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21. Masao Uchibayashi : Studies on Steroids. XIV.*1 Transformation of Steroids by *Pseudomonas*. (1).*2

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Microbiological transformation of steroids, first accomplished by Mamoli and Vercellone[1] in 1937, entered upon its new phase by the announcement of Peterson and Murray[2] in 1952 that molds of the Mucorales order could introduce oxygen into the important 11-position of the steroid ring. In the next few years intensive researches were carried out rapidly and on an extensive scale, and in consequence many papers appeared. A survey of these numerous publications reveals that organisms of interest in the investigation of steroid transformation are preponderantly fungi. Indeed, there is scarcely a steroid bioconversion described in the literature that cannot be achieved by one or other of the fungi. In some cases, of course, bacteria, yeasts, or Actinomycetes afford better results. The molds as a group, however, have certainly provided the most diverse assortment of transformation products, and it might be undeniable that studies of other microörganisms have been regarded, for the most part, as of second importance.

In the course of studies on steroids, an examination was made on the action of one genus of the bacteria, *Pseudomonas*, on steroids in cooperation with the research groups of the Institute for Fermentation, Osaka, and the Osaka Plant of the Takeda Pharmaceutical Industries, Ltd. As to the bioconversion of steroids by *Pseudomonas* species, only the reports of Talalay and his co-workers[3] have been available so far which dealt with the interconversions of estrone and 17β-estradiol, 4-androstene-3,17-dione and testosterone, and androsterone and androstane-3,17-dione as well as Δ^1-dehydrogenation of testosterone and its homologs. Transformation of some steroid substrates using several species of *Pseudomonas* maintained in the Institute was observed and interesting findings were obtained on the action of *Pseudomonas* species on steroids. In the present paper will be described the conversion of Reichstein's Substance S (I) (17α,21-dihydroxy-4-pregnene-3,20-dione) into 17α,20β,21-trihydroxy-1,4-pregnadien-3-one (II) and hydrocortisone (11β,17α,21-trihydroxy-4-pregnene-3,20-dione)(IV) by *Pseudomonas* sp. 109.*4

Incubation of Reichstein's Substance S (I) was carried out with a 24-hour growth culture of *Pseudomonas* sp. 109 at 30° for 17 hours in a synthetic medium containing glycerol, urea, sulfates, and phosphates.*4 Extraction of the culture filtrate with ethyl acetate yielded a powdery steroid extract. Repeated recrystallizations of a portion of the extract from acetone gave material having a fairly sharp melting point. Paper chromatography, however, indicated traces of contaminants. Acetylation of the steroid

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*1 This paper constitutes a part of a series entitled "Studies on Steroids" by Hayao Nawa. Part XIII. H. Nawa, M. Uchibayashi : This Bulletin, **6**, 508(1958).

*2 For a preliminary report of this investigation, see Tetrahedron, **4**, 201(1958).

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*4 The number assigned to the organism is the designation of Rokuro Takeda's laboratory of the Institute for Fermentation, Osaka. This organism was identified as closely akin to *Pseudomonas boreopolis*. Details of the microbiological and fermentational accounts will be reported elsewhere by J. Terumichi.

1) L. Mamoli, A. Vercellone : Z. physiol. Chem., **245**, 93(1937).

