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Microbiological Transformations of Steroids. V. The Oxygenation of 17α-Hydroxyprogesterone to 6β,17α-Dihydroxyprogesterone and 11α,17α-Dihydroxyprogesterone[1]

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Incubation of 17α-hydroxyprogesterone with *Rhizopus arrhizus* Fischer (A.T.C.C. 11145) produced in good yield 6β,17α-dihydroxyprogesterone and in small amounts 11α,17α-dihydroxyprogesterone. Incubation with *Rhizopus nigricans* Ehrb. (A.T.C.C. 6227b) gave excellent yields of 11α,17α-dihydroxyprogesterone and very little of 6β,17α-dihydroxyprogesterone.

Discussion

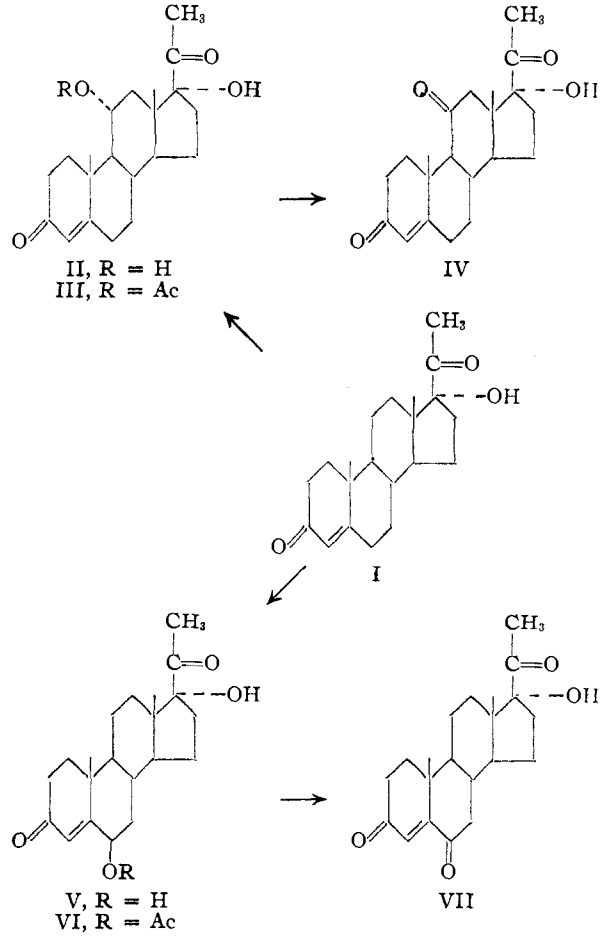
In paper III of this series[2] we reported that *Rhizopus arrhizus* oxygenates desoxycorticosterone in good yield to 6β-hydroxy-11-desoxycorticosterone and produces only very small amounts of 11-epicorticosterone.

Incubation of desoxycorticosterone with *Rhizopus nigricans* gave 11-epicorticosterone in good yield.

When the bioconversion of Reichstein's Compound S was investigated[1a] essentially the same results were obtained, inasmuch as 6β,17α,21-trihydroxy-4-pregnene-3,20-dione and 11α,17α,21-trihydroxy-4-pregnene-3,20-dione (compound 11-epi F) were produced by *Rhizopus arrhizus* as well as by *Rhizopus nigricans*. In conformity with the previous experience *Rhizopus nigricans* performed 11-oxygenation in higher yields than *Rhizopus arrhizus* did. These results pointed to a significant difference between the oxygenation of progesterone in the one case and of desoxycorticosterone and Reichstein's Compound S in the other. The introduction of one or more hydroxy groups into the side chain of the basic substrate molecule, *i.e.*, progesterone, evidently permitted only monohydroxylation at positions 6 or 11, and suppressed almost completely[3] the process of introducing two hydroxyl groups into the substrate. These facts clearly suggested the microbiological conversion of 17α-hydroxyprogesterone.

