparable to those of the most potent ones.

An introduction of a methyl group at the C-6α or C-6β position of the natural substrate androstenedione gives rise to the increased affinity for aromatase. The addition of one more methylene unit to the methyl group markedly increases the affinity. In contrast, further elongation of the alkyl chain up to C_4 decreases it in proportion to its carbon number in which, however, the *n*-butyl derivatives **5d** and **6d** (K_i = 8.8 and 12 nM, respectively) have still higher affinities for the enzyme than the substrate in each series. The isopropyl derivatives **5e** and **6e** are markedly weak inhibitors compared to the corresponding *n*-propyl compounds **5c** and **6c** (K_i = 22 vs 4.6 nM for **5e** vs **5c** and 31 vs 6.7 nM for **6e** vs **6c**, respectively). Analysis of the conformations of the isopropyl function by the molecular modeling with the PM3 calculations indicates that one of two methyls of the 6-isopropyl group orients to the vicinity of the A ring of the 6β-steroids **5e** while it extends to below the plane of the steroid skeleton of the 6α-isomer **6e** in a perpendicular direction. Thus, this methyl moiety may sterically prevent access to the active site, since the geometry of the other two carbons of the substituent is very similar to that of a ethyl function of compounds **5b** and **6b**, respectively. Thus, in a series of the 6β-alkyl derivatives, the inhibitory activity decreases in the order ethyl > propyl > butyl > methyl > isopropyl, whereas in the 6α-isomer series, in the order ethyl > methyl > propyl > butyl > isopropyl. Furthermore, in view of the effect of the configuration of the C-6 substituents on the affinity, the 6β-alkyl derivatives have higher affinities than the corresponding 6α-isomers, except the 6-methyl series.

An introduction of a double bond to the 6β-ethyl moiety of steroid **5b** causes the decrease of the affinity for aromatase (K_i: 5.1 nM for the vinyl steroid **5h**). The 6β-vinyl moiety superimposes very well with the 6β-ethyl function in our modeling. This strongly suggests that an electrostatic effect rather than the steric reasons would principally be involved in the change of the affinity. The 6-methylene steroid **10** (K_i = 4.9 nM) is also an extremely potent competitive inhibitor of the enzyme. The methylene moiety locates nearly in parallel with the plane of the steroid nucleus between the 6α- and 6β-ethyl substituent regions (Figure 2).

The results demonstrate for the first time that there is the accessible volume, hydrophobic binding pocket, in the active site of aromatase in the β-side region, rather than the α-side, of the C-6 position of the substrate androstenedione. If these inhibitors bind to the active site in the same geometry as the natural substrate, the accessible size of the binding pocket would be comparable to that of four methylene units.

On the other hand, the 6β-phenyl and 6β-ethynyl groups of inhibitors **5f** and **5i** extend in a perpendicular direction to the long axis of the β-side of the steroid skeleton (Figure 3) whereas the phenyl ring of the 6β-benzyl inhibitor **5g** locates parallel to the β-side of the steroid nucleus (Figure 2). The phenyl ring of the 6α-phenyl steroid **6f** presents a dihedral angle of *ca.* 87.7° to the B-ring plane, occupying the perpendicular region of the C-6 position. These observations suggest the existence of a sterically forbidden area corresponding to those regions in the active site of aromatase and offers an explanation for the relatively weak activity. However, the aromatic ring of the 6α-benzyl derivative **6g** which has an affinity similar to that of the substrate, lies in the region of the accessible volume suggested by the 6-*n*-alkyl steroid series. These results shows a high degree of bulk tolerance in the 6α-region but a limited one in the 6β-region. The perpendicular region at C-6 especially does not tolerate the access of substituents such as ethynyl- and phenyl groups. However, not only the intrinsic steric factor but also an electronic one may be involved in the lower inhibitory activities of the ethynyl and aryl steroids.

