Aromatase is a unique cytochrome P-450 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 AD isomers, 4-en-6-one 1a, 5-en-4-one 2a, and 5-en-7-one 3a, competitive inhibitors of aromatase with an A, B-ring enone structure to serve as a substrate, we incubated the three inhibitors separately with human placental aromatase in the presence of NADPH in air. The metabolites were analyzed as the methoxime-trimethylsilyl ethers by gas chromatography-mass spectrometry. All of the inhibitors were found to be oxygenated with aromatase to produce the corresponding 19-hydroxy derivatives 1b, 2b, and 3b with rates of 2.0, 51, and 0.3 pmol/min/mg protein, respectively. Only in the experiment with the 5-en-4-one steroid 2a, the production of the 19-oxo metabolite 2c was detected with a rate of 3.1 pmol/min/mg protein. The 19-oxygenation of steroid 2a, the best substrate for aromatase among the three, was kinetically determined to give the $V_{\text{max}}$ value of 40 pmol/min/mg protein and the $K_{m}$ value of 1.43 μM, respectively. The results reveal that a good inhibitor of aromatase is not essentially a good substrate for the enzyme in a series of the A, B-ring enone steroids.

**Key words** aromatase; inhibitor; androstenedione isomer; 19-oxygenation; human placental microsomes; gas chromatography-mass spectrometry

Aromatase is a unique cytochrome P-450 enzyme complex responsible for estrogen biosynthesis in vivo. The aromatase reaction is thought to proceed through three sequential oxygenations at C-19 of the androgens androstenedione (AD) and testosterone. The first two occur at the C-19 position to produce 19-hydroxy and 19-oxo intermediates. In the last step, C-19 and the $\beta$-protons are eliminated as formic acid and water, respectively (Fig. 1). Inhibitors of aromatase are valuable as therapeutic agents in the treatment of breast cancer. For this reason, a number of potent aromatase inhibitors, which are analogs of the substrate AD, have been described by various laboratories.

We previously reported that C₁₉ steroids, having a unique enone structure in a A, B-ring system, such as 4-en-6-one 1a, 5-en-4-one 2a, and 5-en-7-one 3a instead of the 4-en-3-one structure of AD, efficiently inhibit aromatase activity in a competitive manner, even though there is no oxygen function at the C-3 position (Fig. 2). Steroid 1a has the highest affinity for aromatase among the four enones including the substrate AD whereas the 5-en-7-one 3a, with the lowest affinity, inactivates aromatase in a suicide manner. Moreover, it was also reported that 3-deoxy AD as well as its 5-en-3-one isomer, both good inhibitors of aromatase, can serve as a substrate of the enzyme that results in the formation of 19-oxygenated products in each.

In connection with work in our laboratory on the ability of aromatase inhibitors to serve as a substrate, it was of interest to determine whether aromatase can catalyze the 19-oxygenation of the 3-deoxy-enone steroids 1a, 2a, and 3a. This paper describes the identification of 19-oxygenated metabolites formed from the enones 1a, 2a, and 3a with human placental aromatase, as well as the kinetic study of the 19-oxygenation, using gas chromatography-mass spectrometry (GC-MS). All of the enones were oxygenated at C-19 with aromatase, the 5-en-4-one compound 2a was the best substrate for the enzyme.

**MATERIALS AND METHODS**

**Materials** The $\alpha,\beta$-unsaturated ketones 1a, 2a, and 3a, their 19-hydroxy-1b, 2b, and 3b and 19-oxo-1c, 2c, and 3c analogs, androst-5-ene-17β,19-diol

**Fig. 1.** Aromatase Reaction of Androstenedione by Human Placental Aromatase

**Fig. 2.** Structures of C₁₉ Steroids with a A, B-Ring Enone Structure

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were synthesized according to the previously reported methods. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Kojin Co., (Tokyo, Japan) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), dimethylisopropylsilyle (DMIPS) imidazole and methoxyamine hydrochloride from Tokyo Kasei Kogyo (Tokyo, Japan). Human placental microsomes (particles sedimenting at 105000 g for 60 min) were obtained as described by Ryan and they were washed with 0.05 mM dithiothreitol solution, lyophilized, and stored at −20°C.

