Summary

Application of *Sclerotium hydrophilum* to progesterone, 17α-hodroxyprogesterone, deoxycorticosterone, Reichstein's compound S, and corticosterone as the substrate steroids afforded 11α- and 6β-hydroxy compounds from all except corticosterone, and the latter formed 6β- and 15β-hydroxypregn-4-ene-3,11,20-trione.

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33. Makoto Shirasaka : Microbiological Transformation of Steroid. VI.[1] Hydroxylation of Steroid by *Stachylidium bicolor*.

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Hydroxylation of steroid by microörganisms is now known to occur in almost all the positions in the steroidal skeleton.[2] Among such microörganisms, those effecting 11β-hydroxylation are of importance and of practical value because they can directly manufacture steroidal hormones like hydrocortisone, and some fungi have been found to date that carry out 11β-hydroxylation, like *Cunninghamella* sp.[3] and *Curvularia* sp.[4]

During examination of oxidative ability of numerous fungi to steroids, it was found that *Stachylidium bicolor* effected 11β-hydroxylation of Reichstein's compound S and the fungus was applied to deoxycorticosterone and other steroids. It was thereby found that the fungus effected 14α-hydroxylation as well as 11β-hydroxylation, and while the fungus produced hydrocortisone almost solely from the compound S, it formed 14α-hydroxy compound from deoxycorticosterone and only a trace of 11β-hydroxy compound was formed. The fungus carried out 11β-hydroxylation of 14α,21-dihydroxypregn-4-ene-3,20-dione but did not effect 14α-hydroxylation of corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione). These results indicated that this fungus had an extremely marked substrate specificity, and these experiments are described herein.

The cultured cells of *Stachylidium bicolor* were applied to Reichstein's compound S as the substrate and the concentrated extract from the reaction mixture was examined by paper chromatography.[1] One main spot with greater polarity than the compound S and a very weak spot with smaller polarity than that were detected on the chromatogram. These spots were separated by Florisil-column chromatography and a large amount of hydrocortisone (I) was obtained as crystals. Another crop of crystals was obtained but the amount obtained was so small that the substance was not identified. Acetylation of (I) with acetic anhydride and pyridine gave a monoacetate and its oxidation with chromium trioxide afforded a triketone compound. The constants of the oxidation product were

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1) Part V : This Bulletin, **9**, 196 (1961).
2) E. Vischer, A. Wettstein : Advances in Enzymol., **20**, 237 (1959).
3) F. R. Hanson, *et al.* : J. Am. Chem. Soc., **75**, 5369 (1953).
4) G. M. Shull, *et al.* : *Ibid.*, **77**, 763 (1955).

identical with those of cortisone acetate and no depression of the melting point was observed in admixture.

The same fermentation of this fungus with deoxycorticosterone as the substrate and paper chromatographic examination of the concentrated extract showed the presence of two spots with greater polarity than deoxycorticosterone. Of these two spots, color of the one with greater polarity was deeper than the other spot. Their separation through Florisil column chromatography gave a small amount of the unreacted deoxycorticosterone from the initial eluate and crystals (II) of m.p. 177~179° from the second eluate. The final fraction afforded a comparatively large amount of crystals (III) melting at 172~177°. Analytical values of both these products indicated introduction of one hydroxyl into deoxycorticosterone. Acetylation of these products with acetic anhydride and pyridine gave a monoacetate from both and the fact indicated that the newly introduced hydroxyl is not acetylated.

Oxidation of this monoacetate of (II) with chromium trioxide afforded a triketone compound whose constants agreed with those of 11-dehydrocorticosterone 21-acetate and their admixture showed no depression in the melting point. (II) was found to be identical with corticosterone by admixture and comparison of physical data.

