*UDC 547.92:576.882.8.095.3*

# **Makoto Shirasaka and Masako Tsuruta**: Microbiological Transformation of Steroid. IV.[1] 11$\alpha$-Hydroxylation of Steroid by *Gloeosporium kaki* and *Glomerella lagenarium*.

(*Takamine Laboratory, Sankyo Co., Ltd.*[*1])

It is well known that the first example among numerous hydroxylation of steroids by microörganisms and which was responsible for the great development in conversion of steroids by microörganisms is the 11$\alpha$-hydroxylation of steroids by *Rhizopus* sp. discovered by Peterson and others.[2] Moreover, this hydroxylation of 11-position in steroid is an extremely important reaction in that it effects introduction of oxygen into 11-position, which is an important functional group in steroidal hormones such as cortisone, hydrocortisone, and corticosterone. Numerous microörganisms have been found to date which effect this 11-hydroxylation but majority of such microörganisms also carry out other reactions as well, especially 6$\beta$-hydroxylation,[2,3] as well as 11$\beta$-,[4] 17$\alpha$-,[5] and/or 21-hydroxylation.[6] The representative among these microörganisms which carry out 11$\alpha$-hydroxylation alone are *Rhizopus nigricans* of Peterson[2] and *Aspergillus niger* of Fried and others.[7]

During examination of numerous fungi for their hydroxylation ability, a large number were found to effect 11$\alpha$-hydroxylation of steroid but majority was accompanied with other reactions as cited above and only *Gloeosporium kaki* and *Glomerella lagenarium* were found to effect 11$\alpha$-hydroxylation alone. The fungi which would carry out 11$\alpha$-hydroxylation alone is of great value in the manufacture of steroidal hormones like cortisone and hydrocortisone.

These newly found fungi were applied to progesterone, 17$\alpha$-hydroxyprogesterone, deoxycorticosterone, and Reichstein's compound S, and their 11-hydroxy derivatives were successfully obtained.

Fermentation of *Gloeosporium kaki* in potato decoction medium was carried out, as will be described later, with progesterone as the substrate and the concentrate obtained from fermentation liquor by extraction indicated two spots of greater polarity than progesterone by paper chromatographic examination. This concentrate was dissolved in benzene, the insoluble matter was collected, and recrystallization from methanol afforded crystals of dihydroxyprogesterone (I). Its acetylation with acetic anhydride and pyridine in usual manner afforded a diacetate, indicating that the two newly introduced hydroxyls in (I) are both primary or secondary. The constants of (I) and its acetate were in good agreement with those of 6$\beta$,11$\alpha$-dihydroxyprogesterone and its diacetate obtained by Peterson and others with the use of *Rhizopus* sp.[2]

The supernatant of the foregoing benzene solution was submitted to alumina chromatography and a large amount of monohydroxyprogesterone (II) was obtained. Its acetylation afforded a monoacetate, indicating that the newly introduced hydroxyl is primary or secondary. Oxidation of (II) with chromium trioxide in acetic acid gave a trioxo compound, which was found to be identical with the known 11-oxoprogesterone.[8] Since the constants

*1 Nishi-shinagawa, Shinagawa-ku, Tokyo (白坂 亮, 鶴田雅子).

1) Part III: This Bulletin, **9**, 152 (1961).
2) D. H. Peterson, *et al.*: J. Am. Chem. Soc., **74**, 5933 (1952); **75**, 55, 408, 416, 419 (1953).
3) S. H. Epstein, *et al.*: *Ibid.*, **75**, 408 (1953); E. L. Dulaney, *et al.*: Mycologia, **47**, 464 (1955).
4) J. Schmidt-Thome: Angew. Chem., **69**, 238 (1957).
5) E. L. Dulaney, *et al.*: Appl. Microbiol., **3**, 372 (1955); P. D. Meister, *et al.*: J. Am. Chem. Soc., **76**, 4050 (1954).
6) E. Weisz, G. Wix, M. Bodansky: Naturwiss., **43**, 39 (1956).
7) J. Fried, *et al.*: J. Am. Chem. Soc., **74**, 3962 (1952).
8) C. W. Shoppee, T. Reichstein: Helv. Chim. Acta, **24**, 351 (1941).

