Studies on the Microbiological Transformation of Steroids

Part VI. Stereospecific Reduction of the 20-Carbonyl Group by Fungi

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The microbiological reduction of the 20-carbonyl group of steroids has been investigated. *Candida pulcherrima* IFO 0964 and *Sporotrichum gougeroti* IFO 5982 converted the following substrates into the corresponding 20 β -hydroxy derivatives (yields of the products are indicated in parentheses): Reichstein's Compound S (60~70%) and 17 α ,21-dihydroxypregna-1,4-diene-3,20-dione (40~80%). *Rhodotorula glutinis* IFO 0895 converted the following substrates into the corresponding 20 α -hydroxy derivatives: Reichstein's Compound S (65%), 17 α ,21-dihydroxypregna-1,4-diene-3,20-dione (80%), 11 β ,17 α -dihydroxypregn-4-ene-3,20-dione (45%) and 17 α ,19,21-trihydroxypregn-4-ene-3,20-dione (10%).

Microorganisms belonging to genus *Streptomyces*, such as *St. lavendulae*¹⁾, *St. coelicolor*²⁾, *St. hydrogenans*³⁾, *St. griseus*⁴⁾ are well known to be the 20-carbonyl reducing organism; i.e. they reduce most pregnane steroids to the corresponding 20 β -hydroxysteroids. In addition to them *Fusarium*⁵⁾, *Calonectria*⁵⁾, *Pseudomonas*⁶⁾, *Gleospodium*⁷⁾, *Pythium*⁸⁾ and *Stereum*⁹⁾ have been reported to have a reducing power similar to that of *Streptomyces*. *Fusarium*, *Calonectria*, *Pseudomonas* and *Gleospodium*, however, oxidize another site of the same compound concomitantly. On the other hand, *Pythium* and *Stereum* act only on

certain compounds. It may be therefore, said that useful microorganisms for the conversion of the 20-carbonyl group of steroids into the corresponding 20 β -hydroxyl group generally belong to the *Streptomyces* group. As a microorganism which reduces 20-oxosteroids to the corresponding 20 α -hydroxysteroids is known only one microorganism, *Rhodotorula longissima*. In our study on the microbiological transformation of steroids *Candida pulcherrima* IFO 0964 and *Sporotrichum gougeroti* IFO 5982 were found to reduced the 20-carbonyl group to the 20 β -hydroxyl group, and *Rhodotorula glutinis* IFO 0935 to reduce the group to the 20 α -hydroxyl group in good yields. Using these organisms the authors obtained various 20-hydroxysteroids including a new compound from the corresponding 20-oxosteroids.

As to the fermentation of steroids the details will be described in the experimental part but an evidence should be mentioned here that no influence of pH of culture broth on the reduction was observed in the range

1) J. Fried, R.W. Thoma and A. Klingsberg, *J. Am. Chem. Soc.*, **75**, 5764 (1953).

2) E. Vischer, Ch. Meystre and A. Wettstein, *Experientia*, **11**, 465 (1955).

3) F. Lindner, R. Junk, G. Neesemann and J. Schmidt-Thomé, *Hoppe-Seyler's Z. physiol. Chem.*, **313**, 117 (1958).

4) F. Carvajal, O.F. Vitale, M.J. Gentles, H.L. Herzog and B. Hershberg, *J. Org. Chem.*, **24**, 691 (1959).

5) S.A. Szpilfogel, P.A. Van Hemert and M.S. De Winter, *Rec. trav. chim.*, **75**, 1227 (1956).

6) H. Nawa, M. Uchibayashi, R. Takeda, J. Nakanishi, J. Terumichi, M. Uchida,

M. Katsumata, K. Yoshino and H. Fujitani, *Tetrahedron*, **4**, 201 (1958).

7) E. Kondo and E. Masuo, *J. Agr. Chem. Soc. Japan*, **34**, 759 (1960).

8) M. Shirasaka and M. Ozaki, *ibid.*, **35**, 206 (1961).

9) T. Takahashi, Y. Uchibori and T. Hasegawa, *ibid.*, **36**, 67 (1962).

