Synthesis and Structure−Activity Relationships of 6-Substituted Androst-4-ene Analogs as Aromatase Inhibitors

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Series of 6α- and 6*β*-alkyl-substituted androst-4-en-17-ones (**18** and **19**) and their 17*β*-reduced derivatives (**14** and **15**) (alkyl: methyl, ethyl, *n*-propyl, *n*-pentyl, *n*-octyl) were synthesized and evaluated as aromatase inhibitors. Androst-4-en-17-ones having an oxygen function (hydroxy, acetoxy, or methoxy group) at C-6α and C-6*β* (**4** and **5**) were also tested for their abilities to inhibit aromatase. All of the steroids studied inhibited human placental aromatase in a competitive manner. The inhibitory activities of the 6α- and 6*β*-methyl-17-keto steroids **18a** and **19a** (K_i = 3.1 and 5.3 nM, respectively) as well as the 6*β*-alcohol **5a** (K_i = 6.0 nM) were high, and their apparent K_i values were lower than that of the parent 6-unsubstituted 3-deoxy steroid **1** (K_i = 6.8 nM). Elongation of the methyl group decreased affinity for aromatase in relation to carbon number of the alkyl chain in each series, in which the 6α-alkyl steroids **18** essentially had higher affinity for the enzyme than the corresponding 6*β*-isomers **19**. The inhibitory activities of the 17*β*-hydroxy analogs **14** and **15** were less potent than those of the corresponding 17-keto steroids. The 6α-ethyl compound **18b**, the 6α-oxygenated derivatives **4**, and the 6*β*-acetoxy and 6*β*-methoxy analogs **5b** and **5c** were powerful inhibitors (K_i = 12−24 nM). The methyl steroids (**18a** and **19a**) produced "type I" difference spectra upon interaction with aromatase. These results along with molecular modeling with the PM3 method suggest that compounds **18a** and **19a** may produce a thermodynamically stable enzyme−inhibitor complex in the hydrophobic binding pocket with a limited accessible volume. A carbonyl group at C-17 of the 6-alkylandrost-4-enes is essential for the tight binding. Moreover, the binding pocket also tolerates a polar hydroxy group at the 6*β*-position rather than at the 6α-position.

Aromatase is a unique cytochrome P-450 enzyme complex which catalyzes the synthesis of estrone and estradiol from 4-en-3-one androgens androst-4-ene-3,-17-dione (androstenedione) and testosterone.[1] Inhibitors of aromatase have recently become of interest in the treatment of advanced estrogen-dependent breast cancer.[2] A number of potent aromatase inhibitors, which are analogs of the substrate androstenedione, have been described,[2d,e,3] including 4-hydroxy,[4] 19-ethynyl,[5] 6-methylene-Δ^1,[6] and 1-methyl-Δ^1 derivatives[7] of androstenedione which are now under clinical trials.

Recently, we synthesized 6-alkyl (C-1 to C-8)-androstenediones (**6** and **7**) as well as 6-aryl (phenyl and benzyl) analogs,[3h,8] some of which are among the most potent competitive steroidal inhibitors of aromatase reported so far.[3] The structure−activity relationships obtained using these steroids have revealed that aromatase has a hydrophobic binding pocket with a limited accessible volume in the region corresponding to the *β*-side rather than the α-side of the C-6 position of the substrate androstenedione. On the other hand, it has been found that the 3-deoxy derivatives of androstenedione, androst-4-en-17-one (**1**)[9] and its 6α,7α-cyclopropano analog **3**[10] (Figure 1), are excellent competitive inhibitors, although they are lacking a carbonyl group at C-3 which is thought to be essential for a proper binding of the substrate to the active site of aromatase. Moreover, the structure−activity relationships of 16- and 19-substituted androst-4-enes have indicated that a 17-carbonyl function is necessary for effective binding of the 3-deoxy steroids to the active site.[11]

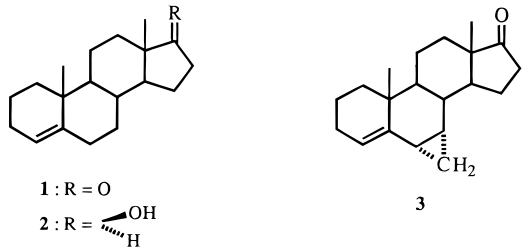

Figure 1. Structures of 3-deoxy steroids.

As a continuing study of the 4-ene steroids as aromatase inhibitors, we were interested in steroids which have a structure combining the 4-ene grouping with an alkyl substituent or an oxygen function at C-6α or C-6*β*. Studies described in this paper focus on the synthesis and biochemical evaluation of 6-alkylandrost-4-en-17-ones (**18** and **19**) (alkyl: methyl, ethyl, *n*-propyl, *n*-pentyl, and *n*-octyl) as well as their corresponding 17*β*-hydroxy derivatives **14** and **15.** Biochemical evaluation of androst-4-en-17-ones **4** and **5** having an oxygen function (hydroxy, acetoxy, or methoxy group) at C-6 is also described. The methyl steroids **18a** and **19a** and the 6*β*-hydroxy steroid **5a** were very potent competitive inhibitors. A 17-carbonyl function is necessary for a tight binding of the 6-alkyl-4-ene steroids to the active site of aromatase.

Results

Chemistry. 6-Methoxy steroids **4c** and **5c** were prepared by treatment of the corresponding hydroxy

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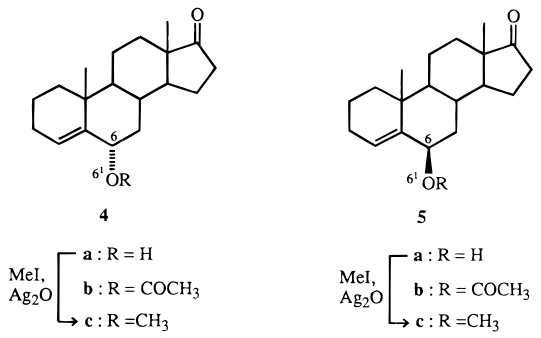
Scheme 1