The relative orientation of heme and steroid in the binding to aromatase and the overall shape of the active-site cavity of the enzyme have extensively been studied principally based on both the crystal structure of P-450$_{cam}$ and inhibitory activities of various inhibitors, suggesting the existence of the hydrophobic binding pocket around

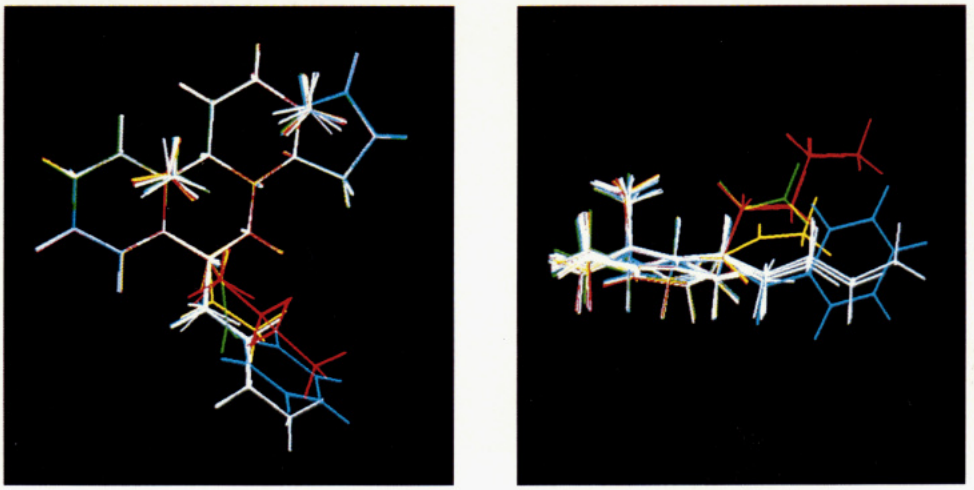


Figure 2. Overlay of 6-substituted inhibitors having a higher affinity to aromatase than androstenedione by superimposing their respective steroid nucleus. Views from the β-side (left) and from the C-3 carbonyl group (right). Red, 6β-n-alkyl steroids **5a–d**; white, 6α-n-alkyl steroids **6a–d**; blue, 6α-benzyl steroid **6g**; green, 6β-vinyl steroid **5h**; yellow, 6-methylene steroid **10**.

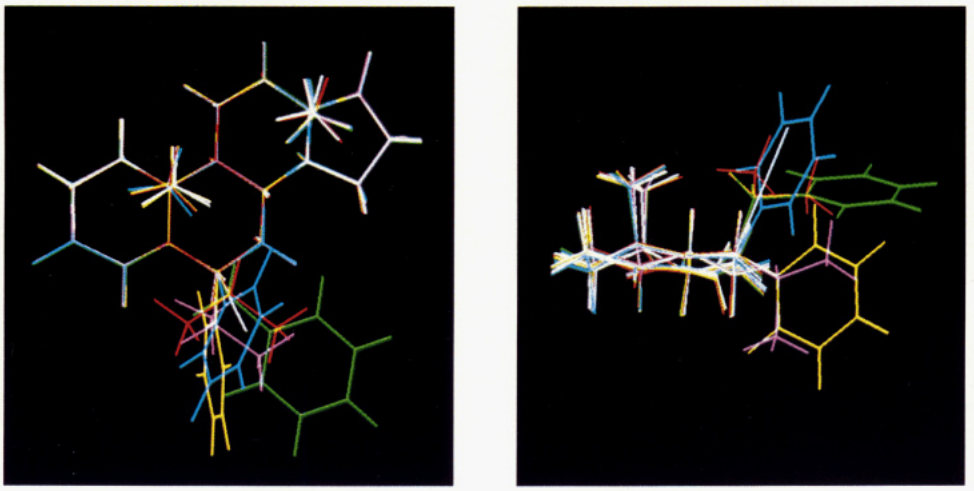


Figure 3. Overlay of 6-substituted inhibitors having a lower affinity to aromatase than androstenedione by superimposing their respective steroid nucleus. Views from the β-side (left) and from the C-3 carbonyl group (right). Red, 6β-isopropyl steroid **5e**; violet, 6α-isopropyl steroid **6e**; blue, 6β-phenyl steroid **5f**; green, 6β-benzyl steroid **5g**; yellow, 6α-phenyl steroid **6f**; white, 6β-ethynyl steroid **5i**.

