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

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As part of studies on the transformation of steroids by *Pseudomonas*, the preceding paper*1 described the conversion of Reichstein's Substance S (I) into 17α,20β,21-trihydroxy-1,4-pregnadien-3-one (II) and hydrocortisone by the action of *Pseudomonas* sp. 109. Such findings as Δ^1-dehydrogenation, 11β-hydroxylation, and 20β-hydrogenation, were so interesting and encouraging for the study of steroid bioconversion by *Pseudomonas* that further investigation was made on the dissimilation ability of other species of *Pseudomonas* genus on steroid compounds. This paper reports the transformation of compound (I) to compound (II) and 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione (III) by the action of *Pseudomonas* sp. 125,*4 and to 17α,20β,21-trihydroxy-4-pregnen-3-one (IV) by the action of *Pseudomonas* sp. M8.*4

Pseudomonas sp. 125 : Fermentation of Reichstein's Substance S (I) was conducted as described previously.*1 Extraction with ethyl acetate, followed by washing with alkaline solution and concentration, afforded a powdery steroid extract. Paper chromatography revealed the extract to contain, besides the unchanged substrate (I), two new compounds which had mobility slower than (I). Fluorescence, coloration with antimony trichloride, and Rf values suggested that the new components were 17α,20β,21-trihydroxy-1,4-pregnadien-3-one (II) and 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione (III), or closely related compounds.

For isolation of the transformation products, the crude extract was acetylated with acetic anhydride and pyridine, and subjected to chromatography over Florisil. The material dissolved in benzene was added to the column and eluted with ether-acetone (increasing amounts of acetone added to ether). Eluates were collected in small portions and examined by paper chromatography and melting point, and there were obtained three crystalline substances. The crystals eluted first possessed a melting point of 234~235°, and the infrared spectrum and mixed melting point showed this compound to be the acetate of the starting material. The second substance crystallized in colorless needles, m.p. 215~218°, and was identified as 17α-hydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione (the acetate of (III)[1]) by infrared spectrum and mixed melting point. The acetate was further converted by treatment with potassium hydrogencarbonate into a free steroid which was confirmed to be 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione (III)[1] by comparison with an authentic sample. The compound of the last fractions obtained as colorless prisms, m.p. 178~179°, was found identical in all respects including infrared spectrum and melting point with 17α-hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one, the acetate of (II) which was produced, as described previously, by the action of *Pseudomonas* sp. 109 on Reichstein's Substance S (I). The product was further converted by

*1 This paper constitutes a part of a series entitled "Studies on Steroids" by Hayao Nawa. Part XIV. M. Uchibayashi : This Bulletin, 8, 112(1960).

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

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*4 The number refers to Rokuro Takeda's collection in the Institute for Fermentation, Osaka. *Ps.* sp. 125 was identified as closely related to *Pseudomonas oleovorans*, and *Ps.* sp. M8 to *Pseudomonas fluorescens*. Detailed microbiological and fermentational accounts will be published elsewhere by J. Terumichi.

1) G. Rosenkranz, J. Pataki, St. Kaufmann, J. Berlin, C. Djerassi : J. Am. Chem. Soc., **72**, 4081 (1950); E. Vischer, Ch. Meystre, A. Wettstein : Helv. Chim. Acta, **38**, 835(1955).

hydrolysis to 17α,20β,21-trihydroxy-1,4-pregnadien-3-one (II).

The compounds (II) and (III) were obtained in this way through acetyl derivatives. It was also possible to isolate the components of the crude steroid extract, without acetylation, by application of paper-chromatographic conditions to column chromatography. The steroid extract was added to a column of cellulose powder, retaining a mixture of propylene glycol and methanol (3:7) as a stationary phase and developed with dioxane-toluene (3:7). Analyses of the eluates were essentially the same as in the case of Florisil chromatography of the acetates, and the unchanged substrate (I), and the compounds (II) and (III) were isolated. This procedure, however, seemd somewhat less effective than acetylation. The ratio of formation of (III) and (II) was roughly two to one.

Pseudomonas sp. M8 : Methods and conditions employed for the microbial conversion followed the previously described ones. The crude steroid extract obtained by conventional extraction procedures was shown, by paper chromatography, to contain the unchanged starting material (I) and a new compound which exhibited similarity to hydrocortisone in fluorescence and coloration to the antimony trichloride reagent, but its Rf value was larger, though slight, than that of hydrocortisone.