TABLE I. THE INFLUENCE OF PH ON THE REDUCING POWER OF THE WASHED CELL SUSPENSIONS

pH*	Microorganism Substrate	<i>Rh. glutinis</i>		<i>Sp. gougeroti</i>	
		S	Δ'-S	S	Δ'-S
2.0	KCl-HCl	65***	64	59	30
4.0	Citrate-Phosphate	62	70	69	22
6.0	"	58	67	67	47
8.0	Tris	67	56	64	22
10.0	Na ₂ CO ₃ -NaHCO ₃	81	66	66	50

* Initial pH.

** Ref. 10.

*** Percent of the reduction product formed.

from pH 2 to pH 10 with any organism (Table I).

Reductions of Reichstein's Compound S (I) and 17 α ,21-dihydroxypregna-1,4-diene-3,20-dione (II) with *C. pulcherrima* IFO 0964, *Sp. gougeroti* IFO 5982 and *Rh. glutinis* IFO 0395 and subsequent paper chromatography

of the products afforded the papergrams shown in Fig. 1, in which (a) is concerned with the experiments using I as substrate and (b) with the experiments using II as substrate. Figs. (a) and (b) show that all the organisms used converted I and II into materials of R_F 0.7 and R_F 0.5 (E-E solvent system), respectively. These spots were regarded as those of reduction products at the C-20 position because of their failure to develop a red color with triphenyltetrazolium chloride (TPTZ) reagent.

Through the fermentation with either *C. pulcherrima* or *Sp. gougeroti* I was converted into a material (III) of m.p. 188~190°C and II into a material (V) of m.p. 193~195°C. On the other hand, in the case of fermentation with *Rh. glutinis*, I was converted into a material (IV) of m.p. 220~222°C and II was

FIG. 1. Paper Chromatograms of Reduction Products.

converted into a material (VI) of m.p. 212~213°C. Both the materials III and IV, analyses of which agreed with the molecular formula of $C_{21}H_{32}O_4$, showed no absorption band of the 20-carbonyl in their infrared spectra and gave androst-4-ene-3,17-dione (VII) by oxidation either with sodium bismuthate or with periodic acid. Based on these observations III and IV were established to be the 20-epimers of 17 α ,20-21-trihydroxypregn-4-en-3-one to each other. Since both the materials V and VI analyses of which fit the same formula $C_{21}H_{30}O_4$, showed no absorption band of the 20-carbonyl group in their infrared spectra and through periodic acid oxidation gave androsta-1,4-diene-3,17-dione (VIII), which was in accord with the

oxidation product of II by sodium bismuthate, they also were characterized as the 20-epimers of 17 α ,20,21-trihydroxypregna-1,4-diene-3-one to each other.

An attempt was then made to determine the 20-configuration of the two sets of 20-epimers. When acetylated, III gave a diacetate (IX) of m.p. 178~180°C and IV gave a diacetate (X) of m.p. 245~247°C. A comparison of properties of III, IV, IX and X with those¹¹⁾ of authentic samples led to the conclusion that III and IX were 17 α ,20 β ,21-trihydroxypregn-4-en-3-one and its 20 β ,21-diacetate, respectively and IV and X were 17 α ,20 α ,21-trihydroxypregn-4-en-3-one and its 20 α ,21-diacetate, respectively.

When another set of 20-epimers, V and VI,

FIG. 2.

11) P.L. Julian, E.W. Meyer, W.J. Karpel and W. Cole, *J. Am. Chem. Soc.*, **73**, 1982 (1951).

were acetylated, V gave a material (XI) of m.p. 178~180°C and VI afforded a diacetate (XII) of m.p. 200~202°C. Since properties of V and XI well agreed with those⁹⁾ of 17 α ,20 β ,21-trihydroxypregna-1,4-dien-3-one and its diacetate, a comparison was made between V and an authentic sample of 17 α ,20 β ,21-trihydroxypregna-1,4-dien-3-one and their identity was established by mixed m.p. and comparison of their infrared spectra. And therefore, the materials VI and XII should be 17 α ,20 α ,21-trihydroxypregna-1,4-dien-3-one and its 20 α ,21-diacetate, respectively. These materials the infrared spectra of which are shown in Fig. 2 are new compounds.

