Time-Dependent Inactivation of Aromatase by 6-Alkylandrosta-1,4-diene-3,17-diones. Effects of Length and Configuration of the 6-Alkyl Group†

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Series of 6α- and 6β-alkylandrosta-1,4-diene-3,17-diones (**3** and **4**) were synthesized and evaluated as time-dependent inactivators of aromatase in human placental microsomes to gain insights to the structure−activity relationship of varying the 6-*n*-alkyl substituents (C-1−C-7) to the time-dependent inactivation activity. All of the inhibitors synthesized were powerful to good competitive inhibitors of aromatase, with apparent K_i's ranging from 4.7 to 54 nM. The 6β-ethyl (**4b**) and 6β-*n*-pentyl (**4e**) compounds were the most potent among them (K_i = 4.7 and 5.0 nM for **4b** and **4e**, respectively). In a series of the 6α-alkyl steroids, the inhibitors **3a**−**d** having C-1−C-4 at the 6-position as well as the 6α-*n*-hexyl steroid **3f** caused a time-dependent inactivation of aromatase while 6α-*n*-pentyl (**3e**) and 6α-*n*-heptyl (**3g**) compounds did not. In contrast, in the 6β-alkyl steroid series, only the methyl analog **4a** inactivated aromatase in a time-dependent manner, and the other alkyl steroids having more than two carbons at C-6β did not. The inactivations were prevented by the substrate androstenedione, and no significant effects of L-cysteine on the inactivation were observed in each case. These results along with molecular modeling with the PM3 method indicate that both length and stereochemistry of a straight alkyl substituent at the C-6 position of androsta-1,4-diene-3,17-dione (**3h**) play an important role in the cause of a time-dependent inactivation of aromatase. No significant correlation between affinity for the enzyme and the inactivation ability in the 6-alkylandrosta-1,4-diene-3,17-diones is observed.

Introduction

Aromatase, a unique cytochrome P-450 enzyme complex, catalyzes the conversion of androst-4-ene-3,17-dione (androstenedione) and testosterone to estrone and estradiol.[1] Aromatization of the androgens appears to involve three oxygenation steps, eventual loss of the angular methyl group at C-19, and the elimination of the 1β- and 2β-hydrogens, resulting in the formation of the estrogens.[2] A potent selective inhibitor of aromatase may be effective in the treatment of estrogen-dependent breast cancer and in the modulation of the reproductive process.[3] For this reason, the specific, irreversible blockade of estrogen biosynthesis via a mechanism-based (suicide) inactivation has been pursued with the goal of developing practical clinical drugs.

Androsta-1,4-diene-3,17-dione (**3h**), which can serve as a substrate for aromatase with higher apparent K_m compared to that for androstenedione,[4] is a prototypical mechanism-based inhibitor of aromatase;[5] the 1,4-dien-3-one structure is responsible for inactivation, although the inactivation mechanism is currently unknown.[3d,5] Various analogs of steroid **3h**, with 1-methyl,[6] 6-methylene,[7] and 7α-thiophenyl[8] groups or with a modified D ring such as reduction or removal of the 17-carbonyl group[9] or conversion to a lactone,[5] are also suicide substrates for aromatase. On the other hand, we have recently reported that 6-alkyl-substituted androstenediones are very powerful competitive inhibitors of aromatase.[10] On the basis of these previous findings, we were interested in the combination of the 1,4-dien-3-one grouping with a 6-alkyl substituent to develop a powerful mechanism-based inhibitor of aromatase. Thus, we prepared 1,4-diene-3,17-dione derivatives **3** and **4** having various lengths of straight alkyl chains (C-1 to C-7) at the C-6α and C-6β positions, respectively, and tested them as competitive and mechanism-based inhibitors of aromatase in human placental microsomes. The 6β-ethyl and 6β-*n*-pentyl compounds **4b** and **4e** were extremely powerful competitive inhibitors. Only 6β-methyl steroid **3a** in the 6β-series as well as all of the 6α-alkyl steroids **4** except *n*-pentyl and *n*-heptyl analogs **4e** and **4g** caused the time-dependent inactivation.

