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Stereochemistry of Microbial Dehydrogenation of 5α-3-Ketosteroid[1]

Toshio Nambara, Shigeo Ikegawa, and Chizuru Takahashi

Pharmaceutical Institute, Tohoku University[2]

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In order to clarify the stereochemistry of hydrogen loss from C-4 during the enzymatic dehydrogenation the stereospecifically labeled 4-d_1-5α-androstane-3,17-diones (III, VI) were synthesized. Each substrate was incubated with the cell-free extract of *Nocardia restrictus* and the biotransformation products, androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione, were separated. Inspection of their mass and nuclear magnetic resonance spectra revealed that elimination of hydrogen from C-4 in the Δ^4 unsaturation process is stereoselectively β.

A variety of microorganisms are capable of introducing a double bond into the 1,2- and 4,5-positions of 5α-, 5β-, and Δ^4-3-ketosteroids.[3] The steric mechanism of unsaturation in ring A has extensively been studied and in consequence the stereochemistry is sufficiently substantiated.[4] With regard to Δ^4 unsaturation of 5α-androstan-3-one the stereoselective loss of 4α hydrogen during the process has previously been demonstrated by Abul-Hajj with the substrates having bromine or methyl group at C-4.[5] This explanation, however, is not necessarily acceptable because the 4-substituted steroids hereby employed are unsuitable for the stereochemical approach. In order to elucidate the stereochemistry the more appropriate substrates should be employed for enzymatic C-4,5 dehydrogenation.

The design of the experiment required 5α-androstane-3,17-diones labeled with the isotope stereospecifically at C-4 as substrates. An initial effort was directed to the preparation of C-4 epimeric 4-d_1-5α-androstane-3,17-diones. The 4α-deuterated substrate was synthesized by utilizing hydroboration toward the Δ^3-olefin. Treatment of androst-3-en-17-one (I) with deuterated diborane prepared from lithium aluminum deuteride and boron trifluoride and then with hydrogen peroxide gave the *cis*-addition products, 4α,17α-d_2-5α-androstane-3α,17β-diol (IIa) and the isomeric 3β- and 4α-hydroxyl derivatives. Separation of the expected 3α,17β-diol was readily attained by leading to the 3,17-bis(dimethyl-*tert*-butylsilyl) ether (IIb),[6] followed by column chromatography on silica gel. Removal of the silyl group was effected by brief exposure to hydrochloric acid in acetone. Subsequent oxidation with chromium trioxide-pyridine complex provided 4α-d_1-5α-androstane-3,17-dione (III) in a satisfactory yield.

The preparation of the epimeric 4β-deuterated substrate was carried out by *trans*-diaxial opening of the 3α,4α-epoxy ring. Reductive cleavage of 3α,4α-epoxy-5α-androstan-17-one (IV) with lithium aluminum deuteride afforded the 4β,17α-dideuterated 3α,17β-diol (V), which on chromium trioxide oxidation was led to the desired 4β-d_1-5α-androstane-3,17-dione (VI).

1) Part CII of "Studies on Steroids" by T. Nambara; Part CI: H. Hosoda, K. Yamashita, H. Sagae, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **23**, 2118 (1975).
2) Location: *Aobayama, Sendai.*
3) H. Iizuka and A. Naito, "Microbial Transformation of Steroids and Alkaloids," University of Tokyo Press, Tokyo, 1967, pp. 101—138.
4) *a*) H.J. Ringold, M. Hayano, and V. Stefanovic, *J. Biol. Chem.*, **238**, 1960 (1961); *b*) R. Jerussi and H.J. Ringold, *Biochemistry*, **4**, 2113 (1965); *c*) H.J. Brodie and P.A. Warg, *Tetrahedron*, **23**, 535 (1967); *d*) T. Nambara, T. Anjyo, M. Ito, and H. Hosoda, *Chem. Pharm. Bull.* (Tokyo), **21**, 1938 (1973); *e*) T. Nambara, S. Ikegawa, and H. Hosoda, *ibid.*, **21**, 2794 (1973).
5) Y.J. Abul-Hajj, *Biochem. Biophys. Res. Commun.*, **43**, 766 (1971).
6) H. Hosoda, K. Yamashita, H. Sagae, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **23**, 2119 (1975).

