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The Steric Mechanisms of Enzymatic Aromatization of 19-Norsteroids[1]

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The stereochemistry of hydrogen removal from C–2 during the placental and microbial aromatization of 19-norsteroids has been investigated. The substrates for this purpose, epimeric [2-^{3}H] estr-4-ene-3,17-diones (IV, VIII), were prepared along the route which had previously been developed. The tritiated compounds were admixed with an appropriate amount of [4-^{14}C] estr-4-ene-3,17-dione and recrystallized until constant isotope ratio was achieved. Each substrate was incubated with the human placental preparation according to the procedure of Ryan. The transformation products, estrone and 1β-hydroxyestr-4-ene-3,17-dione, and recovered substrate were separated by preparative thin-layer chromatography, diluted with the carrier and then recrystallized to constant isotope ratio, respectively. Estrone formed from the substrate with the label at 2β exhibited a 79% loss, while that from the 2α-labeled substrate lost only 20% tritium. The double-isotope labeled substrate was also incubated with respiring cultures of *Bacillus sphaericus*. The aromatized product from the 2β-tritiated substrate lost 80% of the label, whereas the 2α-epimer showed only 11% loss. Elimination of hydrogen from C–2 in the aromatization process with human placenta and microorganism is thus stereoselectively β.

The bioconversion of neutral steroid into estrogen involves the loss of the C-19 methyl group and of hydrogens from both C-1 and C-2.[3] 19-Norsteroids also serve as estrogen precursors in the placental and ovarian tissues[4] and hence are useful model compounds for the studies on the aromatization reactions if the similar mechanisms are involved. In addition the metabolic fate of 19-norsteroids is of particular interest, because they are widely used for the contraceptive purpose. The β-*cis* nature of C-1,2 dehydrogenation in the placental aromatization has recently been established with C_{19} steroids.[5] With regard to 19-norsteroids, however, the evidence only for the elimination of the 1β hydrogen in this process has been demonstrated.[6] 19-Norsteroids having the Δ^4-3-keto structure are also good precursors for aromatization in some microbiological systems, and estr-4-ene-3,17-dione is aromatized with respiring cultures of *Bacillus sphaericus* (ATCC 7055) with loss of the 1β hydrogen.[7]

1) This paper constitutes Part LXIV of the series entitled "Analytical Chemical Studies on Steroids"; Part LXIII: T. Nambara and T. Iwata, *Chem. Pharm. Bull.* (Tokyo), **21**, 899 (1973). Preliminary accounts of this work have been presented: T. Nambara, T. Anjyo, and H. Hosoda, *Chem. Pharm. Bull.* (Tokyo), **20**, 853 (1972); T. Anjyo, M. Ito, H. Hosoda, and T. Nambara, *Chem. Ind.* (London), **1972**, 784.

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However, the stereochemistry of hydrogen loss from C-2 has not as yet been clarified. The comparative studies on the aromatization mechanisms with the human placental preparation and *B. sphaericus* appeared to be an attractive problem. The present paper deals with the stereochemistry of hydrogen removal from C-2 during the enzymatic aromatization of estr-4-ene-3,17-dione.

The design of the experiment required the synthesis of estr-4-ene-3,17-diones stereospecifically labeled with tritium in the 2α and 2β positions which would serve as the substrate for the enzymatic aromatization. The preparation of the desired compounds was carried out along the route which had previously been developed for obtaining epimeric 2-deuterioestr-4-ene-3,17-diones[8] as shown in Chart 1. The tritium label was introduced into the 2β position employing estr-2-ene-5β,17β-diol (I) as a starting material. Hydroboration of I with tritiated diborane, freshly prepared from lithium aluminum tritiide and boron trifluoride, and subsequent oxidation with hydrogen peroxide gave the [2β-^{3}H]3β,5β,17β-triol (IIa). Selective acetylation of secondary hydroxyl groups yielded the 3,17-diacetate (IIb), which on dehydration with thionyl chloride and pyridine was led to [2β-^{3}H]estr-4-ene-3β,17β-diol diacetate (IIIb). Mild alkaline hydrolysis provided the free diol (IIIa), which in turn was oxidized with chromium trioxide-pyridine complex to yield the desired substrate, [2β-^{3}H]estr-4-ene-3,17-dione (IV).

