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Biological aromatization of $\Delta^{4,6}$- and $\Delta^{1,4,6}$-androgens and their 6-alkyl analogs, potent inhibitors of aromatase

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Abstract

Enzymic aromatization of Δ^6- and $\Delta^{1,6}$-derivatives of the natural substrate androstenedione with human placental aromatase was first studied using gas-chromatography-mass spectrometry. The two steroids were aromatized with apparent K_m and V_{max} values of 62 nM and 32 pmol/min/mg protein for the Δ^6-steroid and 167 nM and 10 pmol/min/mg protein for the $\Delta^{1,6}$-steroid, respectively. We next explored the aromatization of a series of 6-alkyl (methyl, ethyl, *n*-propyl, and *n*-pentyl)-substituted Δ^6-androstenediones and their $\Delta^{1,6}$-analogs, potent competitive inhibitors of aromatase, to gain insight into the relationships between the inhibitory activity of the 6-alkyl-C_{19} steroids and their ability to serve as a substrate of aromatase. In a series of the $\Delta^{1,6}$-androstenediones, all the 6-alkyl steroids were more efficient substrates than the parent $\Delta^{1,6}$-steroid in which the aromatization rates of the alkyl steroids were about 2-fold that of the parent steroid, in contrast, all of the 6-alkyl-substituted Δ^6-androstenediones were converted into the corresponding 6-alkyl-Δ^6-estrogens with the rates of less than about a half that of the parent steroid. These results indicate that the 6-alkyl function decreases the aromatization rate of the Δ^6-steroid but enhances that of the $\Delta^{1,6}$-steroid. The relative apparent K_m values for the C_{19} steroids obtained in this study are different from the relative K_i values obtained previously, indicating that a good inhibitor is not essentially a good substrate in the 6-alkyl-substituted Δ^6- and $\Delta^{1,6}$-androstenedione series. © 1999 Elsevier Science Ltd. All rights reserved.

1. 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 [1–3]. Aromatization of the androgens appears to involve three oxygenation steps [4–11]. The first two are sequential hydroxylations of the 19-methyl group to produce 19-hydroxy and 19,19-*gem*-diol intermediates, respectively. Dehydration of this *gem*-diol leads to the readily isolated 19-oxo intermediate. In the third step, C-19 and 1β,2β-protons are eliminated as formic acid and water, respectively, to produce the estrogens. It is currently thought to be likely that the third mole of oxygen attacks on the 19-aldehyde intermediate in a nucleophilic manner to produce a 19-hydroxy-19-ferric peroxide intermediate [7,12,13].

Aromatase is a potential therapeutic target for the selective lowering of estrogen level in patients with estrogen-dependent tumors, including breast cancer [14–20]. Thus, a number of potent aromatase inhibitors, analogs of the substrate androstenedione, have been described by various laboratories. Androsta-1,4,6-triene-3,17-dione, a $\Delta^{1,6}$-analog of androstenedione, is one of the prototypical mechanism-based inhibitors (suicide substrates) of aromatase, although the inactivation mechanism is unknown [17,21–25]. On the other hand, androsta-4,6-diene-3,17-dione, a Δ^6-analog of androstenedione, is a good competitive inhibitor of aromatase but does not cause a mechanism-based inactivation of aromatase [23,26,27]. The triene and diene steroids have lower affinity for aromatase than the substrate androstenedione. We have synthesized 6-alkyl (C_1–C_7)-substituted $\Delta^{1,6}$- or Δ^6-androstenediones,

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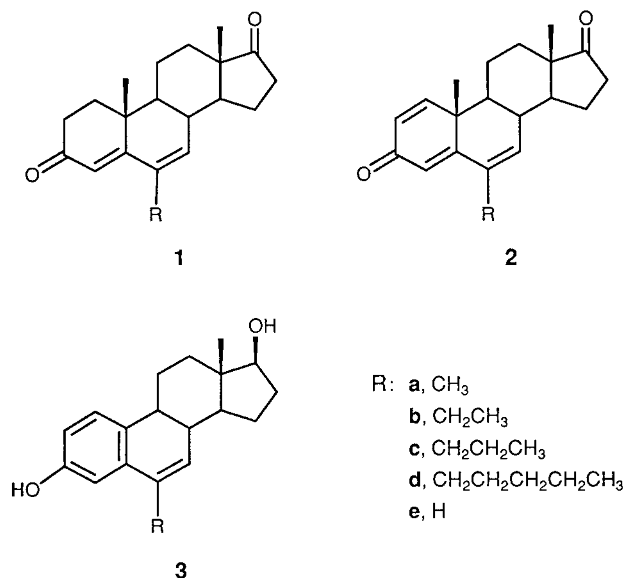