In order to confirm the above assignment, increments of molecular rotation¹²⁾ of III, IV, V and VI on acetylation were measured. The

TABLE II. ASSIGNMENT OF THE 20-CONFIGURATIVE OF THE REDUCTION PRODUCTS BY THE METHOD OF MOLECULAR ROTATION DIFFERENCES

Reduction Product	M_D^{Acetate}	M_D^{Alcohol}	$\Delta^{20} + \Delta^{21}$	Configuration* of the 20-ol
III	588	268	+320	β
IV	100	208	-108	α
V	426	59	+367	β
VI	-95	45	-140	α

$$\Delta = M_D^{\text{Acetate}} - M_D^{\text{Alcohol}}$$

* Since the absolute value of the Δ^{21} is small, the sign of the Δ^{20} is in accord with that of the $\Delta^{20} + \Delta^{21}$. (Ref. 13)

calculation supported the previous assignment that III and V were 20 β -ols and IV and VI were 20 α -ols (Table II).

From the results mentioned above it became clear that *Candida pulcherrima* and *Sporotrichum gougeroti* reduced I and II to the corresponding 20 β -ols (in 40~80% yields) and

FIG. 3. The Infrared Absorption Spectra of (a), 17 α ,20 α ,21-Trihydroxypregna-1,4-dien-3-one (VI) and (b), 20 α ,21-Diacetoxypregna-1,4-dien-17 α -ol-3-one (XII).

12) L.F. Fieser and M. Fieser, "Steroids", Maruzen, 1959, Chapt. 19.

13) D.H.R. Barton and W. Klyne, *Chem. Ind.*, **1948**, 755.

that *Rhodotorula glutinis* converted I and II into the corresponding 20 α -ols (in 60~80% yields).

Further using *Rh. glutinis* an attempt was made to convert 11 β ,17 α -dihydroxypregn-4-ene-3,20-dione (XIII) and 17 α ,19,21-trihydroxypregn-4-ene-3,20-dione (XIV) into the corresponding 20 α -ols. Figs. 1c and 1d show the papergrams of fermentation products of XIII and XIV, respectively. From the fermentation of XIII two materials, R_F 's of which were 0.7 and 0.5 on the papergram (F-C system), were isolated in yields of 40% and 45% through a column chromatography over Florisil. By periodic acid oxidation the less polar product (XV) of m.p. 209~210°C gave rise to a material of m.p. 191~192°C, which was identical with 11 β -hydroxandrost-4-ene-3,17-dione (XVI) produced through oxidation of hydrocortisone with sodium bismuthate. On acetylation XV afforded monoacetate

20 α -acetate. The structure of the more polar products remains to be established.

XIV was converted with difficulty and the conversion ratio was 1/10 after seven day incubation with *Rh. glutinis*. The mobility of the conversion product (XVIII) on the papergram (E-E solvent system) was one-sixth of that of XIV and coincided with that of 17 α ,19,20 α ,21-tetrahydroxypregn-4-en-3-one¹⁴⁾. Negative TPTZ reaction supported that XVIII was a reduction product at the C-20 position of XIV. 11 β ,17 α ,20 α -trihydroxypregn-4-en-3-one and its

EXPERIMENTAL*

Microorganisms: *Candida pulcherrima* IFO 0964
Sporotrichum gougeroti IFO 5982
Rhodotorula glutinis IFO 0395

Fermentation of Steroids with the Microorganisms.

(a) With *C. pulcherrima* or *Rh. glutinis*. Fifty milliliters of a medium containing 100 g of sucrose, 3 g of ammonium sulfate, 2 g of magnesium sulfate, 5 g of potassium phosphate, mono-basic, and 10 ml of vitamin solution¹⁵⁾ in 1 l of tap water were sterilized

FIG. 4.

(XVII) of m.p. 223~228°C. Properties of XV and XVII well agreed with those of

14) T. Takahashi, This Journal, in press.

15) T. Hasegawa and T. Takahashi, *ibid.*, **23**, 137 (1959).