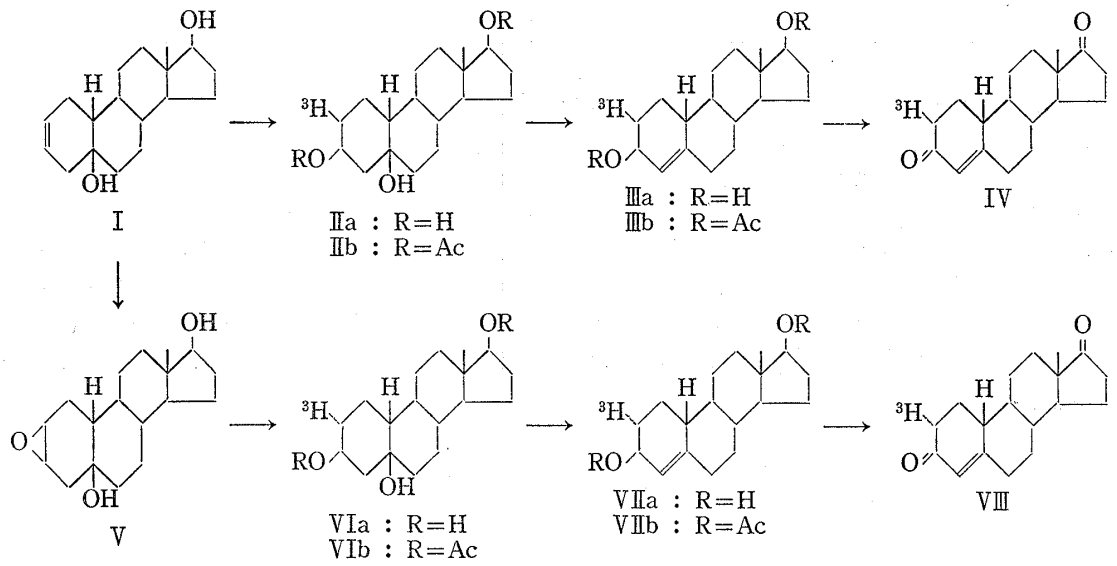

Chart 1

Reductive opening of 2β,3β-epoxyestrane-5β,17β-diol (V) derivable from I with lithium aluminum tritiide afforded the [2α-^{3}H]3β,5β,17β-triol (VIa). Transformation of VIa into the other substrate, [2α-^{3}H]estr-4-ene-3,17-dione (VIII), was performed by a reaction sequence identical with that used for the 2β-labeled substrate *via* the 3,17-diacetate (VIIb) and its free diol (VIIa). Each substrate was admixed with an appropriate amount of [4-^{14}C]estr-4-ene-3,17-dione and recrystallized repeatedly to constant isotope ratio.

The double-isotope labeled substrate was incubated with the human placental aromatase preparation according to the procedure of Ryan.[9] The biotransformation products and recovered substrate were separated by preparative thin-layer chromatography (TLC), diluted with the carrier and then recrystallized until constant isotope ratio was achieved. The results of the incubation experiment are listed in Table I. Estrone formed from the substrate with the label at the 2β position exhibited a 79% tritium loss, while the 2α-labeled substrate lost

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only 20% of tritium initially present. These data unambiguously tell us that the hydrogen removal from C-2 during the aromatization process is highly stereospecific and is β. The 1β-hydroxylated products obtained from both incubations showed almost the same tritium content with only a slight decrease. The retention of tritium in the starting material recovered implies that the hydrogen removal from C-2 is not a reversible process.

