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12. Makoto Shirasaka : Microbiological Transformation of Steroids. II.[1] Hydroxylation of Steroids by *Absidia regnieri*.

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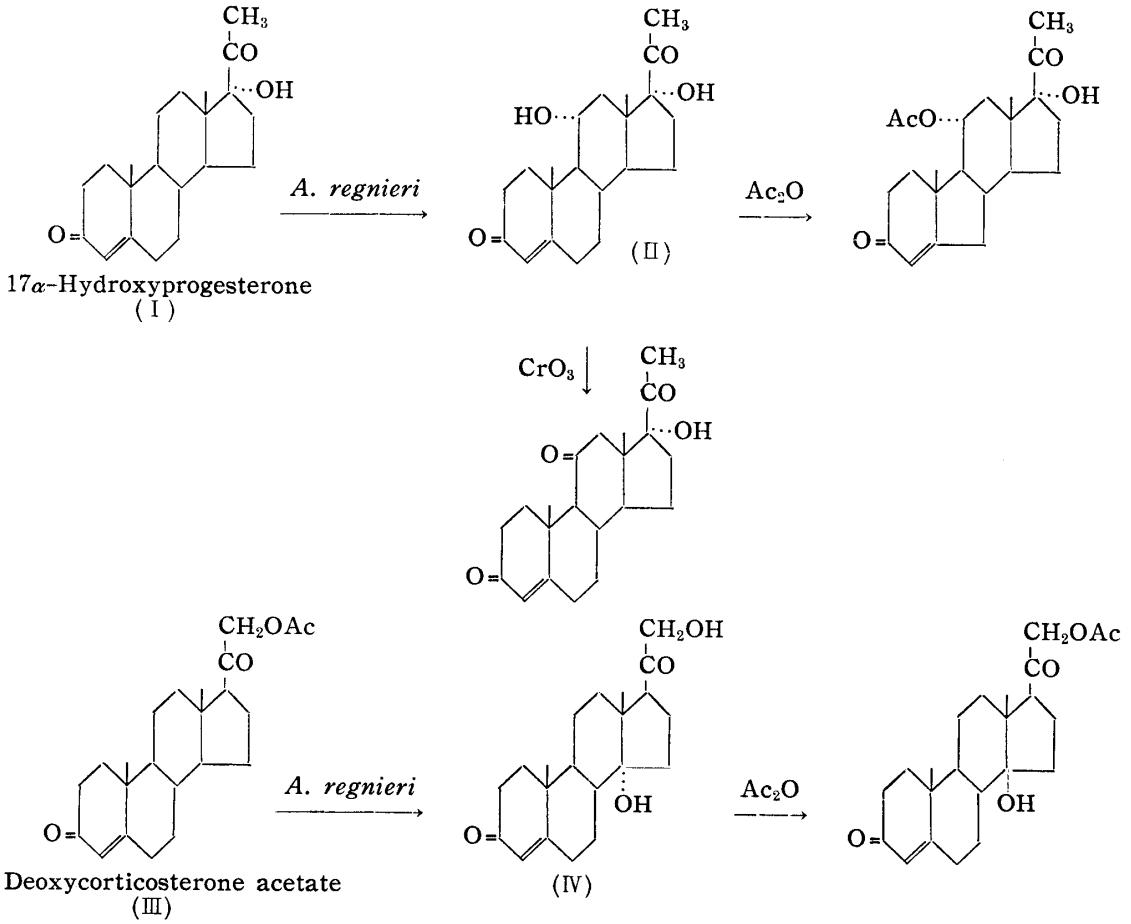
14α-Hydroxylation of steroids by microörganisms is a comparatively common reaction and Mucor spp.[2] are often used. In the course of examination of various fungi, *Absidia regnieri* was found to effect 14α-hydroxylation of progesterone.[3] Application of this fungus strain to 17α-hydroxyprogesterone, Reichstein's compound S, deoxycorticosterone, and corticosterone showed that 14α-hydroxylated compound was formed in only trace amounts in the two former compounds and in its stead, 11α-hydroxylated compound was the major product, while the 14α-hydroxyl compound was obtained from the latter two steroids, as in the case of progesterone.

The foregoing facts showed that this fungus had a marked substrate specificity in the oxidation of steroids.