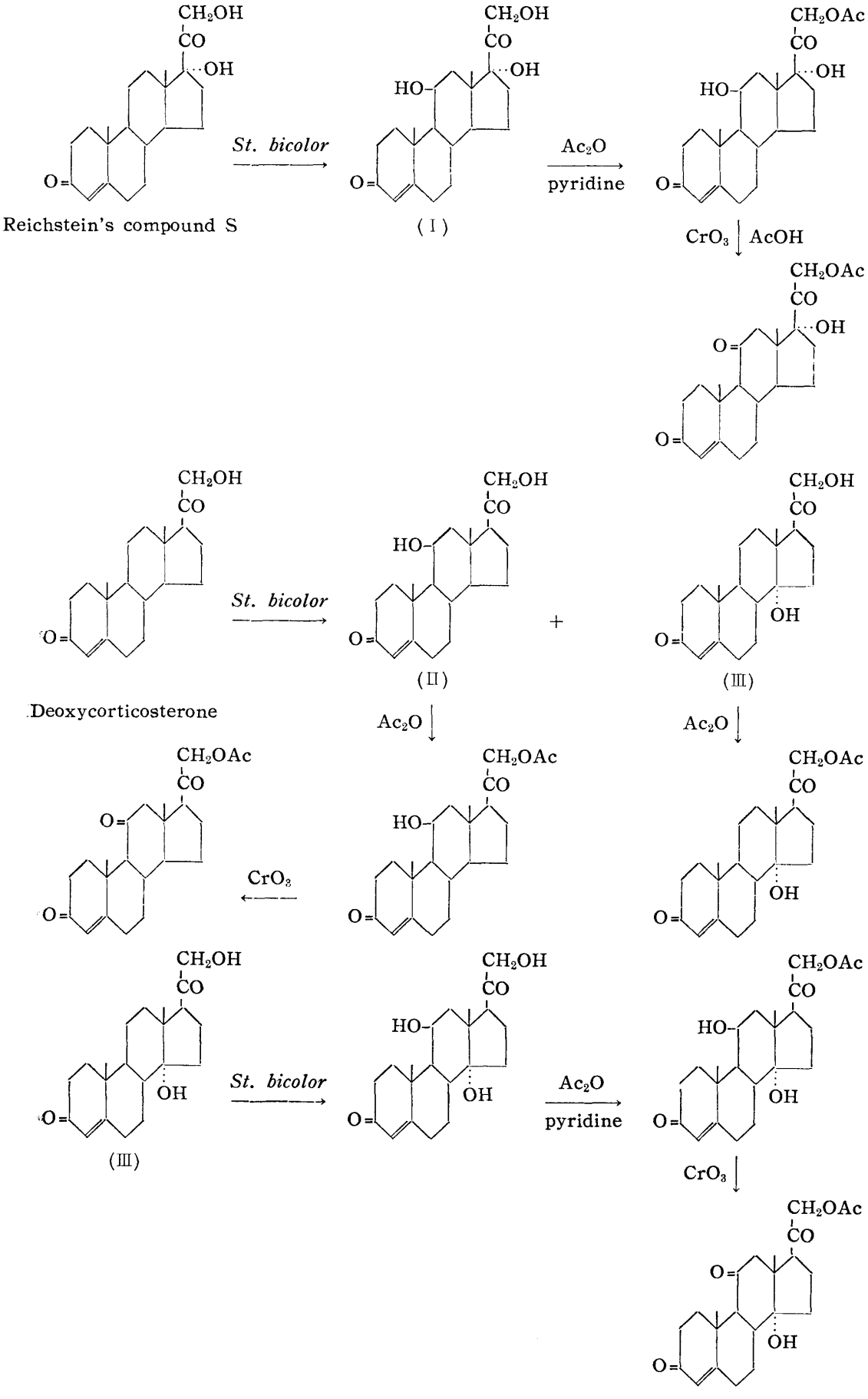
The same oxidation of the monoacetate of (III) ended in the recovery of the starting material, indicating that the newly introduced hydroxyl in (III) is tertiary. The constants and infrared spectra of (III) and its monoacetate were identical with those of 14α,21-dihydroxypregn-4-ene-3,20-dione and its 21-acetate, obtained from deoxycorticosterone by a similar manner using *Absidia regnieri*, reported previously.[5]

Finally, the same fermentation of this fungus with 14α,21-dihydroxypregn-4-ene-3,20-dione (III) as the substrate was carried out and the concentrated extract was recrystallized from acetone to crystals (IV) of m.p. 206~215°. Its analytical values indicated introduction of one hydroxyl into (III) but this hydroxyl was found to be not acetylated, since the acetylation of (IV) with acetic anhydride and pyridine afforded a monoacetate. Oxidation of this monoacetate with chromium trioxide gave a triketone compound and its ultraviolet absorption maximum was located at 237 mμ, which is in a shorter wave-length region than that of ordinary Δ^4-3-keto-steroids. Consequently, 11β-hydroxyl must have been oxidized to a ketone[6] and the newly introduced hydroxyl must be in this 11β-position. These experimental evidences have shown that the structure of (IV) is 11β,14α-dihydroxypregn-4-ene-3,20-dione, which is a new steroid.

Application of this fungus to corticosterone failed to give any oxidation product.

