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Stereospecific Hydrogen Addition at C-4 in Transformation of 4-Androstene-3,17-dione into 5α-Androstane-3,17-dione by *Penicillium decumbens*[1]

Toshio Nambara, Shigeo Ikegawa, Hideki Ishida, and Hiroshi Hosoda

Pharmaceutical Institute, Tohoku University[2]

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The stereochemistry of the hydrogen addition at C-4 in the microbial 5α-hydrogenation of a Δ^4-3-ketosteroid has been investigated. 4-d_1-4-Androstene-3,17-dione was converted by incubation with *Penicillium decumbens* into 5α-androstane-3,17-dione. The position of deuterium retained in the product was elucidated to be 4α by ^{1}H nuclear magnetic resonance spectroscopy using the lanthanide shift reagent. These results led to the conclusion that the hydrogen addition at C-4 occurs stereospecifically from the β-side in this biotransformation.

Keywords——bioconversion mechanism; *Penicillium decumbens*; Δ^4-3-ketosteroid; 5α-hydrogenation; C-4 stereochemistry; 4-d_1-4-androstene-3,17-dione; 4α-d_1-5α-androstane-3,17-dione; *tert*-butyldimethylsilyl ether; ^{1}H NMR spectroscopy; lanthanide shift reagent

The microbial conversion of Δ^4-3-ketosteroids into 5α-3-ketosteroids is of particular interest in connection with the metabolism of steroid hormones in man. In a series of our studies on the microbial transformation of steroids the stereochemistry of dehydrogenation in ring A has been investigated.[3,4] The existence of the enzyme system which is capable of saturating the double bond, was demonstrated with microorganisms,[5] mammalian liver and testis.[6,7] The studies on the origin of hydrogen and the stereochemistry of its sttack in the bioconversion of Δ^4-3-ketosteroids yielding 5α- and 5β-steroids by mammalian tissues have been extensively carried out.[8–11] As for the microbial transformation, however, the mode of the hydrogen addition at C-4 still remains unclear, although it has been demonstrated that hydride ion is donated from a cofactor to the 5β-position in hydrogenation of 4-cholesten-3-one.[12] The present paper describes the stereochemistry of the hydrogen addition at C-4 in the transformation of 4-androstene-3,17-dione into 5α-androstane-3,17-dione by *Penicillium decumbens*.

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Experimental[13]

Preparation of Substrates——4-d_1-4-Androstene-3,17-dione (1),[14] epimeric 4-d_1-5α-androstane-3,17-diones,[4] and 5α-androstane-3β,17β-diol 17-*tert*-butyldimethylsilyl ether[15] were prepared by the methods previously established in these laboratories.

2β-d_1-5α-Androstane-3,17-dione——A solution of 2β-d_1-5α-androstane-3α,17β-diol 17-*tert*-butyldimethylsilyl ether[16] (50 mg) and 5 N HCl (1 ml) in acetone (10 ml) was stirred at room temperature for 1 hr. The resulting solutions was neutralized with 5% $NaHCO_3$, concentrated to its half volume under the reduced pressure, and extracted with AcOEt. The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated. Recrystallization of the crude product from MeOH gave 2β-d_1-5α-androstane-3α,17β-diol (29 mg) as colorless plates, mp 222—224°. Mixed melting point on admixture with 5α-androstane-3α,17β-diol showed no depression. A solution of 2β-d_1-5α-androstane-3α,17β-diol (50 mg) and CrO_3–pyridine complex (1: 10 w/v) (4 ml) in pyridine (4 ml) was stirred at room temperature for 5 hr. The reaction mixture was diluted with ether, washed with 10% AcOH, 10% Na_2CO_3, and H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent the crude product was purified by preparative thin-layer chromatography (TLC) using hexane–AcOEt (4: 1) as developing solvent. Recrystallization of the eluate from acetone–hexane gave 2β-d_1-5α-androstane-3,17-dione (37 mg) as colorless leaflets, mp 131—132°. Mixed melting point on admixture with 5α-androstane-3,17-dione showed no depression. MS *m/e*: 289 (M^+) (98% d_1).

2α-d_1-5α-Androstane-3,17-dione——Hydrolysis of 2α-d_1-5α-androstane-3α,17β-diol 17-*tert*-butyldimethylsilyl ether[16] (100 mg) with 5 N HCl in acetone was carried out in the manner as described with the C-2 epimer. Recrystallization of the crude product from MeOH gave 2α-d_1-5α-androstane-3α,17β-diol (59 mg) as colorless plates, mp 222—224°. Mixed melting point on admixture with 5α-androstane-3α,17β-diol showed no depression. 2α-d_1-5α-Androstane-3α,17β-diol (50 mg) was oxidized with CrO_3–pyridine complex (1: 10 w/v) (4 ml) in the manner as described with the C-2 epimer. The crude product was purified by preparative TLC using hexane–AcOEt (4: 1) as developing solvent. Recrystallization of the eluate from acetone–hexane gave 2α-d_1-5α-androstane-3,17-dione (32 mg) as colorless leaflets, mp 131—132°. Mixed melting point on admixture with 5α-androstane-3,17-dione showed no depression. MS *m/e*: 289 (M^+) (98% d_1).