In the present series of work *Absidia regnieri* was cultured, as will be described later, with 17α-hydroxyprogesterone (I) as the substrate, the culture liquid was extracted, and the extract was concentrated. The residue was examined by paper chromatography and



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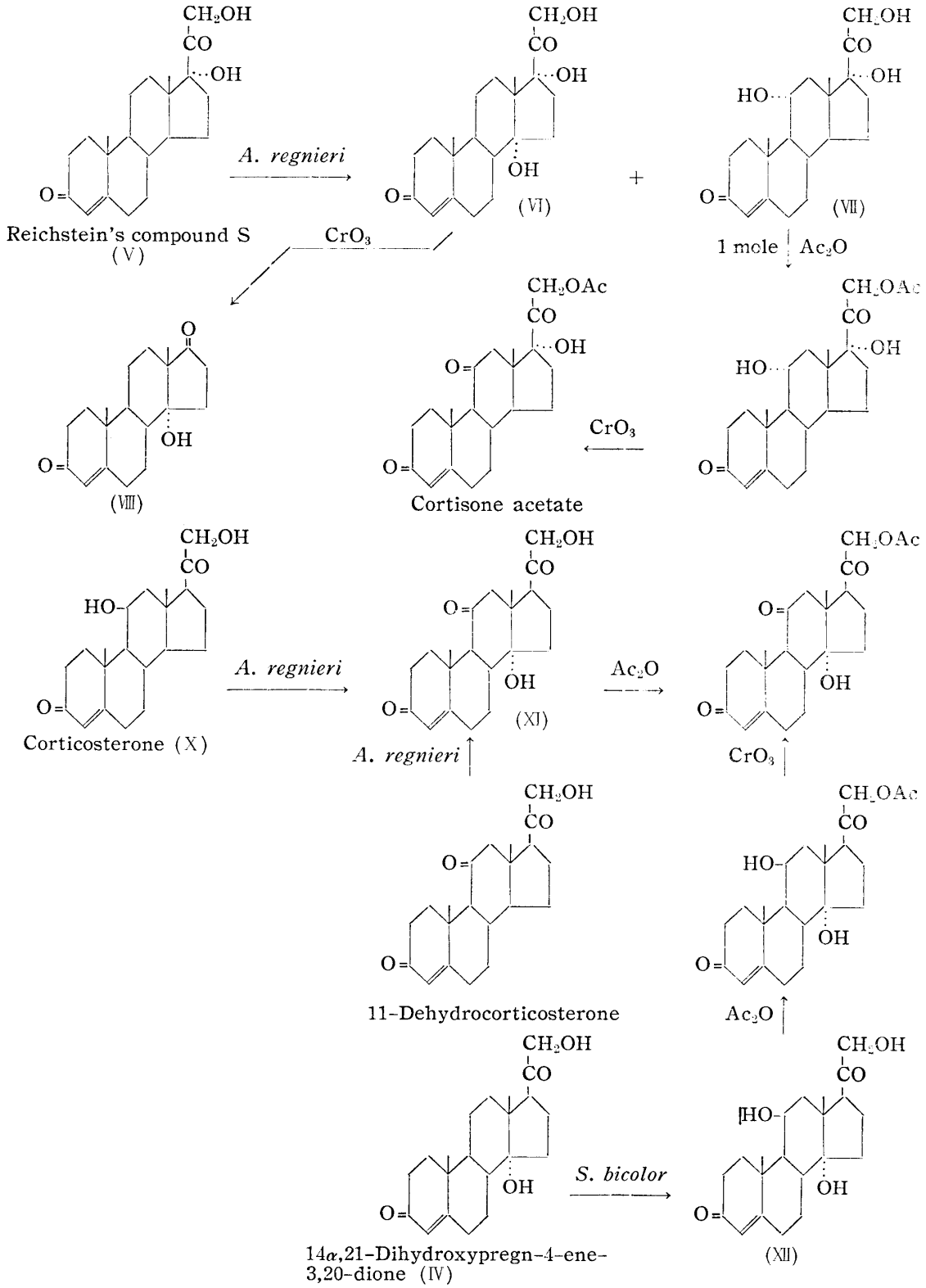

one spot with larger polarity than (I) was detected, besides a few faint spots. For separation of these spots, the residue was submitted to column chromatography through Florisil and some unreacted (I) was recovered from the initial eluate. The solvent was evaporated from the second effluent and the residue was crystallized from acetone, from which a large amount of crystals (II) of m.p. 216~221° was obtained. Analytical data of (II) showed it to be dihydroxyprogesterone, a product formed by introduction of one hydroxyl into

(I). Acetylation of (II) with acetic anhydride and pyridine gave a monoacetate, indicating that the newly introduced hydroxyl is primary or secondary.