Table I. Tritium Loss of Products and Substrates in Placental Aromatization

	Specific activity (dpm/mg)			
	^3H	^{14}C	$^3H/^{14}C$	% ^3H lost
[2β-^{3}H]Estrenedione	2180000	57900	37.6	
Estrone	395	49	8.0	79
1β-Hydroxyestrenedione	5204	178	29.2	
Recovered substrate	39747	1135	35.0	
[2α-^{3}H]Estrenedione	1380000	36600	37.8	
Estrone	1094	36	30.1	20
1β-Hydroxyestrenedione	5942	196	30.3	
Recovered substrate	26580	747	35.6	

Next project was directed to the studies on the microbial aromatization with estr-4-ene-3,17-dione. Each double-isotope labeled substrate was incubated with respiring cultures of *B. sphaericus*. The incubation mixture was extracted with ethyl acetate and the extract was then separated by preparative TLC to provide estrone in fairly good yield. The transformation product was diluted with the carrier and then recrystallized to constant specific activity. The results of the radioactive counting are collected in Table II. Estrone derived from the 2β-tritiated substrate lost 80% of the label, whereas the 2α epimer showed only 11% tritium loss. Elimination of hydrogen from C-2 in the microbial aromatization is thus stereospecifically β.

Table II. Tritium Loss of Products in Microbial Aromatization

	Specific activity (dpm/mg)			
	^3H	^{14}C	$^3H/^{14}C$	% ^3H lost
[2β-^{3}H]Estrenedione	27000	764	35.3	
Estrone	2018	281	7.2	80
[2α-^{3}H]Estrenedione	22600	620	36.4	
Estrone	3987	123	32.5	11

Discussion

The results of the enzymatic aromatization of estr-4-ene-3,17-dione with the human placenta reveal that the hydrogen removal from C-2 is stereoselectively β and in consequence the nature of C-1,2 dehydrogenation is β-*cis*. These findings strongly suggest that the similar mechanisms may be involved in the aromatization with both C_{19} and 19-nor steroids. Current concepts of the aromatization mechanism imply that the hydrogen loss at C-2 occurs first by enolization. The evidences for the preferential elimination of the 2β hydrogen appear to lend a support to the enolization mechanism since the axial hydrogen at 2β would be abstracted with more ease in enol formation. The lack of isotope exchange in the recovered starting material indicates that if enolization is involved in the hydrogen removal from C-2

such a process would not be reversible. Brodie and his co-worker suggested that 1β-hydroxy-estr-4-ene-3,17-dione and estrone would be formed concurrently through a common intermediate which is formed by attack of activated oxygen at the 1β position and is capable of collapsing to yield both products.[6] However, such is not the case here as evidenced by the retention of tritium at C-2 in the 1β-hydroxylated compound.

It is sufficiently substantiated that with *B. sphaericus* there is a *trans* removal of the 1α and 2β hydrogens in Δ^4-3-keto and 5α-3-keto steroids.[10] Elimination of hydrogens from C-1 and C-2 in estr-4-ene-3,17-dione in the aromatization process has proved to be 1α,2β-*trans*. The stereochemistry of C-1,2 dehydrogenation of 19-norsteroids is then the same as that of C_{19} steroids. These results together with the previous findings support the proposed mechanism requiring enolization of the 3-ketone followed by hydride ion removal from C-1.[11]

Experimental

Materials——NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.4.9) were purchased from Sigma Chemical Co. (St. Louis). [4-^{14}C] Estr-4-ene-3,17-dione was prepared from [4-^{14}C] 19-nortestosterone acetate (58.8 mCi/mmole, 50 μCi) obtained from the Radiochemical Centre (Amersham), by refluxing for 1 hr in 2% KOH in 50% aq. MeOH followed by oxidation with CrO_3-pyridine complex.[12] The product was purified by preparative TLC, diluted with the carrier (60 mg) and recrystallized repeatedly to constant specific activity (7.29×10^4 dpm/mg). 1β-Hydroxyestr-4-ene-3,17-dione was prepared from 1β,17β-dihydroxyestr-4-en-3-one by chromic acid oxidation.[13] For preparative TLC silica gel HF_{254} (E. Merck AG, Darmstadt) was used as adsorbent.

