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19-oxygenations of 3-deoxy androgens, potent competitive inhibitors of estrogen biosynthesis, with human placental aromatase

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Abstract

Aromatase is a cytochrome P450 enzyme complex that catalyzes the conversion of androst-4-ene-3,17-dione (AD) to estrone through three sequential oxygenations of the 19-methyl group. To gain insight into the ability of 3-deoxy derivative of AD, compound **1**, and its 5-ene isomer **4**, which are potent competitive inhibitors of aromatase, to serve as a substrate, we studied their 19-oxygenation by human placental aromatase and the metabolites isolated were analyzed by gas chromatography–mass spectrometry. Inhibitors **1** and **4** were found to be oxygenated with aromatase to produce the corresponding 19-hydroxy derivatives **2** and **5** and 19-oxo derivatives **3** and **6** as well as the 17β-reduced 19-hydroxy compounds **7** and **8**. Kinetic studies indicated that the 5-ene steroid **4** was surprisingly a good substrate for the aromatase-catalyzing 19-oxygenation with the V_{max} value of 45 pmol/min per mg prot which was approx. four times higher than that of the other. The relative K_m value for steroids **1** and **4** obtained in this study is opposite from the relative K_i value obtained previously in the inhibition study. The results reveal that there is a difference between a binding suitable for serving as an inhibitor of aromatase and a binding suitable for serving as a substrate of the enzyme in the 3-deoxy steroid series and the C-3 carbonyl group of AD is essential for a proper binding as a substrate to the active site of aromatase.

1. Introduction

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 (AD) and testosterone [1–3]. Aromatization of the androgens appears to involve three sequential oxygenations at the C-19 position, eventual loss of the angular methyl group at C-19 and the elimination of the 1β,2β-hydrogens, resulting in the aromatization of the A-ring of the androgen molecule to form estrogen [4–6]. Inhibitors of aromatase have recently become of interest in the treatment of advanced estrogen-dependent breast cancer [7–9]. For this reason, a number of potent aromatase inhibitors which are analogs of the substrate AD, have been described by various laboratories.

We have previously reported that 3-deoxy AD (**1**) [10] and its 5-ene analog **4** [11] are excellent and good competitive inhibitors of human placental aromatase, respectively, although they are lacking a carbonyl group at C-3 which has been thought to be essential for a proper binding of the substrate to the active site of aromatase (Fig. 1). Moreover, the structure-activity relationships of the 3-deoxy steroids with a 4-ene or 5-ene structure have indicated that a 17-carbonyl function is necessary for a tight binding of the 3-deoxy steroids to the active site [11,12].

Thus, it was of interest to know whether the 3-deoxy inhibitors **1** and **4** can serve as a substrate of aromatase that results in the formation of 19-oxyge-

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nated products, from viewpoint of a correlation between their ability to serve as a substrate and their ability to serve as an inhibitor. This paper describes identification of 19-oxygenated metabolites formed from steroids **1** and **4** with human placental aromatase and kinetic studies of the 19-oxygenations, using gas chromatography–mass spectrometry (GC-MS). Steroids **1** and **4** were oxygenated at the C-19 position with aromatase where the 5-ene inhibitor **4** was more effective substrate for aromatase.

2. Materials and methods

2.1. Materials

3-Deoxy steroids **1** and **4** and their 19-hydroxy-(**2** and **5**) and 19-oxo-(**3** and **6**) analogs and 17β,19-diols **7** and **8** were synthesized according to the previously reported methods [10,11,13]. [3,3,7,7-$^2H_4$]-Compound **7** and [3β,7,7,17α-$^2H_4$]-compound **8** were prepared according to the known methods [14]. NADPH was purchased from Kohjin Co. (Tokyo, Japan), $NaBH_4$ from Aldrich Chemical Co. (Milwaukee, WI, USA), and bistrimethylsilyltrifluoroacetamide (BSTFA), dimethylisopropylsilyl (DMIPS) imidazole and methoxyamine hydrochloride from Tokyo Kasei Kogyo (Tokyo, Japan). The anti-aromatase monoclonal antibody, MAb3-2C2, prepared by Dr Y. Osawa's group [15] with the support of a grant from the National Institute of Child Health and Human Development (HD 04945), was kindly donated. Human placental microsomes (particles sedimenting at 105,000 *g* for 60 min) were obtained as described by Ryan [16] and they were washed with 0.05 mM dithiothreitol solution, lyophilized, and stored at −20°C.