the region of C-6.[19] However, Deisenhofer's group has very recently reported that substantial differences between P-450 BM-3, a bacterial fatty acid monooxygenase, and P-450$_{cam}$ are observed among the substrate binding pockets.[20] On the basis of the present results of the aromatase inhibition by various 6-substituted steroids, a delineation of the available volume around this region of the substrate is proposed as a tight enzyme pocket that can accommodate a hydrophobic 6-substituent up to 6.27 Å in length, 5.25 Å in width, and 7.74 Å in height. It should be noted that the 6β-ethyl function provides the best fit to the substrate binding pocket among those examined in this study. Although the actual geometry of binding of these inhibitors to aromatase is not directly known, fit of the 6β-ethyl group to the pocket of the active site may produce the thermodynamically stable enzyme–inhibitor complex to result in the very potent inhibition of the enzyme activity. Further study on characterizations of the binding pocket is now underway in our laboratory.

Experimental Section

Chemistry. Materials and General Methods. Melting points were measured on a Yanagimoto melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR 1725X spectrophotometer and UV spectra in 95% EtOH solutions on a Hitachi 150-20 spectrophotometer. ^{1}H NMR spectra were obtained in $CDCl_3$ solutions with JEOL GSX 400 (400 MHz) and JEOL EX 270 (270 MHz) spectrometers using tetramethylsilane as an internal standard, and mass spectra with a JEOL JMS-DX 303 spectrometer. Thin-layer chromatography was performed on E. Merck precoated silica gel plates. Column chromatography was conducted with silica gel (E. Merck, 70–230 mesh). Grignard reagents in THF solutions were purchased from Aldrich Chemical Co. High-performance liquid chromatography (HPLC) was carried out using a Waters Model 510 pump, YMC D-ODS-5 column (250 mm × 20-mm i.d.), and a UV detector (270 nm).

3,3:17,17-Bis(ethylenedioxy)androstane 5α,6α-Epoxide (2). *m*-Chloroperbenzoic acid (3.37 g, 19.5 mmol) was added to a solution of 3,3:17,17-bis(ethylenedioxy)androst-5-ene (1)[21] (5.2 g, 13.9 mmol) in CH_2Cl_2 (60 mL), and the mixture was stirred at room temperature for 4 h under dark. After this time, the mixture was washed with 10% $Na_2S_2O_3$ solution, 5% $NaHCO_3$ solution, and water, subsequently, and dried (Na_2SO_4). After removal of the solvent, the residue was subjected to column chromatography (hexane–AcOEt) to afford the 5α,6α-epoxide **2** (2.4 g, 43%) as well as its β-isomer (2.6 g, 48%) as the less polar product.[22] **2**: mp 215–216 °C; ^{1}H NMR (270 MHz) δ 0.73 (3H, s, 18-Me), 1.07 (3H, s, 19-Me), 1.19 (1H, dd, J = 14.0 and 2.0 Hz, 4α-H), 2.36 (1H, d, J = 14.0 Hz, 4β-H), 2.81 (1H, d, J = 4.0 Hz, 6β-H), 3.80–4.06 (8H, m, OCH_2CH_2O × 2). The 5β,6β-epoxide; mp 132–134.5 °C;

^{1}H NMR (270 MHz) δ 0.82 (3H, s, 18-Me), 1.00 (3H, s, 19-Me), 1.24 (1H, dd, J = 13.2 and 2.0 Hz, 4α-H), 2.33 (1H, d, J = 13.2 Hz, 4β-H), 3.07 (1H, d, J = 2.3 Hz, 6α-H), 3.81-4.00 (8H, m, OCH_2CH_2O × 2).

Grignard Reactions of the 5α,6α-Epoxide 2. To a solution of **2** (0.5 g, 1.28 mmol) in THF (20 mL) was added 20 molar equiv of Grignard reagent (RMgBr: R = methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, vinyl, benzyl, or phenyl) in THF (15 mL), and the mixture was heated under reflux for 3 h in a N_2 stream. The reaction with ethynylmagnesium bromide was carried out essentially according to the previous method[23] in which the reagent was prepared by reaction of EtMgBr with acetylene gas. After the solution was cooled, saturated NH_4Cl solution (100 mL) was added to this and the product was extracted with AcOEt (200 mL × 2). The combined organic layers were washed with water to neutrality, dried (Na_2SO_4), and evaporated to dryness leaving the residue which was purified by column chromatography (hexane–AcOEt) and/or recrystallization to yield 6β-substituted 3,3:17,17-bis(ethylenedioxy)androstan-5α-ols (**3**).