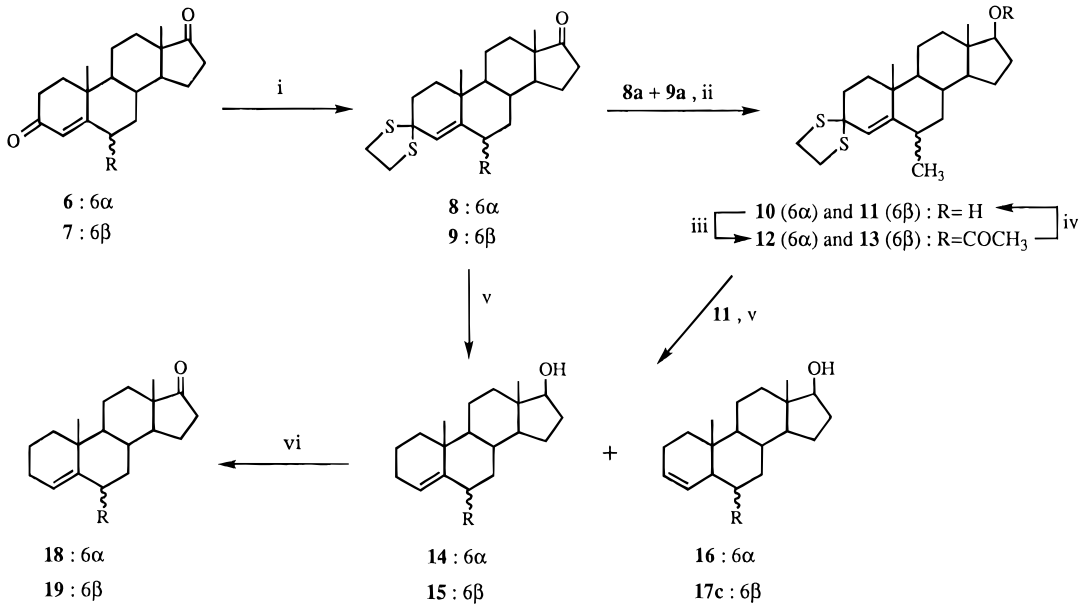


steroids **4a** and **5a**[12] synthesized previously, with CH_3I and Ag_2O under reflux (Scheme 1).

Treatment of 6α- and 6β-alkylated (alkyl: methyl, ethyl, *n*-propyl, *n*-pentyl, and *n*-octyl) androst-4-ene-3,-17-diones (**6** and **7**), which were synthesized according to the methods[3h,8] previously reported, with ethane-1,2-dithiol in the presence of p-toluenesulfonic acid gave the corresponding 3,3-ethylene dithioacetals **8** and **9** in moderate yields (Scheme 2, Table 1). The relatively low yields of the 6β-alkyl steroids **9** were principally due to the partial isomerization (about 20−50%) of the axial alkyl group to the thermodynamically more stable 6α-isomers **8**. The 6β-isomers **9** except the 6β-methyl analog **9a** were isolated from the mixtures of stereoisomeric products by recrystallization and/or reverse-phase HPLC (C_{18} column, MeCN−H_2O). Since the methyl steroid **9a** could not be obtained in a pure form by these separation methods, a *ca.* 1:1 mixture of the 6α- and 6β-methyl compounds **8a** and **9a** was, without further purification, converted into the 17β-acetates **12** and **13** upon treatment with $NaBH_4$ followed by acetylation with acetic anhydride and pyridine. Reverse-phase HPLC (C_{18} column, MeCN−H_2O) was effective for separation of the 6β-methyl acetate **13** from the 6α-isomer **12**. The acetates **12** and **13** were backed to the corresponding 17β-ols **10** and **11** upon treatment with NaOH. Desulfurization of the thioacetals **8**, **9b**−**e**, and **11** with Na metal−liquid NH_3[9,13] yielded the corresponding 6-alkylandrost-4-en-17β-ols **14** and **15** in 30−64% yields. Production of their 3-ene isomers **16** was observed in the reaction with the 6α-alkyl steroids **8** (20−40% yields), based on ^{1}H-NMR spectra of the products (signals of the C-3 double bond: 5.47−5.57 and 5.67−5.74 ppm), similar to the previous report,[13] whereas the 3-ene isomers **17** except the 6β-propyl analog **17c** were not produced in the reaction with the 6β-alkyl steroids **9b**−**e** and **11**. The desulfurized steroids **14a**−**c** and **15** were isolated effectively by recrystallization and/or reverse-phase HPLC (C_{18} column, MeOH−H_2O). On the other hand, selective catalytic reduction of the 3-ene products with Pd on charcoal followed by multiply developed TLC gave a successful result in isolation of the 6α-pentyl and 6α-octyl 4-ene analogs **14d** and **14e**. The long alkyl (pentyl and octyl) chains at C-6 would sterically block access of the catalysis to the vicinity of a C-4−C-5 bond, allowing the selective reduction of a 3-ene group. Oxidation of the 17-ols **14** and **15** with Jones reagent yielded the 6-alkylandrost-4-en-17-ones (**18** and **19**) in good yields.

Biochemical Properties. Inhibition of aromatase activity in human placental microsomes by the 6α- and 6β-alkyl steroids **18** and **19** and their 17β-ol derivatives **14** and **15** as well as the 6-hydroxy analogs **4** and **5** was examined in vitro by enzyme kinetics. The results are shown in Tables 2 and 3. In addition to the above compounds, the parent 3-deoxyandrostenedione (**1**) and its 17β-ol analog **2** are listed for comparision. Aromatase activity in the placental microsomes was determined using a radiometric assay[14] in which tritiated water released from [1β-^{3}H]androstenedione into the incubation medium during aromatization was measured. In order to characterize the nature of inhibitor binding to the active site of aromatase, aromatization

Scheme 2[a]



R : **a** = CH_3 ; **b** = CH_2CH_3 ; **c** = $(CH_2)_2CH_3$; **d** = $(CH_2)_4CH_3$;
e = $(CH_2)_7CH_3$

[a] Reagents: (i) ethane-1,2-dithiol, CH_3COOH or THF, *p*-TsOH; (ii) $NaBH_4$, MeOH; (iii) $(CH_3CO)_2O$, pyridine; (iv) KOH, MeOH; (v) Na metal, liquid NH_3, MeOH or EtOH; (vi) Jones reagent, acetone.