In our earlier work with *Rhizopus nigricans*, 17α-hydroxyprogesterone (I) was incubated for a period of 5–6 days under conditions which were otherwise identical to those reported for the bioconversion of progresterone.[4] Paper chromatography revealed the presence of two major transformation products. After chromatography over a Florisil[2] (a magnesium silicate) column 11α,17α-dihydroxyprogesterone (II) and 6β,17α-dihydroxyprogesterone (V) were isolated in yields of 25–30% and 1–2%, respectively. Later, it was found that a reduction of the incubation period to 24–30 hours increased the yield of compound II to 70–75%. Thus, avoiding chromatography it was possible to isolate compound II directly by following the procedure which was devised for the isolation of 11α-hydroxyprogesterone.[4] This higher yield of II was achieved without any increased formation of V as was shown by papergram analysis of mother liquors of compound II.

When I was incubated with *Rhizopus arrhizus* for 24–48 hours, the extract contained mainly compound II and compound V as evidenced by papergram analyses. Again, chromatography over Florisil afforded a good resolution of V and II. The two components were isolated in crystalline form in 45 and 2% yields, respectively of V and II.



In order to prove the structure of the new biocon-

(1) (a) Paper IV of this series: D. H. Peterson, S. H. Eppstein, P. D. Meister, B. J. Magerlein, H. C. Murray, H. Marian Leigh, A. Weintraub, L. M. Reineke, THIS JOURNAL, **75**, 412 (1953). (b) The transformations recorded in detail in this communication are contained in part in our U. S. Patent 2,602,769 (issued July 8, 1952, based on an original application filed August 19, 1950).

(2) S. H. Eppstein, P. D. Meister, D. H. Peterson, H. C. Murray, H. Marian Leigh, D. A. Lyttle, L. M. Reineke and A. Weintraub, THIS JOURNAL, **75**, 408 (1953).

(3) Papergram studies show that small amounts of highly polar material are formed from desoxycorticosterone and from compound S.

(4) Paper I of this series: D. H. Peterson, H. C. Murray, S. H. Eppstein, L. M. Reineke, A. Weintraub, P. D. Meister and H. Marian Leigh, THIS JOURNAL, **74**, 5933 (1952).

version products,[5] compound II was oxidized with chromic acid to the known 17α-hydroxy-4-pregnene-3,11,20-trione (IV)[6] which was in all details identical to an authentic sample. The monoacetate, III, was easily formed and this was considered sufficient proof for the α-orientation of the 11-hydroxy group.

The position of the 6-hydroxy group in compound V was established by oxidation to 17α-hydroxy-4-pregnen-3,6,20-trione. The presence of the resulting ene–dione chromophore was evident from the spectroscopic data. The ultraviolet absorption maximum at 248 mμ showed the low intensity (*E* 9,000) reported for similar groups.[7] Furthermore, the infrared spectrum showed that the introduction of a keto group into position 6 shifts the $\Delta^{4,5}$-double bond absorption band from 1618 cm.$^{-1}$ for 17α-hydroxyprogesterone to 1602 cm.$^{-1}$ for 17α-hydroxy-4-pregnene-3,6,20-trione.[8]

There is no direct proof for the stereochemical arrangement of the 6-hydroxy group. Indirect evidence for its β-orientation was adduced from the transformation of desoxycorticosterone to 6β-hydroxy-11-desoxycorticosterone.[2] The absolute stereochemical specificity displayed by *Rhizopus* with respect to the oxygenation at position 11 permits this assignment by analogy. In conclusion of our experiments with desoxycorticosterone, Reichstein's Compound S and 17α-hydroxyprogesterone it can be stated that the bioconversion of these compounds or their acetates with *Rhizopus nigricans* leads in good yield to the oxygenation at position 11 of the substrate and in small amounts to oxygenation at position 6. The biooxygenation with *Rhizopus arrhizus* produces predominantly the 6-hydroxylated isomers and minor quantities of the 11-oxygenated material. Polyhydroxylated conversion products are present in either conversion to a minor extent.