Synthesis of Substrate——[2β-^{3}H] Estrane-3β,5β,17β-triol (IIa): To a solution of estr-2-ene-5β,17β-diol (I) (10 mg) in THF (0.2 ml) was added an ethereal solution of $LiAl^3H_4$ (100 mCi/mmole) (1.5 mg in 0.2 ml). To the ice-cooled solution was added dropwise an ethereal solution of BF_3-etherate (220 mg in 0.3 ml) under a stream of N_2 gas over a period of 30 min. After stirring at room temperature for 1 hr the moist ether was added to decompose the excess reagent. The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4 and evaporated. To a solution of the residue obtained in THF (3 ml) were added 10% NaOH (1 ml) and then 30% H_2O_2 (1 ml) under ice-cooling and stirred for 1 hr. The resulting solution was diluted with H_2O and extracted with AcOEt. The organic layer was washed with 10% $NaHSO_3$ and H_2O, dried over anhydrous Na_2SO_4 and evaporated. The residue was submitted to preparative TLC using benzene–ether (1: 4) as developing solvent. The area corresponding to IIa (*Rf* 0.59) was eluted with MeOH-AcOEt and the eluate was submitted to further step.

[2β-^{3}H] Estr-4-ene-3,17-dione (IV): Treatment of IIa with Ac_2O and pyridine in the usual manner gave [2β-^{3}H]estrane-3β,5β,17β-triol 3,17-diacetate (IIb). To a solution of IIb in pyridine (10 drops) was added $SOCl_2$ (5 drops) under ice–cooling and allowed to stand for 5 min. After addition of ice–water to decompose the excess reagent the resulting solution was extracted with $CHCl_3$. The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4 and evaporated to give [2β-^{3}H]estr-4-ene-3β,17β-diol diacetate (IIIb). Hydrolysis of IIIb with 5% methanolic KOH (1.7 ml) in the usual manner gave [2β-^{3}H]estr-4-ene-3β,17β-diol (IIIa). To a solution of IIIa in pyridine (1 ml) was added CrO_3-pyridine complex (0.5 ml) and allowed to stand at room temperature for 5 hr. The resulting solution was extracted with AcOEt. The organic layer was washed with 10% AcOH, 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4 and evaporated. The residue obtained was submitted to preparative TLC using hexane–AcOEt (1: 1) as developing solvent. The area corresponding to the radioactive spot (*Rf* 0.61) was eluted with $CHCl_3$ to give IV (9.00×10^6 dpm).

[2α-^{3}H]Estrane-3β,5β,17β-triol (VIa): To a solution of 2β,3β-epoxyestrane-5β,17β-diol (V) in THF (0.2 ml) was added $LiAl^3H_4$ (100 mCi/mmole, 0.5 mg) and refluxed for 4 hr. After the addition of moist AcOEt and then of 25% Rochelle salt solution the resulting solution was extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4 and evaporated. The residue was submitted to preparative TLC using benzene–ether (1: 4) as developing solvent. The area corresponding to VIa (*Rf* 0.59) was eluted with MeOH–AcOEt and the eluate was submitted to further step.

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[2α-^{3}H]Estr-4-ene-3,17-dione (VIII): Treatment of VIa in the same manner as for its C-2 epimer afforded [2α-^{3}H]estr-4-ene-3β,17β-diol (VIIa) by way of [2α-^{3}H]estrane-3β,5β,17β-triol 3,17-diacetate (VIb) and [2α-^{3}H]estr-4-ene-3β,17β-diol diacetate (VIIb). Oxidation of VIIa with CrO_3-pyridine complex (0.5 ml) followed by purification on TLC in hexane–AcOEt (1: 1) gave VIII (1.56×10^7 dpm).