6β-Methyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (3a): yield, 98%; mp 157.5–158.5 °C (from hexane–AcOEt); IR (KBr) ν_{max} 3500 (OH) cm^{-1}; ^{1}H NMR (400 MHz) δ 0.86 (3H, s, 18-Me), 0.88 (3H, d, J = 3.3 Hz, 6-Me), 1.03 (3H, s, 19-Me), 2.99 (1H, d, J = 14.7 Hz, 4-H), 3.89–4.01 (8H, m, OCH_2CH_2O × 2). Anal. ($C_{24}H_{38}O_5$) C, H.

6β-Ethyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (3b): yield, 86% (oil); IR (neat) ν_{max} 3512 (OH) cm^{-1}; ^{1}H NMR (400 MHz) δ 0.87 (3H, s, 18-Me), 0.90 (3H, t, J = 12.1 Hz, 6-CH_2Me), 0.99 (3H, s, 19-Me), 3.06 (1H, d, J = 14.7 Hz, 4-H), 3.92–4.02 (8H, m, OCH_2CH_2O × 2); exact mass found 420.2874, calcd for $C_{25}H_{40}O_5$ 420.2876.

6β-n-Propyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (3c): yield, 87%; mp 161–162 °C (from acetone); IR (KBr) ν_{max} 3513 (OH) cm^{-1}; ^{1}H NMR (400 MHz) δ 0.88 (3H, s, 18-Me), 0.90 (3H, t, J = 8.8 Hz, 6-*n*-propyl-*Me*), 1.00 (3H, s, 19-Me), 3.90–4.02 (8H, m, OCH_2CH_2O × 2). Anal. ($C_{26}H_{42}O_5$) C, H.

6β-n-Butyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (3d): yield, 90%; mp 151–152 °C (from AcOEt); IR (KBr) ν_{max} 3515 (OH) cm^{-1}; ^{1}H NMR (270 MHz) δ 0.88 (3H, s, 18-Me), 0.89 (3H, t, J = 7.9 Hz, 6-*n*-butyl-*Me*), 1.00 (3H, s, 19-Me), 3.85–3.98 (8H, m, OCH_2CH_2O × 2). Anal. ($C_{27}H_{44}O_5$) C, H.

6β-Isopropyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (3e): yield, 55% (oil); IR (neat) ν_{max} 3515 (OH) cm^{-1};^{1}H NMR (270 MHz) δ 0.84 (3H, s, 18-Me), 0.95 (6H, d, J = 6.6 Hz, 6-CHMe_2), 1.05 (3H, s, 19-Me), 3.85–3.98 (8H, m, OCH_2CH_2O × 2); exact mass found 434.3037, calcd for $C_{28}H_{42}O_5$ 434.3032.

6β-Phenyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (3f): yield, 95% (oil); IR (neat) ν_{max} 3497 (OH) cm^{-1};^{1}H NMR (270 MHz) δ 0.70 (3H, s, 19-Me), 0.99 (3H, s, 18-Me), 3.03 (1H, d, J = 6.2 Hz, 6α-H), 3.86–4.07 (8H, m, OCH_2CH_2O × 2), 7.16–7.43 (5H, m, aromatic protons); exact mass found 468.2864, calcd for $C_{29}H_{40}O_5$ 468.2876.

6β-Benzyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (3g): yield, 91% (oil); IR (neat) ν_{max} 3477 (OH) cm^{-1}; ^{1}H NMR (270 MHz) δ 0.92 (3H, s, 18-Me), 1.11 (3H, s, 19-Me), 2.36 (2H, m, CH_2Ph), 3.82–4.05 (8H, m, OCH_2CH_2O × 2), 7.08–7.38 (5H, m, aromatic protons); exact mass found 482.3048, calcd for $C_{30}H_{42}O_5$ 482.3032.

6β-Vinyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (3h): yield, 91% (oil); IR (neat) ν_{max} 3489 (OH) cm^{-1}; ^{1}H NMR (270 MHz) δ 0.86 (3H, s, 18-Me), 0.97 (3H, s, 19-Me), 3.84–4.01 (8H, m, OCH_2CH_2O × 2), 4.95–5.04 (2H, m, 6-CH=CH_2), 5.98 (1H, m, 6-CH=CH_2); exact mass found 418.2719, calcd for $C_{25}H_{38}O_5$ 418.2766.