Experimental

A. Fermentation of 17α-Hydroxyprogesterone (I) by *Rhizopus nigricans*. I. Isolation of 11α,17α-Dihydroxyprogesterone (II) and 6β,17α-Dihydroxyprogesterone (V) by Chromatography over Florisil.—*Rhizopus nigricans* was grown in shake bottles on four liters of medium H[9] for 30 hours. Two grams of substrate (I), dissolved in 100 ml. of methanol, was added. After a bioconversion period of 5 days the extraction with methylene dichloride gave 8.64 g. of an oily extract which was shown by paper chromatography to contain starting material and two new components. The extract was dissolved in 200 ml. of ethylene dichloride and fractionated over 350 g. of Florisil with 500-ml. portions of the following solvent mixtures: ethylene dichloride (2 fractions); ethylene dichloride–acetone mixtures 25:1, 15:1 (2 fractions); 12:1 and 10:1 (3 fractions); 8:1 and 5:1 (4 fractions); 2:1 (2 fractions); acetone (1 fraction). On the basis of papergram analysis fractions 5–9 (A, 1.2 g.) were combined and fractions 12–16 (B, 1.449 g.) were combined. When fraction A was recrystallized twice from 10 ml. of chloroform–ether 1:1, 589 mg. of crystals was formed, m.p. 220–222°,[10] which were identified as starting material by mixture melting point and infrared spectrum. Sublimation of the mother liquors at 0.05 mm. and 160° increased the recovery to 649 mg.

Fraction B was dissolved in 10 ml. of methanol. The solution was concentrated and gradually diluted with ether. Three recrystallizations in this manner gave 260 mg. of crystals, m.p. 220–222°; mixture melting point with (I) 210–215°. The infrared spectrum confirmed the introduction of a hydroxyl group into the substrate molecule: $\lambda_{max}^{alc.}$ 243 mμ (*E* 16,200), $[\alpha]^{23}D$ +76° (*c* 1.132 in chloroform).

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.85; H, 8.47.

The acetate was prepared by dissolving 41 mg. of compound II in 2 ml. of acetic anhydride–pyridine 1:1. Isolation of the reaction product in the known manner and recrystallization from 2 ml. of acetone–petroleum ether 1:1 gave 25 mg. of 11α-acetoxy-17α-hydroxy-4-pregnene-3,20-dione (III), m.p. 213–215°; mixture melting point with compound II, 195–205°. The infrared spectrum corroborated its structure; $\lambda_{max}^{alc.}$ 240 mμ (*E* 15,700), $[\alpha]^{23}D$ +68° (*c* 1.099 in chloroform).

Anal. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.29; H, 8.37.

Oxidation of II to 17α-Hydroxy-4-pregnene-3,11,20-trione (IV).—Seventy three mg. of 11α,17α-dihydroxyprogesterone was dissolved in glacial acetic acid (3 ml.) and oxidized by dropwise addition of a solution of chromium trioxide (17.9 mg. in 2 ml. of glacial acetic acid–water 9:1). After 4 hours at room temperature the excess oxidant was destroyed with methanol. The solution was then made alkaline with 5% sodium bicarbonate solution and extracted with ether three times. The extracts were washed three times with water, dried over anhydrous sodium sulfate and concentrated at room temperature in an air stream. The resulting crystalline product (52 mg., 72% yield) was recrystallized twice from 1 ml. of acetone–ether, to give 51 mg. of crystals, m.p. 238.5–239.5°. The infrared spectrum, when taken in chloroform solution, showed this compound to be identical to an authentic sample of 17α-hydroxy-4-pregnene-3,11,20-trione (IV), $\lambda_{max}^{alc.}$ 239 mμ (*E* 15,500), $[\alpha]^{23}D$ +181° (*c* 0.6923 in chloroform).