Fig. 1. Structures of 6-alkyl-substituted androgens and estrogens.

some of which are very potent competitive or mechanism-based inhibitors [28]. The structure-activity relationships have revealed that the length of the *n*-alkyl-substituent at C-6 of the 1,4,6-triene, rather than its 4,6-diene analog, plays a critical role in tight binding to the active site of aromatase. No significant correlation was observed between affinity for the enzyme and the inactivation ability of the 6-alkyl-1,4,6-trienes.

Thus, we were interested to know aspects of aromatization of the 6-alkyl-1,4,6-trienes and their 4,6-diene analogs with human placental aromatase in relation to their affinity to the enzyme (Fig. 1). This paper describes the kinetic studies of the aromatization of the 6-alkyl-triene and diene steroids with gas chromatography-mass spectrometry. The 6-alkyl (C_1–C_3 and C_5) analogs of the 1,4,6-triene were aromatized more efficiently than the parent steroid whereas introduction of the 6-alkyl groups decreased the aromatization in the 4,6-diene series. Neither the apparent K_m nor V_{max} values for the aromatization of the alkyl steroids was significantly correlated with the inhibition constants (K_i values).

2. Materials and methods

6-Alkyl-substituted derivatives of androsta-4,6-diene-3,17-dione, androsta-1,4,6-triene-3,17-dione, and Δ^6-estradiol (alkyl = methyl, ethyl, *n*-propyl, and *n*-pentyl), compounds **1**–**3**, were synthesized by previously reported methods [28,29]. [2,4,6,6,7,16,16,17α-$^2H_8$]6-Alkyl-Δ^6-estradiol (**3**) and [2,4,16,16,17α-$^2H_5$]Δ^6-estradiol were prepared from the corresponding non-labeled steroids **3** with the known method [30] [6-methyl compound **3a** d_7, 15%, d_8, 47%, d_9, 38%; 6-ethyl compound **3b** d_7, 28%, d_8, 62%, d_9, 10%; 6-*n*-propyl compound **3c** d_7, 19%, d_8, 81%; 6-*n*-pentyl compound **3 d** d_7, 98%, d_9, 2%; Δ^6-estradiol **3e** d_3, 6%, d_4, 40%, d_5, 54%]. [1β,^{3}H]Androstenedione (specific activity 27.5 Ci/mmol; ^{3}H-distribution 76% at 1β) was obtained from New England Nuclear (Boston, MA, USA). NADPH was purchased from Kohjin Co. (Tokyo, Japan), $NaBH_4$ from Aldrich Chemical Co. (Milwaukee, WI, USA) and bistrimethylsilyltrifluoroacetamide from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

2.1. Enzyme preparation

Human placental microsomes (sedimenting after 60 min at 105,000 g) were obtained as described by Ryan [31]. 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 (1 month) of the study.

2.2. Aromatization studies with gas chromatography-mass spectrometry (GC-MS)

Incubation experiments were carried out essentially according to the previous method [32]. Briefly, a mixture of 1 μM of substrate, 114 μM of NADPH, and 500 μg protein of placental microsomes in 67 mM phosphate buffer (pH 7.4) was incubated at 37°C. After 5 or 30 min of incubation, the deuterated internal standard (50 or 100 ng) was added to each incubation mixture and extracted with ethyl acetate. The extract was reduced with $NaBH_4$ and then placed in a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). The steroid fraction eluted with 80% methanol was placed in Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) in which each aromatized product was obtained in 1–14 ml fraction (benzene/methanol, 95:5). The recovery rates for all the 6-alkyl-Δ^6-estradiols with these procedures were in the range 70–80%.

In the above experiments with Δ^6-androstenedione (**2e**), 250, 500, and 750 μg of the microsomal protein and 5, 15, 30 and 45 min of the incubation time were also employed to determine the linearity of the aromatization rate.