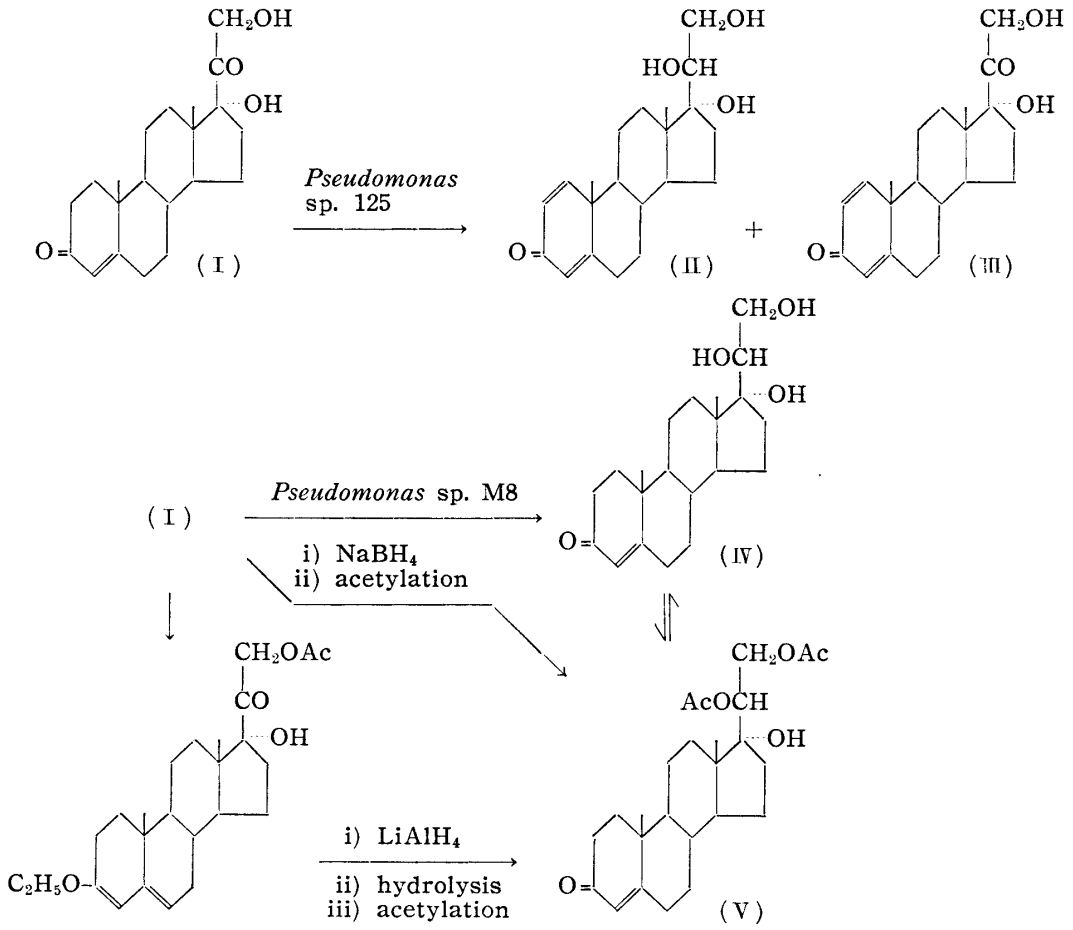
Acetylation of the steroid extract followed by chromatography over Florisil using ether-acetone as a developer resulted in a good separation into the two constituents. The compound eluted first was the acetate of the substrate (I) judged by infrared spectrum, melting point, and mixed melting point. The later fractions afforded colorless needles (V) of m.p. 191~192°, which were hydrolyzed with potassium hydrogencarbonate to yield the free steroid (IV) as colorless needles, m.p. 188~189°. The infrared spectrum showed absorption bands characteristic of 4-en-3-one system and no band corresponding to an isolated carbonyl group. Thus, the compound (IV) was thought to have been produced by hydrogenation of the 20-carbonyl group of the starting material, as in the formation of 17α,20β,21-trihydroxy-1,4-pregnadien-3-one. To substantiate the validity of this supposition, Reichstein's Substance S (I) was subjected to reduction with sodium borohydride under controlled conditions.[2] Acetylation of the product with acetic anhydride and pyridine followed by chromatography over Florisil afforded two crystalline compounds, one having a melting point of 164~166° and the other melting at 191~192°. The latter product was identified as the acetate of (IV), i.e. (V), by comparison of infrared spectra and melting points. Structure of the former compound will be discussed in a subsequent paper.