Results

Chemistry. The 6α- and 6β-alkylandrosta-1,4-diene-3,17-diones (**3a**−**f** and **4a**−**f**: alkyl = CH_3, C_2H_5, *n*-C_3H_7, *n*-C_4H_9, *n*-C_5H_{11}, and *n*-C_6H_{13}) were prepared, in moderate yields, by dehydrogenation of the corresponding 4-en-3-one steroids **1a**−**f** and **2a**−**f** with DDQ according to the known method[11] (Scheme 1). 6α- And 6β-*n*-hept-4-en-3-ones **1g** and **2g** were obtained essentially according to the method[10a] previously reported for the synthesis of steroids **1** and **2** (Scheme 2). Grignard reaction of 3,3:17,17-bis(ethylenedioxy)androstane-5α,6α-epoxide (**5**) with *n*-$C_7H_{15}MgBr$ gave 6β-*n*-heptyl-5α-ol **6** which was treated with 3 M $HClO_4$ in THF followed by dehydration with $SOCl_2$ yielded the 6β-compound **2g**. Treatment of the 5α-ol **6** with 1 M HCl in EtOH under reflux gave the 6α compound **1g** in one step. Both steroids **1g** and **2g** were converted into the 1,4-diene derivatives **3g** and **4g**, respectively, similarly as de-

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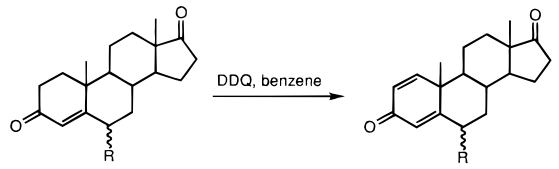
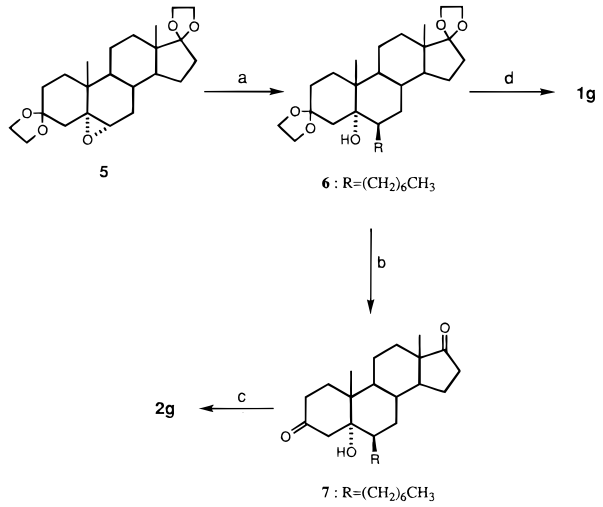
Scheme 1



R : **a**, CH_3 ; **b**, CH_2CH_3 ; **c**, $(CH_2)_2CH_3$; **d**, $(CH_2)_3CH_3$; **e**, $(CH_2)_4CH_3$ **f**, $(CH_2)_5CH_3$; **g**, $(CH_2)_6CH_3$; **h**, H

Scheme 2[a]



[a] Reagent: (a) $CH_3(CH_2)_6MgBr$, THF; (b) 3 M $HClO_4$, THF, room temperature; (c) $SOCl_2$, pyridine; (d) 1 M HCl, EtOH, reflux.

scribed above. Physicochemical data of these 1,4-diene steroids **3** and **4** are shown in Table 1.

Biochemical Properties. Reversible inhibition of aromatase activity in human placental microsomes by the 6α- and 6*β*-*n*-alkyl-substituted steroids **3** and **4** was initially tested by enzyme kinetics under initial velocity conditions. The results are shown in Table 2. The inhibition by the parent steroids **3h** having no alkyl function at C-6 is also listed for comparison. Aromatase activity in the placental microsomes was determined essentially according to the method of Siiteri and Thompson[12] in which the amount of tritiated water released from [1*β*-^{3}H]androstenedione into the inactivation medium during aromatization was used as an index of estrogen formation. IC_{50}'s were initially obtained, and then, in order to characterize the nature of their binding to the active site of aromatase, aromatization was measured at several concentrations of the inhibitors and androstenedione. The results of the studies were plotted in a typical Lineweaver−Burk plot. All the steroids studied exhibited clear-cut competitive-type inhibition. The apparent inhibition constants (K_i), an index of the affinity, were determined by analysis of the Dixon plot. The Lineweaver−Burk plot of aromatase inhibition by the 6*β*-ethyl steroid **4b** is shown in Figure 1. In these studies, the apparent K_m for androstenedione was found to be ca. 20 nM.

Two stereoisomeric series of the 6-alkyl steroids **3** and **4** were then tested for their abilities to cause a time-dependent inactivation of aromatase. In a series of the 6α-alkyl steroids **3**, the inhibitors **3a**−**d** having the alkyl