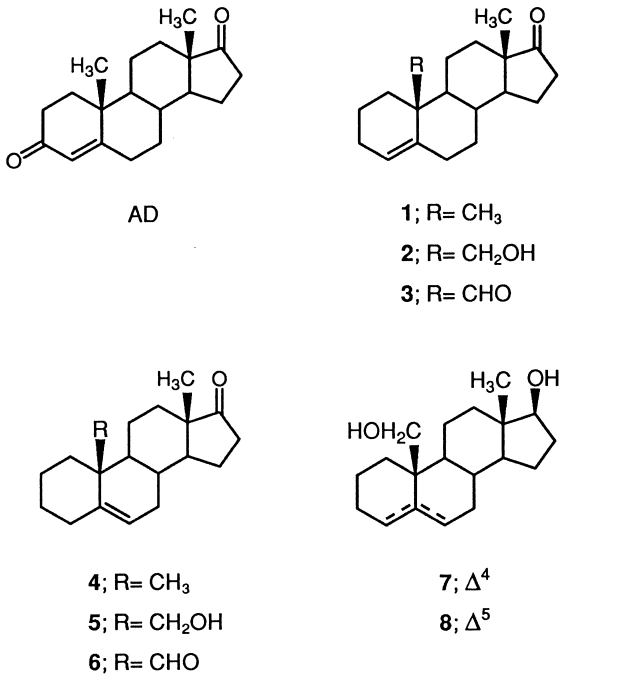

Fig. 1. Structures of the natural substrate AD and 3-deoxy steroid.

2.2. 19-Oxygenation studies with GC-MS

Incubations were conducted in a shaking water bath at 37°C in air with 50 ml Erlenmeyer flasks. Each contained 3.8 μM of steroids **1** and **4**, 300 μM NADPH, 500 or 1000 μg of protein of placental microsomes, 50 μl of methanol and 67 mM phosphate buffer, pH 7.4, in a total volume of 4 ml. For each experiment, four parallel incubations were performed. After 30 min of incubation, metabolites were extracted twice with 5 ml of ethyl acetate. The organic layer was evaporated under reduced pressure to give a residue, which was dissolved in 100 μl of methanol, diluted with water, and placed in a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). After a wash with water (5 ml) followed by 10% methanol (5 ml), the steroid fraction was eluted with 80% methanol (10 ml). For the kinetic analysis, the incubation mixture contained various concentrations of each 3-deoxy steroid, 100 or 200 μg of the microsomal protein and 60 or 150 μM NADPH for experiment with steroid **4** or **1**, 50 μl of methanol, and the 67 mM phosphate buffer, pH 7.4, in a 50 ml Erlenmeyer flask in a total volume 4.0 ml. The mixture containing steroid **1** or **4** was incubated for 5 or 20 min in air, respectively. After adding [3,3,7,7-$^2H_4$] compound **7** (20 ng) or [3β,7,7,17α-$^2H_4$]-compound **8** (100 ng) as an internal standard to each incubation mixture, the steroidal material was extracted twice with 5 ml of ethyl acetate. The organic layer was evaporated to give a residue which was dissolved in methanol (100 μl). $NaBH_4$ (500 μg) was added to the methanolic solution at 0°C [17]. After 30 min of reaction, the mixture was diluted with water (3 ml) and then placed in a Sep-Pak C_{18} cartridge. After the same work-up as described above, the steroid fraction was obtained. The recovery rates for 17β,19-diols **7** and **8** were about 65% in each.

On the treatment with $NaBH_4$, 19-hydroxy-17-keto metabolites **2** and **5** along with 19-oxo-17-keto metabolites **3** and **6** were converted into the corresponding 17β,19-diols **7** and **8** in more than 90% yield.

