Microbial 16 β -Hydroxylation of Steroids with *Aspergillus niger*

Haruo YAMASHITA, Kenyu SHIBATA, Nobuaki YAMAKOSHI,
Yuichiro KUROSAWA and Hiromu MORI

*Department of Chemistry, Research Laboratories, Teikoku Hormone Mfg. Co., Ltd.,
Kawasaki, Kanagawa*

Received August 29, 1975

Microbial 16 β -hydroxylation of some steroids with *Wojnowicia graminis*, *Corticium centrifugum* and *Bacillus megaterium* has been reported, but not 16 β -hydroxylation of normal 17-oxo steroids with *Aspergillus niger*. This time, we tried microbial transformation of dehydroepiandrosterone with this fungus, and obtained 4-androstene-3,17-dione, 17 β -hydroxy-4-androstene-3,16-dione, 16 β ,17 β -dihydroxy-4-androsten-3-one and a new compound, 16 β -hydroxy-4-androstene-3,17-dione. This new compound was also obtained by the fermentation of 4-androstene-3,17-dione and testosterone.

During the studies of microbial conversion of dehydroepiandrosterone (I), we found that *Aspergillus niger* NRRL 599 could directly introduce a 16 β -hydroxyl function into normal 17-oxo steroids. In our knowledge, microbial 16 β -hydroxylation of steroids with *A. niger* has rarely been observed, except the case of digitoxigenin.¹⁾ Incubation of (I) with *A. niger* in Czapek-Dox medium containing yeast extract produced 4-androstene-3,17-dione (II), 16 β -hydroxy-4-androstene-3,17-dione (III), 17 β -hydroxy-4-androstene-3,16-dione (IV) and 16 β ,17 β -dihydroxy-4-androsten-3-one (V). These compounds were also obtained when 4-androstene-3,17-dione and testosterone were used as fermentation substrates. Identification of (II) and (V) was easily achieved by comparison of melting points, thin-layer chromatographic mobilities and infrared absorption spectra with those of authentic samples.* (IV) giving a positive blue tetrazolium test was identified as 17 β -hydroxy-4-androstene-3,16-dione by its melting point and infrared absorption spectrum.

The chemical structure of (III) was determined to be 16 β -hydroxy-4-androstene-3,17-dione on the basis of the following evidence.

* The authentic sample of (V) was prepared by hydrolysis of 16 β ,17 β -dihydroxy-4-androsten-3-one acetone with hydrochloric acid in methanol (K. Shibata, J. Yamada and H. Mori, *Chem. Pharm. Bull.*, **15**, 186 (1967)).

Absorptions at 3420 cm⁻¹ (hydroxyl group), 1758 cm⁻¹ (saturated five-membered ring ketone) and 1668 cm⁻¹ (α , β -unsaturated carbonyl group) in the infrared absorption spectrum (Fig. 1) and a maximum absorption at 240 nm in the ultraviolet absorption spectrum were observed. The presence of α -ketol structure was confirmed by a positive blue tetrazolium test. The molecular ion was observed at m/e : 302 in its mass spectrum. (III) exhibited a triplet signal at δ 3.99 ($J=7$ Hz) in the nuclear magnetic resonance spectrum (Fig. 2). As it seemed that such a signal could be reasonably assigned to C₁₆-hydrogen in the 16-hydroxy-17-oxo structure, the nuclear magnetic resonance spectra of 3,16 α -diacetoxy- and 3,16 β -diacetoxy-1,3,5 (10)-estratrien-17-ones (VIII and IX)^{2,3)} were measured and the signals due to 16-hydrogen were observed as a triplet in (IX) and a quartet in (VIII). Comparison of these nuclear magnetic resonance spectra as well as examination of the molecular model strongly supported the presence of the 16 β -hydroxy-17-oxo partial structure in (III).

Treatment of (III) with dilute sulfuric acid in methanol at room temperature⁴⁾ gave 17 β -hydroxy-4-androstene-3,16-dione identical with an authentic sample,^{5,6)} indicating that (III) must be one of 16,17-ketols. Since it was reported that 16 β -acetoxy-17-oxo steroid was hydrolyzed into 16-oxo-17 β -hydroxy one

FIG. 1. Infrared Absorption Spectrum of Isolated 16 β -Hydroxy-4-androstene-3,17-dione (III).