* All melting points are uncorrected.

under 15 pound steam pressure for fifteen minutes, inoculated with a lump of cells of a given organism, and incubated at 28°C on a rotary shaker for three days. The culture was aseptically poured into an Erlenmeyer flask containing 400 ml of the same medium and further incubated for three days. To the culture 50~100 mg of a substrate as fine powders was added and incubation was continued aerobically for two to seven days. Reduction products were extracted from the culture broth with an equal volume of ethyl acetate. The ethyl acetate extract was washed with 5% sodium bicarbonate solution and water, dried with anhydrous sodium sulfate and evaporated to dryness. The residue was examined through paper chromatography and then was treated with convenient techniques for isolation of the products.

(b) **With *Sp. gougeroti*.** Fifty milliliters of a medium containing 30 g of glucose, 3 g of corn steep liquor, 15 g of polypeptone in 1 l of tap water were inoculated with a lump of mycelium of *Sp. gougeroti* IFO 5982 and incubated at 28°C on a reciprocating shaker for five days. To 400 ml of the same medium the culture was added aseptically and further incubated for three days. At this point of shaking culture 50~100 mg of a substrate was added to the culture and incubation was continued for two to five days. Treatment of the reaction mixture was the same as that in the case of *C. pulcherrima*.

(c) **Influence of pH on the Reduction by the Microorganisms.** In order to examine influences of the pH of a reaction mixture on the reduction process the microbiological reduction was conducted with washed cell suspensions of *Rh. glutinis* or *Sp. gougeroti* in buffer solutions of various pH's. According to the procedures mentioned above, 0.5 l of each culture was incubated. From the culture broths the mycelia were harvested by centrifugation (in the case of *Rh. glutinis*) or by filtration (in the case of *Sp. gougeroti*) and washed with 0.5 l of water two times. Each mycelium thus obtained was divided equally into 10 Erlenmeyer flasks containing 50 ml of a buffer solution. To each flask was added a solution of 5 mg of either Reichstein's Compound S (I) or 17 α ,21-dihydroxypregna-1,4-diene-3,20-dione (II) dissolved in 0.5 ml of ethanol and the mixture was incubated on a rotary shaker for twenty-four hours. For quantitative measurements of reaction products was made use of a method through paper chromatography as mentioned below. The result of this experiment is shown

in Table II. Both organisms showed no remarkable fluctuation in their strong reducing powers between pH 2 and 10. *Sp. gougeroti* was different from *Rh. glutinis*, which easily reduced both I and II, in that the former showed much weaker reducing power on II than on I.

Examination and Quantitative Measurements of Reduction Products through Paper Chromatography.

A reaction mixture was extracted with a solvent and the steroid fraction after evaporation of the solvent was dissolved in acetone. A definite volume of the acetone solution containing 100 γ of steroids was pipetted and applied on the starting point of a paper strip (Whatman No. 1). Then the paper was chromatographed with a solvent system such as ethylene glycol/ethylene chloride (E-E system)¹⁶⁾ or formamide/chloroform (F-C system)¹⁷⁾. For detection of spots UV absorption was used. In order to differentiate spots of reduction products from other spots the color reaction with TPTZ reagent¹⁸⁾ was made use of. The following procedure was used for quantitative evaluation of unreacted substrate and reduction products. A desired spot of the papergram was cut off carefully and eluted with 5 ml of methanol. Absorbancy of the methanol solution was measured spectrophotometrically at the wave length of maximum absorption, e.g. at 240 m μ in the case of α,β -unsaturated oxosteroids. Using the value as well as the molecular extinction coefficient of the compound the amount of the steroid contained in the methanol solution was calculated.

17 α ,20 β ,21-Trihydroxypregna-4-en-3-one (III).

(a) **Reduction of I by *C. pulcherrima*.** According to the procedure mentioned above, 10 l of the culture of *C. pulcherrima* IFO 0964 were prepared. To the culture was added 2.6 g of I and incubation was continued aerobically for seven days. The crude product obtained by extraction of the reaction mixture was dissolved in ethylene chloride, charged on the top of a column prepared by filling up with 100 g of Florisil and developed with solvents varying proportions of acetone in ethylene chloride. Paper chromatography of the eluates showed that small amounts of unreacted substrate was eluted with 2~10% acetone in ethylene chloride and the reduction product desired was eluted

16) T. Takahashi and T. Heasegawa, *J. Agr. Chem. Soc. Japan*, **35**, 1394 (1961).