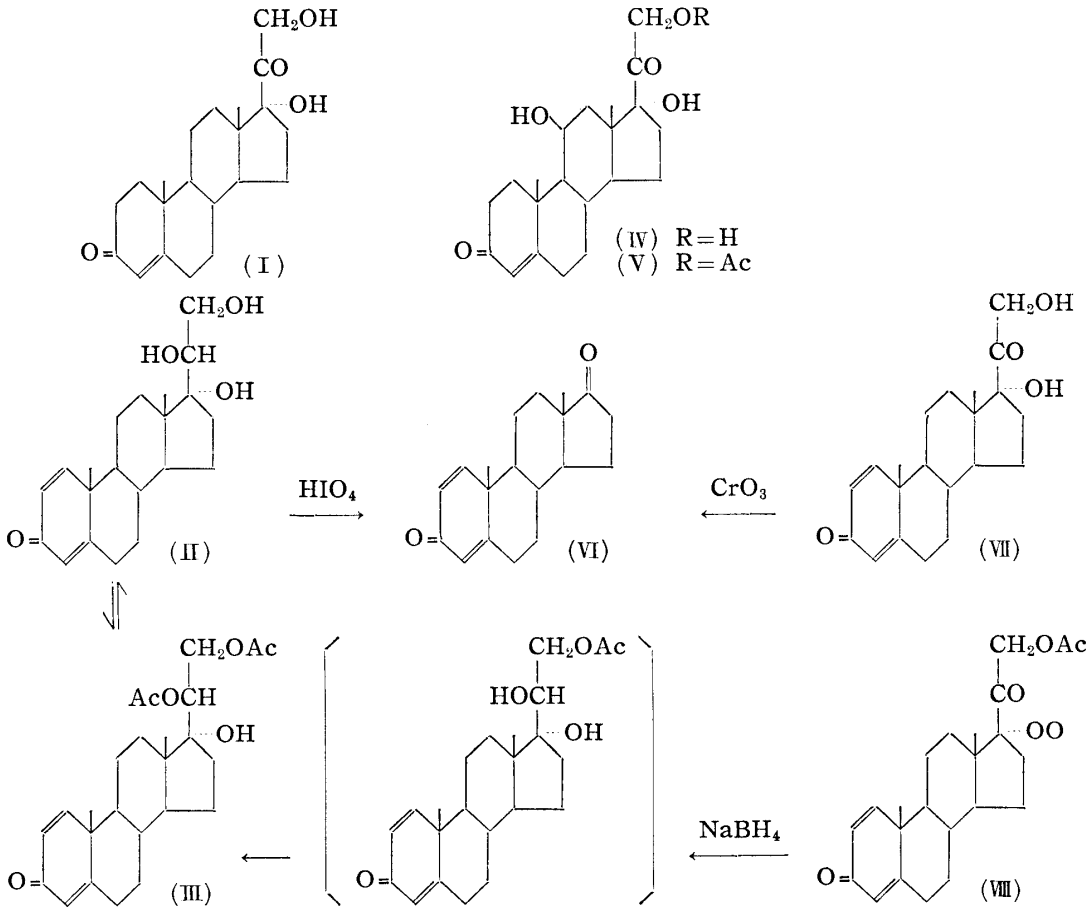
2) D.H. Peterson, H.C. Murray : J. Am. Chem. Soc., **74**, 1871(1952).

3) P. Talalay, M.M. Dobson, D.F. Tapley : Nature, **170**, 620(1952); P. Talalay, M.M. Dobson : J. Biol. Chem., **205**, 823(1953); P. Talalay, P.I. Marcus : Nature, **173**, 1189(1954); H.R. Levy, P. Talalay : J. Am. Chem. Soc., **79**, 2658(1957). See also P. Talalay : Physiol. Rev., **37**, 362(1957); S.H. Eppstein, P.D. Meister, H.C. Murray, D.H. Peterson : Vitamins and Hormones, **14**, 359(1956).

extract was then carried out with acetic anhydride and pyridine, and by direct crystallization of the reaction mixture, compound (III) was obtained as colorless prisms of m.p. 178~179°. On treatment with potassium hydrogencarbonate, (III) was successfully converted to (II) as colorless prisms, m.p. 194~195°. This compound was evidently the main product of microbial conversion by *Pseudomonas* sp. 109.

Structural assignment for the crystalline product (II) was first made. Paper chromatography revealed that fluorescence under ultraviolet light and coloration with the antimony trichloride reagent were distinctive of 1,4-dien-3-one compounds and its Rf value was almost indistinguishable from that of prednisolone (11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione). The infrared spectrum exhibited three strong absorption bands characteristic of a 1,4-dien-3-one system in the 1600~1670 cm^{-1} region and no band corresponding to an isolated carbonyl group at around 1700 cm^{-1}. For the purpose of clarifying the structure of steroid skeleton, the compound (II) was subjected to periodic acid oxidation. The reaction product (VI) obtained by the usual procedures was found to be identical in all respects with the compound prepared by oxidation of 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione (VII) with chromium trioxide. Thus the structure of (VI) was verified as 1,4-androstadiene-3,20-dione.[4]

Nature of the side-chain of the compound (II) was then examined. As a method of selective reduction of the 20-carbonyl group of steroids, Norymberski and Woods[5] reported the use of sodium borohydride for reduction of cortisone acetate, hydrocortisone acetate, and related compounds under certain controlled conditions to the corresponding 20-hydroxy



4) H. H. Inhoffen, G. Zühlsdorff, Huang-Minlon : Chem. Ber., **73**, 451(1940); H. H. Inhoffen : Angew. Chem., **59**, 207(1947); J. Fried, R. W. Thomas, A. Klingsberg : J. Am. Chem. Soc., **75**, 6764(1953).
5) J. K. Norymberski, G. F. Woods : J. Chem. Soc., **1955**, 3426.