EXPERIMENTAL

Fermentation of dehydroepiandrosterone (I) with Aspergillus niger NRRL 599

A. niger spores (maintained on Czapek-Dox agar slants) were inoculated aseptically into 500-ml Erlenmeyer flasks each containing 100 ml of the following medium: 160 g of glucose, 12 g of NaNO₃, 8 g of yeast extract, 4 g of K₂HPO₄, 2 g of KCl, 2 g of MgSO₄·7H₂O, 0.1 g of FeSO₄·7H₂O and distilled water to a total volume of 4 liters. After a 36 hr incubation period at 30°C on a reciprocal shaker, 15 mg of (I) dissolved in 0.4 ml of ethanol was added to each flask and the incubation was continued for 48 hr at 30°C with shaking. The flask contents were combined,

FIG. 2. NMR Spectrum of Isolated 16 β -Hydroxy-4-androstene-3,17-dione (III).

accompanied by isomerization on the same treatment, whereas 16 α -acetoxy-17-oxo steroid gave 16 α -hydroxy-17-oxo one, (III) was considered to be 16 β -hydroxy-4-androstene-3,17-dione. Partial reduction of (III) with sodium borohydride gave 16 β , 17 β -dihydroxy-4-androstene-3-one (V). Similar reduction of (III) and (IV) with sodium borodeuteride gave 17-d-16 β , 17 β -dihydroxy-4-androstene-3-one (VI) and 16-d-16 β , 17 β -dihydroxy-4-androstene-3-one (VII), respectively. This conclusion was based on the lack of signals due to 17 α -proton in (VI) and 16 α -proton in (VII) in their nuclear magnetic resonance spectra. This observation indicated that (III) must be 17-oxo compound. Accordingly, the assignment of the chemical structure was proved unequivocally.

the mycelium was filtered and the filtrate was extracted twice with an equal volume of ethyl acetate after adjusting pH to 6.0 with 1 N HCl. The extracts were dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo* (approximately one gram of oily residue was obtained). A small amount of the oily residue thus obtained was submitted to thin-layer chromatography (developing system: chloroform-methanol (9:1)). The results indicated the presence of two main products (corresponding to (II) and (III)), a small amount each of products (IV) and (V), and traces of some unknown products were found. This oily residue was subjected to chromatography on a silica gel column. The adsorbed materials were eluted with an ethyl acetate-benzene mixture. (II) was eluted with a 19:1 mixture and purified by preparative thin-layer chromatography (chloroform-methanol, 9:1, as the developing solvent) and recrystallized from acetone-ether, 90 mg, mp 172~173°C. Infrared spectrum of (II) was identical with that of 4-androstene-3,17-dione. A mixture of (III), (IV) and (V) (230 mg) was eluted with a 17:3~

SCHEME I. Microbial Transformation of Dehydroepiandrosterone with *A. niger*.

16:4 mixture. By preparative thin-layer chromatography of the mixture and recrystallization from acetone-ether, 110 mg of (III), 10 mg of (IV) and 10 mg of (V) were obtained. (III): mp 127~132°C; $[\alpha]_D^{25} + 169^\circ$ ($c = 1.05$, CHCl_3); UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm(ϵ): 240(16,900). *Anal.* Calcd. for $\text{C}_{19}\text{H}_{28}\text{O}_3$: C, 75.46; H, 8.67. Found: C, 75.17; H, 8.61%. This compound was confirmed to be 16 β -hydroxy-4-androstene-3,17-dione as above-stated. (IV): mp 153~154°C, IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3460 (OH), 1760 (16-CO), 1670, 1618 (Δ^4 -3-CO); giving a positive blue tetrazolium test. (V): mp 177~179°C; IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3490, 3375 (OH), 1680, 1617 (Δ^4 -3-CO). (IV) and (V) were confirmed to be 17 β -hydroxy-4-androstene-3,16-dione and 16 β ,17 β -dihydroxy-4-androstene-3-one, respectively.