Table 1. Physicochemical Properties of 6-Alkyl Steroids **3** and **4**

compd	R	yield, %	mp, °C	recryst solvent	formula	anal.[a]	UV (EtOH), nm (*λ*)	IR (KBr),[b] cm^{-1}	^{1}H-NMR ($CDCl_3$), *δ* 18-Me	19-Me	1-H (*J*, Hz)	2-H (*J*, Hz)	4-H (*J*, Hz)
							6α-Series						
3a	CH_3	54	225−228	ether	$C_{20}H_{26}O_2$	C, H	243.8 (16 600)	1735, 1659	0.95	1.26	7.06 (d, 10.2)	6.26 (dd, 1.7 and 10.2)	6.12 (d, 1.7)
3b	CH_2CH_3	50	147−148	AcOEt	$C_{21}H_{28}O_2$	C, H	244.2 (14 300)	1735, 1663	0.95	1.26	7.05 (d, 10.1)	6.26 (dd, 1.7 and 10.2)	6.11 (d, 1.7)
3c	$(CH_2)_2CH_3$	35	156−157	AcOEt	$C_{22}H_{30}O_2$	C, H	244.2 (14 300)	1735, 1665	0.95	1.26	7.05 (d, 10.2)	6.26 (dd, 1.7 and 10.1)	6.11 (s)
3d	$(CH_2)_3CH_3$	36	110−111	AcOEt	$C_{23}H_{32}O_2$	C, H	244.3 (14 700)	1741, 1664	0.95	1.26	7.06 (d, 10.2)	6.26 (dd, 2.0 and 10.2)	6.11 (d, 1.7)
3e	$(CH_2)_4CH_3$	41	oil[c]		$C_{24}H_{34}O_2$	exact MS	244.4 (16 800)	1740, 1661	0.95	1.26	7.06 (d, 10.2)	6.26 (dd, 1.7 and 10.2)	6.11 (d, 1.7)
3f	$(CH_2)_5CH_3$	28	oil[c]		$C_{25}H_{36}O_2$	exact MS	244.2 (14 300)	1740, 1669	0.95	1.26	7.06 (d, 10.2)	6.26 (dd, 1.7 and 10.2)	6.11 (d, 1.7)
3g	$(CH_2)_6CH_3$	22	oil[c]		$C_{26}H_{38}O_2$	exact MS	244.2 (14 400)	1740, 1660	0.95	1.26	7.06 (d, 9.9)	6.26 (dd, 1.8 and 9.9)	6.11 (s)
							6*β*-Series						
4a	CH_3	68	136−138	ether	$C_{20}H_{26}O_2$	C, H	243.5 (14 800)	1739, 1661	0.98	1.32	7.00 (d, 10.1)	6.20 (dd, 1.9 and 10.1)	6.13 (d, 1.7)
4b	CH_2CH_3	47	153−154	ether	$C_{21}H_{28}O_2$	C, H	243.0 (15 200)	1737, 1660	0.96	1.29	7.00 (d, 10.1)	6.21 (dd, 1.9 and 10.1)	6.11 (d, 1.7)
4c	$(CH_2)_2CH_3$	41	199−200	ether	$C_{22}H_{30}O_2$	C, H	244.2 (15 200)	1737, 1658	0.97	1.30	7.00 (d, 10.2)	6.21 (dd, 1.7 and 10.2)	6.11 (d, 1.7)
4d	$(CH_2)_3CH_3$	36	145−147	AcOEt	$C_{23}H_{32}O_2$	C, H	244.3 (15 400)	1732, 1657	0.97	1.30	7.00 (d, 10.2)	6.21 (dd, 1.7 and 10.2)	6.11 (d, 1.9)
4e	$(CH_2)_4CH_3$	41	oil[c]		$C_{24}H_{34}O_2$	exact MS	244.4 (15 700)	1740, 1662	0.97	1.29	7.00 (d, 10.2)	6.21 (dd, 1.7 and 10.2)	6.11 (d, 1.7)
4f	$(CH_2)_5CH_3$	35	oil[c]		$C_{25}H_{36}O_2$	exact MS	244.0 (15 300)	1740, 1661	0.97	1.29	7.00 (d, 10.2)	6.20 (dd, 1.7 and 10.2)	6.10 (d, 1.7)
4g	$(CH_2)_6CH_3$	41	oil[c]		$C_{26}H_{38}O_2$	exact MS	244.2 (14 600)	1741, 1661	0.97	1.29	7.00 (d, 10.2)	6.21 (dd, 1.7 and 10.2)	6.11 (d, 1.7)

[a] Analytical results obtained for the solid products are within 0.4% of the theoretical values 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 reverse-phase HPLC using ODS column.

Table 2. In Vitro Aromatase Inhibition by 6-Alkyl 1,4-Diene Steroids

compound	IC_{50}, nM[a]	apparent K_i, nM[b]
	6α-Alkyl Steroid	
methyl, **3a**	620	54
ethyl, **3b**	250	19
n-propyl, **3c**	270	22
n-butyl, **3d**	200	16
n-pentyl, **3e**	140	11
n-hexyl, **3f**	280	25
n-heptyl, **3g**	250	20
	6β-Alkyl Steroid	
methyl, **4a**	520	43
ethyl, **4b**	54	4.7
n-propyl, **4c**	60	7.0
n-butyl, **4d**	180	14
n-pentyl, **4e**	54	5.0
n-hexyl, **4f**	76	8.0
n-hepty,l **4g**	60	7.8
	For Comparison	
androstenedione	300	20 (K_m)
Δ^1-androstenedione (**3h**)	530	43

[a] 300 nM of [1β-^{3}H]androstenedione and 20 μg of protein from human placental microsomes were used. [b] Apparent inhibition constant (K_i) was obtained by Dixon plot. All of the inhibitors examined showed a competitive type of inhibition based on analysis of the Lineweaver-Burk plot.

