Aromatization of 19-Oxygenated Androst-4-ene-3,6,17-triones with Human Placental Microsomes

Mitsuteru NUMAZAWA,* Takanori SUGIYAMA, and Masao NAGAOKA

Tohoku College of Pharmacy, 4-4-1 Komatsushima, Aoba-ku, Sendai 981, Japan.

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To gain insight into the aromatization sequence of androst-4-ene-3,6,17-trione (**1**), a suicide substrate of aromatase, the aromatization of its 19-hydroxy and 19-oxo analogs **2** and **3** with human placental microsomes, was studied using GC-MS. Steroids **2** and **3** were separately incubated with the microsomes in the presence of NADPH in air. The GC-MS analysis of the trimethylsilyl derivative of the aromatization product indicated that both the 19-oxygenated steroids **2** and **3** were aromatized to yield 6-oxoestrogens, 6-oxoestrone (**4**) and 6-oxoestradiol (**5**), in each experiment. The aromatization rates of substrates **2** and **3** were 605 ± 48 and 1794 ± 75 pmol/mg protein/10 min, respectively. These relatively higher rates, compared to that of the parent steroid **1** (73.2 ± 6.6 pmol/mg protein/10 min), indicates that the suicide substrate **1** is aromatized through the 19-oxygenated intermediates **2** and **3**.

Key words aromatization; androst-4-ene-3,6,17-trione; 19-oxygenated analog; suicide substrate; GC-MS analysis

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.^{1,2)} Aromatization of the androgens appears to involve three oxygenations at C-19 of the androgens, resulting in the eventual loss of the 19-angular methyl group. The first two occur at the C-19 position to produce 19-hydroxy and 19,19-gem-diol. Dehydration of this gem-diol leads to the readily isolated 19-oxo intermediate.^{3–6)} In the last step, C-19 is eliminated as formic acid to produce the estrogens.

Androst-4-ene-3,6,17-trione (**1**) is one of the earliest discovered suicide substrates of aromatase.^{7–9)} The mechanism for aromatase inactivation by inhibitor **1** has been established by us^{10–12)}; the two initial hydroxylations at C-19 of steroid **1** produce the 19-oxo steroid **3**, of which further oxygenation yields the 4 β ,5 β -epoxy derivative as a reactive electrophile that irreversibly binds to the active site of aromatase. During the course of the inactivation reaction, 6-oxoestrone (**4**) and 6-oxoestradiol (**5**), as well as 19-hydroxy and 19-oxo analogs **2** and **3**, are formed as metabolites of steroid **1**. However, there is no definite evidence with respect to the intermediary role of the 19-oxygenated steroids **2** and **3** in the aromatization reaction. We report here the aromatization of compounds **2** and **3** with human placental microsomes. Both compounds were efficiently aromatized to 6-oxoestrogens **4** and **5**, compared to the parent steroid **1**.

MATERIALS AND METHODS

Materials Compounds **1**,¹³⁾ **2**,¹⁰⁾ **3**,¹⁰⁾ **4**,¹⁴⁾ **5**¹⁴⁾ were synthesized according to the methods previously reported. NADPH was purchased from Kohjin Co., Ltd. (Tokyo) and bis-trimethylsilyltrifluoroacetamide from Tokyo Kasei Kogyo Co., Ltd. (Tokyo).

Enzyme Preparation Human term placental microsomes (sedimented after 60 min at $105000 \times g$) were obtained as described by Ryan.¹⁵⁾ They were washed once with 0.05 mM dithiothreitol solution, lyophilized, and stored at -20°C . No significant loss of activity occurred for two months.

Aromatization Studies Incubation was done in a shak-

ing water bath at 37°C in air using 30-ml Erlenmeyer flasks. Each contained $1.67 \mu\text{M}$ of the 19-methyl steroid **1** and its 19-hydroxy and 19-oxo analogs **2** and **3**, $150 \mu\text{M}$ NADPH, $500 \mu\text{g}$ protein of placental microsomes, methanol ($50 \mu\text{l}$) and 67 mM phosphate buffer, pH 7.4, in a total volume of 4.0 ml. For each experiment, four parallel incubations were carried out. After 10 min of incubation, two internal standards (5 ng of 6 β -methylestradiol and 50 ng of ethynylestradiol) were added to each incubation mixture and extracted with ethyl acetate ($5 \text{ ml} \times 2$). The organic layer was evaporated under reduced pressure to give a residue which was dissolved in methanol ($100 \mu\text{l}$) and diluted with water (3 ml), and the mixture was then subjected to a Sep-Pak C₁₈ cartridge (Waters, Milford, MA, U.S.A.). After washing with water (5 ml), followed by 10% methanol (5 ml), the steroid fraction was obtained as an eluent with 80% methanol (6 ml). The steroid fraction was evaporated to dryness under reduced pressure to afford a residue, which was dissolved in $150 \mu\text{l}$ of a mixture of benzene and methanol (95:5, v/v) and was then subjected to a Sephadex LH 20 column (Pharmacia Fine Chemical, Uppsala, Sweden) ($7.3 \text{ cm} \times 0.6 \text{ cm i.d.}$). The column was developed with the same solvent mixture in which each aromatized product was obtained in 1–14 ml fractions. The recoveries for **4** and **5** using these procedures were $95 \pm 7.2\%$ and $80 \pm 3.2\%$, respectively. In the above incubation experiments with compounds **1** and **2**, 100 and $250 \mu\text{g}$ of protein of placental microsomes were also employed to deter-

Fig. 1. Structures of Androst-4-ene-3,6,17-triones and 6-Oxoestrogens

Fig. 2. Mass Spectra of the Aromatized Products Obtained by Incubation of the 19-Hydroxy Steroid **2** with Human Placental Microsomes and Their Authentic Samples

The products were converted to trimethylsilyl ethers. **A** and **B**, the product and the authentic 6-oxoestrone, respectively. **C** and **D**, the product and the authentic 6-oxoestradiol, respectively. The 6-oxoestrogens produced from the other steroids, **1** and **3**, were also identical with the corresponding authentic samples (data not shown).

mine the linearity of their aromatization rates.