Placental Incubation——Human placenta obtained immediately after delivery was processed at 4° according to Ryan's procedure.[9] The placental tissue (500 g) was teased free of large blood vessels, put through a meat glinder and homogenized in 0.05 M phosphate buffer (pH 7.0) (160 ml) containing sucrose and nicotinamide for 1 min. The homogenate was centrifuged at $10000 \times g$ for 30 min and the supernatant (320 ml) was separated.

[2β-^{3}H]Estr-4-ene-3,17-dione (IV) (9.00×10^6 dpm) was admixed with [4-^{14}C]estr-4-ene-3,17-dione (7.29×10^4 dpm/mg, 2.779 mg) and recrystallized until constant isotope ratio was achieved (^{3}H 2.18×10^6 dpm/mg; ^{14}C 5.79×10^4 dpm/mg). To each of ten flasks containing this substrate (40—50 μg) were added placental preparation (10 ml) equivalent to 16 g wet weight of tissue, NADP (3 μmoles), glucose-6-phosphate (10 μmoles), glucose-6-phosphate dehydrogenase (1.7 K units) and 0.05 M phosphate buffer (pH 7.0) (0.9 ml), and incubation was carried out in air at 37° for 1 hr. The reaction was terminated by the addition of acetone (5 ml) containing estrone (500 μg), 1β-hydroxyestr-4-ene-3,17-dione (500 μg) and estr-4-ene-3,17-dione (500 μg). The incubation mixture was collected and extracted with AcOEt, which was washed with H_2O, dried over anhydrous Na_2SO_4 and evaporated. The residue was submitted to preparative TLC and developed first in hexane–AcOEt (4: 1) followed by hexane–AcOEt (4: 3), and the appropriate bands were eluted. To each eluate was added the carrier steroid (5—8 mg) and recrystallized repeatedly to constant specific activity. Estrone, 1β-hydroxyestr-4-ene-3,17-dione and estr-4-ene-3,17-dione were recrystallized from EtOH, CH_2Cl_2-hexane and acetone–hexane, respectively.

[2α-^{3}H]Estr-4-ene-3,17-dione (VIII) (1.56×10^7 dpm) was admixed with [4-^{14}C]estr-4-ene-3,17-dione (7.29×10^4 dpm/mg 4.565 mg) and recrystallized until the constant isotope ratio was achieved (^{3}H 1.38×10^6 dpm/mg ;^{14}C 3.66×10^4 dpm/mg). The incubation was carried out exactly as above.

Microbial Incubation——The labeled substrate was incubated with a two-day culture of *B. sphaericus* in 100 ml of nutrient broth in a flask at 30°. The nutrient broth was prepared as follows: yeast extract 0.3 g, N–Z–Case peptone 0.5 g and distilled water 100 ml.[14] A solution of [2β-^{3}H-4-^{14}C]estr-4-ene-3,17-dione (^{3}H 2.70×10^4 dpm/mg; ^{14}C 764 dpm/mg, 7.7 mg) or [2α-^{3}H-4-^{14}C]estr-4-ene-3,17-dione (^{3}H 2.38×10^4 dpm/mg; ^{14}C 635 dpm/mg, 6.4 mg) in acetone–EtOH (1: 1) (0.6 ml) was added to each culture and the incubation was carried out with shaking for 48 hr. After the addition of acetone (30 ml) to terminate the reaction the broth was extracted with AcOEt and the combined extract was washed with H_2O, dried over anhydrous Na_2SO_4 and evaporated below 30°. The residue obtained was submitted to preparative TLC using hexane–AcOEt (7: 3) as developing solvent. The area corresponding to estrone (*Rf* 0.47) was eluted with acetone and the elute was diluted with the carrier steroid (*ca.* 10 mg) and recrystallized from MeOH repeatedly to constant isotope ratio.

Counting of Radioactivity——Samples were counted in Packard Tri-Carb Model 3380 liquid scintillation spectrometer. Toluene containing 2,5-diphenyloxazole (4 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (400 mg/liter) was used as a scintillant.

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