Table 3. Kinetic Analysis of Time-Dependent Inactivation of Aromatase Caused by 6-Alkylandrosta-1,4-diene Derivatives **3** and **4**[a]

	K_I, nM		k_{inact}, min^{-1}	
R	**3** (6α)	**4** (6β)	**3** (6α)	**4** (6β)
a, methyl	800	757	0.048	0.061
b, ethyl	196	NT [b]	0.065	NT
c, *n*-propyl	339	NT	0.044	NT
d, *n*-butyl	571	NT	0.029	NT
e, *n*-pentyl	NT	NT	NT	NT
f, *n*-hexyl	650	NT	0.050	NT
g, *n*-heptyl	NT	NT	NT	NT
	For Comparison			
h, H[c]	952	–	0.059	–

[a] Apparent K_I and k_{inact} were obtained by Kitz-Wilson plot.[13] [b] NT: the time-dependent inactivation was not observed. [c] The previously reported K_I and k_{inact} values for **3h** are 320 nM and 0.055 min^{-1} (ref 5) and 260 nM and 0.056 min^{-1} (ref 9), respectively.

groups being constituted with less than four carbons as well as the *n*-hexyl compound **3f** showed the time-dependent inactivation when they were incubated in the presence of NADPH under aerobic condition, whereas the *n*-pentyl and *n*-heptyl analogs **3e** and **3g**, at concentrations employed (190, 480, and 950 nM), did not. In the other series, only the 6β-methyl steroid **4a** inactivated aromatase in a time-dependent manner, but the other derivatives **4b**–**g** did not. Pseudo-first-order kinetics were obtained during the first 12 min of the incubation of the inhibitors when the kinetics data were analyzed according to the method of Kitz and Wilson[13] (Figure 2). Double-reciprocal plots of k_{obs} versus inhibitor concentration gave k_{inact}'s and K_I's,[14] respectively, for the inhibitors (Table 3). The kinetics of the inactivation by the parent compound **3h** were also determined and are listed for comparison.

NADPH was essential for the time-dependent activity loss by the irreversible inhibitors (Figure 3A). The substrate androstenedione blocked the inactivation while a nucleophile, L-cysteine, had no significant effect on it in each case (Figure 3B).

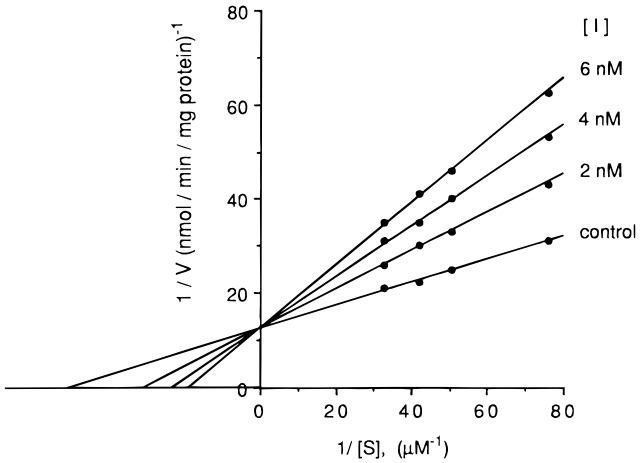


Figure 1. Lineweaver–Burk plot of 6β-ethylandrosta-1,4-diene-3,17-dione (**4b**). Each point represents the mean of two determinations which varied by less than 5% of the mean. The inhibition experiments with all the other steroids examined gave essentially similar plots to Figure 1 (data not shown).