2.3. Derivatization of the 19-oxygenated products

2.3.1. Identification experiment

The 19-oxygenated products obtained by the solid-phase extraction with a Sep-Pak C_{18} cartridge were dissolved in pyridine (50 μl) containing 5% methoxyamine hydrochloride and the mixture was heated at 60°C for 30 min. After this time, the mixture was diluted with ethyl acetate (6 ml), washed with water

(2 ml), evaporated to dryness under a stream of N_2 to yield a residue; in this reaction, the carbonyl compound was converted into the methoxime (MO) derivative. The residue was further treated with BSTFA according to the method previously reported [14]; on this treatment, the trimethylsilyl (TMS) derivative was produced from the hydroxy compound. On the other hand, DMIPS-imidazole (20 μl) was added to the mixture containing the MO derivative obtained as above and then the resulting mixture was further heated at 60°C for 30 min, and then placed in a Sephadex LH-20 column (5.0 × 0.6 cm i.d.; Pharmacia, Uppsala, Sweden). The column was developed with 2.5 ml of $CHCl_3$/hexane/methanol (10:10:1) and evaporation of the solvent gave a residue containing the DMIPS derivatives. This was dissolved in hexane (25 μl) containing 0.5% pyridine and subjected to the GC-MS analysis.

2.3.2. Retention times of the 19-oxygenated steroids

The 4-ene series: 19-hydroxy-17-ketone **2**, MO-TMS and MO-DMIPS derivatives 12.38 and 14.17 min, respectively; 17,19-dione **3**, MO derivative 12.59 min; 17β,19-diol **7**, TMS and DMIPS derivatives 12.21 and 16.57 min, respectively. The 5-ene series: 19-hydroxy-17-ketone **5**, MO-TMS and MO-DMIPS derivatives 12.57 and 15.01 min, respectively; 17,19-dione **6**, MO derivative 12.59 min; 17β,19-diol **8**, TMS and DMIPS derivatives 12.48 and 17.39 min, respectively.

2.3.3. Kinetic study

The 17β,19-dihydroxy product obtained on the treatment of the incubation products with $NaBH_4$, 17β,19-diol **7** or **8**, was directly converted into the bis-TMS derivative with BSTFA and was injected to GC-MS as described above in each experiment to obtain the aromatase-catalyzing 19-oxygenation rate of each substrate.

2.4. GC-MS

A Finnigan MAT SSQ GC-MS (San Jose, CA, USA) and a HP 5970B GC-MS (Palo Alto, CA, USA) instruments were used. GC and MS (electron impact, EI-MS) conditions were essentially similar to those previously reported [17]. Methane was used as a reagent gas for the chemical ionization method (CI-MS).

The quantitative analysis of the 17β,19-bis-TMS derivatives of the 17β,19-diols was conducted with a selected ion monitoring method (EI mode) with a base peak ion, m/z 344 ($M^+ - 90$).

2.5. Aromatization assay

The aromatization rate of AD was determined by measuring the amount of tritiated water released from [1β-^{3}H]-AD, a substrate for aromatase, into the incubation medium during aromatization, according to the method previously reported [18].

3. Results and discussion

19-Oxygenation studies on the 3-deoxy steroids **1** and **4** were initially carried out to determine whether they are converted into the corresponding 19-hydroxy-(**2** and **5**) and/or 19-oxo-(**3** and **6**) compounds by human placental aromatase (Fig. 1). The incubation products were subjected to reaction with methoxyamine followed by silylation with BSTFA or DMIPS-imidazole, and then analyzed using GC-MS. Mass spectra and retention times of the derivatives, the MO-TMS and MO-DMIPS derivatives of the 19-hydroxy-17-ketones **2** and **5**, the bis-TMS and bis-DMIPS derivatives of the 17β,19-diols **7** and **8**, and the bis-MO derivatives of the 19-oxo-17-ketones **3** and **6**, were identical with those of the corresponding authentic samples, respectively. A molecular ion (M^+) was not observed in the EI-MS spectra of all of the silyl derivatives of the 19-hydroxy steroids **2**, **5**, **7**, and **8**, whereas the methoximes of the 19-oxo steroids **3** and **6** showed a molecular ion (M^+, m/z = 344) in each EI-MS spectrum. On the other hand, the CI-MS spectra showed a pseudo molecular ion ($M^+ + 1$) in all cases and were also identical with those of the corresponding authentic samples (Figs. 2 and 3). The results revealed that the 3-deoxy steroids **1** and **4**, aromatase inhibitors, were converted into the corresponding 19-oxo steroids **3** and **6** through two sequential oxygenations at their 19-methyl group by human placental microsomes, as seen in the aromatization sequence of the substrate AD [4–6]. However, we did not observe any production of the 19-demethylated metabolite in the GC-MS spectrum of each incubation. The 19-ols **2** and **5** may be converted into their 17β-hydroxy analogs **7** and **8** by the action of 17β-hydroxysteroid dehydrogenase [19].