17) A. Zaffaroni, R.B. Burton and E.H. Keutmann, *Science*, **111**, 6 (1950).

18) R.B. Burton, A. Zaffaroni and E.H. Keutmann, *J. Biol. Chem.*, **188**, 763 (1951).

with 15~40% acetone in ethylene chloride. The fractions containing the product were combined and concentrated to give 1.6 g of crude crystals which were recrystallized from acetone to yield 17 α ,20 β ,21-trihydroxypregn-4-en-3-one (III) of m.p. 188~190°C, $[\alpha]_D^{21} + 77^\circ$ ($c = 1.0$, chloroform), $\lambda_{\max}^{\text{EtOH}}$ 241 m μ (ϵ , 15,800), $\nu_{\max}^{\text{KBr}}$ 3450 (OH), 1650, 1618 cm $^{-1}$ (Δ^4 -3-CO). *Anal.* Found: C, 72.36; H, 9.17. Calcd. for C $_{21}$ H $_{32}$ O $_4$: C, 72.38; H, 9.26%.

(b) **Reduction of I by *Sp. gougeroti*.** To 4 l of the culture of *Sp. gougeroti* IFO 5982 was added I (1 g) and incubation was continued aerobically for a period of three days. The ethyl acetate extract from the reaction mixture was chromatographed over Florisil using ethylene chloride-acetone mixture for elution. Fractions containing the reduction product were combined and concentrated with concurrent crystallization of the product. The crude crystal (520 mg) was recrystallized from acetone to give 17 α ,20 β ,21-trihydroxypregn-4-en-3-one (III) of m.p. 188~190°C. This material had an infrared spectrum identical with that of the reduction product by *C. pulcherrima* and the mixed melting point of these two samples showed no depression.

17 α ,20 α ,21-Trihydroxypregn-4-en-3-one (IV).

To 4 l of the culture of *Rh. glutinis* IFO 0395 was added I (1 g) dissolved in 30 ml of ethanol and aerobic incubation was continued for three days. Extraction of the reduction mixture with ethyl acetate, followed by concentration gave crude crystals (650 mg) which by recrystallization from ethanol afforded 17 α ,20 α ,21-trihydroxypregn-4-en-3-one (IV) of m.p. 220~222°C, $[\alpha]_D^{22} + 60^\circ$ ($c = 1.0$, ethanol), $\lambda_{\max}^{\text{EtOH}}$ 242 m μ (ϵ , 15,500), $\nu_{\max}^{\text{KBr}}$ 3330 (OH), 1661, 1618 cm $^{-1}$ (Δ^4 -3-CO). A mixture of this material and III showed depression in M.p. *Anal.* Found: C, 72.41; H, 9.26. Calcd. for C $_{21}$ H $_{32}$ O $_4$: C, 72.38; H, 9.26%.

17 α ,20 β ,21-Trihydroxypregna-1,4-dien-3-one (V).

(a) **Reduction of II by *C. pulcherrima*.** To 5 l of the culture of *C. pulcherrima* was added II (1.2 g) dissolved in 120 ml of ethanol and incubation was continued for forty-eight hours. After extraction with ethyl acetate, the reduction product was purified through column chromatography over Florisil. Fifty percent acetone in methylene chloride and acetone eluted a crude crystal (850 mg), which was recrystallized from methanol-*n*-hexane to give 17 α ,20 β ,21-trihydroxypregna-1,4-dien-3-one (V) containing one mole

of methanol of crystallization, m.p. 191~193°C. *Anal.* Found: C, 70.12; H, 9.24. Calcd. for C $_{21}$ H $_{30}$ O $_4$ ·CH $_3$ OH: C, 69.81; H, 9.05%.