6β-Ethynyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (3i): yield, 89%; mp 216–217 °C; IR (KBr) ν_{max} 3469 (OH), 2366 (C≡C) cm^{-1}; ^{1}H NMR (270 MHz) δ 0.91 (3H, s, 18-Me), 1.26 (3H, s, 19-Me), 2.12 (1H, s, 6-C≡CH), 3.93–4.03 (8H, m, OCH_2CH_2O × 2). Anal. ($C_{25}H_{36}O_5$) C, H.

Hydrolysis of Bis(ethylenedioxy) Steroids 3. $HClO_4$ (3 M) (4 mL) was added to a solution of compound **3** (1.2 mmol) in THF (10 mL), and the reaction mixture was stirred at room temperature for 3 h. After this time, the mixture was diluted with AcOEt (150 mL), washed with 5% $NaHCO_3$ solution and water, dried (Na_2SO_4), and evaporate to give the residue of which column chromatography followed by recrystallization yielded the corresponding 3,17-diketone **4**.

6β-Methyl-5α-hydroxyandrostane-3,17-dione (4a): yield, 97%; mp 172–174 °C (from acetone); IR (KBr) ν_{max} 3416 (OH), 1741 and 1703 (C=O) cm^{-1}; ^{1}H NMR (400 MHz) δ 0.92 (3H, s, 18-Me), 1.12 (3H, d, J = 7.7 Hz, 6-Me), 1.28 (3H, s, 19-Me). Anal. ($C_{20}H_{30}O_3$) C, H.

6β-Ethyl-5α-hydroxyandrostane-3,17-dione (4b): yield, 95%; mp 189–192 °C (from acetone); IR (KBr) ν_{max} 3412 (OH), 1739 and 1704 (C=O) cm^{-1}; ^{1}H NMR (400 MHz) δ 0.91 (3H, s, 18-Me), 0.94 (3H, t, J = 7.3 Hz, 6-CH_2Me), 1.21 (3H, s, 19-Me). Anal. ($C_{21}H_{32}O_3$) C, H.

6β-n-Propyl-5α-hydroxyandrostane-3,17-dione (4c): yield, 96%; mp 175–177 °C (from AcOEt); IR (KBr) ν_{max} 3435 (OH), 1744 and 1717 (C=O) cm^{-1}; ^{1}H NMR (400 MHz) δ 0.91 (3H, s, 18-Me), 0.92 (3H, t, J = 7.4 Hz, 6-*n*-propyl-*Me*), 1.22 (3H, s, 19-Me). Anal. ($C_{22}H_{34}O_3$) C, H.

6β-n-Butyl-5α-hydroxyandrostane-3,17-dione (4d): yield, 85%; mp 186–188 °C (from AcOEt): IR (KBr) ν_{max} 3441 (OH), 1746 and 1710 (C=O) cm^{-1}; ^{1}H NMR (270 MHz) δ 0.91 (3H, s, 18-Me), 0.93 (3H, t, J = 6.9 Hz, 6-*n*-butyl-*Me*), 1.22 (3H, s, 19-Me). Anal. ($C_{23}H_{36}O_3$) C, H.

6β-Isopropyl-5α-hydroxyandrostane-3,17-dione (4e): yield, 65%; mp 198–201 °C (from AcOEt); IR (KBr) ν_{max} 3448 (OH), 1739 and 1702 (C=O) cm^{-1}; ^{1}H NMR (270 MHz) δ 0.90 (3H, s, 18-Me), 1.00 and 1.02 (3H, each, d, J = 7.6 Hz, 6-CHMe_2), 1.29 (3H, s, 19-Me). Anal. ($C_{22}H_{34}O_3$) C, H.

6β-Phenyl-5α-hydroxyandrostane-3,17-dione (4f): yield, 98%; mp 214–216 °C (from AcOEt); IR (KBr) ν_{max} 3452 (OH), 1736 and 1714 (C=O) cm^{-1}; ^{1}H NMR (270 MHz) δ 0.97 (3H, s, 19-Me), 1.02 (3H, s, 18-Me), 2.92 (1H, d, J = 6.6 Hz, 6α-H), 7.21–7.42 (5H, m, aromatic protons). Anal. ($C_{25}H_{32}O_3$) C, H.