Fractions 10 and 11 from the chromatogram described above contained by paper chromatography a compound with a mobility different from the one of compound II. These two fractions (200 mg.) were combined with the mother liquors of compound II. The oil (2.333 g.) was rechromatographed over 150 g. of Florisil. Two hundred and twenty-ml. portions of the following solvent mixtures were collected: ethylene–dichloride (1 fraction); ethylene dichloride–acetone 15:1 and 12:1 (2 fractions each time); ethylene dichloride–acetone 10:1 (3 fractions); ethylene dichloride–acetone 2:1 (2 fractions); and acetone (1 fraction). Fractions 7–9 (180 mg.) were crystalline. After two recrystallizations from 3 ml. of ether–hexane 3:1, 25 mg. of compound V, m.p. 250–252°, was isolated. The infrared spectrum was in all details identical to the spectrum of V as obtained below; $[\alpha]^{23}D$ +4° (*c* 0.775 in chloroform).

When fractions 11–14 of this chromatogram (445 mg.) were recrystallized from 5 ml. of acetone–ether 2:1 an additional 102 mg. of compound II was recovered. The yield of II was, therefore, 27% (taking into consideration the recovery of starting material).

II. Isolation of 11α,17α-Dihydroxyprogesterone from a Fermentation with *Rhizopus nigricans* by Direct Crystallization.—To 12 l. of a 19-hour growth of *Rhizopus nigricans* in medium H was added 3 g. of compound I dissolved in 300 ml. of ethanol. After an incubation period of 24 hours, our standard procedure of extraction with methylene dichloride yielded a semicrystalline residue weighing 5.30 g. This material was washed four times, each time with

(5) After the issuance of U. S. Patent 2,602,769, J. Fried, *et al.*, THIS JOURNAL, **74**, 3962 (1952), described the conversion of 17α-hydroxyprogesterone to 11α,17α-dihydroxyprogesterone by *Aspergillus niger*.

(6) L. H. Sarett, *ibid.*, **70**, 1454 (1948); T. H. Kritchevsky, D. L. Garmaise and T. F. Gallagher, *ibid.*, **74**, 483 (1952); J. Fried, *et al.*, *ibid.*, **74**, 3962 (1952). We are indebted to Dr. T. F. Gallagher, of the Sloan–Kettering Institute for Cancer Research, New York, for an authentic sample of this compound.

(7) L. F. Fieser and Mary Fieser, "Natural Products Related to Phenanthrene," Reinhold Publishing Corp., New York, N. Y., 1949, p. 193.

(8) This characteristic shift of the double bond absorption band was first observed in the case of 4-androstene-3,6,17-trione.[12] A forthcoming communication by Dr. J. L. Johnson, of our laboratories, will report similar observations in detail.

(9) For exact details of the fermentation and extraction procedures see Paper I (reference 4).

(10) All melting points are uncorrected.

5 ml. of ether. After trituration the solvent was decanted in each case. The resulting crystalline residue weighed 2.359 g., m.p. 214–217° (75% yield). The infrared spectrum and papergram analysis showed the product to be of high purity.

B. Bioöxygenation of Compound I by *Rhizopus arrhizus*.—*Rhizopus arrhizus* was grown on 12 l. of medium H for 24 hours in the stir bottle assembly. After addition of 3 g. of substrate (I) in acetone solution (150 ml.) the bioconversion was continued for an additional 28 hours. The crude extract weighed 9.547 g. and was shown by paper chromatography to contain small amounts of highly polar material and two new components, one of which could not be distinguished from compound II.

