[Chem. Pharm. Bull. 21(12)2794—2796(1973)]

UDC 547.92.04 : 542.98

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

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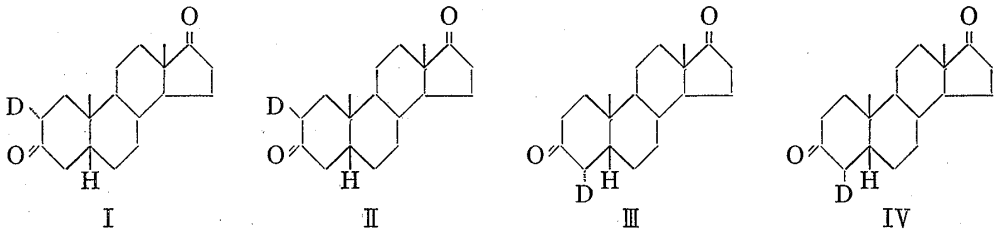
(Received June 14, 1973)

It is well known that introduction of the 1,2 and 4,5 double bonds into 5α- and 5β-3-ketosteroids is accomplished by a variety of microorganisms.[3] With regard to 5β-androstan-3-one the stereospecific elimination of 4α and 5β hydrogens in the Δ^4 unsaturation process has been demonstrated with the substrates having bromine or methyl group at C-4.[4] The deuterium or tritium labeled compounds, however, are much more suitable as the substrate for obtaining the precise knowledge on the steric mechanism of enzymatic dehydrogenation. The present paper deals with the stereochemistry of microbial ring A dehydrogenation of 5β-3-ketosteroid.



I II III IV

Chart 1

The required substrates for this purpose, two pairs of epimeric 2- and 4-deuterio-5β-androstane-3,17-diones (I—IV), were synthesized by the route previously established in this laboratory.[5] 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.*[3a,b] Each substrate was incubated with the cell-free extract employing phenazine methosulfate as the artificial electron acceptor.

The biotransformation products were extracted with ethyl acetate and then purified by preparative thin-layer chromatography (TLC), followed by recrystallization. The desired androsta-1,4-diene-3,17-dione and androst-4-ene-3,17-dione were thus obtained in a satisfactory yield. The deuterium contents of these two products were determined by inspection of the molecular ion peak in the mass spectra. The results of the isotope retention are listed in Table I.

Both androsta-1,4-diene-3,17-dione and androst-4-ene-3,17-dione formed from the 4α-deuterated substrate (III) showed almost the complete loss of the isotope while those derived

1) This paper constitutes Part II of the series entitled "Studies on Microbial Transformation Products Derived from Steroids"; Part I: T. Nambara, K. Shimada, Y. Fujii, and M. Katō, *Chem. Pharm. Bull.* (Tokyo), **20**, 336 (1972). This work has been presented as a preliminary account: S. Ikegawa and T. Nambara, *Chem. Ind.* (London), **1973**, 230.

2) Location: *Aobayama, Sendai.*

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4) Y.J. Abul-Hajj, *Biochem. Biophys. Res. Commun.*, **43**, 766 (1971).

5) T. Nambara, H. Hosoda, T. Anjyo, and S. Ikegawa, *Chem. Pharm. Bull.* (Tokyo), **20**, 2256 (1972).

Table I. Deuterium Retention of Products in Microbial Dehydrogenation

Product	Substrate (%)			
	2α-D(I)	2β-D(II)	4α-D(III)	4β-D(IV)
Androsta-1,4-diene-3,17-dione	89	0	2	78
Androst-4-ene-3,17-dione	92	85	6	77

from the 4β-epimer (IV) retained *ca.* 80% of the label. As for the $\Delta^{1,4}$-unsaturated products obtained from the C-2 deuterated substrates (I, II) the isotope labeled at 2β was completely lost whereas 2α deuterium retained substantially intact.

It has proved that in the C-4,5 dehydrogenation of 5β-androstane-3,17-dione elimination of hydrogen from C-4 is stereoselectively α. This result is in good accord with the stereochemistry of enzymatic removal of C-4 hydrogen from 4-bromo- or 4-methyl-5β-androstane-3,17-dione with *Nocardia restrictus*. In addition the stereospecific removal of 2β hydrogen is consistent with the recent finding on the C-1,2 dehydrogenation of 5β-pregnane-3,11,20-trione by *Septomyxa affinis*,[6] although the reaction sequence of ring A unsaturation leading to androsta-1,4-diene-3,17-dione still remains unsolved.