Table 1. Physicochemical Properties of 6-Substituted Androst-4-enes

								^{1}H NMR ($CDCl_3$), δ			
compd	R	yield, %	mp, °C	recryst solvent	formula	anal.[a]	IR,[b] cm^{-1}	18-Me	19-Me	4-H	other signals
					6α-Series						
4c	OCH_3	41	138−139	acetone	$C_{20}H_{30}O_2$	C, H	1735	0.89	1.03	5.67 (t, J = 2.6 Hz)	3.42 (s, 6-OMe), 3.69 (m, 6β-H)
8a	CH_3	59	164−167	AcOEt	$C_{22}H_{32}OS_2$	C, H, S	1737	0.89	1.05	5.48 (s)	1.04 (d, J = 6.6 Hz, 6-Me), 3.19−3.43 (m, $-S(CH_2)_2S-$)
8b	CH_2CH_3	76	122−123	AcOEt	$C_{23}H_{34}OS_2$	C, H, S	1737	0.89	1.04	5.48 (s)	0.95 (t, J = 7.3, 7.6 Hz, 6-CH_2*Me*), 3.13−3.46 (m, $-S(CH_2)_2S-$)
8c	$(CH_2)_2CH_3$	69	125−126	AcOEt	$C_{24}H_{36}OS_2$	C, H, S	1733	0.89	1.04	5.48 (s)	0.93 (t, J = 6.9, 7.3 Hz, 6-$(CH_2)_2$*Me*), 3.34−3.45 (m, $-S(CH_2)_2S-$)
8d	$(CH_2)_4CH_3$	76	49−51	acetone	$C_{26}H_{40}OS_2$	C, H, S	1737	0.89	1.04	5.48 (s)	0.90 (t, J = 6.6 Hz, 6-$(CH_2)_4$*Me*), 3.36−3.41 (m, $-S(CH_2)_2S-$)
8e	$(CH_2)_7CH_3$	66	oil		$C_{29}H_{46}OS_2$	exact MS	1740	0.89	1.04	5.48 (s)	0.89 (t, J = 6.6 Hz, 6-$(CH_2)_7$*Me*), 3.33−3.44 (m, $-S(CH_2)_2S-$)
14a	CH_3	30	139−141	AcOEt	$C_{20}H_{32}O$	C, H	3306	0.76	1.02	5.29 (d, J = 2.3 Hz)	0.96 (d, J = 6.6 Hz, 6-Me), 3.62 (t, J = 8.6 Hz, 17α-H)
14b	CH_2CH_3	32	168−169	AcOEt	$C_{21}H_{34}O$	C, H	3235	0.77	1.01	5.29(t, J = 2.0 Hz)	0.90 (t, J = 7.3 Hz, 6-CH_2*Me*), 3.62 (t, J = 8.3, 8.6 Hz, 17α-H)
14c	$(CH_2)_2CH_3$	30	oil		$C_{22}H_{36}O$	exact MS	3435	0.77	1.02	5.29 (t, J = 2.0 Hz)	0.90 (t, J = 6.9, 7.2 Hz, 6-$(CH_2)_2$*Me*), 3.62 (t, J = 8.3, 8.6 Hz, 17α-H)
14d	$(CH_2)_4CH_3$	32	oil		$C_{24}H_{40}O$	exact MS	3273	0.77	1.01	5.29 (t, J = 2.3, 2.6 Hz)	0.89 (t, J = 5.0, 6.9 Hz, 6-$(CH_2)_4$*Me*), 3.62 (t, J = 8.3, 8.6 Hz, 17α-H)
14e	$(CH_2)_7CH_3$	57	oil		$C_{27}H_{46}O$	exact MS	3306	0.77	1.01	5.29 (t, J = 2.3, 2.6 Hz)	0.88 (t, J = 6.3, 6.9 Hz, 6-$(CH_2)_7$*Me*), 3.62 (t, J = 8.3, 8.6 Hz, 17α-H)
18a	CH_3	80	134−137	acetone	$C_{20}H_{30}O$	C, H	1743	0.90	1.04	5.33 (t, J = 2.1, 2.3 Hz)	0.99 (d, J = 6.6 Hz, 6-Me)
18b	CH_2CH_3	93	80−81	hexane	$C_{21}H_{32}O$	C, H	1738	0.90	1.03	5.31 (t, J = 2.6 Hz)	0.94 (t, J = 7.3 Hz, 6-CH_2*Me*)
18c	$(CH_2)_2CH_3$	91	87	acetone	$C_{22}H_{34}O$	C, H	1732	0.90	1.03	5.32 (t, J = 2.3, 2.6 Hz)	0.92 (t, J = 7.3 Hz, 6-$(CH_2)_2$*Me*)
18d	$(CH_2)_4CH_3$	90	oil		$C_{24}H_{38}O$	exact MS	1742	0.90	1.03	5.32 (t, J = 2.6 Hz)	0.90 (t, J = 5.6, 7.9 Hz, 6-$(CH_2)_4$*Me*)
18e	$(CH_2)_7CH_3$	85	oil		$C_{27}H_{44}O$	exact MS	1740	0.89	1.03	5.32 (t, J = 2.6 Hz)	0.89 (t, J = 6.9 Hz, 6-$(CH_2)_7$*Me*)
					6β-Series						
5c	OCH_3	21	185−186	MeOH	$C_{20}H_{30}O_2$	C, H	1737	0.90	1.12	5.57 (dd, J = 2.3, 5.0 Hz)	3.17 (s, 6-OMe), 3.56 (t, J = 2.6 Hz, 6α-H)
9b	CH_2CH_3	42	134−137	AcOEt	$C_{23}H_{34}OS_2$	C, H, S	1738	0.90	1.08	5.51 (s)	0.87 (t, J = 6.3, 7.3 Hz, 6-CH_2*Me*), 3.18−3.43 (m, $-S(CH_2)_2S-$)
9c	$(CH_2)_2CH_3$	29	67−70	acetone	$C_{24}H_{36}OS_2$	exact MS	1732	0.90	1.09	5.50 (s)	0.91(t, J = 6.9, 7.3 Hz, 6-$(CH_2)_2$*Me*), 3.39−3.45 (m, $-S(CH_2)_2S-$)
9d	$(CH_2)_4CH_3$	42	109−111	ether	$C_{26}H_{40}OS_2$	C, H, S	1740	0.90	1.08	5.50 (s)	0.90 (t, J = 5.6, 8.0 Hz, 6-$(CH_2)_4$*Me*), 3.20−3.28 (m, $-S(CH_2)_2S-$)
9e	$(CH_2)_7CH_3$	46	oil		$C_{29}H_{46}OS_2$	exact MS	1741	0.90	1.08	5.50 (s)	0.93 (t, J = 6.6 Hz, 6-$(CH_2)_7$*Me*), 3.34−3.46 (m, $-S(CH_2)_2S-$)
15a	CH_3	54	semisolid	acetone[c]	$C_{20}H_{32}O$	C, H	3294	0.79	1.10	5.33 (d, J = 3.6 Hz)	1.11 (d, J = 7.6 Hz, 6-Me), 3.64 (t, J = 8.6 Hz, 17α-H)
15b	CH_2CH_3	59	122−124	AcOEt	$C_{21}H_{34}O$	C, H	3424	0.77	1.05	5.29 (t, J = 3.6 Hz)	0.82 (t, J = 7.2, 7.3 Hz, 6-CH_2*Me*), 3.63 (dd, J = 2.6, 16.8 Hz, 17α-H)
15c	$(CH_2)_2CH_3$	30	semisolid	acetone[c]	$C_{22}H_{36}O$	C, H	3396	0.78	1.06	5.28 (t, J = 3.6 Hz)	0.88 (t, J = 6.9, 7.3 Hz, 6-$(CH_2)_2$*Me*), 3.64 (t, J = 8.3, 8.6 Hz, 17α-H)
15d	$(CH_2)_4CH_3$	53	73−76	hexane	$C_{24}H_{40}O$	C, H	3400	0.78	1.05	5.28 (t, J = 3.6 Hz)	0.88 (t, J = 6.6, 6.9 Hz, 6-$(CH_2)_4$*Me*), 3.64 (t, J = 8.6 Hz, 17α-H)
15e	$(CH_2)_7CH_3$	64	oil		$C_{27}H_{46}O$	exact MS	3369	0.78	1.05	5.28 (t, J = 3.6 Hz)	0.88 (t, J = 5.0, 6.9 Hz, 6-$(CH_2)_7$*Me*), 3.63 (t, J = 8.3, 8.6 Hz, 17α-H)
19a	CH_3	90	157−162	AcOEt	$C_{20}H_{30}O$	C, H	1742	0.92	1.11	5.36 (t, J = 3.6, 4.0 Hz)	1.15 (d, J = 7.6 Hz, 6-Me)
19b	CH_2CH_3	92	98−101	AcOEt	$C_{21}H_{32}O$	C, H	1741	0.90	1.06	5.32 (t, J = 3.6 Hz)	0.85 (t, J = 7.3 Hz, 6-CH_2*Me*)
19c	$(CH_2)_2CH_3$	95	83−84	AcOEt	$C_{22}H_{34}O$	C, H	1732	0.91	1.07	5.31 (t, J = 3.3, 4.0 Hz)	0.90 (t, J = 6.9, 7.3 Hz, 6-$(CH_2)_2$*Me*)
19d	$(CH_2)_4CH_3$	94	oil		$C_{24}H_{38}O$	exact MS	1742	0.91	1.06	5.31 (t, J = 3.6 Hz)	0.89 (t, J = 6.3, 7.3 Hz, 6-$(CH_2)_4$*Me*)
19e	$(CH_2)_7CH_3$	86	oil		$C_{27}H_{44}O$	exact MS	1742	0.91	1.06	5.31 (t, J = 3.6 Hz)	0.89 (t, J = 5.1, 6.6 Hz, 6-$(CH_2)_7$*Me*)