When recrystallized from ethyl acetate, this material gave rise to V of m.p. 193~195°C, $[\alpha]_D^{21} + 17^\circ$ ($c = 1.0$, chloroform), $\lambda_{\max}^{\text{EtOH}}$ 246 m μ (ϵ , 15,200), $\nu_{\max}^{\text{KBr}}$ 3390 (OH), 1656, 1613, 1600 cm $^{-1}$ (Δ^1 , Δ^4 -3-CO). *Anal.* Found: C, 72.92; H, 8.92. Calcd. for C $_{21}$ H $_{30}$ O $_4$: C, 72.80; H, 8.73%.

(b) **Reduction of II by *Sp. gougeroti*.** From 4 l of the culture of *Sp. gougeroti* the mycelium was harvested through filtration, washed twice with 4 l of water and suspended in 4 l of a citrate-phosphate buffer solution (pH 6.0). To the cell suspension was added II (1 g) as fine powders and incubation was continued on a reciprocating shaker for twenty-four hours. An ethyl acetate extract from the reaction mixture was chromatographed over Florisil using methylene chloride and methylene chloride-acetone mixture. Elution with methylene chloride-acetone gave 290 mg of 17 α ,20 β ,21-trihydroxypregna-1,4-dien-3-one (V), m.p. 192~193°C. This material showed no depression in melting point on admixture with the material produced through the action of *C. pulcherrima* on II and the infrared spectra of the two were identical. In addition to V a considerable amount of the starting material was recovered in this experiment.

17 α ,20 α ,21-Trihydroxypregna-1,4-dien-3-one (VI).

To 4 l of the culture of *Rh. glutinis* was added II (1 g) dissolved in 30 ml of ethanol and incubation was continued for seven days. The ethyl acetate extract from the reaction mixture was concentrated and cooled to give a crude crystal, which was recrystallized from acetone to give rise to 550 mg of 17 α ,20 β ,21-trihydroxypregna-1,4-dien-3-one (VI), m.p. 212~213°C, $[\alpha]_D^{21} + 13^\circ$ ($c = 0.8$, chloroform), $\lambda_{\max}^{\text{EtOH}}$ 245 m μ (ϵ , 15,400), $\nu_{\max}^{\text{KBr}}$ 3390 (OH), 1670, 1630, 1608 cm $^{-1}$ (Δ^1 , Δ^4 -3-CO). *Anal.* Found: C, 72.89; H, 8.73. Calcd. for C $_{21}$ H $_{30}$ O $_4$: C, 72.80; H, 8.73%.

Androst-4-ene-3,17-dione (VII).

(a) Two hundred milligrams of I dissolved in 10 ml of glacial acetic acid and 10 ml of water was allowed to react with 2 g of sodium bismuthate¹⁹⁾ with stirring for two hours. The reaction mixture was neutralized with potassium hydroxide and extracted with methylene chloride. The methylene chloride

19) C.J.W. Brooks and J.K. Norymberski, *Biochem. J.*, **55**, 371 (1953).

sidue after recrystallization from acetone afforded androst-4-ene-3,17-dione (VII), m.p. 178~180°C, $[\alpha]_D^{21} + 193^\circ$ ($c=10$, chloroform), $\lambda_{\max}^{\text{EtOH}}$ 239 μ (ϵ , 16,200), $\nu_{\max}^{\text{KBr}}$ 1739 (17-CO), 1667, 1618 cm^{-1} (Δ^4 -3-CO). *Anal.* Found: C, 79.45; H, 8.92. Calcd. for $\text{C}_{19}\text{H}_{26}\text{O}_2$: C, 79.68; H, 9.15%.

(b) To 105 mg of III dissolved in 4.5 ml of dioxane was added a solution of 150 mg of periodic acid²⁰⁾ in 1.5 ml of water. After being kept at room temperature for eighteen hours, the reaction mixture was extracted with methylene chloride. The residue obtained after evaporation of the methylene chloride extract was purified through a column chromatography on Florisil and crystallized from ether-*n*-hexane to yield a material of m.p. 166~168°C. A mixture of this material with VII showed no depression in melting point and its infrared spectrum was identical with that of VII.