Julian and his co-workers[3] reported that the reduction with lithium aluminum hydride of the 3-enol ether of 21-acetoxy-16,17-epoxy-4-pregnene-3,20-dione gave both 17α,20α,21-trihydroxy-4-pregnen-3-one and its 20β isomer (IV), whereas the reduction of the 3-enol ether of the acetate of Reichstein's Substance S yielded only the 20β isomer. The 20β compound (IV) was prepared by their method, starting from the acetate of (I) and this (IV) was confirmed to be identical in every detail with the product obtained by the microbiological conversion by *Pseudomonas* sp. M8 and by the sodium borohydride reduction of (I). Thus, the bioconversion product of Substance S (I) by *Pseudomonas* sp. M8 was definitely proved to be 17α,20β,21-trihydroxy-4-pregnen-3-one (IV). The melting points and optical rotations of (IV) and (V) corresponded well with those found in the literature,[3,4] and the infrared spectrum of (IV) was superimposable on the spectrum reported by other researchers.[5] The production of compound (IV) by microbial transformation of (I) with *Epicoccum oryzae*[6] and *Streptomyces coelicolor*[7] has been cited, and

2) J.K. Norymberski, G.F. Woods : J. Chem. Soc., **1955**, 3426.
3) P.L. Julian, E.W. Meyer, W.J. Karpel, W. Cole : J. Am. Chem. Soc., **73**, 1982(1951).
4) W. Logemann : Naturwissenschaften, **27**, 196(1939); C.W. Shoppee : Helv. Chim. Acta, **23**, 925 (1940).
5) H.J. Hübener, J. Schmidt-Thomé : Z. physiol. Chem., **299**, 244(1955).

since completion of this work, a report appeared, which dealt with the preparation of compound (IV) from (I) by the action of *Streptomyces hydrogenans*.[8]

In this way, it was demonstrated that *Pseudomonas* sp. 125 effected microbiological transformation of Reichstein's Substance S (I) to yield 17α,20β,21-trihydroxy-1,4-pregnadien-3-one (II) and 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione (III), and *Pseudomonas* sp. M8 to give 17α,20β,21-trihydroxy-4-pregnen-3-one (IV), and that these *Pseudomonas* species had the ability of effecting Δ^1-dehydrogenation and 20β-hydrogenation. It may be interesting to note that these transformations take place singly or separately in *Pseudomonas* sp. 125 and *Pseudomonas* sp. M8, whereas they occur together in *Pseudomonas* sp. 109.



Experimental[*5]

Transformation of Reichstein's Substance S (I) by *Pseudomonas* sp. 125—The microbiological conversion of Reichstein's Substance S (I) by *Pseudomonas* sp. 125 was essentially the same as described in the preceding paper.[*1] The extraction with AcOEt, followed by washing with 5% Na_2CO_3 solution and concentration, afforded a crude steroid extract, which was shown by paper chromatography to contain two new components as well as the unchanged starting material (I). The mobility of the new components was slower than that of (I).

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

6) G. M. Shull, B. M. Bloom, unpublished work, cited from G. M. Shull: Transactions N. Y. Acad. Sci., Ser. II. **19**, 147(1956).
7) E. Vischer, Ch. Meystre, A. Wettstein, unpublished data, cited in A. Wettstein: Experientia, **11**, 465(1955).
8) F. Lindner, R. Junk, G. Nesemann, J. Schmidt-Thomé: Z. physiol. Chem., **313**, 117(1958).

Isolation of 17α,20β,21-Trihydroxy-1,4-pregnadien-3-one (II) and 17α,21-Dihydroxy-1,4-pregnadiene-3,20-dione (III)—Through a column packed with 170 g. of cellulose powder was passed a solvent mixture of propylene glycol and MeOH (3:7) and excess of solvent mixture was removed by water-pump suction. A 460-mg. sample of the crude steroid extract dissolved in a mixture of MeOH and $CHCl_3$ (2:1) was added to the column and developed with a mixture of dioxane and toluene (3:7). Eluates were collected in small portions and analyzed by paper chromatography. Thus, the three components, viz. the substrate (I) and the compounds (III) and (II), were found to be eluted separately in the order mentioned. This method, however, was not satisfactory in yield and purity of the products, as compared with the acetylation method stated below.