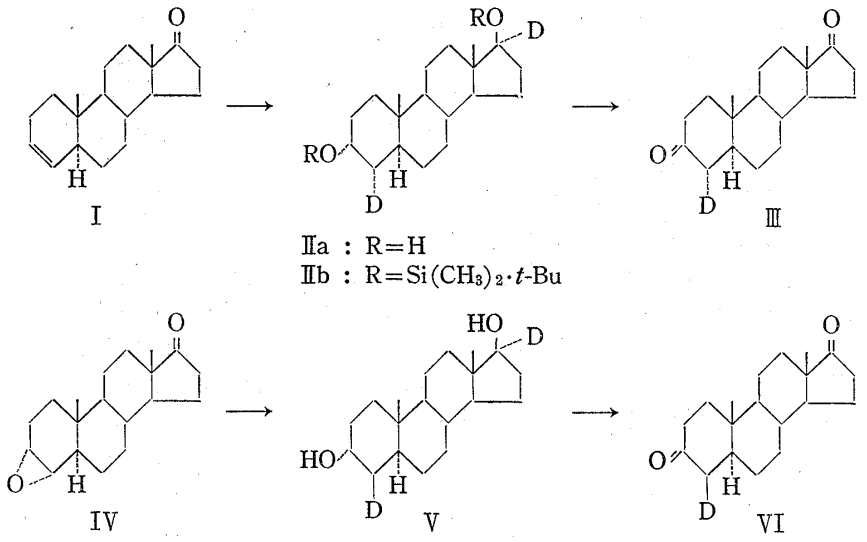

Chart 1

The infrared (IR) spectra of non-labeled 5α-androstane-3,17-dione and epimeric 4-deuterated compounds (III, VI) were different one another in the finger print region. The quantity of the isotope in these labeled substrates was determined by means of mass spectrometry. Inspection of the molecular ion peak, which appeared at *m/e* 289 with an increment of one mass unit, revealed that the deuterium contents of the labeled compounds were both more than 95%.

Microbial transformation of these deuterated substrates was then undertaken. The cell-free extract of *Nocardia restrictus* (ATCC 14887), which is capable of introducing 1,2- and 4,5-double bonds into the steroid nucleus, was prepared according to the procedure of Sih, *et al.*[7] and used as an enzyme source. Each of the labeled substrates was incubated with the cell-free extract in the presence of phenazine methosulfate as an electron acceptor. The biotransformation products were extracted with ethyl acetate and separated by preparative thin-layer chromatography (TLC) to afford androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione. The deuterium contents in these products were determined by means of mass spectrometry. As listed in Table I the biotransformation products formed from the 4β-deuterated substrate showed the complete loss of the isotope, while those derived from the 4α-epimer retained the label almost intact. In addition, the locality of the retained isotope was confirmed by inspection of the nuclear magnetic resonance (NMR) spectra.

Table I. Deuterium Retention of Products in Microbial Dehydrogenation

Product	Substrate (%)	
	4α-D (III)	4β-D (VI)
Androst-4-ene-3,17-dione	98	0
Androsta-1,4-diene-3,17-dione	98	0

It is evident from the data that during the process of C-4,5 dehydrogenation 4β hydrogen is stereospecifically eliminated. The present finding is incompatible with the Abul-Hajj's reported involving the stereoselective loss of 4α hydrogen in Δ^4 unsaturation. This conflict is obviously ascribable to the difference in the substrates used for microbial transformation. 5α-Androstane-3,17-diones substituted with a bulky group such as methyl or bromine at C-4

7) C.J. Sih and R.E. Bennett, *Biochim. Biophys. Acta*, **38**, 378 (1960); *idem, ibid.*, **56**, 584 (1962).

are not always pertinent as substrates for the stereochemical approach. In our study the deuterated substrates were employed and hence any significant influence of the label on the enzymatic dehydrogenation was not anticipated. Although no plausible explanation for the influence of the bulky substituent at C-4 is presently available, the generalized conclusion by the American investigator should be corrected. Thus we can arrive at the generalization that the stereochemistry of enzymatic Δ^4 unsaturation proceeds by a *trans*-diaxial removal of 4,5-hydrogens in both 5α- and 5β-series.[4a]