For kinetic analysis, the incubation mixture contained various concentrations of each steroid, 50 μg of the microsomal protein for all the experiments, except for that with the 4,6-diene steroid **1e** or the 6-*n*-pentyl-1,4,6-triene **2 d** in which 25 or 100 μg of the protein was used, respectively, 120 μM NADPH for all the experiments, except for that with the steroid **1e** or **2 d** in which 60 or 240 μM NADPH was used in each, 100 μl of MeOH, and 0.9 ml of the 67 mM phosphate buffer (pH 7.4). The mixture was incubated at 37°C for 5 min and the aromatized product was purified as described above. When

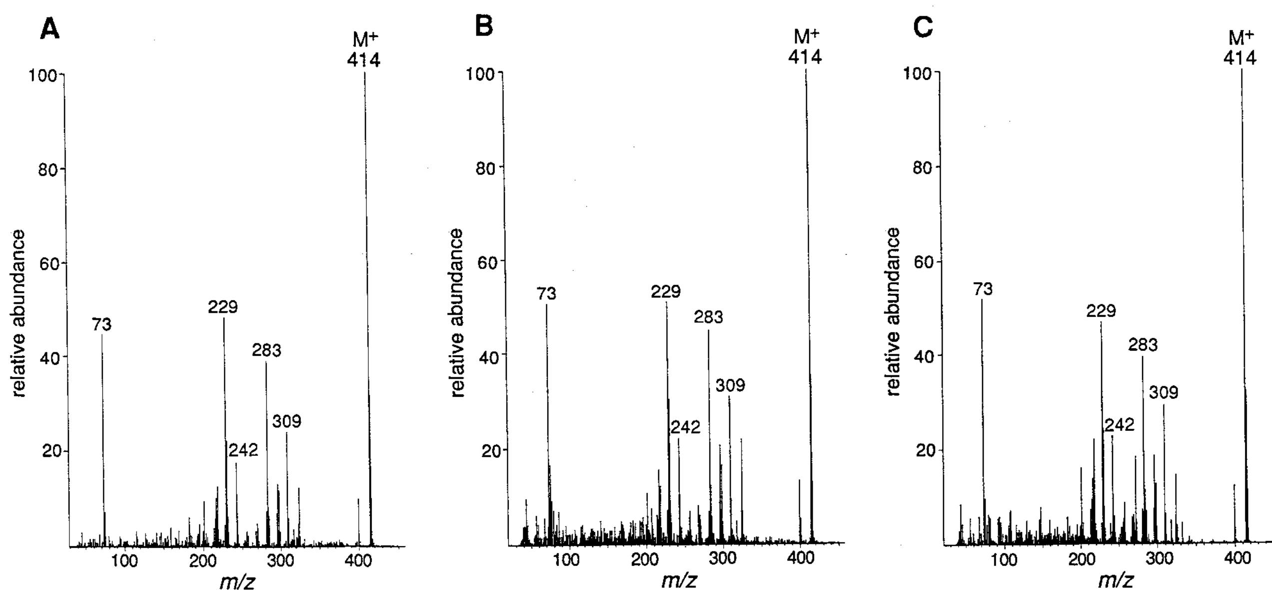

Fig. 2. Mass spectra of the aromatized products obtained by incubation of the 4,6-diene steroid **1e** (**A**) and its 1,4,6-triene analog **2e** (**B**) with human placental microsomes and the authentic Δ^6-estradiol (**C**). The androgens (1 μM) were separately incubated with the microsomes (500 μg protein) for 30 min at 37°C. The products were converted into the 17β-hydroxy derivatives by reduction with $NaBH_4$ and then analyzed as Δ^6-estradiol using GC–MS, as described in Section 2.

[1β,^{3}H]androstenedione was used as the substrate in the kinetic analysis, the aromatization rate was determined by measuring the amount of ^3H_2O released from the labeled substrate into the incubation medium during aromatization according to the method previously reported [33].