Oxidation of (II) with chromic acid by the usual method gave a triketo-steroid whose constants agreed with those of 17α-hydroxypregn-4-ene-3,11,20-trione.[4,5] The constants of (II) and its acetate agreed with those of 11α,17α-dihydroxyprogesterone and its acetate and the structure of (II) was established as 11α,17α-dihydroxypregn-4-ene-3,20-dione.

A few more crystalline products were obtained from the final eluate but the amount was too small for further identification.

The same culture of this strain with deoxycorticosterone (III) as the substrate was carried out but the growth of the organism was markedly inhibited and oxidation was very weak. It was considered that the free deoxycorticosterone had a kind of toxicity against this fungus, as was stated in the preceding paper.[1] Consequently, in this case also, use of its acetate rather than the free deoxycorticosterone was found to effect oxidation. Examination of the product by paper chromatography indicated the presence of one spot with larger polarity than (III). Further purification of the residue by column chromatography through Florisil gave a small amount of free (III) and a large amount of a new product (IV). Analytical results indicated (IV) to be dihydroxyprogesterone, a product obtained by introduction of one hydroxyl into (III). Acetylation of (IV) with acetic anhydride and pyridine gave a monoacetate and oxidation of (IV) with chromium trioxide ended in recovery of the starting compound, from which the newly introduced hydroxyl must be tertiary. Absence of Porter-Silber reaction[6] in (IV) indicated that this hydroxyl is not in 17-position and, therefore, this tertiary hydroxyl must be in either 14α, 9α, or 8β-position. Eppstein and others[2] had previously obtained 14α-hydroxyl and 8β(or 9α)-hydroxyl compounds from deoxycorticosterone by the action of *Helicostylum* sp. The constants of these compounds obtained by Eppstein and others are compared with those of (IV) in Table I and the constants of (IV) and its acetate are found to agree with those of 14α,21-dihydroxypregn-4-ene-3,20-dione and its 21-acetate.

Table I. Comparison of the Constants for 14α- and 8β(or 9α)-Hydroxy Derivatives of Deoxycorticosterone

Compound	m.p. (°C)	$[\alpha]_D$ (in $CHCl_3$)
14α,21-Dihydroxypregn-4-ene-3,20-dione	167~170.5	+190°
Its acetate	158~161	+192°
8β(or 9α),21-Dihydroxypregn-4-ene-3,20-dione	180~183	+167°
Its acetate	212~214	+177°
Compound (IV)	170~175	+171°
Its acetate	160~161	+200°

The same culture of this fungus with Reichstein's compound S (V) as the substrate and paper chromatographic examination of its product indicated the presence of one spot with greater polarity than (V) and another spot with slightly larger polarity than (V). For the separations of these products, the residue was submitted to column chromatography through Florisil. The solvent was evaporated from the initial eluate and recrystallization of the residue from acetone afforded a very minute amount of a product (VI).

From the next-eluted fraction, a large amount of main oxidation product was obtained as crystals (VII) of m.p. 206~209°. Both were found, from their analytical values, to have one hydroxyl newly introduced into (V).

The usual acetylation of (VI) with acetic anhydride and pyridine gave a monoacetate and its oxidation with chromium trioxide failed to afford any oxidation product, entirely

4) L. H. Sorett : J. Am. Chem. Soc., **70**, 1454 (1948); F. Fried, *et al.* : *Ibid.*, **74**, 3962 (1952).
5) P. D. Meister, *et al.* : *Ibid.*, **75**, 416 (1953).
6) C. C. Porter, R. H. Silber : J. Biol. Chem., **185**, 201 (1950); *ibid.*, **210**, 923 (1951).

recovering the starting material. The newly introduced hydroxyl in (VI), therefore, is tertiary. Treatment of (VI) with chromium trioxide in acetic acid gave a monohydroxyandrostenedione (VIII), whose constants agreed with those of 14α-hydroxyandrost-4-ene-3,17-dione,[7] and the product (VI) was identified as 14α,17α,21-trihydroxypregn-4-ene-3,20-dione.