The foregoing fermentation experiments with *Stachylidium bicolor*, using various steroids as a substrate, incidated that this fungus mainly effected 11β- and 14α-hydroxylation. However, it was found that only 11β-hydroxylation occurred in the case of the compound S while 14α-hydroxylation mainly occurred in deoxycorticosterone and these facts indicated that this fungus has an extremely marked substrate specificity. The reason why 14α-hydroxylation does not occur in the compound S is probably due to its chemical structure and the 17α-hydroxyl group in this steroid must have some kind of steric hindrance against this reaction. The 14α-hydroxylation of deoxycorticosterone may be due to stronger reactivity of 14α-hydroxylation than 11β-hydroxylation. Although this fungus effected 11β-hydroxylation of the 14α-hydroxylated compound (III) of deoxycorticosterone, it failed to effect 14α-hydroxylation of the 11β-hydroxylated compound (corticosterone). Consequently, there seems to be a kind of substrate specificity in these two reactions by this fungus.

5) Part II. M. Shirasaka : This Bulletin, **9**, 59 (1961).
6) F. Sondeheimer, *et al.* : J. Am. Chem. Soc., **76**, 5020 (1954); L. Dorfman : Chem. Revs., **53**, 72 (1953).



Experimental

Fermentation and Extraction—A medium consisting of 5% of glucose, 2% of peptone, and 0.5% of corn-steep liquor was placed in twenty 500-cc. shake flasks, 100 cc. to each flask, and sterilized. Each flask was inoculated with *Stachylidium bicolor* and the flasks were shaken at 26° for 48~72 hr. The culture liquid was filtered to separate fungal cells, which were washed with water, and mixed with 2 L. of water. To this mixture, 20 cc. of 2.5% MeOH solution of the substrate steroid was added, mixed thoroughly, and the mixture was poured into twenty shake flasks, 100 cc. to each flask. After shaking the flasks for 48~72 hr. at 26°, the mixture was filtered to separate the fungal cells, which were extracted with AcOEt, and the extract was combined with the filtrate. The mixture was extracted with AcOEt, the extract was washed with 2% $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and the solvent was evaporated in diminished pressure. The residue was crystallized from a suitable solvent or purified by column chromatography.

Hydroxylation of Reichstein's Compound S—The foregoing fermentation of *Stachylidium bicolor* was carried out with 1 g. of Reichstein's compound S as the substrate and 1.3 g. of concentrated extract was obtained. This residue was dissolved in 100 cc. of $C_2H_4Cl_2$ and the solution was passed through a column of 80 g. of Florisil. The column was successively eluted with various mixtures of $C_2H_4Cl_2$ and Me_2CO. The residue obtained by evaporation of the initial fraction was crystallized from Me_2CO and 520 mg. of crude crystals of hydrocortisone (I) was obtained. Recrystallization from Me_2CO gave crystals of m.p. 202~208°; $[\alpha]_D$ +162° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found: C, 69.44; H, 8.30. UV: λ_{max}^{MeOH} 242 mμ (ε 14,000). IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 1713 (20-CO), 1645, 1615 (Δ^4-3-CO). These constants and infrared spectral data were identical with those of hydrocortisone.

21-Acetate of (I): Usual acetylation of (I) with Ac_2O and pyridine gave the 21-monoacetate of m.p. 205~210°; $[\alpha]_D$ +150° (MeOH). *Anal.* Calcd. for $C_{23}H_{32}O_6$: C, 68.29; H, 7.97. Found: C, 68.77; H, 7.41.

Oxidation of 21-Acetate of (I) with CrO_3—To a solution of 50 mg. of the monoacetate of (I) dissolved in 7 cc. of AcOH, 2 cc. of AcOH containing 27 mg. of CrO_3 was added in small portions and the mixture was allowed to stand for 6 hr. at room temperature. The mixture was extracted with CH_2Cl_2 in the usual manner, the extract was washed with $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and evaporated. The residue was recrystallized from Me_2CO to 27 mg. of cortisone acetate, m.p. 235~242°; $[\alpha]_D$ +180° (Me_2CO). *Anal.* Calcd. for $C_{23}H_{30}O_6$: C, 68.63; H, 7.51. Found: C, 68.81; H, 7.70. UV: λ_{max}^{MeOH} 238 mμ (ε 15,800). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3442 (OH), 1730 (acetyl CO), 1710 (20-CO), 1670, 1621 (Δ^4-3-CO). These constants and spectral data were identical with those of authe ntic sample of cortisone acetate.

Hydroxylation of Deoxycorticosterone—The same fermentation of this fungus was carried out with 1 g. of corticosterone as the substrate and 1.5 g. of the concentrated residue thereby obtained was purified by Florisil-column chromatography. The initial eluate furnished the unreacted deoxycorticosterone in a small amount. The residue from the second fraction was recrystallized from Me_2CO and furnished 110 mg. of crude crystals of corticosterone (II), which were recrystallized from the same solvent to crystals of m.p. 177~179°; $[\alpha]_D$ +220° (EtOH). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.74. Found: C, 71.80; H, 8.11. IR ν_{max}^{KBr} cm^{-1}: 3432 (OH), 1700 (20-CO), 1630, 1625 (Δ^4-3-CO).