compounds. Referring to this method, 17α-hydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione (Ⅷ) was submitted to reduction with sodium borohydride and the product obtained after acetylation with acetic anhydride and pyridine was completely identical with the acetate of (Ⅱ), i.e. (Ⅲ), with respect to infrared spectrum and mixed melting point. Difference in molecular rotation data between a free steroid and its acetate have been used to define the configuration of a hydroxyl group at 20-position; a positive shift of molecular rotation produced by acetylation is attributed to a 20β-hydroxy compound and a negative shift to a 20α-hydroxy isomer.[6] The molecular rotation difference of the compounds (Ⅱ) and (Ⅲ) is +316°, clearly indicating the β-orientation. This finding was in good agreement with the accepted view that the sodium borohydride reduction of a 20-carbonyl group provides in general a 20β-hydroxyl.[7] The compound (Ⅱ) was thus proved to possess a structure of 17α,20β,21-trihydroxy-1,4-pregnadien-3-one and the diacetate (Ⅲ) to be 17α-hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one.

Sutter and his collaborators[8] reported the formation of (Ⅱ) by the transformation of Reichstein's Substance S (I) with *Mycobacterium lacticola*, but gave only the melting points and the infrared spectral data of the free steroid and its diacetate.

In order to isolate other substances from the crude steroid extract, the mother liquor, left after the transformation products were subjected to acetylation followed by direct crystallization of (Ⅲ), was chromatographed over Florisil. The material dissolved in benzene was added to a Florisil column and developed with a solvent mixture of benzene and chloroform (1:1). Eluates were collected in small portions and analyzed by paper chromatography. A crystalline fraction gave colorless granules (V) melting at 214~216°. Non-crystalline fractions were combined and repeatedly chromatographed on Florisil, using ether-acetone as a developer to yield the acetate of the starting material and additional amounts of (V) and (Ⅲ). Paper chromatography suggested the compound (V) most likely to be hydrocortisone acetate and the comparison of the infrared spectra of (V) and authentic hydrocortisone acetate gave the unquestionable evidence of their identity. Elementary analysis, melting point, and mixed melting point also confirmed the identity of the compound (V) and hydrocortisone acetate. The formation ratio of the compounds (Ⅱ) and (Ⅳ) was approximately 10 to 1.

Thus, microbiological transformation of Reichstein's Substance S (I) by *Pseudomonas* sp. 109 resulted in the production of 17α,20β,21-trihydroxy-1,4-pregnadien-3-one (Ⅱ) and hydrocortisone (Ⅳ). From these results it is seen that *Pseudomonas* sp. 109 possesses enzyme systems capable of performing Δ^1-dehydrogenation, 11β-hydroxylation, and 20-hydrogenation and, above all, the production of hydrocortisone may be worthy of special attention as one of the rare examples of 11β-hydroxylation accomplished by microorganisms other than fungi.

Experimental*5

Transformation of Reichstein's Substance S (17α, 21-Dihydroxy-4-pregnene-3,20-dione) (I) by *Pseudomonas* sp. 109—To a 24-hr. growth culture of *Pseudomonas* sp. 109 in a synthetic medium containing glycerol, urea, $MgSO_4$, KH_2PO_4, and $FeSO_4$, 10 g. of Reichstein's Substance S (I) dissolved in EtOH was added. After an incubation of 17 hr. at 30°, the culture filtrate was extracted with AcOEt. The extract was concentrated to 1 L., washed with 5% Na_2CO_3 solution and water, dried over anhyd. Na_2SO_4, and concentrated to dryness below 50°. The powdery residue weighed 3.4 g.

*5 All m.p.s are uncorrected and the infrared spectra were measured in Nujol mulls.

6) L. H. Sarett : J. Am. Chem. Soc., **71**, 1175(1949); W. Klyne, D. H. R. Barton : *Ibid.*, **71**, 1500 (1949).
7) E. P. Oliveto, E. B. Hershberg : *Ibid.*, **75**, 488(1953).
8) O. Sutter, W. Charney, P. L. O'Neil, F. Carvajal, H. L. Herzog, E. B. Hershberg : J. Org. Chem., **22**, 578(1957).