Acetylation of (VII) with acetic anhydride and pyridine gave a diacetate, indicating the newly introduced hydroxyl to be primary or secondary. Acetylation of (VII) with equivalent of acetic anhydride in pyridine and oxidation of the acetate with chromium trioxide afforded the acetate (IX) of the triketo-steroid. The physical constants of this acetate and its infrared spectrum were in good agreement with those of cortisone acetate. However, these data were different from those of hydrocortisone so that (VII) must be its epimer and (VII) was identified as 11α,17α,21-trihydroxypregn-4-ene-3,20-dione. The infrared absorption of (VII) was identical with that of authentic sample of epihydrocortisone.

The same culture of this fungus with corticosterone (X) as the substrate and examination of its product by paper chromatography indicated the presence of one spot with greater polarity than (X). The product was recrystallized directly from acetone and crystals (XI) of m.p. 210~215° were obtained. Its analytical values indicated introduction of one hydroxyl in (X). Acetylation of (XI) with acetic anhydride and pyridine gave a monoacetate and its oxidation with chromium trioxide only resulted in the recovery of the starting compound, so that the newly introduced hydroxyl must be tertiary. The result of this treatment with chromium trioxide also indicated that the 11-position of (XI) had already been converted to a ketone group by oxidation. (XI) was also obtained by the culture of the present fungus with 11-dehydrocorticosterone as the substrate, which also indicated that the 11-position had been converted to a ketone group.

In order to find the position of the newly introduced hydroxyl group, 14α,21-dihydroxypregn-4-ene-3,20-dione (IV) was used as the substrate for the culture of *Stachylidium bicolor*[*2] which had previously been found to perform 11β-hydroxylation. The sole product in this reaction was an oxidized steroid (XII), whose acetylation, followed by oxidation with chromium trioxide in acetic acid, gave the acetate whose physical constants and infrared spectrum were in good agreement with those of the acetate of (XI), and the melting point was not depressed on mixed fusion. Consequently, the structure of (XI) was established as 14α,21-dihydroxypregn-4-ene-3,11,20-trione. This is a new steroidal compound.

The foregoing results indicated that this *Absidia regnieri* had the action of effecting dehydrogenation of the 11β-hydroxyl group as well as the usual hydroxylation reaction. Consequently, this fungal strain was cultured with epicorticosterone as the substrate, in order to examine whether this reaction was also effected in the isomeric 11α-hydroxylated compound, and the results showed that there was no evidence of dehydrogenation of the 11α-hydroxyl group.

Thus, the application of *Absidia regnieri* to various steroids has shown that this fungus possessed the action of chiefly effecting both 14α- and 11α-hydroxylation (Table II), and it

Table II. Hydroxylation of Various steroids by *Absidia regnieri*

Substrate steroid	Position hydroxylated
Progesterone	14α (6β, 7α, 15β, etc.)
17α-Hydroxyprogesterone	11α
Deoxycorticosterone	14α
Compound S	11α (14α)
Corticosterone	14α, 11-CO

Numbers in parentheses indicate slight hydroxylation.

[*2] Oxidation of steroids with *Stachylidium bicolor* will be reported in subsequent papers.

is clear that this reaction shows a marked difference according to the kind of substrate steroid used. The fungus effects practically 11α-hydroxylation alone in steroids possessing 17α-hydroxyl group, such as 17α-hydroxyprogesterone and Reichstein's compound S, while 14α-hydroxylation alone occurs in other steroids. This fact shows that 14α-hydroxylation of this fungus is in some way sterically hindered by the 17α-hydroxyl group of the substrate steroid, considering the steric structure of the steroid, and the reaction is markedly inhibited by this 17α-hydroxyl group. The fact also shows that this fungus has a very marked substrate specificity in oxidation of steroids.

This fungus was found to form free oxidized steroid from deoxycorticosterone 21-acetate and this might suggest that this fungus has a comparatively powerful esterase action. It was also found from present series of experiments that this fungus has a marked dehydrogenation action besides the foregoing hydroxylation action. Moreover, this action occurs easily in 11β-hydroxyl group but not in its epimer, 11α-hydoxyl. It may therefore be said that this dehydrogenation of 11-hydroxyl group has a kind of stereochemical specificity, reaction taking place only with one configuration (β) of the two steric configurations of the 11-hydroxyl group.