Isolation of 17α-Hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one and 17α-Hydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione—A solution of 1 g. of the crude steroid extract dissolved in 12.5 cc. of pyridine and 7.5 cc. of Ac_2O, was allowed to stand at room temperature for 24 hr. and then warmed at 50° for 1 hr. After concentration of the solution *in vacuo*, the residue was taken up in $CHCl_3$ and the extract was washed successively with dil. HCl, $NaHCO_3$ solution, and water, and dried over anhyd. Na_2SO_4. Removal of the solvent gave an oily residue which was then chromatographed over 280 g. of Florisil using Et_2O-Me_2CO as a developer.

The fractions from the eluates of Et_2O-Me_2CO (95:5) yielded 0.5 g. of the acetate of unchanged (I), m.p. 234~235°. The infrared spectrum and mixed melting point characterized this compound.

The fractions eluted by Et_2O-Me_2CO (92:8) were recrystallized from Me_2CO to 0.2 g. of colorless needles, m.p. 215~218°. IR ν_{max} cm^{-1}: 3333(OH), 1739(acetate), 1718(carbonyl), 1658, 1613, 1600, 893 (1,4-dien-3-one). *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.48; H, 7.82. Found: C, 71.26; H, 7.78.

Comparison of this product by mixed melting point and infrared spectrum[1] with an authentic sample of 17α-hydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione showed these compounds to be identical.

Further, the fractions from the Et_2O-Me_2CO (85:15) eluates were purified by recrystallization from Me_2CO to afford 0.1 g. of colorless prisms, m.p. 178~179°. IR ν_{max} cm^{-1}: 3484(OH), 1730(acetate), 1656, 1623, 1575, 885(1,4-dien-3-one). *Anal.* Calcd. for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96. Found: C, 69.49; H, 7.88.

Infrared spectrum showed the compound to be identical with 17α-hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one which was described in the preceding paper. Mixed melting point was not depressed.

17α,20β,21-Trihydroxy-1,4-pregnadien-3-one (II) from 17α-Hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one—17α-Hydroxy-20β,21-diacetoxy-1,4-pregnadien-3-one was subjected to hydrolysis as described in the preceding paper. The colorless prisms, m.p. 194~195°, did not depress the melting point of an authentic sample of (II).

17α,21-Dihydroxy-1,4-pregnadiene-3,20-dione (III) from 17α-Hydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione—To a solution of 400 mg. of 17α-hydroxy-21-acetoxy-1,4-pregnadiene-3,20-dione in 40 cc. of MeOH, 420 mg. of $KHCO_3$ dissolved in 5 cc. of water was added. After being refluxed for 1 hr., the mixture was concentrated *in vacuo* to dryness and extracted with $CHCl_3$. The extract was washed with water, dried over anhyd. Na_2SO_4, and evaporated. The residue was recrystallized from Me_2CO to 245 mg. of colorless plates, m.p. 225~228° (decomp.). IR ν_{max} cm^{-1}: 3333(OH), 1724(carbonyl), 1667, 1618, 1608, 896(1,4-dien-3-one). *Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found: C, 73.01; H, 8.13. The infrared spectrum was identical with that of authentic (III) and the mixed melting point showed no depression.[1]

Transformation of Reichstein's Substance S (I) by *Pseudomonas* sp. M8 and Isolation of 17α-Hydroxy-20β,21-diacetoxy-4-pregnen-3-one (V)—The bioconversion of Reichstein's Substance S (I) by *Pseudomonas* sp. M8 was conducted in a similar manner to that of *Pseudomonas* sp. 125. The AcOEt extraction provided a crude steroid extract which contained a new compound in addition to the unchanged substrate (I), as shown by paper chromatography.