On the basis of relative total ion volumes in the GC-MS spectra of the metabolites, it was indicated that the 5-ene substrate **4** was more efficiently oxygenated at C-19 than the 4-ene isomer **1** where the 19-hydroxy-17-ketones **2** and **5** were main metabolites, respectively. The relative amount of the 19-oxo steroid to the 19-alcohol was less than 0.2, in each experiment. In addition, the 17β-hydroxy metabolites **7** and **8** were produced in an amount of about 10% of the corresponding 17-ketones **2** and **5**. Thus the 19-oxygenation products were converted into the 17β,19-dihydroxy compounds **7** and **8** on treatment with $NaBH_4$ in each experiment, which was then purified and analyzed as the bis-TMS ether by GC-MS (EI mode), and the production rate of the bis-TMS ether of the 17β,19-dihy-

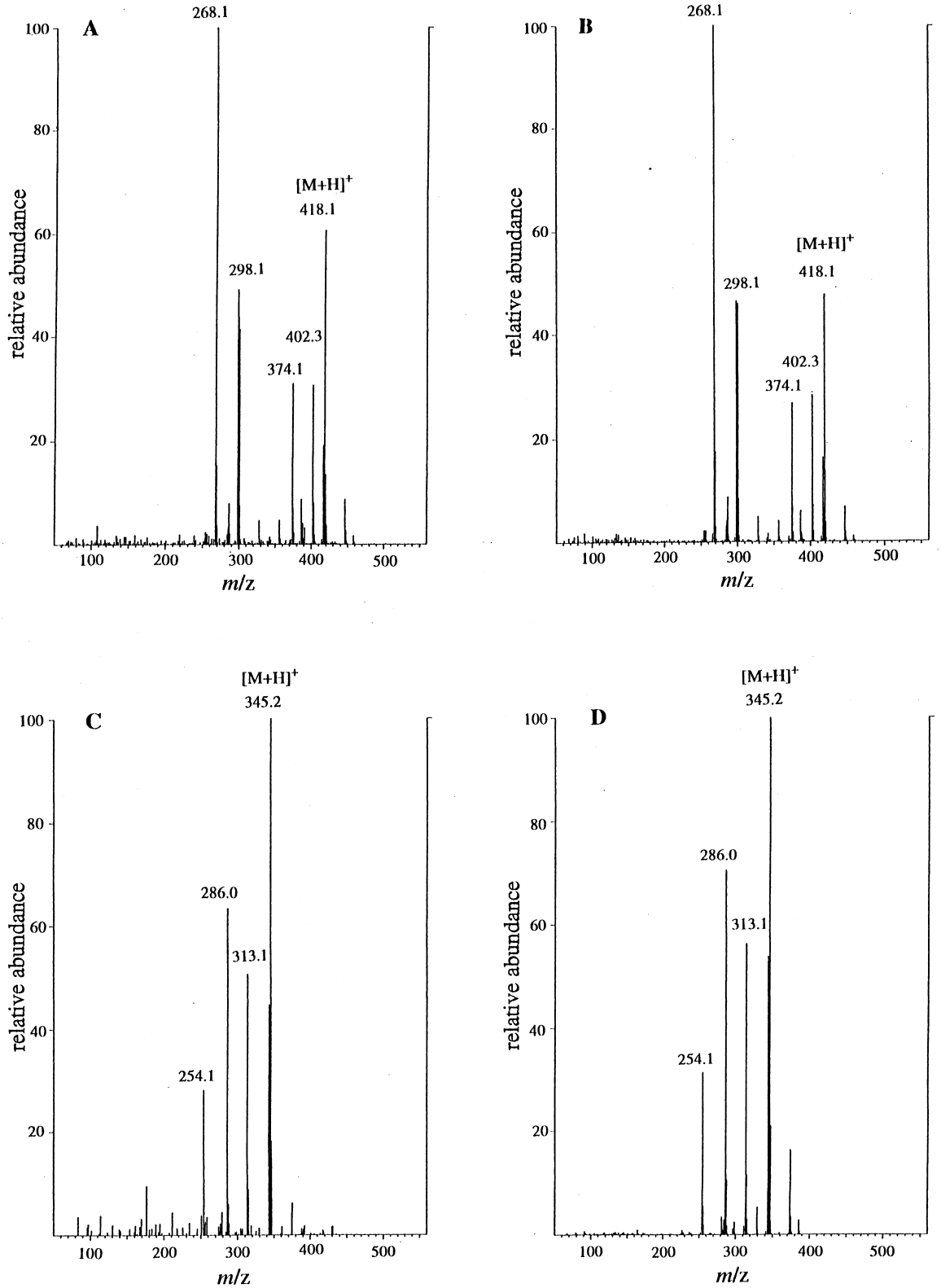

Fig. 2. CI-Mass spectra of the 19-oxygenated products obtained by incubation of the 5-ene steroid **4** and their authentic samples. (A, B) The MO-DMIPS derivatives of the 19-hydroxy-17-keto product **5** (A) and the authentic sample (B). (C, D) The MO derivatives of the 19-oxo product **6** (C) and the authentic sample (D).