Experimental

Fermentation and Extraction—A medium containing 5% of glucose, 2% of peptone, and 0.3% of corn-steep liquor was placed in shake flasks, 100 cc. to each flask, and sterilized. Each flask was inoculated with *Absidia regnieri* and shake-cultured at 28° for ca. 40~50 hr. To each of the flasks, 2 cc. of 2.5% MeOH solution of substrate steroid (50 mg. as the steroid) was added and shake-culture was continued. After completion of the fermentation, the fermentation mixture was separated into fungal cells and filtrate, the cells were extracted with Me_2CO and AcOEt, and the extracts were combined with the filtrate. The filtrate was further extracted twice with AcOEt. The combined extract was washed with 2% $NaHCO_3$ and H_2O, dried over anhyd. Na_2SO_4, and concentrated.

Paper Chromatography—As described in the preceding paper,[1] paper chromatography was carried out by the modified Zaffaroni method, with propylene glycol-MeOH (1:2) as the stationary phase and toluene-dioxane mixture (72:22) as the developing solvent, by the descending method.

Hydroxylation of 17α-Hydroxyprogesterone (I)—*Absidia regnieri* was cultured as above with 1 g. of (I) as the substrate and 1 g. of concentrated residue was obtained. This residue was dissolved in 100 cc. of $C_2H_4Cl_2$ and submitted to column chromatography over 80 g. of Florisil. The column was eluted with various mixtures of $C_2H_4Cl_2$ and Me_2CO. The solvent was evaporated from the initial effluent and the residue was recrystallized from Me_2CO, affording 250 mg. of unreacted (I).

The fractions eluted with 3:1 and 2:1 mixtures of $C_2H_4Cl_2$ and Me_2CO were recrystallized from MeOH and afforded 430 mg. of 11α,17α-dihydroxyprogesterone (II). Further recrystallization from MeOH gave crystals of m.p. 216~221°; $[\alpha]_D$ +80° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.74. Found: C, 72.91; H, 8.66. UV λ_{max}^{MeOH}: 243 mμ (ε 15,800). IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 1690 (20-one), 1667, 1610 (Δ^4-3-CO).

11α-Acetate of (II): Acetylation of 50 mg. of (II) with 2 cc. each of Ac_2O and pyridine gave 40 mg. of the acetate, m.p. 212~214°; $[\alpha]_D$ +70° ($CHCl_3$). *Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.21; H, 8.10. UV: λ_{max}^{MeOH} 240 mμ (ε 16,000).

17α-Hydroxypregn-4-ene-3,11,20-trione—To a solution of 70 mg. of (II) dissolved in 3 cc. of AcOH, 20 cc. of 90% AcOH containing 18 mg. of CrO_3 was added and the mixture was allowed to stand for 5 hr. at room temperature. The mixture was extracted in a usual manner and 40 mg. of crude crystals was obtained. Its recrystallization from Me_2CO gave 17α-hydroxypregn-4-ene-3,11,20-trione, m.p. 236~240°; $[\alpha]_D$ +174° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found: C, 73.11; H, 8.34. UV: λ_{max}^{MeOH} 239 mμ (ε 15,000).

Hydroxylation of Deoxycorticosterone 21-Acetate—The same culture of the fungus was carried out with 1 g. of 21-acetate of (III) as the substrate and 1.6 g. of concentrated residue was obtained. This was submitted to the column chromatography as described above and the fraction eluted with 12:1 mixture of $C_2H_4Cl_2$ and Me_2CO afforded 45 mg. of (III). The next fraction, eluted with 8:1 mixture of the same solvents, afforded 380 mg. of crude crystals of (IV) which was recrystallized from Me_2CO to 14α,21-dihydroxypregn-4-ene-3,20-dione (IV) of m.p. 170~175°; $[\alpha]_D$ +171° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.72. Found: C, 72.70, H, 8.61. UV: λ_{max}^{MeOH} 240 mμ (ε 16,500). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1710 (20-one), 1679, 1660 (3-one), 1615 (Δ^4).