2.3. Derivatization of the aromatized products, 6-alkyl-Δ^6-estradiols, and GC-MS

The aromatized product, Δ^6-estradiol or its 6-alkyl analog, was converted to the bistrimethylsilyl ether with bistrimethylsilyltrifluoroacetamide and then sub-

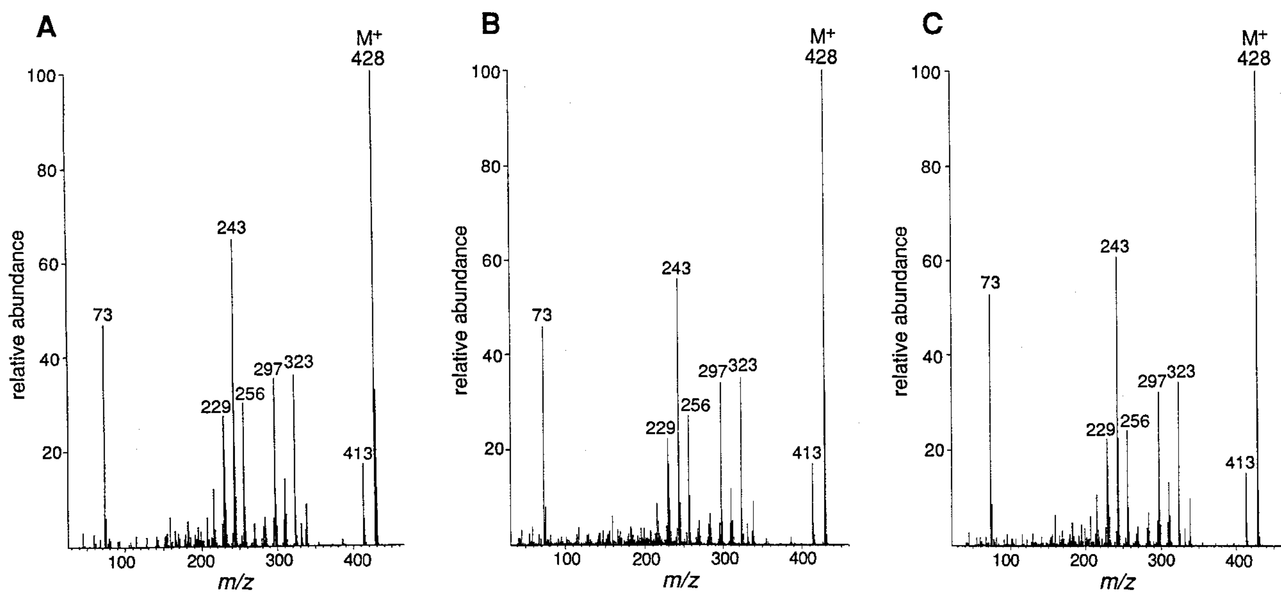

Fig. 3. Mass spectra of the aromatized products obtained by incubation of the 6-methyl-4,6-diene steroid **1a** (**A**) and its 1,4,6-triene analog **2a** (**B**) with human placental microsomes and the authentic 6-methyl-Δ^6-estradiol (**C**). The mass spectra were obtained similarly as shown in Fig. 2. The 6-alkylestrogens produced from the other 6-alkylandrogens were also identical with the corresponding authentic samples (data were not shown).

Table 1
Aromatization of 6-alkyl-substituted Δ^6- and $\Delta^{1,6}$- androstenediones with human placental microsomes[a]

6-Alkyl substituent (R)	Aromatization rate (pmol/min/mg protein)	
	30 min incubation	5 min incubation
Δ^6-androstenediones **1**		
a, CH_3	14.5 ± 0.37	14.9 ± 0.40
b, CH_2CH_3	11.4 ± 0.11	12.1 ± 0.23
c, $CH_2CH_2CH_3$	4.9 ± 0.22	–[b]
d, $CH_2CH_2CH_2CH_2CH_3$	2.1 ± 0.16	–
e, H	28.2 ± 0.50	30.0 ± 0.61
$\Delta^{1,6}$-androstenediones **2**		
a, CH_3	12.9 ± 0.17	18.7 ± 0.32
b, CH_2CH_3	10.2 ± 0.47	21.6 ± 0.74
c, $CH_2CH_2CH_3$	8.1 ± 0.46	17.8 ± 0.52
d, $CH_2CH_2CH_2CH_2CH_3$	7.8 ± 0.51	16.0 ± 0.32
e, H	4.5 ± 0.09	8.6 ± 0.07
For comparison		
androstenedione[c]	120 ± 11	–

[a] All steroids (1.0 μM) were incubated separately with human placental microsomes (500 μg of protein) at 37°C for 5 or 30 min in the presence of NADPH (114 μM) in 2.1 ml of 67 mM phosphate buffer, pH 7.4. The aromatized products were analyzed as the bistrimethylsilyl derivatives by GC-MS with the deuterated internal standard in each case. Results are means SD (n = 4).