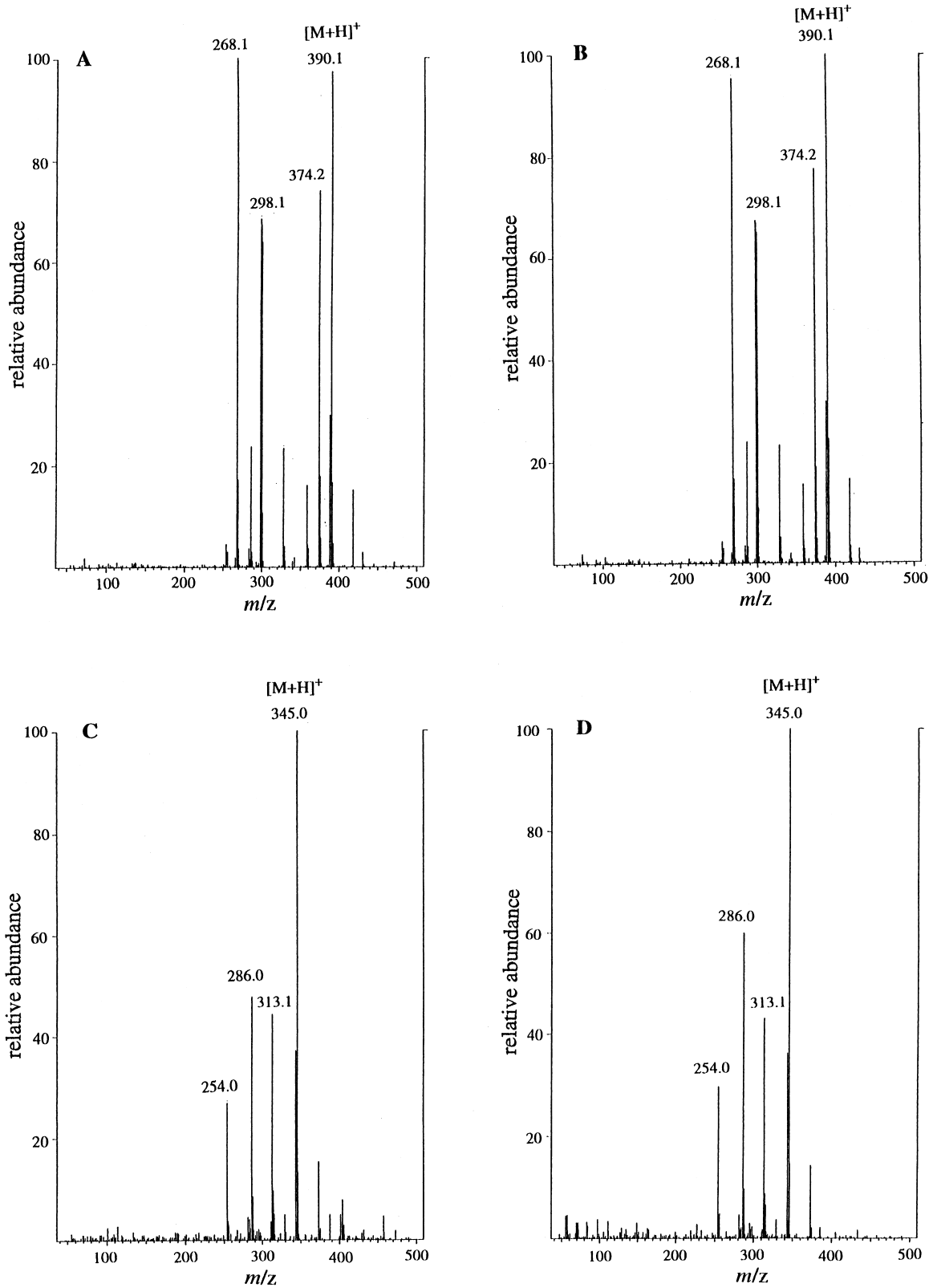

Fig. 3. CI-Mass spectra of the 19-oxygenated products obtained by incubation of the 4-ene steroid **1** and their authentic samples. (A, B) The MO-TMS derivatives of the 19-hydroxy-17-keto product **2** (A) and the authentic sample (B). (C, D) The MO derivatives of the 19-oxo product **3** (C) and the authentic sample (D).

droxy steroid **7** or **8** was used as an index of the 19-oxygenation rate in each case.

The 19-oxygenation rates of steroids **1** and **4** at a concentration of 200 nM increased linearly with increasing amounts of placental microsomes (up to 400 and 600 μg of protein, respectively) and with incubation time (up to 45 min). The 19-oxygenations were efficiently prevented by the anti-aromatase monoclonal antibody (Fig. 4), respectively, indicating that the oxygenations are catalyzed by aromatase in placental microsomes.

The 19-oxygenations were further studied to characterize the affinity (K_m) for aromatase and the conversion rate (V_{max}) under initial velocity conditions in which the conversions of the steroids were less than 5%. The two steroids 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 as shown in Table 1. In this study, the apparent K_m