21-Acetate of (IV): (IV) was acetylated in a usual manner with Ac_2O and pyridine, and afforded its

21-monoacetate of m.p. 160~161°; $[\alpha]_D$ +200° ($CHCl_3$). *Anal.* Calcd. for $C_{22}H_{32}O_5$: C, 71.10, H, 8,30. Found: C, 71.00; H, 8.47.

Hydroxylation of Reichstein's Compound S (V)—The same culture of this fungus was carried out with 1 g. of (V) as the substrate and 2.1 g. of concentrated residue was obtained. This residue was submitted to column chromatography through Florisil as described above and the fraction eluted with 1:1 and 8:1 mixtures of $C_2H_4Cl_2$ and Me_2CO afforded a small amount of 14α,17α,21-trihydroxypregn-4-ene-3,20-dione (VI) as crude crystals from Me_2CO. Recrystallization from Me_2CO gave crystals of m.p. 213~218°; $[\alpha]_D$ +155° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found: C, 69.40; H, 8.10. UV: λ_{max}^{EtOH} 241 mμ (ε 11,000). IR ν_{max}^{KBr} cm^{-1}: 3500, 3300 (OH), 1710 (20-CO), 1650, 1615 (Δ^4-3-CO).

21-Acetate of (VI): Usual acetylation of (VI) with Ac_2O and pyridine gave the 21-monoacetate of m.p. 222~227°; $[\alpha]_D$ +177° ($CHCl_3$). *Anal.* Calcd. for $C_{23}H_{32}O_6$: C, 68.24; H, 7.97. Found: C, 68.40, H, 7.81.

14α-Hydroxyandrost-4-ene-3,17-dione (VIII)—To a solution of 70 mg. of (VI) dissolved in 5 cc. of AcOH, 5cc. of 85% AcOH containing 63 mg. of CrO_3 was added in small portions and the mixture was allowed to stand for 16 hr. at room temperature. This was extracted and the extract was concentrated in a usual manner. The residue therefrom was recrystallized from Me_2CO and afforded 32 mg. of (VIII), m.p. 255~260°; $[\alpha]_D$ +168° ($CHCl_3$). *Anal.* Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.66. Found: C, 75.30; H, 8.41. UV: λ_{max}^{MeOH} 241 mμ (ε 16,000).

The fractions eluted with 2:1 and 1:1 mixtures of $C_2H_4Cl_2$ and Me_2CO afforded 730 mg. of epihydrocortisone (VIII) and its recrystallization from Me_2CO gave crystals melting at 206~209°; $[\alpha]_D$ +120° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.50; H, 8.34. Found: C, 69.20; H, 8.18. UV: λ_{max}^{MeOH} 242 mμ (ε 14,500). IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 1715 (20-CO), 1657, 1615 (Δ^4-3-CO).

11α,21-Diacetate of (VII): Usual acetylation of (VII) with Ac_2O and pyridine gave the diacetate of m.p. 205~207°; $[\alpha]_D$ +125° (MeOH). *Anal.* Calcd. for $C_{25}H_{34}O_7$: C, 69.24; H, 7.65. Found: C, 67.10; H, 7.50.

Formation of Cortisone Acetate (IX) from (VII)—A solution of 130 mg. of (VII) dissolved in 5 cc. of pyridine and added with 45 mg. of Ac_2O was allowed to stand for 4 hr. at room temperature. The reaction mixture was poured into cold water, the precipitate formed was collected, and recrystallized, but no crystals were obtained.

This product was dissolved in 10 cc. of AcOH and 3 cc. of AcOH containing 25 mg. of CrO_3 was added. The reaction mixture was allowed to stand for 5 hr. at room temperature, concentrated, and the residue was recrystallized from Me_2CO, affording 80 mg. of cortisone 21-acetate (IX), m p. 238~244°; $[\alpha]_D$ +170° (Me_2CO). *Anal.* Calcd. for $C_{23}H_{30}O_6$: C, 68.63; H, 7.2. Found: C, 68.31; H, 7.71. UV: λ_{max}^{MeOH} 237.5 mμ (ε 15,300). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3440 (OH), 1750 (acetyl CO), 1730 (11-CO), 1710 (20-CO), 1670, 1620 (Δ^4-3-CO).

This product showed no depression of m.p. on admixture with cortisone 21-acetate.