It is generally accepted that in microbial C-1,2 dehydrogenation of the 5α- and Δ^4-3-ketosteroids 2β-hydrogen is stereoselectively removed.[8] In the preceding paper of this series it was also demonstrated that 5β-androstane-3,17-dione underwent $\Delta^{1,4}$ unsaturation with the stereospecific elimination of 2β and 4α hydrogens, when incubated with the cell-free extract of *Nocardia restrictus*.[4a] In the present biotransformation yielding androsta-1,4-diene-3,17-dione an initial dehydrogenation would occur at the 4,5-position with loss of 4β-axial hydrogen resulting in formation of Δ^4-3-ketosteroid, in which 2β hydrogen is of quasi-axial nature. From these results it may be deduced that microbial dehydrogenation in ring A proceeds along the stereoselective course with loss of axial hydrogen at the α-position. However, there has been reported only an exceptional instance that C-1,2 dehydrogenation of 5β-pregnane-3,11,20-trione by *Septomyxa affinis* involves an elimination of 2β-equatorial hydrogen.[9] It seems to be worthwhile to determine whether the orientation of α-hydrogen removal by microorganisms is consistent with that by the chemical agent or not. Further studies are being conducted in these laboratories and the details will be reported in the near future.

Experimental[10]

Syntheses of Substrates

4α,17α-d_2-5α-Androstane-3α,17β-diol (IIa)——To a solution of IIb (170 mg) in acetone (20 ml) was added 5N HCl (2 ml) and allowed to stand at 60° for 30 min. After evaporation of the solvent the residue was extracted with AcOEt. The organic phase was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. Recrystallization of the residue from MeOH gave IIa (91 mg) as colorless leaflets. mp 217—219°. IR ν_{max}^{KBr} cm^{-1}: 2170 (C–D). Mixed melting point on admixture with the non-labeled authentic sample showed no depression. Mass Spectrum *m/e*: 294 (M^+) (98% d_2).

4α,17α-d_2-5α-Androstane-3α,17β-diol Bis (dimethyl-*tert*-butylsilyl) Ether (IIb)——To a stirred solution of 5α-androst-3-en-17-one (I)[11] (306 mg) and $LiAlD_4$ (500 mg) in ether (40 ml) was added BF_3-etherate (5 g) in ether (16 ml) at 0° over a period of 30 min under a stream of N_2 gas. After stirring at room temperature for 1 hr the excess reagent was carefully decomposed with moist ether and then the resulting mixture was extracted with ether. The organic phase was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. To a solution of the residue in THF (30 ml) were added dropwise 10% NaOH (16 ml) and 30% H_2O_2 (30 ml) under ice-cooling and stirred at 0° for 1 hr. The resulting solution was diluted with H_2O and extracted with AcOEt. The organic phase was washed with 5% $NaHSO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The residue was treated with dimethyl-*tert*-butylsilyl chloride (600 mg) and imidazole (800 mg) in DMF (2 ml) at room temperature for 2 hr. The reaction mixture was diluted with H_2O and extracted with ether. The organic phase was washed with H_2O and dried over anhydrous Na_2SO_4. After evaporation of solvent the crude product was submitted to column chromatography on silica gel. Elution with hexane and recrystallization of the eluate from acetone gave IIb (200 mg) as colorless plates. mp 123—124°. Mixed melting point on admixture with the non-labeled authentic sample showed no depression.

8) R. Bentley, "Molecular Asymmetry in Biology," Vol. II, Academic Press, New York, 1970, pp. 334—340.
9) Y.J. Abul-Hajj, *J. Biol. Chem.*, **247**, 686 (1972).
10) All melting points were taken on a hot-stage apparatus and are uncorrected. IR spectra were run on a JASCO Model IRA-1 spectrometer. NMR spectra were recorded on a JEOL Model PS-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Abbreviation used s=singlet, d=doublet, and dd=doublet of doublets. Mass spectral measurements were run on a Hitachi Model RMU-6E-spectrometer under the following conditions: ionization voltage 80 eV, accelerator voltage 1.4 kV, temperature of ionization chamber 170°, and width of collector slit 0.4 mm.
11) L. Caglioti, G. Cainelli, G. Maina, and A. Selva, *Tetrahedron*, **20**, 957 (1964).