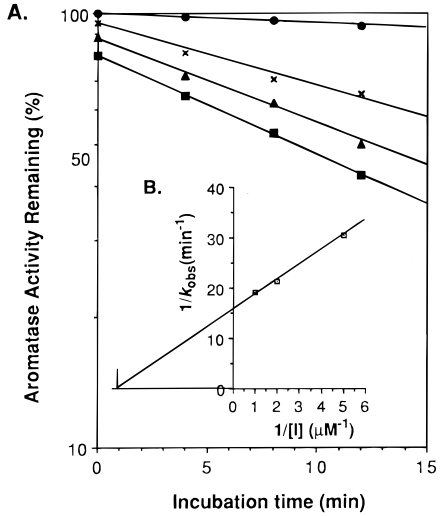


Figure 2. Time-dependent inactivation (A) and concentration dependent inactivation (B) of human placental aromatase by 6α-ethylandrosta-1,4-diene-3,17-dione (**3b**) in the presence of NADPH in air. Concentrations of the inhibitor: control (0 μM), ●; 0.2 μM, ×; 0.5 μM, ▲; 1.0 μM, ■. Each point represents the mean of two determinations which varied by less than 5% of the mean. The time-dependent inactivation experiments with the other 6α-alkyl compounds **3a**, **3c**, **3d**, and **3f** as well as the 6β-methyl analog **4a** gave essentially similar plots to Figure 2 (data not shown).

Molecular Modeling. The minimum-energy conformations of all the inhibitors, assayed in this study, along with the parent steroid **3h** were determined by the MOPAC package using PM3 Hamiltonian. The 6-*n*-alkyl chains of all of the 1,4-diene steroids analyzed in this study were linearly extended in a geometry similar to that previously reported for the alkyl chains of androst-4-ene-3,17-diones, of which the long axis is inclined at an angle of about 76° in the case of the 6β-series or about 70° in the case of the 6α-series to the C-6 and C-7 edge the parent steroid **3h**.

Discussion

The 6α- and 6β-*n*-alkylandrosta-1,4-dien-3-ones **3** and **4** having various lengths of straight alkyl chains ranging from a methyl to a *n*-heptyl group were synthesized, and the effect of introducing the alkyl chains at the C-6

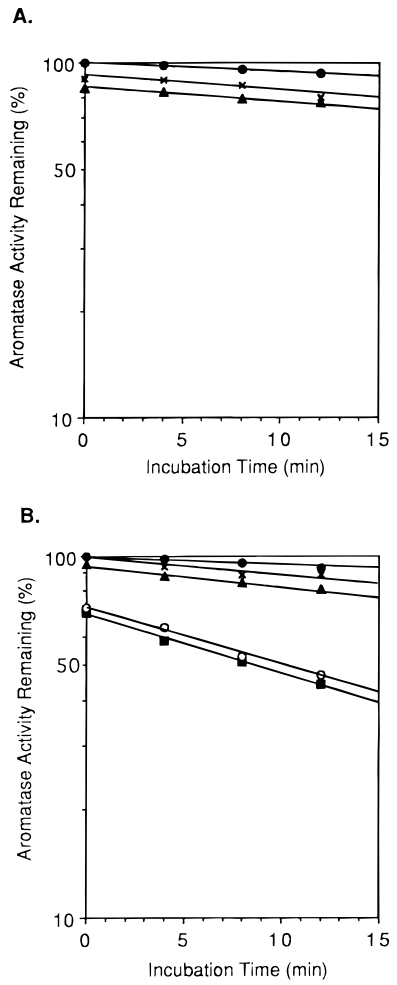
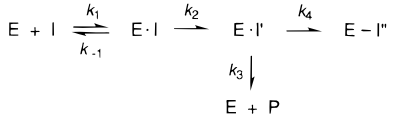


Figure 3. Inactivation of human placental aromatase by the 6α-ethyl steroid **3b** under various conditions. (A) In the absence of NADPH, the inhibitor at concentrations of 0.5 μM (×) and 1.0 μM (▲) failed to cause the inactivation. Control sample (●) contained no inhibitor. (B) Androstenedione (5.4 μM) (▲) or L-cysteine (0.5 mM) (○) was incubated with the placental aromatase, the inhibitor (1.0 μM), and NADPH, and androstenedione protected the enzyme from the inactivation by the inhibitor (■) whereas L-cysteine did not to a significant extent. Control sample with androstenedione (●) or L-cysteine (×) contained no inhibitor. Each point represents the mean of two determinations which varied by less than 7% of the mean. The inactivation experiments with compounds **3a**, **3c**, **3d**, **3f**, and **4a** gave essentially similar results to Figure 3 (data not shown).

positions of androsta-1,4-diene-3,17-dione (**3h**) on both affinity for aromatase and rate of inactivation of the enzyme was examined. The 6β-alkyl derivatives **4** except the methyl and *n*-butyl compounds **4a** and **4d** were very potent competitive inhibitors of aromatase in human placental microsomes with apparent K_i's ranging from 4.7 to 8.0 nM. Moreover, compound **4d** as well as all the 6α-alkyl analogs **3** except the methyl compound **3a** were still good inhibitors (K_i 11−29 nM). On the other hand, the 6α-alkyl steroids **3a**−**d** and **3f** having the alkyl functions being constituted with C-1−C-4 and C-6 along with the 6β-methyl steroid **4a** inactivated aromatase in a mechanism-based manner with k_{inact} ranging from 0.029 to 0.065 min^{-1}. The rate of inactivation decreased when the substrate androstenedione was included in the incubation mixture while L-cysteine, a nucleophile, failed to protect aromatase from the inactivation by the inactivators, in each case. Thus,