[b] Not determined.

[c] Aromatization rate was obtained with a radiometric assay using [1β-^{3}H] androstenedione as a substrate.

jected to analysis according to the previous method [32].

A Finnigan MAT SSQ GC-MS instrument (San Jose, CA, USA) was used and the GC-MS conditions were the same as those previously used for analysis of the 6-alkylestradiols [30]. The quantitative analysis of the aromatized product was performed with a selected ion monitoring method (electron impact mode); the molecular ion (M^+) was the base peak ion for all of the Δ^6-estradiol analogs and then the amount of the estrogen formed was obtained by the relative abundance of the molecular ion of the product to that of the deuterated internal standard in each experiment.

3. Results

Aromatization of series of androsta-4,6-diene-3,17-diones and their Δ^1-derivatives was initially studied to determine whether they are converted into the corresponding estrogens by human placental aromatase. The aromatization product was postulated to be a mixture of Δ^6-estrone derivative and its 17β-reduced compound, Δ^6-estradiol derivative, produced by the action of 17β-reduced hydroxysteroid dehydrogenase [34,35], in each incubation. Thus, the aromatization product was reduced to Δ^6-estradiol analog **3** on treatment with $NaBH_4$ in each experiment. The 17β-hydroxy steroid **3** was then purified and analyzed as the bistrimethylsilyl ether by GC–MS (EI mode). Mass spectra and retention times of the aromatized products of all of the 4,6-diene and 1,4,6-triene steroids **1** and **2** were identical with those of the corresponding authentic Δ^6-estradiol and its 6-alkyl derivatives **3**, respectively (Figs. 2 and 3). The mass spectra of the bistrimethylsilyl ethers of all the aromatized products showed a molecular ion (M^+) as the base peak with characteristic ions of M^+–105, M^+–131, and M^+–185. The abundant fragment ion (M^+–105) is the favored trimethylsiloxybenztropylium ion produced by two sequential benzylic cleavages [36].

The aromatization rate of the parent androsta-4,6-diene steroid **1e** at concentration of 1 μM increased linearly with increasing amounts of placental microsomes (up to 750 μg of protein) and with incubation time (up to 45 min). The aromatization rates of series of the 4,6-diene and 1,4,6-triene steroids were first determined in the experiments with a 30 min incubation time and 500 μg of placental microsomes (Table 1). Under the conditions, the conversion rate was less than 12% of each substrate used for the incubation. The aromatization rate of the natural substrate androstenedione to estradiol was 120 pmol/min/mg protein in the radiometric assay with [1β-^{3}H]androstenedione as a substrate. The aromatization of the 6-ethyl-1,4,6-triene **2b** was efficiently

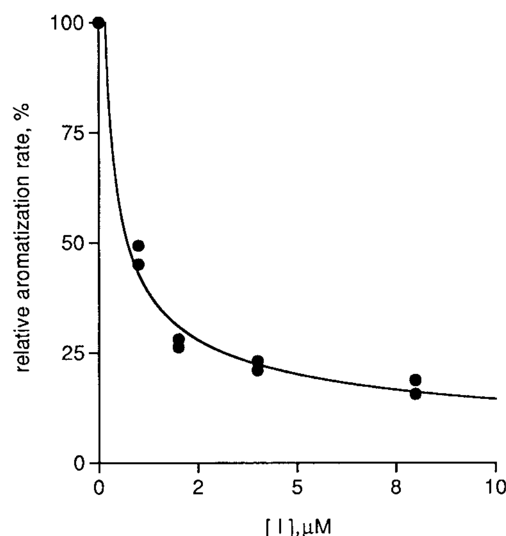

Fig. 4. A dose-dependent inhibition of amortization of the 6-ethyl-1,4,6-triene steroid **2b** by 19-hydroxyandrost-4-en-17-one, a potent competitive inhibitor of aromatase. The steroid **2b** (1 μM) was incubated with human placental microsomes (500 μg of protein) and NADPH (114 μM) for 5 min in the presence of various concentrations (1, 2, 4, and 8 μM) of the 19-hydroxy steroid. Each experiment was carried out in duplicate. Variation between two determinations was less than 10%.