[a] Analytical results obtained for the solid products were 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* Trituration solvent.

was measured at several inhibitor and substrate concentrations. The results of these studies were plotted on a typical Lineweaver−Burk plot.

All the steroids exhibited clear-cut competitive inhibition. The apparent inhibition constants (K_i), which characterize enzyme affinity, were obtained by Dixon plots. The Lineweaver−Burk plot of aromatase inhibition by steroid **18a** is shown in Figure 2. In these studies, the apparent K_m for the substrate androstenedione was found to be about 33 nM.

The nature of the complexes formed between the 4-ene steroids **1**, **18a**, and **19a** and microsomal aromatase P-450 was studied using UV−vis difference spectroscopy. Upon interaction with the P-450, they produced a so-called type I difference spectrum characterized by an absorption maximum at 390−392 nm and an absorption minimum at 418−422 nm, in each, similar to androstenedione (390 and 420 nm, respectively).

Molecular Modeling. The minimum-energy conformations of all the 17-keto steroids **4**, **5**, **18**, and **19**, except the *n*-octyl analogs **18e** and **19e**, assayed in this study were determined by the MOPAC package using Hamiltonian. The results indicated that the 6-*n*-alkyl

Table 2. Aromatase Inhibition by 6α- and 6β-Substituted 17-Keto Steroids[a]

R	IC_{50},[b] μM 6α	IC_{50},[b] μM 6β	apparent K_i,[c] nM 6α	apparent K_i,[c] nM 6β	relative K_i 6α/6β
6-Alkyl Series **18** and **19**					
a, CH_3	0.037	0.049	3.1	5.3	0.6
b, CH_2CH_3	0.11	0.32	14	43	0.3
c, $(CH_2)_2CH_3$	0.23	0.44	30	51	0.6
d, $(CH_2)_4CH_3$	9	12	400	1200	0.3
e, $(CH_2)_7CH_3$	>50 (6.5)[d]	>50 (3.0)[d]	4100	2600	1.6
6-Hydroxy Analogs **4** and **5**					
a, OH	0.19	0.050	21	6.0	3.5
b, $OCOCH_3$	0.16	0.26	18	24	0.8
c, OCH_3	0.12	0.13	12	12	1.0
For Comparison					
1, H	0.060		6.8		

[a] Inhibition type was determined by Lineweaver−Burk plot. All the 17-keto steroids examined inhibited aromatase in a competitive manner. [b] Substrate, [1β-^{3}H]androstenedione (300 nM); 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 33 nM. Human placental microsomes, 20 μg of protein; incubation time, 5 min. [d] The inhibitor concentration required for 20% inhibition of the aromatase activity was given in the parentheses.

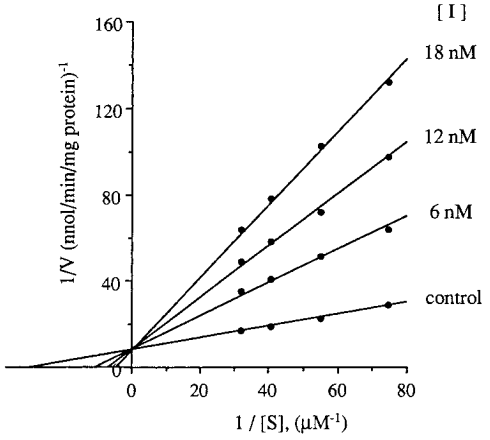


Figure 2. Lineweaver−Burk plot of aromatase inhibition by 6α-methylandrost-4-en-17-one (**18a**). Each point represents the mean of two determinations which varied by less than 5% of the mean. The inhibition experiments with all the other steroids examined gave essentially similar plots to Figure 2 (data not shown).

chains of compounds **18** and **19** were linearly extended, in which long axis is inclined at about 64° of dihedral angle between the C-6−C-7 and C-6^1−C-6^2 bonds (Figure 3), in contrast, in series of compounds **4** and **5** having an oxygen function at C-6, the 6α-methoxy and 6α- and 6β-acetoxy groups are inclined at about 147° of dihedral angle between the C-6−C-7 and O-6^1−C-6^2 bonds and the dihedral angle of the 6β-methyl group is about 162° (Figure 4).