Microbial Transformation——The fermentation was carried out under the conditions similar to those described by Miller, *et al.*[17] except the use of a potato medium containing glucose. 4-d_1-4-Androstene-3,17-dione (1) (124 mg) dissolved in EtOH (2.5 ml) was gently added to a 24 hr culture (500 ml) and incubated aerobically for 72 hr with continuous shaking at 27°. After incubation the broth was extracted with AcOEt and the extract was purified by preparative TLC on silica gel H (E. Merck AG, Darmstadt) using hexane–AcOEt (2: 1) as developing solvent. Recrystallization of the eluate from acetone–hexane gave 4α-d_1-5α-androstane-3,17-dione (2) (101 mg), mp 130—131.5°. Mixed melting point on admixture with 5α-androstane-3,17-dione showed no depression. Mass spectral analysis showed a 92% deuterium content.

Derivatization of 2 into the Substrate for NMR Study——4α-d_1-5α-Androstane-3,17-dione (2) (95 mg) obtained by the microbial transformation was reduced with $LiAlH_4$ (100 mg) in anhydrous ether (20 ml) to give the 3,17-diol, which on treatment with *tert*-butyldimethylsilyl chloride (500 mg) and imidazole (800 mg) in dimethylformamide (2 ml)–pyridine (1 ml) was led to the 3β,17β-bis(*tert*-butyldimethylsilyl) ether (130 mg), mp 152—153.5°. Subsequently selective desilylation of C-3 in the 3,17-disilyl ether by the procedure previously established[15] afforded the 17-monosilyl ether (3) (46 mg), mp 164—165°. Mixed melting point on admixture with 5α-androstane-3β,17β-diol 17-*tert*-butyldimethylsilyl ether showed no depression.

Results and Discussion

4-d_1-4-Androstene-3,17-dione (1) was incubated with *Penicillium decumbens* under the conditions similar to those described by Miller, *et al.*[17] The incubation mixture was extracted with ethyl acetate and the extract was purified by preparative TLC to provide 5α-androstane-

13) Melting points were taken on a micro hot-stage apparatus and are uncorrected. Infrared (IR) spectral measurements were run on a JASCO Model IRA-1 spectrometer. Mass spectra (MS) were obtained on a Hitachi Model RMU-7 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL Model PS-100 spectrometer at 100 MHz with *ca.* 0.08 M substrate in CCl_4. Chemical shifts are quoted as ppm (δ) downfield from $Si(CH_3)_4$ used as an internal standard. The shift reagents were stored *in vacuo* over P_2O_5 until the use. The abbreviations are used for the shift reagents: tris(1,1,1,-2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium (III), Eu(fod)$_3$; tris-(dipivalomethanato)-europium (III), Eu(dpm)$_3$.

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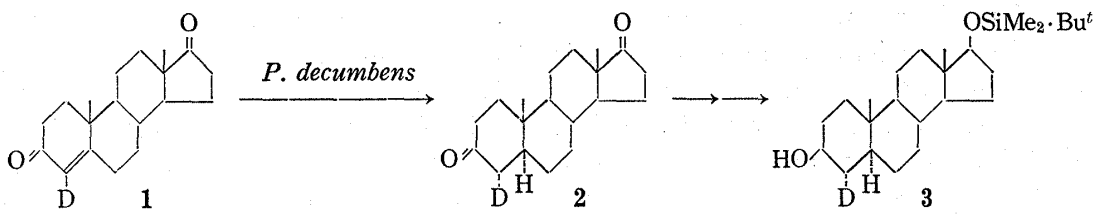

Chart 1

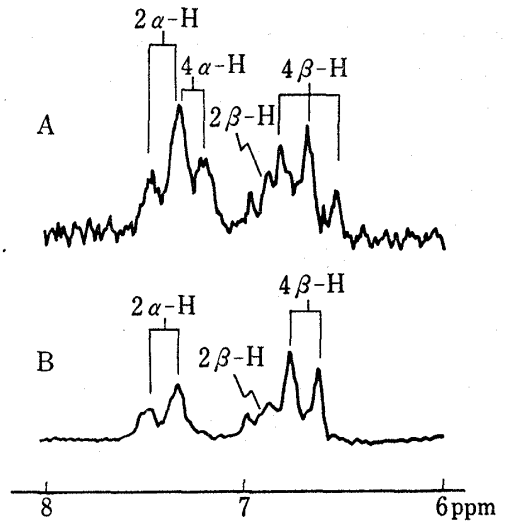

Fig. 1. The ^{1}H NMR Spectra of (A) 5α-Androstane-3,17-dione and (B) Microbial Transformation Product (**2**) with 0.57 Mole Equivalent Each of Eu(fod)$_3$ in Carbon Tetrachloride at 28°