Scheme 3



covalent-bound formation between aromatase and the reactive intermediate appears to occur at the active site, therefore, preventing diffusion of the activated inhibitors, a reactive electrophile, in the surrounding media. Covey [3d] has suggested previously that aromatase inactivates itself because the mechanism-based aromatase inhibitors induce the enzyme to autoxidize itself. Since we have no evidence for covelent modification of aromatase by the 6-alkyl dienones, a substrate analog-induced autoxidation mechanism for inactivation would be considered as an alternate explanation for the observed time-dependent inactivation.

An introduction of a methyl function at the C-6α and C-6β positions of the parent steroid **3h** does not change to a significant extent affinity for the activity site of aromatase (K_i 54 and 43 nM for **3a** and **4a** vs 43 nM for **3h**). The addition of one methylene unit to the methyl group increases the affinity, respectively (K_i 19 and 4.7 nM for **3b** and **4b**). In the 6β-series, elongation of the ethyl group up to C_4 decreases the affinity in relation to its carbon number; in contrast, further elongation with one methylene increases but that with more than two methylenes decreases. These relative affinities are essentially similar to those obtained previously in the 6β-alkylandrostenedione series.[10] On the other hand, in the 6α-series, the 6α-*n*-pentyl compound **3e** (K_i = 11 nM) was the most potent inhibitor among them and the apparent K_i values (16−25 nM) of the other steroids having more than one methylene unit are not distinctly different from each other. These results do not correspond well to those previously obtained in the 6α-alkylandrostenedione series.[10] In addition, the 6β-alkyl steroids **4** are more potent competitive inhibitors than the corresponding 6α-isomers. Taken together, it seems to be likely that at least a series of 6β-alkyl steroids **4** may bind to the pocket of the active site in the similar geometry to that involved in the binding of the 6β-alkylandrostenediones. Comparison of the minimum-energy conformations of the 6β-alkyl steroids **4** with those of 6β-alkylandrostenedione analogs previously obtained support this.

The methyl steroids **3a** and **4a** in the both series inactivated aromatase in a mechanism-based manner in which the K_I (800 and 757 nM for **3a** and **4a**) and k_{inact} (0.048 and 0.061 min^{-1} for **3a** and **4a**) values are close to that of the parent steroid **3f** (K_I = 952 nM, k_{inact} = 0.059 min^{-1}), indicating that a methyl function at the C-6α and C-6β positions also does not affect the process of enzyme-mediated inactivation caused by the parent steroid **3h**. The addition of more than one methylene unit to the 6β-methyl function causes the apparent loss of the time-dependent inactivation. This clearly indicates that in thermodynamically stable enzyme-inhibitor complexes produced by fit of the 6β-alkyl group (C-2−C-7) to the pocket of the active site, the inhibitors **4b**−**g** align in a geometry which is not proper for either aromatase-mediated activation reaction of the inhibitors (production of the reactive electrophiles; k_2, Scheme 3) or the dienone-induced autoxidation of aromatase. In contrast, substitution of a methyl group at C-6α by an

ethyl group increases efficiency of the enzyme-mediated inactivation (k_{inact} = 0.065 for the 6α-ethyl compound **3b**). Elongation of the 6α-ethyl function up to *n*-butyl group decreases the rate of inactivation, accompanied by an increase of the K_I value, in relation to the alkyl chain length, and finally the 6α-*n*-pentyl steroid **3e** does not cause the irreversible inactivation. However, surprisingly, further elongation of the 6α-pentyl group to the 6α-hexyl group recovers the ability of the time-dependent inactivation, whereas that to the 6α-heptyl group does not. Although the reason why the 6α-alkyl function plays such a critical role in the inactivation reaction, is not clear, a steric reason would be at least in part operative in either the aromatase-catalyzed activation reaction of the 6α-alkyl dienones or the dienone-induced autoxidation of aromatase. All of the K_I's obtained from the inactivation experiments are more than 10 times higher than the corresponding apparent K_i's from the competitive inhibition experiments. The similar tendency has previously been reported in the inactivation experiments using other 1,4-diene steroids.[5,8] When the observed inactivation is based on the aromatase reaction *per se*, this relation of the K_I value to the apparent K_i value suggests that binding of the activated inhibitor (I′) to the nucleophlic residue of the active site (k_4, Scheme 3) rather than activation of the inhibitor (k_2) becomes rate determining or partial rate determining.[14]

The present results imply that two components of binding would be involved in the interactions of the 6-alkyl 1,4-diene steroids with the active site of aromatase. The first manner is responsible for a tight binding to the active site but is not suitable for a mechanism-based inactivation of the aromatase; this is operative principally in the binding of a series of the 6β-alkyl steroids. The other is suitable for the mechanism-based inactivation where the binding affinity is lower than the other; this is operative principally in a series of the 6α-isomers. Both length and stereochemistry of a 6-alkyl substituent would determine which is a preferred binding geometry for each steroid.