Discussion

In order to define the effect of varying the C-6 substitution of androst-4-en-17-one (**1**) on the activity of aromatase inhibition, two stereoisomeric series of inhibitors **18** and **19** with methyl, ethyl, *n*-propyl, *n*-pentyl, and *n*-octyl groups at C-6 were synthesized, and their inhibitory activities were determined. Another series of the 3-deoxy compounds **4** and **5** having an oxygen function such as a hydroxy, acetoxy, or methoxy group at C-6α and C-6β were also tested as aromatase inhibitors. The 6α- and 6β-methyl steroids **18a** and **19a** as well as the 6β-hydroxy compound **5a** were potent competitive inhibitors of aromatase in human placental microsomes with apparent K_i's of 3.1 nM for **18a**, 5.3 nM for **19a**, and 6.0 nM for **5a**. The 6α-methyl steroid **18a** bound to aromatase with about 11 times the affinity of the substrate androstenedione (K_m = *ca.* 33 nM). Its inhibitory activity was comparable to that of 6β-ethylandrostenedione (K_m/K_i = *ca.* 13), which is the most powerful inhibitor in a series of 6-alkylandrostenediones.[3h] On the other hand, the inhibitory activities of the 6α-ethyl (**18b**) and 6α-*n*-propyl (**18c**) steroids along with the 6α-hydroxy (**4a**), 6α- and 6β-acetoxy (**4b** and **5b**), and 6α- and 6β-methoxy (**4c** and **5c**) analogs were weaker than the above ones, but were still potent inhibitors with the apparent K_i's (12−30 nM) being smaller than the apparent K_m value.

Introduction of a methyl group at the C-6α and C-6β positions of the 3-deoxy steroid **1** gave rise to the increased affinity for aromatase (K_i = 6.8 nM for **1**). Elongation of the alkyl chain up to C-8 decreased the affinity in relation to carbon number in each series. Furthermore, in view of the effect of the configuration of the C-6 alkyl chain on the affinity, the 6α-alkyl derivatives had higher affinities than the corresponding 6β-isomers, except the 6-*n*-octyl series of which apparent K_i values were extremely large. Our previous studies[3h,8] regarding the structure−activity relationships of the 6α- and 6β-alkylandrostenediones as aromatase inhibitors had revealed that introduction of a methyl group at the C-6α and C-6β positions of androstenedione also gave rise to the increased affinity for aromatase. The addition of one more methylene unit to the methyl group markedly increased the affinity, in contrast, elongation of the alkyl chain up to C-4 decreased it in relation to its carbon number. Surprisingly, the addition of one more methylene to the *n*-butyl group markedly increased the affinity. Further elongation the alkyl chain up to C-8 decreased the affinity. Thus, in view of effects

Table 3. Aromatase Inhibition by 6α- and 6β- Alkyl 17β-Ols **14** and **15**[a]

R	IC_{50},[b] μM **14** (6α)	IC_{50},[b] μM **15** (6β)	apparent K_i,[b] μM **14** (6α)	apparent K_i,[b] μM **15** (6β)	relative K_i 6α/6β
a, CH_3	3.7	1.6	0.38	0.14	2.7
b, CH_2CH_3	1.3	3.3	0.18	0.34	0.5
c, $(CH_2)_2CH_3$	14	43	2.9	5.5	0.5
d, $(CH_2)_4CH_3$	>50 (13)[c]	>50 (24)[c]	44	100	0.44
e, $(CH_2)_7CH_3$	>50 (>50)[d]	>50 (2.5)[d]	ND[e]	ND[e]	
For Comparison					
2, H	0.36		0.045		

[a] Inhibition type was determined by Lineweaver−Burk plot. All the steroids examined inhibited aromatase in a competitive manner. [b] IC_{50} and K_i values were obtained similarly as described in Table 2. [c] The inhibitor concentration required for 20% inhibition of the aromatase activity was given in the parentheses. [d] The inhibitor concentration required for 10% inhibition. [e] Not determined.

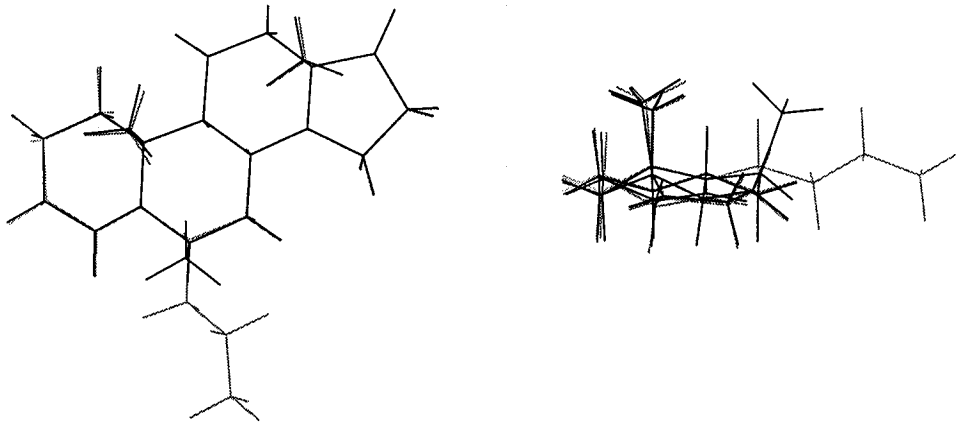


Figure 3. Overlay of 6α-*n*-propyl (light line) and 6*β*-methyl (dark line) steroids **18c** and **19a** having a higher affinity to aromatase than androstenedione by superimposing their respective steroid nucleus. Views from the *β*-side (left) and from the C-2−C-3−C-4 edge (right). The 6-alkyl chains of the 6α-methyl and 6α-ethyl steroids **18a** and **18b** which also have the higher affinity were excellently superimposed on that of the 6α-*n*-propyl steroid **18c**.