Derivatization of the Aromatized Products, 6-Oxoestrogens, and Their Analysis with GC-MS The aromatized products **4** and **5** obtained from the Sephadex LH 20 column chromatography was converted to trimethylsilyl derivatives with bistrimethylsilyltrifluoroacetamide and then subjected to GC-MS analysis with a Finnigan MAT SSQ GC-MS instrument (San Jose, CA, U.S.A.) according to the method previously reported.¹⁶ Retention times of the steroids analyzed were as follows: 6-oxoestrone, 16.49 min; 6-oxoestradiol, 17.34 min; ethynylestradiol, 15.06 min; 6 β -methylestradiol, 14.22 min.

Quantitative analysis of the aromatized product was carried out using a selected ion monitoring method; the molecular ion was the base peak ion for all of the 6-oxo estrogens **4** and **5**, 6 β -ethylestradiol, and ethynylestradiol. Then, the amount of the estrogen produced was obtained by comparing the relative abundance of the molecular ion of the product to those of the two internal standards.

RESULTS AND DISCUSSION

Aromatization studies were initially conducted on 19-oxygenated androst-4-ene-3,6,17-triones (**2** and **3**) to determine whether they are really converted to the corresponding estrogen, 6-oxoestrone, by human placental aromatase. It was postulated that the aromatized product would be a mixture of 6-oxoestrone and its 17 β -reduced estrogen, 6-oxoestradiol, produced by the action of 17 β -hydroxysteroid dehydrogenase,^{17,18} in each incubation. The aromatization products of compounds **2** and **3** were purified and analyzed as a trimethylsilyl ether derivative using GC-MS (electron impact mode). Mass spectra and retention times of the aromatized products were identical to those of the corresponding authentic estrogens, 6-oxoestrone and 6-oxoestradiol, respectively (Fig. 2).

The aromatization rates of the 19-hydroxy and 19-oxo steroids **2** and **3**, as well as the parent 19-methyl compound **1** at a concentration of 1.67 μ M increased linearly with increasing amounts of placental microsomes (up to 500 μ g of protein). In contrast, the rates did not increase linearly with increasing incubation time, since all of the 4-ene-3,6-dione steroids examined are known to be mechanism-based inhibitors of aromatase, which cause a time-dependent loss of the aromatase activity during the incubation.^{7–12} Therefore, to minimize the time-dependent loss of the initial aromatization rate, a short incubation time should be employed. Based

on this, along with the detection limits of the 6-oxoestrogens by GC-MS analysis, the rates were determined in the experiments using a 10-min incubation time and 500- μ g of placental microsomes (Table 1). The conversions of all the substrates were less than 13%.

The present results indicate that the aromatization rates of a series of the 4-ene-3,6-dione steroids decrease in the order 19-oxo > 19-hydroxy > 19-methyl. Considering the inactivation constants of the three suicide substrates **1–3** (k_{inact} values: 0.145, 0.222, and 0.076 min⁻¹ for the 19-methyl **1**, the 19-hydroxy **2**, and the 19-oxo **3**, respectively),^{10,19} the initial rates at the 1.67 μ M concentration of the substrates can be extrapolated to be about 1.7 times (in the cases of compounds **1** and **2**) or about 1.2 times (in the case of compound **3**) that with a 10-min incubation experiment; the order of the initial rates should be the same as above.

These relative rates of aromatization of a series of 4-ene-3,6-dione steroids correspond to those reported previously for a series of natural substrates androstenedione^{2,20,21} and its 16 α -hydroxy analog.^{2,21,22} Moreover, it is presumed that the initial aromatization rates of steroids **1–3** may be similar to those of a series of 16 α -hydroxyandrostenediones. These facts, together with our recent studies^{11,12} establish that further oxygenation of the 19-oxo steroid **3**, produced by the two initial oxygenations at C-19 of steroid **1**, yields not only 6-oxoestrogens but also a reactive electrophile that immediately binds to the active site of aromatase in an irreversible manner, resulting in the inactivation of aromatase. It is also likely that steroids may bind to the active (binding) site of aromatase in a similar fashion to that involved in the binding of the natural substrate, 16 α -hydroxyandrostenedione, rather than androstenedione. The C-6 carbonyl function of steroid **1** may play a similar role in the binding to that of the 16 α -hydroxy function.

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Table 1. Aromatization of 19-Oxygenated Steroids **2** and **3** and Their Parent Steroid **1** with Human Placental Aromatase

Steroid	Product (pmol/mg protein/10 min) ^a		Total aromatization ^b (pmol/mg protein/10 min)
	6-Oxoestrone	6-Oxoestradiol	
19-Hydroxy 2	314 $\pm$ 22	291 $\pm$ 26	605 $\pm$ 48
19-Oxo 3	1011 $\pm$ 133	783 $\pm$ 42	1794 $\pm$ 75
	For comparison		
19-Methyl 1	13.4 $\pm$ 1.4	59.6 $\pm$ 5.2	73.2 $\pm$ 6.6

a) The values are means $\pm$ S.D. (n=4). b) Total aromatization: the sum of the 6-oxoestrone and 6-oxoestradiol productions.

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