4α-d_1-5α-Androstane-3,17-dione(III)——To a stirred solution of IIa (90 mg) in pyridine (2 ml) was added CrO_3–pyridine complex (1: 10 w/v) (4 ml) and allowed to stand at room temperature for 8 hr. The reaction mixture was diluted with ether, washed with 10% AcOH, 10% Na_2CO_3, and H_2O, successively and dried over anhydrous Na_2SO_4. After usual work-up the crude product was submitted to preparative TLC using hexane–AcOEt (4: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.38) and recrystallization of the eluate from acetone–hexane gave III (47 mg) as colorless leaflets. mp 129—131°. Mixed melting point on admixture with the non-labeled authentic sample showed no depression. Mass Spectrum *m/e*: 289 (M^+) (97% d_1).

4β,17α-d_2-5α-Androstane-3α,17β-diol(V)——To a solution of 3α,4α-epoxy-5α-androstan-17-one (IV)[12] (179 mg) in THF (12 ml) was added $LiAlD_4$ (150 mg) and refluxed for 5 hr. After addition of moist AcOEt to decompose the excess reagent the resulting solution was diluted with 20% Rochelle salt solution and extracted with AcOEt. The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. Recrystallization from acetone gave V (172 mg) as colorless leaflets. IR ν_{max}^{KBr} cm^{-1}: 2170 (C–D). mp 219—222°. Mixed melting point on admixture with the non-labeled authentic sample showed no depression. Mass Spectrum *m/e*: 294 (M^+) (98% d_2).

4β-d_1-5α-Androstane-3,17-dione(VI)——Treatment of V (170 mg) with CrO_3–pyridine complex (1: 10 w/v) (10 ml) in pyridine (5 ml) in the manner as described in III. Recrystallization of the crude product from acetone–hexane gave VI (51 mg) as colorless leaflets. mp 131.5—132.5°. Mixed melting point on admixture with the non-labeled authentic sample showed no depression. Mass Spectrum *m/e*: 289 (M^+) (95% d_1).

Preparation of Cell-free Extract——The cells of *Nocardia restrictus* (ATCC 14887) were grown on the following medium: corn steep liquor 0.6%, $NH_4H_2PO_4$ 0.3%, $CaCO_3$ 0.25%, soybean oil 0.22%, yeast extract 0.25%, and glucose 1%. After a week growth at 27° the cells were harvested by centrifugation at $3000 \times g$ for 30 min and washed with 0.03M phosphate buffer (pH 7.0). The cell-free extract was prepared by placing a cell suspension in the sonic field of a 10 kc magnetostrictive oscillator for 20 min. The cell debris was removed by centrifugation at $3000 \times g$ for 30 min. The supernatant (*ca.* 50 ml) was separated by decantation and used directly as an enzyme source.

Incubation with Cell-free Extract——To a solution of 4-d_1-5α-androstane-3,17-dione (5 mg) in DMF (0.1 ml) were added phenazine methosulfate (30 mg) and the cell-free extract (1 ml), and the total volume was brought to 50 ml with 0.03M phosphate buffer (pH 7.0). Incubations were carried out at 27° for 15 hr with continuous shaking.

Separation of Biotransformation Products——The incubation mixture was extracted with AcOEt. The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product was submitted to preparative TLC on silica gel HF_{254} using benzene–ether (1: 1) as developing solvent. Elution of the adsorbent corresponding to the spots (*Rf* 0.32, 0.20), followed by recrystallization of the eluate from acetone–hexane gave androst-4-ene-3,17-dione (mp 172.5—173°) and androsta-1,4-diene-3,17-dione (mp 139—141°) in *ca.* 20% yield, respectively. The NMR spectral data of the biotransformation products obtained in $CDCl_3$ were as follows:

Product	Chemical shift (δ) ppm		
	C_1–H	C_2–H	C_4–H
4α-D Substrate (III)			
Δ^4	—	—	none
$\Delta^{1,4}$	7.00 (d, J=10.5 Hz)	6.20 (d, J=10.5 Hz)	none
4β-D Substrate (VI)			
Δ^4	—	—	5.76(s)
$\Delta^{1,4}$	7.00 (d, J=10.5 Hz)	6.20 (dd, J=10.5, 1.5 Hz)	6.06 (d, J=1.5 Hz)

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12) W. Nagata, M. Yoshioka, and T. Okumura, *J. Chem. Soc.* (*C*), **1970**, 2365.