Fermentation of 4-androstene-3,17-dione (II) with *A. niger* NRRL 599

4-Androstene-3,17-dione (II, 600 mg) was incubated with *A. niger* for 48 hr as above described and one gram of oily product was obtained by the usual work-up. This oily product was chromatographed on 40 g of silica gel. A mixture of (III), (IV) and (V) (390 mg) was eluted with a 17:3~16:4 mixture. By preparative thin-layer chromatography of the mixture and recrystallization from acetone-ether, 150 mg of (III), 15 mg of (IV) and 40 mg of (V) were obtained. (III):

mp 127~131°C; IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3420 (OH), 1758 (17-CO), 1668, 1618 (Δ^4 -3-CO). (IV): mp 153~154°C; IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3460 (OH), 1760 (16-CO), 1670, 1618 (Δ^4 -3-CO). (V): mp 177~179°C; IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3490, 3375 (OH), 1680, 1617 (Δ^4 -3-CO). These crystals were identical in all respects with those obtained as described above.

Fermentation of testosterone with *A. niger* NRRL 599

Testosterone (600 mg) was incubated with *A. niger* for 48 hr as above mentioned, and the usual work-up gave 600 mg of oily product. This oily product was chromatographed on 24 g of silica gel. (II) was eluted with a 19:1~18:2 mixture and recrystallized from acetone-ether, 45 mg, mp 171~172°C. A mixture of (III), (IV) and (V) (170 mg) was eluted with a 17:3~16:4 mixture. By preparative thin-layer chromatography of the mixture and recrystallization from acetone-ether, 60 mg of (III); mp 128~131°C; 10 mg of (IV); mp 153~155°C and 35 mg of (V); mp 177~179°C were obtained. These compounds were identified by infrared analysis and comparison against standards.

Partial reduction of 16 β -hydroxy-4-androstene-3,17-dione (III) with sodium borohydride

To a solution of (III) (10 mg) in methylene chloride (0.5 ml) and ethanol (0.25 ml) was added a solution of

sodium borohydride (1.25 mg) in ethanol (0.25 ml) at 0°C. The combined solution was stirred for 30 min at the same temperature. Ice-cold 10% hydrochloric acid was added and the product was extracted with ether. The organic layer was washed with an aqueous solution of 5% sodium carbonate and water, and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and the residue was submitted to preparative thin-layer chromatography (developing solvent: benzene-acetone 9:1). 16 β ,17 β -Dihydroxy-4-androsten-3-one, 2.52 mg, mp 178~179°C, identical with (V) obtained above, resulted predominantly.

Partial reduction of 17 β -hydroxy-4-androstene-3,16-dione (IV) with sodium borodeuteride

17 β -Hydroxy-4-androstene-3,16-dione (IV) was reduced with sodium borodeuteride in the same manner as described in the above experiment (reaction time: 1.5 hr) to give 16-d-16 β ,17 β -dihydroxy-4-androsten-3-one (VII), 5 mg, mp 177~178.5°C. MS *m/e*: 305 (M^+) (d_0 4%; d_1 96%). NMR $\delta_{Me_4Si}^{CDCl_3}$: 0.87 (3H, s, 18-CH₃), 1.21 (3H, s, 19-CH₃), 3.39 (1H, s, 17-H), 5.76 (1H, d like, 4-H).

Partial reduction of 16 β -hydroxy-4-androsterone-3,17-dione (III) with sodium borodeuteride

17 β -Hydroxy-4-androsterone-3,17-dione (III) (10 mg) was reduced with sodium borodeuteride (1.25 mg) in the same manner as described in the above experiment to give 17-d-16 β ,17 β -dihydroxy-4-androsten-3-one (VI), 5 mg, mp 177~178.5°C from the main fraction. MS *m/e*: 305 (M^+) (d_0 3%; d_1 97%). NMR $\delta_{Me_4Si}^{CDCl_3}$: 0.87 (3H, s, 18-CH₃), 1.21 (3H, s, 19-CH₃), 4.16 (1H, q, $J=4.5$ and 7 Hz, 16-H), 5.76 (1H, d like, 4-H).

3,16 α -Diacetoxy-1,3,5 (10)-estratrien-17-one (VIII)³⁾

NMR $\delta_{Me_4Si}^{CDCl_3}$: 1.00 (3H, s, 18-CH₃), 2.13 and 2.28 (6H, s, -OAc).

3,16 β -Diacetoxy-1,3,5 (10)-estratrien-17-one (IX)³⁾

NMR $\delta_{Me_4Si}^{CDCl_3}$: 1.00 (3H, s, 18-CH₃), 2.13 and 2.28 (6H, s, -OAc), 5.05 (1H, t, $J=9$ Hz, 16 α -H).