Paper chromatography indicated the formation of a large amount of a new compound with small amounts of two other compounds, one of which was more polar and the other less polar than the main product, as well as the presence of some unchanged starting material. Attempts to isolate the main component of the steroid extract by recrystallization failed.

Isolation of 17α-Hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one (III) by Direct Crystallization—A solution of 3.4 g. of the steroid extract dissolved in 47 cc. of pyridine and 29 cc. of Ac_2O was allowed to stand at room temperature for 22 hr., warmed at 50° for 1 hr., and evaporated *in vacuo*. The residue was taken up in Et_2O, washed successively with dil. HCl, $NaHCO_3$ solution, and water, and dried over anhyd. Na_2SO_4. After removal of the solvent, the residue was crystallized from Et_2O-petr. ether to yield 0.75 g. of colorless prisms, m.p. 175~177°. Recrystallization twice from Et_2O gave material of analytical quality, m.p. 178~179°; $[\alpha]_D^{20}$ +100° (c=1.0 in $CHCl_3$); M_D +430°; UV λ_{max}^{EtOH} 243.5 mμ (ε 15,900); IR ν_{max} cm^{-1}: 3509 (OH), 1739 (acetate), 1664, 1626, 1608, 886 (1,4-dien-3-one). *Anal.* Calcd. for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96. Found: C, 69.52; H, 7.92.

The physical constants for this compound reported by Sutter *et al.*[8] are: m.p. 175~177°; IR ν_{max} cm^{-1}: 3484 (OH), 1739 (acetate), 1664, 1629, 1608 (1,4-dien-3-one), 1225 (C-O-C of acetate).

Isolation of Hydrocortisone Acetate (11β,17α-Dihydroxy-21-acetoxy-4-pregnene-3,20-dione) (V) and (III) by Chromatography—The non-crystalline residue from the mother liquor, left after isolation of (III) by direct crystallization from the acetylation mixture of the steroid extract, was dissolved in benzene and chromatographed over 100 g. of Florisil using benzene-$CHCl_3$ (1:1) mixture as a developer. The combined crystalline fraction was recrystallized from MeOH-Me_2CO to yield 20 mg. of colorless granules, m.p. 214~216°. The mother liquor from the recrystallization was rechromatographed on 10 g. of Florisil and elution with Et_2O-Me_2CO (96:4) mixture gave an additional 40 mg. of the same crystals. The infrared spectrum was identical in all details with that of authentic hydrocortisone acetate and the melting point was undepressed on admixture with an authentic sample. IR ν_{max} cm^{-1}: 3390, 3279 (OH), 1739 (acetate), 1709 (carbonyl), 1626 (4-en-3-one). *Anal.* Calcd. for $C_{23}H_{32}O_6$: C, 68.29; H, 7.79. Found: C, 67.91; H, 7.86.

The oily fractions from the benzene-$CHCl_3$ (1:1) eluates of the above chromatography were combined and again fractionated over 200 g. of Florisil. Increasing amount of Et_2O was added to the elution solvent, benzene; benzene-Et_2O ratio of 98:2, then 96:4, 92:8, 90:10, 80:20, 60:40, and finally pure Et_2O, but no residue remained on evaporation of the eluates. Elution was continued with a mixture of Et_2O and Me_2CO, and the residue of the Et_2O-Me_2CO (98:2) eluates was recrystallized from Et_2O-Me_2CO to give 260 mg. of colorless plates, m.p. 232~234°. The infrared spectrum and the mixed melting point showed this compound to be the acetate of the unchanged substrate.

The non-crystalline fractions eluted by Et_2O-Me_2CO (90:10) were partitioned once more over 50 g. of Florisil and elution with Et_2O-Me_2CO (96:4) yielded, after recrystallization, 30 mg. of (V) melting at 210~215°.

Finally, crystals eluted by Et_2O-Me_2CO (80:20) were recrystallized to give 330 mg. of (III), m.p. 177~179°. A component detected as a less polar one than (II) on the paper chromatogram of the crude steroid extract could not be isolated by the above column chromatography.