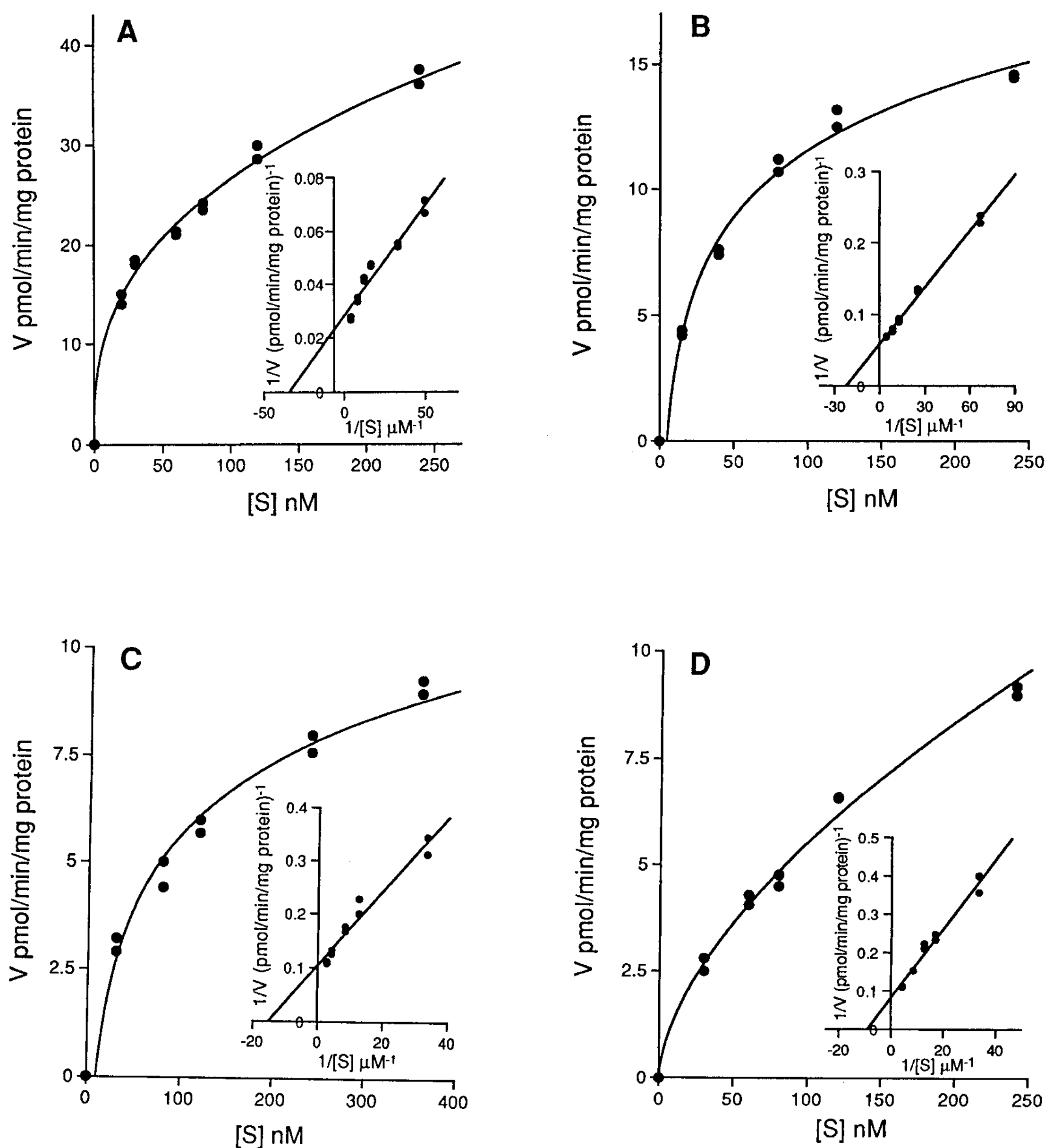

Fig. 5. Kinetic analysis of aromatization of the 4,6-diene steroids **1a** and **1e** and the 1,4,6-triene steroids **2a** and **2e** with human placental aromatase. Various concentrations of the 4,6-diene **1e** (**A**) and its 6-methyl analog **1a** (**B**) and the 1,4,6-triene **2e** (**C**) and its 6-methyl analog (**D**) were separately incubated with the microsomes (25 μg protein for the 4,6-diene **1e** and 50 μg protein for the other three steroids, respectively) for 5 min at 37°C and the aromatization rates were analyzed using GC–MS with an internal standard method as described in Section 2. Each experiment was carried out in duplicate. Variation between two determinations was less than 10%.

blocked in the presence of a competitive inhibitor of aromatase, 19-hydroxyandrost-4-en-17-one [37], in a dose-dependent manner (Fig. 4). A series of 6-alkyl-$\Delta^{1,6}$-androstenediones **2** are known to be suicide substrates of aromatase that cause a time-dependent loss of the aromatase activity during the incubation. Thus, to minimize the time-dependent loss of the initial aromatization rate, a short (5 min) incubation time was employed for the aromatization of this series of steroids. The aromatization rates obtained with the short incubation time were higher than those obtained with the longer incubation time. When the parent steroid **2e** at 1 μM concentration was preincubated with placental microsomes in the presence of NADPH at 37°C, the remaining aromatase activity was about 94–96 % of the initial rate obtained with no preincubation. This finding indicates that the aromatization rate of the suicide substrate, obtained from the 5 min incubation experiment, would be almost the same as the initial rate, as previously reported in the aromatization of the other suicide substrates, 6-alkyl-Δ^1-androstenediones [32]. The aromatization of the 4,6-diene and 1,4,6-tri-

Table 2