A portion of 200 mg. of the crude steroid extract dissolved in 2.5 cc. of pyridine and 1.5 cc. of Ac_2O was allowed to stand at room temperature for 24 hr. and then warmed at 50° for 1 hr. The solution was concentrated to dryness *in vacuo* and the residue was taken up in $CHCl_3$. The extract was 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 dissolved in benzene and chromatographed on 50 g. of Florisil, being developed with Et_2O-Me_2CO. The eluates of Et_2O-Me_2CO (98:2) gave 50 mg. of the acetate of (I), m.p. 234-235°, which was characterized by infrared spectrum, melting point, and mixed melting point. The fractions eluted by the solvent mixtures of Et_2O-Me_2CO (94:6, 92:8 and 90:10) were combined and recrystallized from $CHCl_3$-MeOH and then from Me_2CO to yield 150 mg. of colorless needles, m.p. 191~192°. $[\alpha]_D^{20}$ +133° (c=1.0, in dioxane); M_D +575°. IR ν_{max} cm^{-1}: 3390(OH), 1733(acetate), 1650, 1610(4-en-3-one). *Anal.* Calcd. for $C_{25}H_{36}O_6$: C, 69.42; H, 8.39. Found: C, 69.60; H, 8.34.

This compound was identical in all respects with an authentic sample of (V) obtained by the method described below. The melting point and optical rotation corresponded well with those found in the literature.[3,4]

17α,20β,21-Trihydroxy-4-pregnen-3-one (IV) from (V)—One hundred mg. of the diacetate (V) was dissolved in 10 cc. of MeOH and refluxed for 1 hr. with a solution of 200 mg. of $KHCO_3$ in 1 cc. of water. The mixture was concentrated *in vacuo* and extracted with $CHCl_3$. After being washed with water and dried over anhyd. Na_2SO_4, the $CHCl_3$ solution was evaporated and the residue was recrystallized from Me_2CO to afford 80 mg. of colorless needles, m.p. 188~189°; $[\alpha]_D^{20}$ +67° (c=1.0, in dioxane); M_D +233°; IR ν_{max} cm^{-1} : 3390(OH), 1645, 1610(4-en-3-one). *Anal.* Calcd. for $C_{21}H_{32}O_4$: C, 72.38; H, 9.26. Found : C, 72.21; H, 9.18. The melting point and optical rotation coincided with those reported in the literature,[3,4] and the infrared spectrum was superimposable on the reported one.[5]

(V) from (I)—To a solution of 1.00 g. of the compound (I) in 200 cc. of MeOH was added 126 mg. of $NaBH_4$ at 1° and stirring was continued for 1 hr. After addition of 2 cc. of glacial AcOH, the reaction mixture was concentrated to dryness *in vacuo*. The residue was dissolved in a mixture of 12 cc. of pyridine and 7 cc. of Ac_2O, left over-night at room temperature, and warmed to 50° for 1 hr. Evaporation of the solution gave an oily residue which was dissolved in benzene and added to a column of 90 g. of Florisil.

The fractions from the Et_2O eluates were recrystallized from AcOEt to yield 700 mg. of colorless needles, m.p. 164~166°.

The eluates of Et_2O-Me_2CO (98:2) gave material which, on recrystallization from Me_2CO, yielded 200 mg. of colorless needles, m.p. 191~192°. IR ν_{max} cm^{-1} : 3390(OH), 1727(acetate), 1650, 1613(4-en-3-one). *Anal.* Calcd. for $C_{25}H_{36}O_6$: C, 69.42; H, 8.39. Found : C, 69.60; H, 8.34. The compound was completely identical with (V) obtained as a microbiological conversion product as well as with (V) prepared by the following method.

(V) from 3-Ethoxy-17α-hydroxy-21-acetoxy-3,5-pregnadien-20-one—According to Julian's procedure,[3] the acetate of Reichstein's Substance S (I) was converted-to 3-ethoxy-17α-hydroxy-21-acetoxy-3,5-pregnadien-20-one, m.p. 166~169°. Reduction of the 3-enol ether with $LiAlH_4$ in Et_2O-benzene mixture, followed by hydrolysis with HCl and acetylation with Ac_2O and pyridine furnished a crystalline substance which on chromatography over Florisil (developer : Et_2O-Me_2CO) gave colorless short needles, m.p. 190~191°(from Me_2CO). IR ν_{max} cm^{-1} : 3413(OH), 1739(acetate), 1650, 1613 (4-en-3-one). The infrared spectrum and mixed melting point determination showed this compound to be identical with (V) obtained by the above two methods.

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Summary

Microbiological transformation of Reichstein's Substans S (I) to 17α,20β,21-trihydroxy-1,4-pregnadien-3-one (II) and 17α,21-dihydroxy-1,4-pregnadiene-3,20-dione (III) by the action of *Pseudomonas* sp. 125, and to 17α,20β,21-trihydroxy-4-pregnen-3-one (IV) by *Pseudomonas* sp. M8 was described.

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