The crude extract was chromatographed in ethylene dichloride solution over 350 g. of Florisil. Seven hundred-ml. fractions of the following solvent mixtures were eluted: ethylene dichloride, ethylene dichloride–acetone 25:1 and 15:1 (2 fractions each time); ethylene dichloride–acetone 12:1 (3 fractions); ethylene dichloride–acetone 10:1 (4 fractions); ethylene dichloride–acetone 8:1 and 5:1 (3 fractions each time); ethylene dichloride–acetone 3:1, 1:1 and acetone (1 fraction in each instance). Paper chromatography indicated that 6β,17α-dihydroxyprogesterone (V) appeared in fractions 6–11 and 11α,17α-dihydroxyprogesterone (II) in fractions 12–15. Traces of various highly polar components were present in fractions 16–20. Fractions 6–11 were combined (2.805 g.) and dissolved in 10 ml. of methanol–chloroform 5:1. The solution was concentrated and gradually diluted with ether until the beginning of crystallization. Several steps of crystallization (including trituration of semi-crystalline mother liquors) from various solvent mixtures such as methanol ether 1:1 and chloroform–ether 1:1 gave 1.437 g. of crystals (45.8% yield), m.p. 243–245°. A small sample was recrystallized once more to give crystals, m.p. 244–246°. Infrared spectrum showed the compound to be identical to 6β,17α-dihydroxyprogesterone which was described in section A, above; $\lambda_{max}^{alc.}$ 238 mμ (*E* 12,600), $[\alpha]^{23}D$ +6° (*c* 0.931 in chloroform).

Anal. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.87; H, 8.72.

6β-Acetoxy-17α-hydroxy-4-pregnene-3,20-dione (VI).—Two hundred mg. of 6β,17α-dihydroxyprogesterone was acetylated with 4 ml. of acetic anhydride–pyridine 1:1 at room temperature. After 18 hours the solution was diluted with ice-water to give a colorless precipitate which was filtered off to give 222 mg. of crystals. After two recrystallizations, each time from 2 ml. of acetone, 165 mg. of compound VI was obtained, m.p. 95–100° and 192–197°. The infrared spectrum was in complete agreement with the proposed structure; $\lambda_{max}^{alc.}$ 236 mμ (*E* 12,500); $[\alpha]^{23}D$ +14° (*c* 0.604 in chloroform).

Anal. Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.36, 71.23; H, 8.41, 8.06.

Oxidation of V to 17α-Hydroxy-4-pregnene-3,6,20-trione (VII).—Two hundred mg. of compound V was dissolved in 5 ml. of glacial acetic acid. To the stirred solution 45 mg. of chromium trioxide in 3 ml. of 90% acetic acid was added dropwise. After two hours at room temperature the solution was cooled to 5° overnight. After destroying the excess oxidant with methanol (10 ml.) the reaction mixture was concentrated *in vacuo* at 35° bath temperature. The residue was taken up in 30 ml. of water and extracted with ether–chloroform 5:1. The extract was washed twice with 5% sodium bicarbonate solution, three times with water and dried over anhydrous sodium sulfate. The residue (191 mg.) was recrystallized once from acetone to give 141 mg. of compound VII, m.p. 234–243°. A sample was recrystallized twice from methanol to give crystals, m.p. 242–245°. The infrared spectrum of the oxidation product was consistent with the structure of 17α-hydroxy-4-pregnene-3,6,20-trione. The double bond absorption band which is at 1614–1618 cm.$^{-1}$ for α,β-unsaturated ketones, was shifted to 1602 cm.$^{-1}$. This characteristic shift is attributed to the presence of a keto group in position 6; $\lambda_{max}^{alc.}$ 248 mμ (*E* 9,000), λ_{max} 312 mμ (*E* 1,000).[11] $[\alpha]^{23}D$ −62° (*c* 0.550 in chloroform).

Anal. Calcd. for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found: C, 73.29, 73.36; H, 8.10, 8.42.

Fractions 12–15 from the Florisil chromatogram described above were dissolved in 10 ml. of acetone. Then the solvent was allowed to evaporate at room temperature. This procedure was repeated until all fractions contained some crystalline product. The fractions were then triturated each with 10 ml. of ether and the crystalline residues were combined and recrystallized once from 5 ml. of methanol–ether 3:1. Forty-five mg. of compound II (1.5% yield) was isolated in this way, m.p. 219–220°. Mixture melting point with authentic II, 219–221°. The infrared spectrum corroborated its structure.

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(11) L. F. Fieser and Mary Fieser, ref. 7, give for similar groups in ring A and B; $\lambda_{max}^{CHCl_3}$ 252 mμ (*E* 10,000).