and V_{max} values for AD, which were obtained by a radiometric assay with [1β-^{3}H]-AD as a substrate, were 33 nM and 124 pmol/min per mg prot, respectively. The apparent K_m values (4.8 and 7.1 nM) for the substrates **1** and **4** were much lower compared to that for the AD aromatization. The K_m for the 4-ene steroid **1** was similar to the K_i obtained in the previous study [20], whereas the K_m for the other was extremely lower than the K_i [11] (K_i/

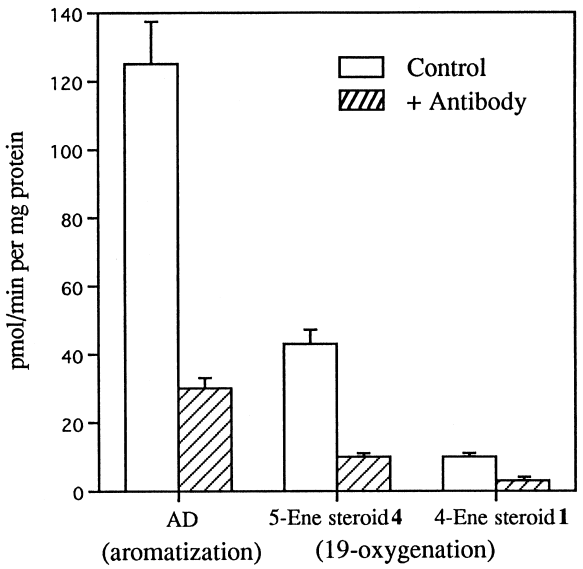

Fig. 4. Effect of the anti-aromatase antibody on the 19-oxygenation of 4-ene- and 5-ene-steroids **1** and **4** and on the aromatization of AD. Human placental microsomes (100 μg of protein) were preincubated with the anti-aromatase monoclonal antibody (MAb 3-2C2) (200 μg of IgG protein) for 45 s at 37°C. The 19-oxygenation rate then was determined under the kinetic analysis conditions described in the Materials and methods section. In the aromatization experiment, 25 μg of microsomal protein and 50 μg of IgG protein were used [17] and the aromatization activity was determined using a radiometric assay [18]. The antibody suppressed the 19-oxygenation or the aromatization to a significant extent ($P < 0.01$, $N = 4$).