(c) Fifty-three milligrams of IV was oxidized with 1 g of sodium bismuthate in the 50% aqueous acetic acid solution. The oxidation product was crystallized from methylene chloride-ether to afford a material melting at 160~166°C. The infrared spectrum of this material was also identical with that of VII.

Androsta-1,4-diene-3,17-dione (VIII).

(a) Five hundred milligrams of II was oxidized with 5 g of sodium bismuthate in the 50% aqueous acetic acid solution. Purification of the oxidation product through a column chromatography on Florisil and subsequent crystallization from methylene chloride-ether gave rise to androsta-1,4-diene-3,17-dione (VIII), m.p. 139~141°C, $[\alpha]_D^{21} + 114^\circ$ ($c=1.0$, chloroform), $\lambda_{\max}^{\text{EtOH}}$ 243 μ (ϵ , 14,800), $\nu_{\max}^{\text{KBr}}$ 1745 (17-CO), 1667, 1631, 1610 cm^{-1} ($\Delta^1,4$ -3-CO). *Anal.* Found: C, 80.51; H, 8.45. Calcd. for $\text{C}_{19}\text{H}_{24}\text{O}_2$: C, 80.24; H, 8.51%.

(b) Fifty milligrams of V in dioxane was oxidized with 80 mg of periodic acid. The reaction product was crystallized from acetone-*n*-hexane to give a material of m.p. 110~115°C, which was identical with the material produced by oxidation of VI with periodic acid. This material had the infrared spectrum identical with that of VIII.

20 β ,21-Diacetoxypregn-4-en-17 α -ol-3-one (IX).

One hundred and thirty-five milligrams of III was acetylated with 4 ml of acetic anhydride and 2 ml of pyridine. Crystallization of the product from acetone

gave 20 β ,21-diacetoxypregn-4-en-17 α -ol-3-one (IX) of m.p. 178~180°C, $[\alpha]_D^{21} + 136^\circ$ ($c=1.0$, chloroform), $\lambda_{\max}^{\text{EtOH}}$ 241 μ (ϵ , 15,600), $\nu_{\max}^{\text{KBr}}$ 3484 (OH), 1751 (acetyl-CO), 1667, 1629 (Δ^4 -3-CO), 1242, 1224 cm^{-1} (C-O-C). *Anal.* Found: C, 69.45; H, 8.57. Calcd. for $\text{C}_{26}\text{H}_{36}\text{O}_6$: C, 69.42; H, 8.39%.

20 α ,21-Diacetoxypregn-4-en-17 α -ol-3-one (X).

Four hundred milligrams of IV was dissolved in 4 ml of pyridine and 2 ml of acetic anhydride. The solution was allowed to stand at room temperature overnight. Dilution of the mixture with water and subsequent cooling gave 20 α ,21-diacetoxypregn-4-en-17 α -ol-3-one (X) of m.p. 245~247°C, $[\alpha]_D^{22} + 23^\circ$ ($c=1.0$, chloroform), $\nu_{\max}^{\text{KBr}}$ 3401 (OH), 1718 (acetyl-CO), 1675, 1618 (Δ^4 -3-CO), 1253, 1227 cm^{-1} (C-O-C). *Anal.* Found: C, 69.27; H, 8.21. Calcd. for $\text{C}_{26}\text{H}_{36}\text{O}_6$: C, 69.42; H, 8.39%.

20 β ,21-Diacetoxypregna-1,4-dien-17 α -ol-3-one (XI).

One hundred milligrams of V was acetylated with 4 ml of pyridine and 4 ml of acetic anhydride. Crystallization of the product from methylene chloride-ether gave 20 β ,21-diacetoxypregna-1,4-dien-17 α -ol-3-one (XI) of m.p. 178~180°C, $[\alpha]_D^{21} + 99^\circ$ ($c=1.0$, chloroform), $\nu_{\max}^{\text{KBr}}$ 3521 (OH), 1739 (acetyl-CO), 1667, 1631, 1608 ($\Delta^1,4$ -3-CO), 1241, 1225, 1217 cm^{-1} (C-O-C). *Anal.* Found: C, 69.61; H, 7.75. Calcd. for $\text{C}_{25}\text{H}_{34}\text{O}_6$: C, 69.74; H, 7.96%.