6β-Benzyl-5α-hydroxyandrostane-3,17-dione (4g): yield, 94%; mp 211–214 °C; IR (KBr) ν_{max} 3437 (OH), 1746 and 1712 (C=O) cm^{-1}; ^{1}H NMR (270 MHz) δ 0.97 (3H, s, 18-Me), 1.35 (3H, s, 19-Me), 2.41 (2H, m, 6-CH_2Ph), 7.10–7.34 (5H, m, aromatic protons). Anal. ($C_{26}H_{34}O_3$) C, H.

6β-Vinyl-5α-hydroxyandrostane-3,17-dione (4h): yield, 76%; mp 189–190 °C (from AcOEt): IR (KBr) ν_{max} 3410 (OH), 1740 and 1703 (C=O) cm^{-1}; ^{1}H NMR (270 MHz) δ 0.91 (3H, s, 18-Me), 1.22 (3H, s, 19-Me), 5.06 (2H, m, 6-CH=CH_2), 6.04 (1H, m, 6-CH=CH_2). Anal. ($C_{21}H_{30}O_3$), C, H.

6β-Ethynyl-5α-hydroxyandrostane-3,17-dione (4i): yield, 98%; mp 226–228 °C (from acetone); IR (KBr) ν_{max} 3441 (OH), 2361 (C≡C), 1729 and 1707 (C=O) cm^{-1}; ^{1}H NMR (400 MHz) δ 0.94 (3H, s, 18-Me), 1.48 (3H, s, 19-Me), 2.20 (1H, s, C≡CH). Anal. ($C_{21}H_{28}O_3$) C, H.

Dehydration of 5α-Hydroxy Steroids 4. Thionyl chloride (0.55 mL) was added to a chilled solution of compound **4** (0.96 mmol) in dry pyridine (6 mL), and the mixture was stirred for 3 min at 0 °C, poured into 50 mL of ice–water, and extracted with AcOEt (50 mL × 2). The combined organic layers were washed with water, dried (Na_2SO_4), and evaporated to afford the crude product which was purified by column chromatography (hexane–AcOEt) and recrystallization, giving the corresponding 4-ene-3,17-dione **5**, respectively.

Isomerization of 6β-Substituted Steroids 5 to Their 6α-Derivatives 6. Compound **5** (0.19 mmol) was dissolved in 95% EtOH (3 mL), 1 M HCl (0.3 mL) was added to the solutions, and the mixtures were heated under reflux for 3 h. After removing most of the solvent, the mixture was diluted with AcOEt (100 mL), washed with 5% $NaHCO_3$ solution and water, dried (Na_2SO_4), and evaporated. The residues were subjected to column chromatography followed by recrystallization or HPLC to afford the corresponding 6α-substituted 4-ene-3,17-dione **6**.

6α-n-Propylandrost-4-ene-3,17-dione (6c): HPLC, t_R 22.9 min (acetonitrile–water, 4/1, 7.0 mL/min) (t_R of the 6β-isomer **5c**, 21.2 min); exact mass found 328.2412, calcd for $C_{22}H_{32}O_2$ 328.2417.

6α-n-Butylandrost-4-ene-3,17-dione (6d): HPLC, t_R 29.6 min (acetonitrile–water, 4/1, 7 mL/min) (t_R of the 6β-isomer **5d**, 26.4 min); exact mass found 342.2559, calcd for $C_{23}H_{34}O_2$ 342.2557.

6α-Phenylandrost-4-ene-3,17-dione (6f): HPLC, t_R 24.7 min (MeOH–water, 85/15, 5 mL/min) (t_R of the 6β-isomer **5f**, 22.5 min); exact mass found 362.2245, calcd for $C_{25}H_{30}O_2$ 362.2247.

6α-Benzylandrost-4-ene-3,17-dione (6g): HPLC, t_R 24.4 min (acetonitrile–water, 3/1, 6 mL/min) (t_R of the 6β-isomer **5g**, 28 min); exact mass found 376.2411, calcd for $C_{26}H_{32}O_2$ 376.2402.