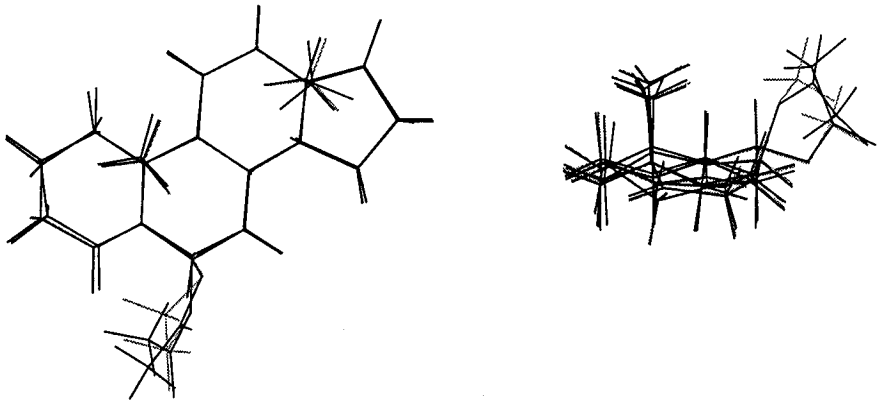


Figure 4. Overlay of 6α- and 6*β*-acetoxy steroids **4b** and **5b** (dark line) and 6α- and 6*β*-methoxy steroids **4c** and **5c** (light line) by superimposing their respective steroid nucleus. Views from the *β*-side (left) and from the C-2−C-3−C-4 edge (right). Oxygen function at C-6 of 6-hydroxy steroids **4a** and **5a** was excellently superimposed on that [O(6^1)] of the 6-acetates **4b** and **5b**.

of stereochemistry and length of the C-6 alkyl substituent on the affinity, there are marked differences between the 3-deoxy steroids **18** and **19** and the androstenedione analogs **6** and **7**.

Introduction of a hydroxy group at the C-6*β* position of the parent compound **1** did not significantly change affinity for aromatase whereas that of the 6α-hydroxy group decreased it (K_i for the 6α-ol **4a** = 21 nM). Substitution of the 6α-hydroxy group with a lipophilic function, an acetoxy or a methoxy group, slightly enhanced the affinity (K_i = 18 and 12 nM for the acetate **4b** and the methoxide **4c**, respectively); in contrast, the same modification of the 6*β*-ol **5a** decreased it (K_i = 24 and 12 nM for the acetate **5b** and the methoxide **5c**, respectively). We had previously reported that in the androstenedione series, the 6α- and 6*β*-hydroxy analogs had lower affinities for aromatase than the parent steroid and acetylation of the 6α-hydroxy group enhanced the affinity whereas that of the 6*β*-one did not significantly affect the affinity.[15] The apparent K_i values for the 6α- and 6*β*-hydroxy steroids in these studies were 210 and 130 nM, respectively. Thus, there is a marked difference of effects of the 6-oxygen functions on affinity for aromatase between the androstenedione and 3-deoxyandrostenedione series. We have previously reported that the 19-hydroxy derivative of compound **1** has very high affinity for aromatase[9] and that its 19-hydroxy group is oriented over the A ring, based on molecular modeling.[16] Robinson's group[17] has explained this from a point of view of anchoring of the 19-hydroxy group by a Glu-302 residue of the active site of aromatase through hydrogen bonding. On the other hand, recently, Graham-Lorence *et al.*[18] have proposed, based on a three-dimensional modeling of aromatase, that both Asp-309 and Thr-310 residues of the active site, instead of the Glu-302, are possibly important in maintaining the catalytic activity of the enzyme. Considering the molecular feature of the 6*β*-ol **5a** in which the axial 6*β*-hydroxy group is positioned in the vicinity of the 19-hydroxy group, hydrogen bonding between the 6*β*-hydroxy group and a hydrophilic functional group of such amino acids could be involved in a tight binding of the inhibitor **5a** to the active site in analogy with the 19-hydroxy analog. The fact that either acetylation or methylation of the 6*β*-hydroxy group decreased the affinity may support this. The 17*β*-ols **14** and **15** were weak to poor competitive inhibitors with apparent K_i's ranging from 140 nM to 100 *μ*M, in which the 6α-ethyl steroid **14b** (K_i = 180 nM) and the 6*β*-methyl steroid **15a** (K_i = 140 nM) were the most potent inhibitors in each series. It should be noted that in the 17*β*-hydroxy steroid series there was no increased effect of the alkyl substitution at either C-6α or C-6*β* on affinity for aromatase, but the alkyl substitution decreased the affinity [K_i for the parent compound, 3-deoxytestosterone (**2**) = 45 nM]. The results along with those[9,11] obtained with other 3-deoxy 4-ene steroids clearly indicate that anchoring of a 17-carbonyl group by a

hydrophilic residue of the active site through hydrogen bonding plays a critical role in a tight binding of the 3-deoxyandrost-4-ene steroid analogs. A 17-carbonyl group is also required for effective binding of 3-keto steroids.[19]

It has been known that the substrate androstenedione[20,21] as well as steroidal reversible inhibitors of aromatase produce a "Type I" difference spectrum upon interaction with the enzyme. The methyl steroids **18a** and **19a** as well as the parent steroid **1** induced the type I spectra, showing that these steroids interact with the substrate binding site and coordinate with the heme iron atom.

On the basis of the present results of the aromatase inhibition, a delineation of the available volume around the C-6 region of the 3-deoxy steroid **1** is proposed as a tight enzyme pocket that accommodates a hydrophobic 6-alkyl substituent up to 4.7 Å in length, 4.3 Å in width, and 3.8 Å in height and an oxygen function up to 4.0 Å in length, 2.0 Å in width, and 3.2 Å in height. This available volume is markedly different from those[3h,8] of the binding pocket around the C-6 region of the natural substrate androstenedione previously proposed.

In conclusion, introduction of a methyl group at C-6α and C-6β of 3-deoxyandrostenedione (**1**) increased affinity for aromatase whereas elongation of the methyl group decreased the affinity in relation to carbon number. Binding of the 6α-methyl group rather than the 6β-methyl group to the pocket of the active site of aromatase may produce the thermodynamically stable enzyme−inhibitor complex to result in the very potent inhibition of the enzyme activity. A 6β-axial hydroxy group also plays an important role in the formation of the stable complex probably through hydrogen bonding to the hydrophilic amino acid residue of the active site. A 17-carbonyl function is essential for a tight binding of the compound **1** analogs to the active site. These results suggest that a binding geometry of the 3-deoxy steroid **1** to the active site of aromatase would be different from that of the substrate androstenedione principally in the region of a A−B ring system of the steroid nucleus. This difference should be due to a lack of a 3-carbonyl group which is involved in a proper binding of androstenedione to the active site[17,18,22] through hydrogen bonding. The inhibitors examined in this study are promising not only to understand conformational aspects of the active site but also to develop a potent aromatase inhibitor. An important aspect of useful aromatase inhibitors is that the candidate compounds and/or their metabolites *in vivo* should not have sex steroid hormonal activities. Thus, studies on the metabolism and hormonal activities of the 3-deoxy compounds should be essential for further development of the useful inhibitors.