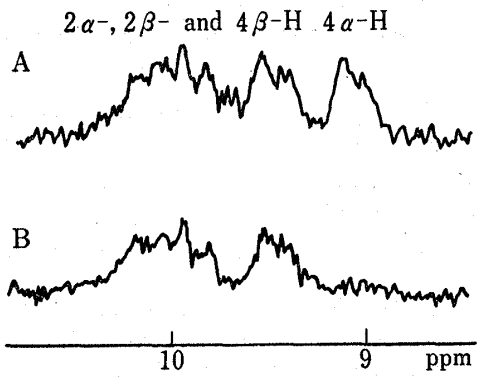

Fig. 2. The ^{1}H NMR Spectra of (A) 5α-Androstane-3β,17β-diol 17-*tert*-Butyldimethylsilyl Ether and (B) Compound **3** with 0.56 Mole Equivalent Each of Eu(dpm)$_3$ in Carbon Tetrachloride at 28°

3,17-dione in *ca.* 80% yield. The deuterium content of the transformation product was determined to be 92% by mass spectrometry. This result indicated that most of deuterium would be retained intact in the biotransformation process, since the substrate used for incubation contained 98% deuterium. It is generally accepted that the IR spectrum of a deuterated compound is distinguishable from those of its epimer and the non-deuterated substrates.[18] In the present case, however, the IR spectroscopy was incapable of providing the precise knowledge of the isomeric purity because the characteristic absorption band useful for the analysis was unavailable.

For the purpose of clarifying the isomeric purity, inspection of the ^{1}H NMR spectra of **2** and its derivative with the lanthanide shift reagent was undertaken. The ^{1}H NMR spectra of non-deuterated 5α-androstane-3,17-dione and **2** with Eu(fod)$_3$ are illustrated in Fig. 1A and 1B, respectively. The use of epimeric 2- and 4-monodeuterated compounds permitted us to assign unequivocally the signals due to C-2 and C-4 protons in 5α-androstane-3,17-dione. In Fig. 1A the signal of the axial 4β-proton appeared at 6.66 ppm as a triplet (J=14 Hz), partly overlapping with the multiple signal of the 2β-proton, whereas the equatorial 4α- and 2α-protons resonated at 7.26 and 7.39 ppm as a broad doublet, respectively due to geminal coupling (J=15 Hz) and small vicinal coupling (J=2—4 Hz). On the other hand, in Fig. 1B the signal of the 4α-proton disappeared and that of the 4β-proton turned into a doublet (J= 14 Hz) from the triplet. These data strongly suggested that deuterium would be located at

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the 4α-position in compound **2**. However, the definite evidence for the stereospecificity at C-4 was still insufficient, because the signals of the 4α- and 4β-protons were overlapped in part with those of the 2α- and 2β-protons even when a larger amount of $Eu(fod)_3$ was added.

It has already been shown that the signal of the 4α-proton of 5α-androstane-3β,17β-diol 17-*tert*-butyldimethylsilyl ether was well separated from those of the 2α-, 2β- and 4β-protons when $Eu(dpm)_3$ was used as a shift reagent.[16] Compound **2**, therefore, was led to 4-d_1-5α-androstanediol 17-monosilyl ether (**3**) by the sequential reactions involving metal hydride reduction, bissilylation, and selective desilylation. The ^1H NMR spectra of 5α-androstanediol 17-monosilyl ether and **3** were measured in a 0.56 molar ratio of $Eu(dpm)_3$ to the substrate in carbon tetrachloride at 28° (Fig. 2A and 2B). The signal of the 4α-proton which was observed at 9.05 ppm with the non-deuterated derivative, almost disappeared in Fig. 2B. The integration trace of the spectrum of **3** revealed that the signal due to the 4β-proton was equivalent to one proton and the 4α-proton was less than one-tenth proton in comparison with the magnitude of the 19-methyl proton signal. Thus the proportion of the 4α-deuterated species in the transformation product proved to be more than 90%.

These results led to the definite conclusion that the addition of hydrogen at C-4 in the microbial saturation of 4-androstene-3,17-dione occurs stereospecifically from the β-side and in consequence the nature of Δ^4 saturation is of *trans*-diaxial.

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