17α,20β,21-Trihydroxy-1,4-pregnadien-3-one (II) from (III)—To a solution of 0.65 g. of the diacetate (III), m.p. 175~177°, in 50 cc. of MeOH, 1.2 g. of $KHCO_3$ dissolved in 5 cc. of water was added and the solution was heated under reflux for 1 hr. After evaporation of the solution *in vacuo*, the residue was taken up in a small volume of water and extracted with Et_2O. The extract was washed with water, dried over anhyd. Na_2SO_4, and concentrated to give colorless prisms which after recrystallization from Me_2CO-Et_2O melted at 187~190° (weight, 0.22 g.). Two more recrystallizations yielded analytically pure material having a melting point of 194~195°; $[\alpha]_D^{20}$ +33° (c=1.0 in $CHCl_3$); M_D +114°. UV λ_{max}^{EtOH} 244.5 mμ (ε 14,200); IR ν_{max} cm^{-1}: 3333 (OH), 1667, 1613, 1600, 885 (1,4-dien-3-one). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.53; H, 8.43.

The physical constants for this compound reported by Sutter, *et al.*[8] are: m.p. 190~193°; IR ν_{max} cm^{-1}: 3311 (OH), 1661, 1616, 1603 (1,4-dien-3-one). Paper chromatographic behavior of this compound judged by fluorescence and coloration with the antimony trichloride reagent is characteristic of 1,4-dien-3-one compounds and its Rf value is almost indistinguishable from that of prednisolone (11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione).

1,4-Androstadiene-3,17-dione (VI) from (II)—A solution of 100 mg. of (II) dissolved in 5 cc. of dioxane was subjected to oxidation with 218 mg. of $HIO_4 \cdot 2H_2O$ in 4 cc. of water. After standing the mixture at room temperature for 16 hr., the solution was concentrated *in vacuo* and the residue was extracted with Et_2O. The Et_2O extract was washed with dil. $NaHCO_3$ solution and water, and dried over anhyd. $MgSO_4$. After removal of the solvent, the residue was recrystallized from Et_2O to 50 mg. of colorless plates, m.p. 135~137°. IR ν_{max} cm^{-1}: 1733 (carbonyl), 1658, 1618, 1600, 889 (1,4-dien-3-one). The infrared spectrum and mixed melting point determination showed this product to be identical with authentic (VI) obtained by the following method.

Formation of (VI) from 17α,21-Dihydroxy-1,4-pregnadiene-3,20-dione (VII)—To a solution of 500 mg. of (VII) in 50 cc. of glacial AcOH, 660 mg. of CrO_3 dissolved in 66 cc. of glacial AcOH and 1 cc. of water was added at room temperature over a period of 30 min. After standing for 2 days at room temperature, the solution was diluted with 20 cc. of EtOH and evaporated *in vacuo*. The residue was extracted with Et_2O, the extract was washed with dil. Na_2CO_3 solution and water, and dried over anhyd. $MgSO_4$. The Et_2O solution, after evaporation and recrystallization from Et_2O, yielded 100 mg. of colorless plates, m.p. 137°; $[\alpha]_D^{20}$ +112°(c=1.0 in $CHCl_3$); IR ν_{max} cm^{-1} : 1736(carbonyl), 1656, 1618, 1600, 890(1,4-dien-3-one). These properties agreed well with those described for (VI) in the literature.[4] *Anal.* Calcd. for $C_{19}H_{24}O_2$: C, 80.24; H, 8.51. Found : C, 79.94; H, 8.33.

Formation of (III) from 17α-Hydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione (VIII)—A solution of 500 mg. of (VIII) dissolved in 100 cc. of EtOH was subjected to reduction by addition of 80 mg. of $NaBH_4$ at 3°. After stirring for 2 hr., the solution was concentrated in vacuum at low temperature, the residue was mixed with 10 cc. of water and 1 cc. of 1% HCl, and extracted with $CHCl_3$. The residual solid from the $CHCl_3$ solution was dissolved in 6 cc. of pyridine and 3.5 cc. of Ac_2O, and allowed to stand at room temperature for 40 hr. After being warmed at 50° for 1 hr., the solution was concentrated *in vacuo* and the residue was extracted with $CHCl_3$. The $CHCl_3$ extract was washed with dil. HCl and water, and evaporated. The crystalline product was purified by recrystallization from Me_2CO to afford 20 mg. of colorless plates, m.p. 177°. IR ν_{max} cm^{-1} : 3484(OH), 1730(acetate), 1656, 1623, 1600, 885(1,4-dien-3-one). By comparison of infrared spectrum and melting point, and by the mixed melting point determination, this compound was found to be identical with (III) obtained from the acetylation mixture of the crude steroid extract described above.

Summary

Microbiological transformation of Reichstein's Substance S (I) by *Pseudomonas* sp. 109 afforded hydrocortisone (IV) and 17α,20β,21-trihydroxy-1,4-pregnadien-3-one (II), and structure of the latter was verified.

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