Experimental Section

Materials and General Methods. Androst-4-en-17-one (**1**) and its 17β-reduced derivative **2**,[11] 6α-hydroxyandrost-4-en-17-one (**4a**) and its 6α-acetate **4b**,[12] 6β-hydroxyandrost-4-en-17-one (**5a**) and its 6β-acetate **5b**,[12] and 6α- and 6β-*n*-alkylandrost-4-ene-3,17-diones (alkyl: CH_3, C_2H_5, C_3H_7, C_5H_{11}, and C_8H_{17}) (**6** and **7**)[3h,8] were prepared according to the known methods. [1β-^{3}H]Androstenedione (27.5 Ci/mmol) (^{3}H distribution: 74−79% at 1β) was purchased from New England Nuclear Corp. (Boston, MA) and NADPH from Kohjin Co., Ltd. (Tokyo, Japan).

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. ^{1}H NMR spectra were obtained in $CDCl_3$ solutions with a JEOL EX 270 (270 MHz) spectrometer using tetramethylsilane as an internal standard. Mass spectra (MS) were determined with a JEOL JMS-DX 303 spectrometer. Thin-layer chromatography (TLC) was performed on E. Merck precoated TLC silica gel plates. Column chromatography was conducted with silica gel (E. Merck, 70−230 mesh). High-performance liquid chromatography (HPLC) was carried out using a Waters 510 pump, YMC-D-ODS-5 column (250 mm × 20 mm i.d.), and a UV detector (220 nm).

Methylation of the 6α- and 6β-Hydroxy Steroids 4a and 5a with Methyl Iodide. The 6α-ol **4a** or its β-isomer **5a** (150 mg, 0.52 mmol) was dissolved in CH_3CN (11 mL) and CH_3I (6 mL). Ag_2O (200 mg, 0.86 mmol) was added to this solution, and the mixture was refluxed with stirring for 15 h (for the reaction of **4a**) or 80 h (for the reaction of **5a**). After dilution with AcOEt (100 mL), the reaction mixture was filtered. The filtrate was condensed to give an oily product, in each case, which was purified by silica gel column chromatography (hexane−AcOEt) followed by recrystallization to give the 6α-methoxide **4c** (64 mg, 41%) or 6β-methoxide **5c** (32 mg, 21%). The starting material **4a** or **5a** was recovered in 27% or 66% yield, respectively.

General Procedure for Reaction of the 6-Alkyl 4-En-3-ones 6 and 7 with Ethane-1,2-dithiol. A solution of ethane-1,2-dithiol (62 mg, 0.66 mmol) in acetic acid (1.8 mL) was added to a solution of the 4-en-3-ones **6** and **7** (0.6 mmol), except the pentyl steroids **6d** and **7d**, and *p*-toluenesulfonic acid (58 mg, 0.33 mmol) in acetic acid (8 mL) and the mixture was stirred at room temperature for 3 h, then poured into water (15 mL), and extracted with ethyl acetate (100 mL × 2). The combined organic layer was washed with saturated $NaHCO_3$ solution and saturated NaCl solution and dried over Na_2SO_4. Evaporation of the solvent gave the crude product. In the same reaction of compounds **6d** and **7d**, THF was employed as the reaction solvent instead of acetic acid according to the method recently reported by Robinson's group[13] (THF, 2.4 mL for dilution of the thiol reagent and 10 mL for dissolving the steroids and the sulfonic acid catalysis), and the other conditions were the same as above. ^{1}H NMR analysis of the products (19-methyl protons δ 1.04−1.05 ppm for the 6α-alkyl products **8** and 1.08−1.09 ppm for the 6β-isomers **9**) showed that isomerization of the 6-alkyl substituents occurred during the reactions of the 6β-alkyl steroids **7** in about 20−50% yields, but this was not observed in the reaction of the 6α-isomers **6**. Silica gel column chromatography (hexane−AcOEt) of the crude products followed by recrystallization afforded the corresponding 6-alkyl-3,3-(ethylenedithio)androst-4-en-17-ones **8** and **9b**,**c**,**e**. The 6β-*n*-pentyl analog **9d** was isolated by reverse-phase HPLC (CH_3CN-H_2O = 90:10, 8 mL/mim): t_R = 71.4 min for **9d** and 72.5 min for the 6α-isomer **8d**. However, the 6β-methyl compound **8a** could not be separated from the isomerized product **9a** by HPLC; therefore a *ca.* 1:1 mixture of the 6α- and 6β-isomers **8a** and **9a** was, without further purification, converted into a mixture of the 17β-acetates **12** and **13** which could be separated by HPLC.

Conversion of the Mixture of the 6-Methyl Compounds 6a and 7a into the 6-Methyl 17β-Acetates 12 and 13. The *ca.* 1:1 mixture of the 17-ones **6a** and **7a** (240 mg, 0.64 mmol) was dissolved in MeOH (20 mL). $NaBH_4$ (23 mg, 0.59 mmol) was added to this solution at 0 °C, and the mixture was stirred at 0 °C for 30 min. After the usual workup, a mixture of the 17β-reduced products **10** and **11** was obtained as an oil (207 mg, 86%). They could not be separated by HPLC. The mixture of the 17β-ols **10** and **11** (205 mg, 0.55 mmol) was acetylated by acetic anhydride (0.9 mL) and pyridine (1.8 mL) at 60 °C for 3.5 h. After dilution with AcOEt (50 mL), the reaction mixture was sequentially washed with 5% $NaHCO_3$ solution, 5% HCl, and H_2O and dried over Na_2SO_4. The crude mixture of acetates **12** and **13** (260 mg) was obtained after evaporation of the solvent. Compounds **12** and **13** (*ca.* 60 mg each) were successfully separated by reverse-phase HPLC (MeCN−H_2O = 85:15, 8 mL/min): t_R = 99.1 min for

the α-acetate **12** and 97.3 min for the β-isomer **13**. 6α-Methyl-3,3-(ethylenedithio)androst-4-en-17β-yl acetate (**12**): mp 163−165 °C (from acetone); ^{1}H NMR δ 0.81 (3H, s, 18-Me), 1.01 (3H, d, J = 7.6 Hz, 6α-Me), 1.02 (3H, s, 19-Me), 2.04 (3H, s, 17β-OCOMe), 3.17−3.46 (4H, m, $-SCH_2CH_2S-$), 4.57 (1H, dd, J = 7.6 and 1.7 Hz, 17α-H), and 5.46 (1H, s, 4-H); FT-IR (KBr) 1734 (C=O) cm^{-1}. Anal. ($C_{24}H_{36}O_2S_2$) C, H, S. 6β-Methyl-3,3-(ethylenedithio)androst-4-en-17β-yl acetate (**13**): mp 196−197 °C (from acetone); ^{1}H NMR δ 0.83 (3H, s, 18-Me), 1.11 (3H, s, 19-Me), 1.23 (3H, d, J = 8.1 Hz, 6β-Me), 2.04 (3H, s, 17β-OCOMe), 3.17−3.42 (4H, m, $-SCH_2CH_2S-$), 4.58 (1H, dd, J = 7.8 and 1.3 Hz, 17α-H), and 5.51 (1H, s, 4-H); FT-IR (KBr) 1727 (C=O) cm^{-1}. Anal. ($C_{24}H_{36}O_2S_2$) C, H, S.