19-Oxygenation Studies with GC-MS Incubations were conducted in a shaking water bath at 37°C in air with 30 ml Erlenmeyer flasks. Each contained 4.7 µM of steroids 1a, 2a, and 3a, 114 µM NADPH, 500 µg of protein of placental microsomes, 100 µl of methanol and 67 mM phosphate buffer, pH 7.4, with a total volume of 2.1 ml. For each experiment, four parallel incubations were performed. After 30 min of incubation, androst-5-ene-17β,19-diol (100 ng) as an internal standard was added to each incubation mixture. The metabolites as well as the internal standard 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 (3 ml), and placed in a Sep-Pak C18 cartridge (Waters, Milford, MA, U.S.A.). 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 0.50, 1.0, 2.5, or 5.0 µM concentration of the 5-en-4-one steroid 2a, 100 µg of the microsomal protein and 57.1 µM NADPH, 100 µl of methanol, and the 67 mM phosphate buffer, pH 7.4, in a 30 ml Erlenmeyer flask with a total volume 1.0 ml. The mixture was incubated separately for 5 min in air, and then the 17β,19-diol steroid (25 ng) as an internal standard was added to each incubation mixture, and 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), and diluted with water (3 ml) and then placed in a Sep-Pak C18 cartridge. After the same work-up as described above, the steroid fraction was obtained. The recovery rates for the 19-ols 1b, 2b, and 3b and the 19-oxo analogs 2c, as well as the internal standard, were about 65—70%.

Derivatization of the 19-Oxygenated Products The 19-oxygenated products obtained by the solid-phase extraction with a Sep-Pak C18 cartridge were dissolved in pyridine and oxygenated products obtained by the solid-phase extraction, were about 65—70%.

After the same work-up as described above, the residue which was dissolved in methanol (100 µl) and the steroidal material was extracted twice with 5 ml of methanol (10 ml). For the kinetic analysis, the incubation mixture contained 0.50, 1.0, 2.5, or 5.0 µM concentration of the 5-en-4-one steroid 2a, 100 µg of the microsomal protein and 57.1 µM NADPH, 100 µl of methanol, and the 67 mM phosphate buffer, pH 7.4, in a 30 ml Erlenmeyer flask with a total volume 1.0 ml. The mixture was incubated separately for 5 min in air, and then the 17β,19-diol steroid (25 ng) as an internal standard was added to each incubation mixture, and 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), and diluted with water (3 ml) and then placed in a Sep-Pak C18 cartridge. After the same work-up as described above, the steroid fraction was obtained. The recovery rates for the 19-ols 1b, 2b, and 3b and the 19-oxo analogs 2c, as well as the internal standard, were about 65—70%.

Aromatization Assay The aromatization rate of AD was determined by measuring the amount of tritiated water released from [1β-3H]AD, a substrate for aromatase, into the incubation medium during aromatization, according to the method previously reported. The quantitative analysis of MO-TMS derivatives of the 19-ols 1b, 2b, and 3b, as well as the tris-MO derivative of the 19-oxo steroid 2c, was conducted with a selected ion monitoring method with a base peak ion, in each case. The base peak ion was m/z 329 (M+−103) for 1b and 2b, m/z 267 (M+−165) for 3b, and m/z 356 (M+−31) for 2c. The internal standard androst-5-ene-17β,19-diol was analyzed as the 17β,19-bis-TMS derivative with a base peak ion, m/z 344 (M+−90).

RESULTS AND DISCUSSION

19-Oxygenation studies of the A, B-ring enone steroids 1a, 2a, and 3a were initially carried out to determine whether they are converted into the corresponding 19-hydroxy- or 19-oxo-compounds by human placental aromatase in the presence of NADPH under an aerobic condition. 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 steroids 1b, 2b, and 3b, and the tris-MO derivatives of the 19-oxo-5-en-4-one 2c were identical with those of the corresponding authentic samples, respectively (Figs. 3 and 4). In contrast, in the incubation experiments using the 4-en-6-one 1a and the 5-en-7-one 3a, any production of their 19-oxo metabolites 1c and 3c was not detected under the assay conditions employed, respectively (lower detection limit : about 100 pg for 1c and 3c). The results revealed that the 3-deoxy-enones 1a, 2a, and 3a, good aromatase inhibitors, were oxygenated at their 19-methyl group by human placental microsomes, yielding their 19-hydroxy metabolites 1b, 2b, and 3b. In addition, the 5-en-4-one steroid 2a was converted into the 19-
oxo compound 2c through two sequential oxygenations at the 19-methyl, as seen in the aromatization sequence of the substrate AD⁴⁻⁷ as well as the aromatase reaction of 3-deoxy AD and its 5-ene isomer.¹³) However, the production of the 17β-hydroxy derivatives of the 19-ols was not observed by the GC-MS analysis in each incubation experiment, suggesting that these enones would be poor substrates for 17β-hydroxysteroid dehydrogenase.