Covey's group has proposed two sequences for the inactivation of aromatase by a 1,4-dien-3-one steroid with no evidence:[3d,5] The first mechanism involves oxygenations at C-19 and the other does removal of an electron from the C-1 double bond by perferry oxygen. The positioning of the heme to the C-1 and/or C-19 of the 6-alkyl 1,4-diene steroids which cause a mechanism-based inactivation is presumed to be similar to that predicted to the inhibitor **3h**. Indeed, in our preliminary study, the 6α-ethyl steroid **3b**, a suicide substrate of aromatase, is aromatized to 6α-ethylestrogens by the enzyme in human placental microsomes to almost a similar extent to that of steroid **3h**, while the 6β-isomer **4b**, which does not cause the time-dependent inactivation, is a poor substrate for the microsomal aromatase (M. Numazawa, A. Yoshimura, and M. Oshibe, to be reported elsewhere). This would support the above presumption. Further study is currently underway in our laboratory to determine more precise aspects of the nature of the binding of the 6-alkyl 1,4-diene steroids.

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 1725 spectrophotometer and UV spectra in 95% EtOH solution on a Hitachi 150−20 spectrophotometer. ^{1}HNMR spectra were obtained in $CDCl_3$ solutions with a JEOL EX 270 (270 MHz) spectrometer using tetramethylsilane as an internal standard, and mass spectra were obtained with a JEOL JMS-DX 303 spectrometer. 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.; Kyoto, Japan), and a UV detector (270 nm). 6α-Alkylandrost-4-ene-3,17-diones (**1a**−**f**) and their 6β-isomers **2a**−**f** were synthesized according to known methods.[10]

Dehydration of 6-Alkylandrost-4-enes 1 and 2 to Their 1,4-Diene Derivatives 3 and 4. A solution of the 6-alkyl-4-enes **1** and **2** (0.2 mmol) and DDQ (0.3 mmol) in dry benzene (3.5 mL) was separately heated under reflux for 18 h in a stream of N_2 according to the known method.[11] After this time, the reaction mixture was subjected to a column of Al_2O_3 (6 g). Elution with hexane−AcOEt gave the crude product which was purified by recrystallization from an appropriate solvent or by the reverse-phase HPLC, yielding the corresponding 1,4-diene steroids **3** and **4**.

6β-*n*-Heptyl-3,3:17,17-bis(ethylenedioxy)androstan-5α-ol (6). A solution of $C_7H_{15}MgBr$ in THF (1 M, 32 mL, 32 mmol) was added to a solution of 3,3:17,17-bis(ethylenedioxy)-androstane-5α,6α-epoxide (**5**)[10a] (600 mg, 1.54 mmol) in THF (24 mL), and the mixture was refluxed for 6 h under a N_2 stream. After the solution was cooled, saturated NH_4Cl solution (150 mL) was added, and the resulting product was extracted with ethyl acetate (250 mL × 2). The combined organic layers were washed to neutrality with water, dried with Na_2SO_4, and evaporated to dryness. The residue was purified by column chromatography (SiO_2, hexane−ethyl acetate) to give compound **6** (680 mg, 90%) as an oil: ^{1}H NMR δ 0.86 (3H, s, 18-Me), 0.88 (3H, t, J = 6.3 Hz, 6-$(CH_2)_6$*Me*), 0.97 (3H, s, 19-Me), 3.81−4.02 (8H, m, OCH_2CH_2O × 2); FT-IR (neat) 3514 (OH) cm^{-1}; exact mass found 490.3613, calcd for $C_{30}H_{50}O_5$ 490.3658.

6β-*n*-Heptyl-5α-hydroxyandrostane-3,17-dione (7). Perchloric acid (3 M, 4 mL) was added to a solution of compound **6** (450 mg, 0.92 mmol) in THF (9.4 mL). This mixture was stirred at room temperature for 3 h, diluted with ethyl acetate (200 mL), washed with 8% $NaHCO_3$ solution and water, dried with Na_2SO_4, and evaporated to dryness; the residue was subjected to column chromatography (SiO_2, hexane−ethyl acetate) followed by recrystallization from ethyl acetate to give compound **7** (320 mg, 85%): mp 148−149 °C; ^{1}H NMR δ 0.89 (3H, t, J = 6.9 Hz, 6-$(CH_2)_6$*Me*), 0.91 (3H, s, 18-Me), 1.22 (3H, s, 19-Me), 3.06 (1H, d, J = 14.5 Hz, 4-H); FT-IR (KBr) 3405 (OH) and 1742 and 1703 (C=O) cm^{-1}. Anal. ($C_{26}H_{42}O_3$) C, H.