DISCUSSION

Reports have been made on microbial 16 β -hydroxylation of some steroids with *Woinowicia graminis*,⁵⁾ *Corticium centrifugum*,⁷⁾ and *Bacillus megaterium*.⁸⁾ Herzog *et al.*⁵⁾ obtained nine crystalline transformation products containing (II), (IV) and (V) by the fermentation of testosterone with *W. graminis*, but the major product was 16 α , 17 β -dihydroxy-4-androsten-3-one. Dodson and Mizuba⁷⁾ isolated (IV) and

(V) as the fermentation products of 4-androstene-3,17-dione with *C. centrifugum*. But they did not isolate (III), and suggested that 16 β ,17 β -dihydroxy-4-androsten-3-one (V) might have resulted from 16 α -hydroxylation of 4-androstene-3, 17-dione, followed by isomerization of this 16,17-ketol into 16-oxo-17 β -hydroxy steroid (IV) and reduction of the resulting 16-keto group. Sax *et al.*,⁸⁾ who isolated 16 β - and 16 α -hydroxy-4-estren-3-one, and 4-estrene-3,16-dione as the fermentation products of estren-3-one with *B. megaterium* and *Cephalosporium acremonium*, suggested that 16 β -hydroxy-4-estren-3-one might have resulted first and subsequently been converted into 4-estrene-3,16-dione and 16 α -hydroxy-4-estren-3-one. In general, microbial hydroxylation of steroids at C-16 gives 16 α - and 16 β -hydroxy steroids, and it is suggested that the interconversion of 16 α - and 16 β -epimers occurred *via* the 16-ketone. In hydroxylation of 4-androstene-3,17-dione with *A. niger*, (III) appears to be formed first by 16 β -hydroxylation and subsequently to be converted into (IV) non-enzymatically. Although it is justifiable to consider that (V) is formed from (IV) by 16-keto-reductase of *A. niger*, the direct pathway (III $\rightarrow$ V) cannot be excluded. *A. niger* NRRL 599 (this strain is the same as *A. niger* ATCC 9142) was reported by Auret and Holland⁹⁾ to form 18-hydroxy-4-androstene-3, 17-dione from (II), but in this experiment we could not isolate 18-hydroxy compound.

Acknowledgement. The authors wish to express their thanks to Dr. S. Udagawa, Department of Microbiology, National Institute of Hygienic Sciences, for generously supplying the strain, to Dr. M. Iida, Institute of Applied Microbiology, the University of Tokyo, for supplying the NMR spectrum of 16 α -hydroxy-4-androstene-3,17-dione, and Drs. T. Miyata, S. Matsushima and M. Sawai, Teikoku Hormone Mfg. Co., Ltd., for their kind suggestions during this work. We are also indebted to Mr. Y. Tsuneda for his help in analyzing the NMR spectra.

REFERENCES

- 1) S. Kikuchi, T. Yamano and T. Kamitani, Japan Patent 22878 (1963) [*C.A.*, **60**, 2302 (1964)].

- 2) N.S. Leeds, D.K. Fukushima and T.F. Gallagher, *J. Amer. Chem. Soc.*, **76**, 2943 (1954).
 - 3) W. R. Biggerstaff and T. F. Gallagher, *J. Org. Chem.*, **22**, 1220 (1957).
 - 4) W. S. Johnson, B. Gastambide and R. Pappo, *J. Amer. Chem. Soc.*, **79**, 1991 (1957).
 - 5) H. L. Herzog, M. J. Gentles, A. B. W. Coscarelli, M. E. A. Zeitz and W. Charney, *J. Org. Chem.*, **25**, 2177 (1960).
 - 6) A. S. Meyer and M. C. Lindberg, *J. Amer. Chem. Soc.*, **76**, 3033 (1954).
 - 7) R. M. Dodson and S. Mizuba, *J. Org. Chem.*, **27**, 698 (1962).
 - 8) K. J. Sax, R. H. Blank, R. H. Evans, Jr., L. I. Feldman and E. Holmland, *ibid.*, **29**, 2351 (1964).
 - 9) B. J. Auret and H. L. Holland, *Chem. Commun.*, 1157 (1971).
-