20 α ,21-Diacetoxypregna-1,4-dien-17 α -ol-3-one (XII).

Sixty milligrams of the VI was dissolved in 1 ml of pyridine and 2 ml of acetic anhydride. The solution was kept at room temperature for 24 hours and crystallization of the product from acetone-*n*-hexane gave rise to 20 α ,21-diacetoxypregna-1,4-dien-17 α -ol-3-one (XII) of m.p. 200~202°C, $[\alpha]_D^{21} - 22^\circ$ ($c=0.5$, chloroform), $\lambda_{\max}^{\text{EtOH}}$ 245 μ (ϵ , 15,500), $\nu_{\max}^{\text{KBr}}$ 3472 (OH), 1739, 1727 (acetyl-CO), 1678, 1637, 1613 ($\Delta^1,4$ -3-CO), 1259, 1232 cm^{-1} (C-O-C). *Anal.* Found: C, 69.72; H, 7.74. Calcd. for $\text{C}_{25}\text{H}_{34}\text{O}_6$: C, 69.74; H, 7.96%.

11 β ,17 α ,20 α -Trihydroxypregn-4-en-3-one (XV).

To 4.5 l of the culture of *Rh. glutinis* was added 1 g of fine powders of 11 β ,17 α -dihydroxypregn-4-ene-3,20-dione (XIII) and incubation was continued for seven days. The reaction product was extracted with ethyl acetate. After evaporation, the ethyl acetate extract gave an oily residue, which was heated with 30 ml of

20) A. Larden, *Helv. Chim. Acta*, **34**, 756 (1951).

n-hexane, and the solvent was removed by decantation. Then the residue was chromatographed over Florisil using a mixture of ethyl acetate and methylene chloride. A material (A) which showed R_F 0.7 in paper chromatography (F-C system) was eluted with 30% ethyl acetate in methylene chloride and then a material (B) of m.p. 176~178°C which showed R_F 0.5 in the PPC was eluted with 50% ethyl acetate in methylene chloride. Recrystallization of the material A from acetone gave rise to 11 β ,17 α ,20 α -trihydroxypregn-4-en-3-one (XV), m.p. 209~210°C, $[\alpha]_D^{21} + 94^\circ$ ($c=0.5$, chloroform). Anal. Found: C, 72.38; H, 9.43. Calcd. for $C_{27}H_{44}O_3$: C, 72.38; H, 9.26%. XV was acetylated with acetic anhydride and pyridine to give 20 α -acetoxy-11 β ,17 α -diol-3-one (XVII) of m.p. 223~228°C. The physical properties of XV and XVII were identical with those of the corresponding authentic samples²¹⁾. Oxidation of 50 mg of XV with periodic acid in methanol gave 11 β -hydroxyandrost-4-ene-3,17-dione (XVI), m.p. 191~192°C, which was identical with that produced through oxidation of hydrocortisone with sodium bismuthate. The structure of the material B remains to be elucidated.

21) G.I. Poos, *J. Am. Chem. Soc.*, **77**, 4932 (1955).

Fermentation of 7 α ,19,21-Trihydroxypregn-4-ene-3,20-dione (XIV) with *Rh. glutinis*.

To 50 ml of the culture of *Rh. glutinis* was added 10 mg of 17 α ,19,21-trihydroxypregn-4-ene-3,20-dione and incubation was continued for five days. Extraction and subsequent paper chromatography (E-E system) of the product gave a paper chromatogram as shown in Fig. 1 (d). The mobility of the spot of the product was 1/6 of that of the substrate and was identical with that of 17 α ,19,20 α ,21-tetrahydroxypregn-4-en-3-one (XVIII) produced by one of the authors through microbiological transformation of 17 α ,20 α ,21-trihydroxypregn-4-en-3-one by *Pellicularia filamentosa* f. s. *microsclerotia*¹⁴⁾. Since the spot of the product showed negative color reaction with TPTZ, it was estimated to be 17 α ,19,20 α ,21-tetrahydroxypregn-4-en-3-one (XVIII).

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