Hydroxylation of Corticosterone (X)—The same culture of the same fungus with 1 g. of (X) as the substrate and usual after-treatment afforded 1.6 g. of concentrated residue. Direct recrystallization of this product from Me_2CO afforded 420 mg. of crude crystals of 14α,21-dihydroxypregn-4-ene-3,11,20-trione (XI). Another recrystallization from the same solvent raised the m.p. to 210~215°; $[\alpha]_D$ +69° (MeOH). *Anal.* Calcd. for $C_{21}H_{28}O_5$: C, 69.97; H, 7.83. Found: C, 69.70; H, 7.78. UV: λ_{max}^{MeOH} 237.5 mμ (ε 15,000). IR ν_{max}^{KBr} cm^{-1}: 3480 (OH), 1705 (20-CO), 1660, 1620 (Δ^4-3-CO).

21-Acetate of (XI): Usual treatment of (XI) with Ac_2O and pyridine afforded a monoacetate of m.p. 204~205°; $[\alpha]_D$ +81° (MeOH). *Anal.* Calcd. for $C_{23}H_{30}O_6$: C, 68.63; H, 7.52. Found: C, 68.31; H, 7.62. IR ν_{max}^{KBr} cm^{-1}: 3560 (OH), 1750 (acetyl CO), 1735 (20-CO), 1690, 1625 (Δ^4-3-CO).

Hydroxylation of 11-Dehydrocorticosterone—The same culture of the same fungus with 1 g. of 11-dehydrocorticosterone afforded 1.3 g. of concentrated residue. This was recrystallized from Me_2CO to 380 mg. of crude crystals which were recrystallized from Me_2CO to crystals of m.p. 208~214°. Its infrared absorption spectrum agreed with that of (XI) and admixture of these two showed no depression in m.p. The product was therefore established as 14α,21-dihydroxypregn-4-ene-3,11,20-trione.

Hydroxylation of 14α,21-Dihydroxypregn-4-ene-3,20-dione (IV) by *Stachylidium bicolor*—From 1 L. of the culture filtrate of *Stachylidium bicolor*, cultured as above for 48 hr., cells were collected by filtration and dispersed in 1 L. of water. To this dispersion, 10 cc. of MeOH solution containing 250 mg. of (IV) was added and 100 cc. of this mixture was placed in each of 10 shake flasks. The flasks were shaken at 28° for 70 hr., the reaction mixture was extracted with AcOEt, and the extract afforded 370 mg. of concentrated residue. Recrystallization of this residue from Me_2CO gave 96 mg. of 11β,14α,21-trihydroxypregn-4-ene-3,20-dione (XII), m.p. 206~215°; $[\alpha]_D$ +180° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found: C, 70.10; H, 8.30. UV: λ_{max}^{MeOH} 241.5 mμ (ε 16,500). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1710 (20-one), 1650, 1620 (Δ^4-3-CO).

Formation of 14α,21-Dihydroxypregn-4-ene-3,11,20-trione (XI) Acetate from (XII)—Acetylation of (XII) by the usual method with Ac_2O and pyridine afforded a monoacetate of m.p. 185~187°; $[\alpha]_D$

+167° (MeOH). *Anal.* Calcd. for $C_{23}H_{12}O_6$: C, 68.29; H, 7.97. Found : C, 68.00; H, 7.66.

To a solution of 57 mg. of this acetate dissolved in 5 cc. of AcOH, 2 cc. of AcOH solution of 18 mg. of CrO_3 was added and the mixture was allowed to stand for 25 hr. at room temperature. The usual treatment of this reaction mixture and recrystallization of the residue from Me_2CO afforded 21 mg. of crystals, m.p. 202~206°, $[\alpha]_D$ +77° (MeOH). The physical constants and infrared absorption data of this product agreed well with those of the acetate of (XI) and no depression of m.p. occurred on their admixture.

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Summary

Cultivation of *Absidia regnieri* with steroidal substrate, 17α-hydroxyprogesterone, deoxycorticosterone, Reichstein's compound S, and 11-dehydrocorticosterone, and isolation and identification of the oxidation product from each showed that the 11α-hydroxylated compound is obtained from 17α-hydroxyprogesterone and compound S, and 14α-hydroxylated compound from other steroids. This fungus was found to have dehydrogenation action of 11β-hydroxyl group, besides the above hydroxylation. The enzyme which effects this latter action was found to have a marked stereochemical specificity since it had no action on 11α-hydroxyl compound.

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