Treatment of the Acetates 12 and 13 with KOH. A 0.5 M KOH solution in ethanol (0.9 mL, 0.45 mmol) was added to a solution of the acetate **12** or **13** (30 mg, 0.07 mmol) in MeOH (60 mL), and the mixture was refluxed for 1 h under N_2. After this time, the mixture was neutralized by adding 10% HCl, condensed to about 10 mL under reduced pressure, and then diluted with AcOEt (100 mL), sequentially washed with 5% HCl, 5% $NaHCO_3$ solution, and H_2O, and dried over Na_2SO_4. Evaporation of the solvent gave a crude product which was recrystallized from AcOEt to give compound **10** (25 mg, 92%) or **11** (22 mg, 81%).

6α-Methyl-3,3-(ethylenedithio)androst-4-en-17β-ol (10): mp 104−106 °C; ^{1}H NMR δ 0.76 (3H, s, 18-Me), 1.00 (3H, d, J = 6.3 Hz, 6α-Me), 1.03 (3H, s, 19-Me), 3.19−3.42 (4H, m, $-SCH_2CH_2S-$), 3.65 (1H, t, J = 8.4 Hz, 17α-H), and 5.46 (1H, s, 4-H); FT-IR (KBr) 3469 (OH) cm^{-1}. Anal. ($C_{22}H_{34}OS_2$) C, H, S.

6β-Methyl-3,3-(ethylenedithio)androst-4-en-17β-ol (11): mp 161−163 °C; ^{1}H NMR δ 0.78 (3H, s, 18-Me), 1.11 (3H, s, 19-Me), 1.12 (3H, d, J = 5.0 Hz, 6β-Me), 3.15−3.67 (4H, m, $-SCH_2CH_2S-$), and 5.51 (1H, s, 4-H); FT-IR (KBr) 3370 (OH) cm^{-1}. Anal. ($C_{22}H_{34}OS_2$) C, H, S.

Desulfurization of the 3,3-Ethylenedithio Compounds 8, 9b−e, and 11. Condition A. A solution of the 3,3-ethylenedithio steroids **8a**−**c**,**f**, **9b**,**c**,**f**, and **11** (0.5 mmol) in THF (4 mL) was, separately, added to a solution of sodium metal (830 mg, 36 mmol) in 28 mL of liquid NH_3 at −78 °C. The reaction mixtures were stirred for 35 min. After most of the ammonia was removed at room temperature and 4 mL of MeOH and AcOEt (100 mL) were carefully added, the mixture was sequentially washed with saturated NH_4Cl solution, 5% $NaHCO_3$ solution, and H_2O and dried over Na_2SO_4.

Condition B. The 6-*n*-pentylethylenedithio compounds **8d** and **9d** (0.5 mmol) were desulfurized according the method[13] reported previously (Na metal: 370 mg, 16 mmol; liquid NH_3, 70 mL; reaction time 2 min; under Ar). EtOH (35 mL) was carefully added to the reaction mixtures. After allowing the mixture for 1.5 h to stir at room temperature, it was adjusted to pH 5 by adding 10% HCl, diluted with AcOEt (150 mL), washed with 5% $NaHCO_3$ solution and H_2O, and dried over Na_2SO_4.

Evaporation of the solvent gave the crude products in each case. The crude products **14** and **15** obtained were initially purified by silica gel column chromatography (hexane−AcOEt). The 6α- and 6β-methyl (**14a** and **15a**), 6α- and 6β-ethyl (**14b** and **15b**), 6β-*n*-pentyl (**15d**), and 6β-*n*-octyl (**15e**) compounds could be isolated by repeated recrystallization from an appropriate solvent. The 6α- and 6β-*n*-propyl analogs **14c** and **15c** were separated efficiently from their 3-ene derivatives **16c** and **17c**, respectively, by reverse-phase HPLC (MeOH−H_2O = 92:8, 7 mL/min; t_R = 71.2 min for **14c** and 67.6 min for its probable 3-ene isomer **16c**, t_R = 67.2 min for **15c** and 65.0 min for its probable 3-ene isomer **17c**).

Isolation of the 6α-*n*-Pentyl and 6α-*n*-Octyl 4-En-17β-ols 14d and 14e. Pd on charcoal (5%, 10 mg) was added to a solution of the crude 4-ene product **14d** or **14e** (20 mg) obtained by silica gel column chromatography as above, in EtOH (1 mL), and the mixture was stirred vigorously for 1.5 h under H_2. After removal of the catalyst by filtration, the filtrate was evaporated to give an oil which was purified by multiple developed-preparative TLC (hexane−AcOEt), yielding an analytical sample of the 17β-ol **14d** or **14e** as an oil in about 50% yield.

Jones Oxidation of the 17β-ols 14 and 15. Jones reagent was, separately, added dropwise to solutions of the 17β-ols **14** and **15** (0.08 mmol) in acetone (30 mL) at 0 °C with stirring until the orange color of the reagent remains, and the mixture were stirred for 3 min. After this time, the mixtures were poured into water and the products were extracted with AcOEt (150 mL × 2). The combined organic layer was washed with 5% $NaHCO_3$ solution and water, dried over Na_2SO_4, and evaporated to afford the corresponding 17-ketones **18** and **19** which were purified by recrystallization or preparative TLC (hexane−AcOEt), giving the analytical samples.

Biochemical Studies. Enzyme Preparation. Human placental microsomes (particles sedimenting at 105000*g* for 60 min) were obtained using the method reported by Ryan.[23] 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 Procedure. 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.[11] 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 in the presence of NADPH in air.

Spectral Studies. A suspension of human placental microsomes (135 μg of protein) in 65 mM phosphate buffer (pH 7.4, 400 μL), initially at 0 °C, was divided into two 200 μL portions and placed in two 1-cm path length cells and allowed to equilibrate to ambient temperature for 5 min. All ligands (200 μM) were made up in solutions of MeOH, and 5 μL portions were added to the experimental cell. The reference cell received MeOH only. All measurements were taken at room temperature on a Beckman DU-650 spectrophotometer.

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 Insight II, version 95.0 software (Biosystem Technologies, San Diego, CA). 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. Low-energy conformations were overlapped within Insight II which uses a least squares fitting algorithm to minimize the displacement between matching atoms in the structures that are superimposed.

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