Kinetic analysis of aromatization of selected 6-alkyl androgens with human placental microsomes[a]

6-alkyl substituent (R)	K_i (nM)[b] from ref.	K_m, nM	V_{max} (pmol/min/mg protein)
Δ^6-androstenediones **1**			
a, CH_3	44	32	15.1
b, CH_2CH_3	50	20	13.0
c, $CH_2CH_2CH_3$	30	6.9	2.9
e, H	61	62	32.0
$\Delta^{1,6}$-androstenediones **2**			
a, CH_3	80	115	16.5
b, CH_2CH_3	3.4	28	19.2
c, $CH_2CH_2CH_3$	12	34	17.0
d, $CH_2CH_2CH_2CH_2CH_3$	15	20	18.7
e, H	47	167	10.0

[a] All incubations were carried out at 37°C for 5 min with various concentrations of the Δ^6- and $\Delta^{1,6}$-steroids and human placental microsomes (25, 50 or 100 μg of protein) in the presence of NADPH (60, 120 or 240 μM), respectively, as described in the experimental section. In the experiment with [1β-^{3}H]androstenedione as a substrate, the apparent K_m for androstenedione was about 33 nM.

[b] Apparent K_i values listed for comparison are corrected ones by the application of a factor (33/24) for the K_i values obtained previously [28], since the K_m for androstenedione in the previous study is 24 nM.

ene steroids **1** and **2** was further studied to characterize the affinity (K_m) for aromatase with a 5 min incubation time. All the steroids, except for the pentyl-4,6-diene **1 d** of which the conversion rate was very low, showed a typical saturation curve with increasing substrate concentration in each case (Fig. 5), and the Lineweaver–Burk plot gave the apparent K_m and V_{max} values shown in Table 2.

4. Discussion

Δ^6- and $\Delta^{1,6}$-androstenediones (**1e** and **2e**) as well as their 6-alkyl derivatives (**1a**–**d** and **2a**–**d**) were aromatized by human placental microsomes to the corresponding Δ^6-estrogens. The conversion of the 6-ethyl-1,4,6-triene steroid **2b** into 6-ethyl-Δ^6-estradiol (**3b**) were efficiently inhibited by 19-hydroxyandrost-4-en-17-one, a potent competitive inhibitor of aromatase, showing that the aromatization of all the steroids examined should be catalyzed by aromatase.