6-Ethyleneandrost-4-ene-3,17-dione (10): yield, 78% from **5h**; mp 130–133 °C (from AcOEt); IR (KBr) ν_{max} 1738 and 1672 (C=O) cm^{-1}; UV λ_{max} (ε) 279.3 nm (10 200); ^1H NMR (400 MHz) δ 0.92 (3H, s, 18-Me), 1.08 (3H, s, 19-Me), 5.73 (1H, q, J = 12.6 Hz, C=C*H*Me), 5.84 (1H, s, 4-H). Anal. ($C_{21}H_{28}O_2$) C, H.

Treatment of Compound 5f with KOH in MeOH. Compound **5f** (50 mg, 0.138 mmol) was dissolved in 15 mL of 1% methanolic KOH solution, and the reaction mixture was allowed to stand at room temperature for 3 days in N_2 gas. After neutralization with 1 M HCl, the mixture was condensed under reduced pressure to about 5 mL, diluted with AcOEt (100 mL), washed with 5% $NaHCO_3$ and saturated NaCl solutions subsequently, and then dried (Na_2SO_4). Evaporation of the solvent yielded the crude product which was subjected to column chromatography to afford two isomeric 6-phenyl 6-ols 8.

6β-Phenyl-6α-hydroxyandrost-4-ene-3,17-dione (8a): yield, 33%; mp 248–253 °C (from acetone); IR (KBr) ν_{max} 3416 (OH), 1730 and 1604 (C=O) cm^{-1}; UV λ_{max} (ε) 244.5 nm (15 900); ^1H NMR (270 MHz) δ 0.57 (3H, s, 19-Me), 0.89 (3H, s, 18-Me), 2.91 (1H, dd, J = 13.5 and 3.0 Hz, 7β-H), 6.78 (1H, s, 4-H), 7.39–7.47 (5H, m, aromatic protons). Anal. ($C_{25}H_{30}O_3$) C, H.

6α-Phenyl-6β-hydroxyandrost-4-ene-3,17-dione (8b): yield, 15%; mp 190–194 °C (from AcOEt); IR (KBr) ν_{max} 3436 (OH), 1736 and 1656 (C=O) cm^{-1}; UV λ_{max} (ε) 232 nm (11 300); ^1H NMR (270 MHz) δ 0.98 (3H, s, 18-Me), 1.57 (3H, s, 18-Me), 5.39 (1H, s, 4-H), 7.28–7.36 (5H, m, aromatic protons). Anal. ($C_{25}H_{30}O_3$) C, H.

Biochemical Studies. Chemicals. [1β-^{3}H]Androstenedione (24.1 Ci/mmol) (^3H distribution: β/α = 69.8/30.2) was purchased from New England Nuclear Corp. (Boston, MA) and NADPH from Kohjin Co., Ltd. (Tokyo, Japan).

Enzyme Preparation. Human placental mirosomes (particles sedimenting at 105000g for 60 min) were obtained as described by Ryan.[24] They were washed once with 0.05 mM dithiothreitol solution, lyophilized, and stored at −20 °C. No significant loss of activity occurred over the period of the study.

Aromatase Assay Procedure. Aromatase activity was measured according to the original procedure of Thompson and Siiteri.[13] The screening assay and time-dependent assay procedures are principally the same as those described in our previous work[23] in which, however, 20 μg of protein of the lyophilized microsomes and a 20-min incubation time for the screening assay and 10 μg of protein of the microsomes and 5-min incubation time for the kinetic assay, respectively, were employed in this study.

Molecular Modeling 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 version 6, Quantum Chemistry Program No. 455) using the 3D graphic option of the MOLGRAPH software (Daikin, Tokyo, Japan). 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. There is an energy barrier between the other minimum-energy conformation in the 6β-substituted steroids **5b**, **5c**, **5d**, and **5g** (6.13, 3.31, 3.28, and 7.18 kcal/mol, respectively) and in the 6α-isomers **6e** (7.32 kcal/mol) and **6g** (7.73 kcal/mol), while there are two energy barriers ranging from 2.46 to 12.69 kcal/mol in the 6α-substituted compounds **6b**, **6c**, and **6d**. On the other hand, a single, broad potential energy well is observed in compounds **5e**, **5f**, **6f**, and **6g**. Low-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.

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