6α-*n*-Heptylandrost-4-ene-3,17-dione (1g). Hydrochloric acid (1 M, 1.2 mL) was added to a solution of steroid **6** (200 mg, 0.41 mmol) in 95% ethanol (12 mL), and the resulting mixture was refluxed for 7 h. After most of the solvent was removed, the mixture was diluted with ethyl acetate (200 mL), washed with 8% $NaHCO_3$ solution and water, dried with Na_2SO_4, and evaporated. The residue was purified by column chromatography (SiO_2, hexane−ethyl acetate) followed by reverse-phase HPLC (acetonitrile−water, 87/13, 7.5 mL/min), yielding compound **1g** (80 mg, 51%) as an oil: HPLC t_R = 46.8 min; ^{1}H NMR δ 0.89 (3H, t, J = 6.9 Hz, 6-$(CH_2)_6$*Me*), 0.92 (3H, s, 18-Me), 1.20 (3H, s, 19-Me), 5.81 (1H, d, J = 1.3 Hz, 4-H); FT-IR 1740 and 1681 (C=O) cm^{-1}; exact mass found 384.3033, calcd for $C_{26}H_{40}O_2$ 384.302 80.

6β-*n*-Heptylandrost-4-ene-3,17-dione (2g). $SOCl_2$ (0.42 mL) was added to a chilled solution of compound **7** (250 mg, 0.62 mmol) in dry pyridine (5 mL). The resulting mixture was stirred for 3 min at 0 °C, poured into 50 mL of ice−water, and extracted with ethyl acetate (50 mL × 2). The combined organic layers were washed with water, dried with Na_2SO_4, and evaporated to yield the crude product, which was purified by column chromatography (SiO_2, hexane−ethyl acetate) followed by reverse-phase HPLC (acetonitrile−water, 87/13, 7.5 mL/min) to give compound **2g** (190 mg, 80%) as an oil: HPLC t_R = 40.4 min; ^{1}H NMR δ 0.89 (3H, t, J = 6.9 Hz, 6-$(CH_2)_6$*Me*), 0.94 (3H, s, 18-Me), 1.25 (3H, s, 19-Me), 5.74 (1H, s, 4-H); FT-

IR (neat) 1740 and 1677 (C=O) cm^{-1}; exact mass found 384.3023, calcd for $C_{26}H_{40}O_2$ 384.302 80.

Biochemical Studies. Chemicals. Androsta-1,4-diene-3,17-dione (**3h**) was purchased from Aldrich Chemical, [1β-^{3}H]androstenedione (27.5 Ci/mmol) (^{3}H distribution: 74−79% at 1β) from New England Nuclear Corp. (Boston, MA), and NADPH from Kohjin Co., Ltd. (Tokyo, Japan).

Enzyme Preparation. Human placental microsomes (particles sedimenting at 105000*g* for 60 min) were obtained using the method reported by Ryan.[4a] 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.[12] The screening assay for determination of IC_{50} value, the kinetic assay, and the time-dependent assay were carried out essentially according to the assay methods described in our previous work.[15] Briefly, 20 μg of protein of the lyophilized microsomes and 20-min incubation time for the screening assay, and 20 μg of protein of the microsomes and 5-min incubation time for the kinetic assay, respectively, were employed in this study, and the assays were carried out in 67 mM phosphate buffer in the presence of NADPH in air. In the time-dependent inactivation experiment, 1/10 of the incubation mixture was used for assays of the remaining aromatase activity.

Molecular Modeling Studies. By use of MOL-CRYS soft (Daikin, Tokyo, Japan), an initial structure for androsta-1,4-dien-3,17-dione (**3h**) was generated from coordinates obtained by an X-ray crystallographic determination of 17β-hydroxyandrosta-1,4-dien-3-one.[16] Molecular models were constructed on a Silicon Graphics IRIS 4D workstation using the 3D graphic option of MOL-GRAPH (Daikin, Tokyo, Japan). Each compound discussed in this study was subjected to the Confsearch option using Monte Carlo analysis to determine all of its minimum-energy conformations. Finally, some conformations near the minimum-energy ones were selected to further analyze the lowest energy conformations using semiempirical molecular orbital calculations with PM3 method (MOPAC version 6, Quantum Chemistry Program No. 455). The version 6 was obtained through the Japan Chemistry Program Exchange (Tokyo). Geometries were considered minimized when the energy change between two subsequent structures was less than 0.001 kcal/mol.

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