A qualitative comparison of the amounts of phenolic products produced from metabolism of androstenedione and its Δ^1-, Δ^6-, and $\Delta^{1,6}$-analogs with human placental microsomes has been carried out to reveal that the presence of the C_6–C_7 double bond in the 4,6-diene and 1,4,6-triene steroids **1e** and **2e** markedly reduces the conversion of these compounds to phenolic products [31,38,39]. However, the structures of the products were not determined at that time. The present results unambiguously proved for the first time that compounds **1e** and **2e** having a double bond at C-6 were aromatized to produce Δ^6-estrone and/or Δ^6-estradiol, on the basis of the GC-MS experiments. The aromatization rates of the two steroids (28.2 and 8.6 pmol/min/mg protein for **1e** and **2e**, respectively) were much lower than those of the corresponding steroids without the C_6–C_7 double bond (120 and 97 pmol/min/mg protein for androstenedione and its Δ^1-analog, respectively) [32]. An introduction of the double bond in the B-ring of steroid nucleus would change the binding alignment in the active site of aromatase to result in the less proper binding for the aromatase reaction probably through the steric and/or electronic reason(s).

When we employed a 5 min incubation time for the aromatization experiment with the 1,4,6-triene steroids **2** which cause a time-dependent inactivation of aromatase, the rate of aromatization increased to be about 1.5 to 2-fold that obtained with the 30 min incubation experiment in each. In contrast, there was no significant difference in the aromatization rates between the 5 and 30 min incubation experiments with the 4,6-diene steroids **1a**, **1b** and **1e** which are not suicide substrates.

The introduction of a methyl group at the C-6 position of the parent 4,6-diene steroid **1e** markedly decreased the capacity to serve as a substrate of aromatase by about 49% whereas the same modification in the 1,4,6-triene series produced about two-fold higher aromatization rate. Elongation of the 6-methyl group decreased the aromatization essentially in proportion to the carbon number of the straight alkyl chain at C-6 in the Δ^6-androstenedione series where the aromatization of the 6-pentyl analog **1 d** decreased to about one-tenth that of the parent compound **1e**. On the other hand, elongation of the 6-methyl group of the 1,4,6-triene steroid **2a** up to a *n*-pentyl group did not change to a significant extent the aromatization.

To gain further insights into the binding aspects of the 4,6-diene and 1,4,6-triene analogs **1** and **2** at the

active site of aromatase, the aromatization of series of steroids **1** and **2** were then analyzed kinetically (Table 2). The apparent K_m values for series of the 1,4,6-triene steroids **2**, obtained in this study, were about 1.3–8.2 times higher than the corresponding K_i values (inhibition constants) obtained previously by the inhibition study [28]. In contrast, the K_m values for all the 4,6-diene steroids, except for the parent steroid **1e** of which the K_m was similar to the K_i value, were lower than the corresponding K_i values. The K_m for the propyl compound **1c** was extremely low (6.9 nM) and about one quarter of the K_i. In addition, there is no significant correlation between the relative K_i and relative K_m values in each series of the steroids.

Covey's group has previously reported that aromatization of the 19-hydroxy derivative of the 4,6-diene steroid **1e** proceeds in the same stereochemical course as that involved in the aromatization of 19-hydroxy derivative of the natural substrate androstenedione, suggesting that a series of the 4,6-diene steroids **1** would be aromatized through the same mechanism to that observed in the androstenedione aromatization [40]. The 6-alkyl function would interfere with the aromatization of the diene steroids probably through a steric reason. In contrast, there is no knowledge regarding the mechanisms for the aromatization as well as a time-dependent inactivation of aromatase in the case of the 1,4,6-triene steroid **2e**. It is thought that the 6-alkyl function in the triene series would change an alignment of the 19-methyl group in the active site to be more proper position for the cause of the catalytic function of aromatase compared to that of the parent steroid **2e**.

In conclusion, the aromatization of Δ^6-androstenedione **1e** and its Δ^1-analog **2e** with human placental aromatase was definitely established using GC-MS. Effects of various lengths of the 6-alkyl group on the aromatization did not significantly correlate with those on the inhibition of the aromatase activity. The 6-alkyl steroids examined would be able to play an important role for understanding the catalytic function of aromatase.

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