On the basis of relative total ion volumes in the GC-MS spectra of the 19-hydroxy metabolites to that of the internal standard, it was indicated that the 5-en-4-one substrate 2a was a proper substrate for the 19-hydroxylation while the other two were poor ones. The production rates of the 19-hydroxy metabolites 1b, 2b, and 3b were then determined using the selected ion monitoring method (EI mode) with a base peak ion, m/z 329 (M⁺−103), of the MO-TMS derivatives in each case (Table 1). The 19-hydroxylation rates of steroids 1a and 2a at a concentration of 4.7 μM increased linearly with increasing amounts of placental microsomes (up to 600 μg of protein for 2a and 1000 μg of protein for 1a) and with incubation time (up to 60 min in each). The 19-hydroxylations of steroids 1a and 2a along with the formation of the 19-oxo metabolite 2c were significantly prevented by AD (5.0 μM), the natural substrate of aromatase. These results indicate that the 19-oxygenations are catalyzed by aromatase in placental microsomes.

The 19-hydroxylation of steroid 2a was further studied to characterize the affinity ($K_m$) for aromatase and the conversion rate ($V_{max}$) under initial velocity conditions in which the conversion of the substrate 2a to the 19-ol 2b was less than 5% in each case. This 19-hydroxylation showed a typical saturation curve with an increasing substrate concentration (Fig. 5), and the Lineweaver–Burk plot gave apparent $K_m$ and $V_{max}$
Table 1. 19-Hydroxylation of α,β-Unsaturated Ketones 1a—3a with Human Placental Microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>19-Hydroxy</td>
</tr>
<tr>
<td>4-En-6-one 1a</td>
<td>2.0±0.11</td>
</tr>
<tr>
<td>4-En-6-one 1a^c</td>
<td>0.7±0.10</td>
</tr>
<tr>
<td>5-En-4-one 2a</td>
<td>51±3.6</td>
</tr>
<tr>
<td>5-En-4-one 2a^c</td>
<td>4.0±0.30</td>
</tr>
<tr>
<td>5-En-7-one 3a</td>
<td>0.3±0.04</td>
</tr>
</tbody>
</table>

^a All steroids (4.7 μM) were incubated separately with human placental microsomes (500 μg of protein) at 37 °C for 30 min in the presence of NADPH (114 μM) in 2.1 ml of 67 mM phosphate buffer, pH 7.4. The 19-hydroxy and 19-oxo products were analyzed by a radiometric assay with the MO-TMS and MO derivative, respectively, with an internal standard as described in the Experimental. Results are mean ± S.D. (n=4). b ND: not detected. The detection limit of the 19-oxo steroids. 1a, 2a, and 3a was about 0.5 pmol/min/mg protein in each. c AD (5.0 μM) was added to the incubation mixture and the 19-oxygenated products were analyzed as described above.

Fig. 5. Lineweaver–Burk Plot of the 19-Hydroxylation of Steroid 2a by Human Placental Aromatase

values of 1.43 μM and 40 pmol/min/mg protein, respectively. In this study, the apparent \( K_m \) and \( V_{max} \) values for the natural substrate AD, which were obtained by a radiometric assay with [1β-3H]AD as a substrate, were 33 nm and 120 pmol/min/mg protein, respectively. The apparent \( K_m \) value for steroid 2a was much higher compared to that for the AD aromatization and about twelve times higher than its own inhibition constant (\( K_i; 120 \text{ nm} \)) obtained in the previous study. The fact that the \( V_{max} \) value for steroid 2a was 33% of that for the AD aromatization, indicates that this steroid was a good substrate for aromatase, although there is no 4-en-3-one structure in the A, B-ring system. The 4-en-6-one steroid 1a, the most potent competitive inhibitor of aromatase (\( K_i; 21 \text{ nm} \)) among the three enones, was a poor substrate, of which the 19-hydroxylation rate was less than 5% of the isomer 2a.

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 series of the A, B-ring enone steroids, as seen in the 6-alkyl and 3-deoxy androgen series. The 5-en-4-one steroid 2a would bind to the active site in geometry similar 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 other two enones, of which the 5-en-7-one 3a is a suicide substrate for aromatase, would be obviously different from that of AD and be less suitable for the 19-oxygenation. The reason for these structural features observed in the aromatase-catalyzed 19-oxygenation of the enones is currently unknown. We previously suggested that the suicide substrate 3a might inactivate the enzyme in a mechanism-based manner through the 19-oxygenation. The fact that the inactivator 3a is oxygenated at C-19 by aromatase, although the oxygenation rate is not very high, would support the involvement of the 19-oxygenation mechanism for the inactivation by compound 3a.

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