21-Acetate of (III): Usual acetylation of (III) with Ac_2O and pyridine afforded the monoacetate of m.p. 160~162°; $[\alpha]_D$ +197° (MeOH). *Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 71.80; H, 8.31.

Oxidation of 21-Acetate of (II) with CrO_3—Oxidation of (II) acetate in AcOH with CrO_3 by the usual procedure furnished 11-hydrocorticosterone 21-acetate.

Hydroxylation of 14α,21-Dihydroxypregn-4-ene-3,20-dione (III)—As reported in Part II of this series,[5] application of this fungus to (III) furnished 11β,14α,21-trihydroxypregn-4-ene-3,20-dione (IV), m.p. 206~215°; $[\alpha]_D$+ 180° (MeOH), and its acetylation with Ac_2O and pyridine gave the 21-monoacetate, m.p. 185~187°; $[\alpha]_D$ +167° (MeOH).

Oxidation of the Acetate of (IV) with CrO_3—To a solution of 57 mg. of the 21-acetate of (IV) dissolved in 5 cc. of AcOH, 2 cc. of AcOH containing 18 mg. of CrO_3 was added and the mixture was allowed to stand for 5 hr. at room temperature. A small amount of MeOH was added to it and the solvent was evaporated in reduced pressure. The residue was diluted with 20 cc. of water and the mixture was extracted twice with CH_2Cl_2. The extract was washed with $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and the solvent was evaporated in reduced pressure. Recrystallization of this residue from Me_2CO furnished 24 mg. of 14α,21-dihydroxypregn-4-ene-3,11,20-trione 21-acetate, m.p. 202~206°; $[\alpha]_D$ +77° (MeOH). *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 68.63; H, 7.52. Found: C, 68.81; H, 7.21. UV: λ_{max}^{MeOH} 237 mμ (ε 15,300), IR ν_{max}^{KBr} cm^{-1}: 3480 (OH), 1447 (acetyl CO), 1730 (20-CO), 1690, 1625 (Δ^4-3-CO).

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Summary

Cultured cells of *Stachylidium bicolor* was applied to Reichstein's compound S, deoxycorticosterone, 14α,21-dihydroxypregn-4-ene-3,20-dione, and corticosterone. The compound S produced hydrocortisone, deoxycorticosterone produced 11β- and 14α-hydroxylated compounds, and 14α,21-dihydroxypregn-4-ene-3,20-dione gave the 11β-hydroxy compound. Oxidation product was not obtained from corticosterone.

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34. Makoto Shirasaka and Masako Tsuruta: Microbiological Transformation of Steroid. VIII.[1] 1-Dehydrogenation of Steroid by Fungi.

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1-Dehydrogenation of steroid is one of the frequent reactions among transformation of steroids by microörganisms. The recent discovery that the activity of adrenocortical hormone increases by the introduction of a double bond in 1-2 position of the steroidal structure and appearance of new hormones like prednisone and prednisolone have made this 1-dehydrogenation reaction quite an important and practicable reaction. Most of the microörganisms which are known to carry out this reaction are bacteria but a few fungi have been reported as effecting this reaction.[2,3] One of them is *Didimella lycopersia*[3] which produces 1-dehydro compound from hydrocortisone.

During examination of various fungi for 1-dehydrogenation of steroids, it was found that *Gliocladium roseum*, *Helminthosporium turcicum*, and *Ophiobolus heterostropus* effected 1-dehydrogenation of Reichstein's compound S. Application of these fungi to other steroids, deoxycorticosterone, corticosterone, and cortisone resulted in formation of their 1-dehydro compounds. However, none of the fungi were able to produce the 1-dehydro compound from hydrocortisone and it was found that these fungi had a marked substrate specificity in the 1-dehydrogenation reaction of steroids.

First, the fermentation of *Gliocladium roseum* was carried out, as will be described later, with deoxycorticosterone as the substrate and the concentrated extract of the culture liquid was examined by paper chromatography, using propylene glycol-toluene system as the developing solvent. The chromatogram contained one spot with somewhat greater polarity than deoxycorticosterone. Purification of the concentrated extract by Florisil-column chromatography afforeded some crystals (I) of m.p. 185~191°; $[\alpha]_D$ +110° (MeOH), whose analytical values showed that there was no increase in the oxygen content. The

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1) Part Ⅶ: This Bulletin, **9**, 238 (1961).
2) E. Visher, *et al.*: Helv. Chim. Acta, **38**, 835 (1955).
3) *Idem*: *Ibid.*, **38**, 1502 (1955).