It is hoped that the present finding will be helpful for disclosing the enzymatic mechanism involved in dehydrogenation of 5β-3-ketosteroid.

Experimental

Material——Epimeric 2- and 4-deuterio-5β-androstane-3,17-diones (I—IV) were prepared by the method previously developed in this laboratory.[5] Mass spectral analysis of these substrates indicated that the deuterium contents were all *ca.* 98%.

Preparation of Cell-free Extract——Cells of *Nocardia restrictus* 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×*g* for 30 min and washed with 0.03M phosphate buffer (pH 7.0). Cell-free extract was prepared by placing a cell suspension in the sonic field of a 10 kc magnetostrictive oscillator for 30 min. The cell debris was removed by centrifugation at 105000×*g* for 30 min. The supernatant (*ca.* 100 ml) was directly used as the source of enzymes.

Dehydrogenation with Cell-free Extract——To a solution of deuterio-5β-androstane-3,17-dione in dimethylformamide (DMF) were added phenazine methosulfate (PMS) and cell-free extract, and then diluted with 0.03M phosphate buffer (pH 7.0) to contain 6 mg protein/ml as follows:

	Substrate (mg)	DMF (ml)	PMS (mg)	Supernatant (ml)	Total volume (ml)
I	20.5	0.5	120	12	200
II	11.0	0.4	60	6	100
III	10.0	0.3	60	6	100
IV	22.5	0.6	150	15	240

The mixture was then incubated at 27° with continuous shaking for 20 hr.

Separation of Biotransformation Products——The incubation mixture was extracted with AcOEt, and the organic phase was separated, washed with H_2O, and dried over anhydrous Na_2SO_4. After evaporation of solvent an oily residue was submitted to preparative TLC on the plate of silica gel H (E. Merck, AG) using benzene–ether (1: 1) as developing solvent. Elution of the adsorbent corresponding to the spots (*Rf* 0.32, 0.20) and recrystallization of the eluate from acetone–hexane gave androst-4-ene-3,17-dione (mp 171—172°) and androsta-1,4-diene-3,17-dione (mp 139—140°) as colorless prisms in *ca.* 40% yield, respectively.

6) Y.J. Abul-Hajj, *J. Biol. Chem.*, **247**, 686 (1972).

Mass Spectrometry——Mass spectral measurements were run on Hitachi Model RMU-6E spectrometer under the following conditions: ionization voltage 70 eV, accelerator voltage 1800 V, temperature of ionization chamber 210°, and width of collector slit 0.4 mm.

Acknowledgement The authors are indebted to Teikoku Hormone Mfg. Co. for obtaining the mass spectra. This work was supported in part by a Grant-in-Aid from Takeda Scientific Research Foundation, which is gratefully acknowledged.

[Chem. Pharm. Bull. 21(12)2796—2798(1973)]

UDC 547.436.09 : 615.36.076.9

Effect of Taurine on the Capacity of the Bile to Solubilize Cholesterol in Lithogenic Hamsters

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(Received August 6, 1973)

It is the recent knowledge that the ratio (bile acids+phospholipids)/cholesterol determines cholesterol solubility in the bile.[2] Whether cholesterol will be in the micellar phase or the crystalline phase can be represented as one point obtained by plotting the relative quantities of the three components on a triangular diagram.

At present, chenodeoxycholic acid treatment seems likely to be the most hopeful in cholelithiasis therapy in order to improve cholesterol solubility in the bile as shown by an increase in the above ratio and by a reduction in the molar ratio of cholesterol.[3] In the preceding paper,[4] the authors reported that taurine-treatment promoted the biliary excretion of intravenously infused deoxycholic acid in the form of the taurine conjugate in normal and vitamin B_6-deficient rats.

Dam and Christensen[5] succeeded in producing experimentally cholesterol-gallstones in hamsters by feeding a diet free of fat and rich in carbohydrate. In the bile of such animals a marked decrease in the ratio bile acids/cholesterol has been noted.[6] On the other hand, a prophylactic effect of taurine on experimentally induced cholelithiasis has been reported in rats and rabbits.[7]

The purpose of the present study is to investigate the effect of administered taurine on gallstone formation and cholesterol solubility in bile in lithogenic hamsters.

Experimental

Male golden hamsters approximately weighing 50 g were kept on the lithogenic diet prepared according to the method of Dam and Christensen[8] for 5 weeks. They were divided into four groups of seven animals

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