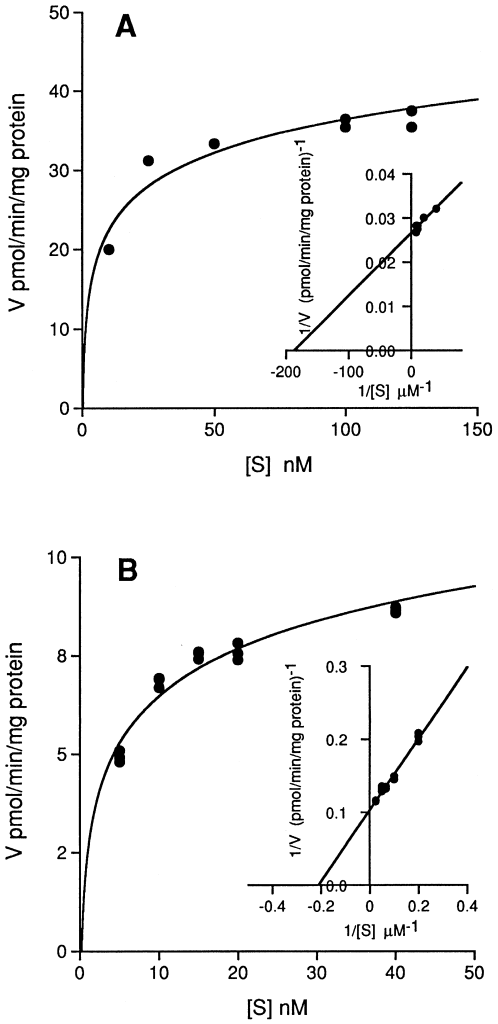

Fig. 5. Kinetic analysis of 19-oxygenation of 3-deoxy steroids **1** and **4** with human placental microsomes. Various concentrations of the 5-ene **4** (A) and the 4-ene isomer **1** (B) were incubated with human placental microsomes in the presence of NADPH, as described in the Materials and methods.

Table 1
Kinetic analysis of 19-oxygenation of 3-deoxy steroids **1** and **4** with human placental microsomes[a]

Steroid	K_i (nM)	K_m (nM)	V_{max} (pmol/min per mg prot)
4-Ene compound **1**	6.8	4.8	9.7
5-Ene compound **4**	120	7.1	45

[a] All incubations were performed at 37°C for 5 min with various concentrations of the 3-deoxy steroids and human placental microsomes (100 μg prot for the 5-ene steroid **4** and 200 μg prot for the 4-ene isomer **1**) in the presence of NADPH, as described in the Materials and methods. Apparent K_i values obtained previously [11,20] are also listed for comparison. In the previous experiments, the apparent K_m value for AD was in a range between approximately 20 and 30 nM.

$K_m = 16.9$). On the basis of the V_{max} values, it was found that the 5-ene compound **4** was surprisingly a good substrate for aromatase, although there is no oxygen function in the A, B-ring system, and its 19-oxygenation proceeded approx. four times faster than that of the 4-ene isomer **1**. Moreover, the V_{max}/K_m ratio for the 5-ene substrate **4** is approx. three times higher than that for the 4-ene isomer **1**.

Thus, there is no significant correlation between the ability to serve as an inhibitor of aromatase and the ability to serve as a substrate of the enzyme in the 3-deoxy androgen series, as seen in the 6-alkyl AD and its Δ^1-analog series [17]. We have previously reported that the relative aromatase inhibitory activities of steroid **1** and its 19-oxygenated derivatives **2** and **3** [10] are significantly different from those of the AD series [2,3], whereas the relative activities of the 5-ene series **4–6** [11] are similar to those of the AD series. These previous findings [10, 11] obtained from the inhibition studies along with the present 19-oxygenation results strongly suggest that the 5-ene steroid **4** would bind to the active site in the similar geometry to that involved in the binding of the natural substrate AD in which the 19-methyl group orients in a proper position for the cause of the catalytic function of aromatase, in contrast, the binding geometry of the 3-deoxy AD (**1**) would be obviously different from that of AD and be unsuitable for the 19-oxygenation. Transposition of a double bond at the C-4 position to the C-5 position causes a conformational change of the A,B-ring structure of a steroid nucleus, which markedly affects the binding manner in the active site. The 3-carbonyl group of AD is not essential for a tight binding to the active site but plays a critical role in a proper binding as a substrate.

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