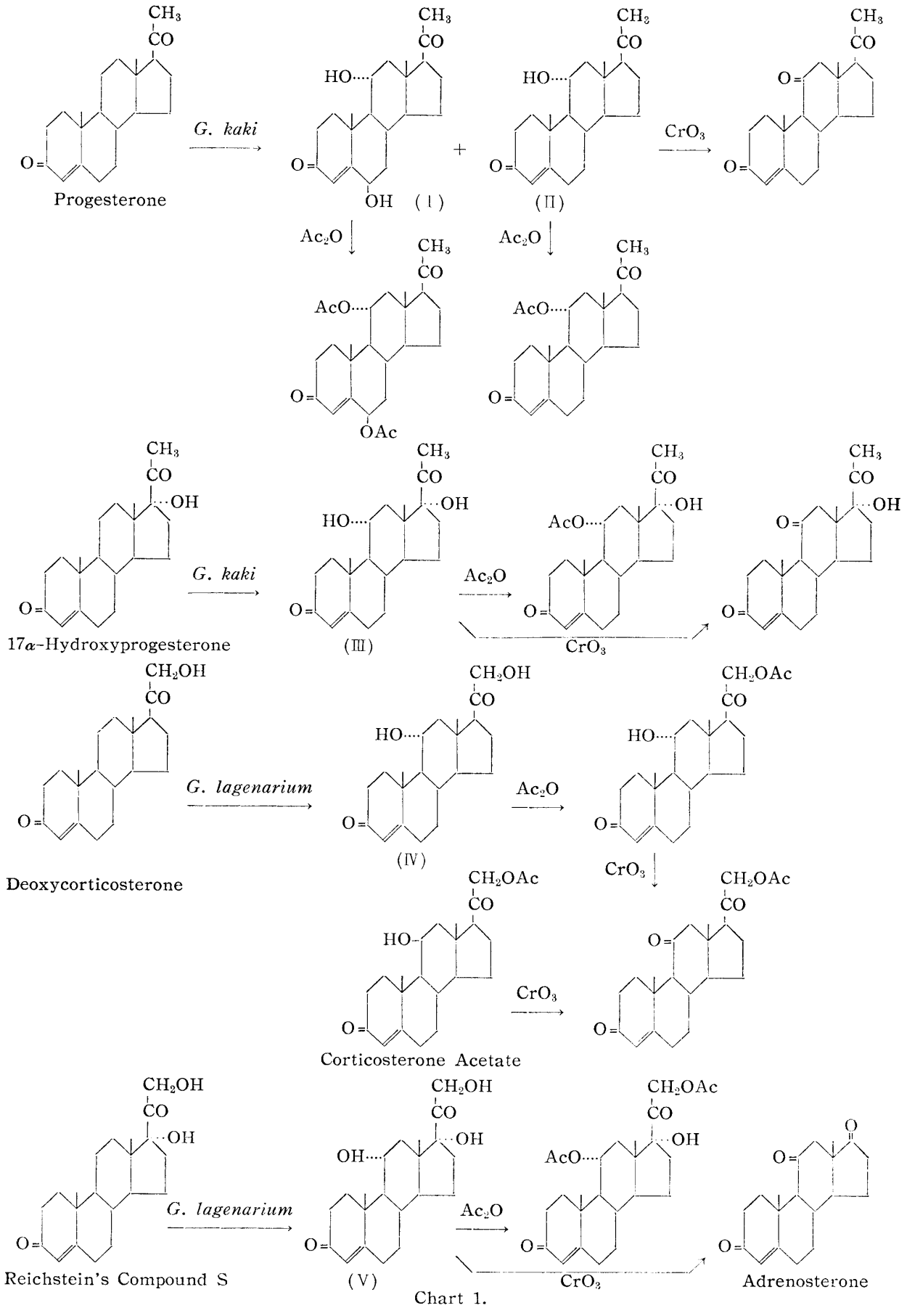


Chart 1.

of (II) were different from those of 11$\beta$-hydroxyprogesterone, it must be 11$\alpha$-hydroxyprogesterone. The constants of (II) and its monoacetate were in good agreement with the constants of 11$\alpha$-hydroxyprogesterone and its 11-acetate obtained by Peterson and others.[2]

The same fermentation of *Gloeosporium kaki* with 17$\alpha$-hydroxyprogesterone as the substrate and paper chromatographic examination of its product indicated two spots, one of unreacted 17$\alpha$-hydroxyprogesterone and the other with greater polarity than that. Separation of these two substances by Florisil column chromatography afforded some crystals of unreacted 17$\alpha$-hydroxyprogesterone and a new crystalline substance (III). Elemental analytical values showed (III) to be a dihydroxyprogesterone and its acetylation with acetic anhydride and pyridine gave a monoacetate, indicating that the newly introduced hydroxyl is primary or secondary. Oxidation of (III) with chromium trioxide in acetic acid formed a trioxo compound of m.p. 234~240°; $[\alpha]_D$ +170°($CHCl_3$). These constants are in good agreement with those of 11$\alpha$,17$\alpha$-dihydroxyprogesterone and its corresponding derivatives,[9] and (III) was therefore established as 11$\alpha$,17$\alpha$-dihydroxyprogesterone.

The usual fermentation was carried out with *Glomerella lagenarium*, with deoxycorticosterone as the substrate and paper chromatographic examination of the product indicated only one spot with greater polarity than deoxycorticosterone. Recrystallization of this product from ethyl acetate gave crystals of dihydroxypregn-4-ene-3,20-dione (IV), with introduction of one hydroxyl in deoxycorticosterone. Acetylation of (IV) with acetic anhydride and pyridine failed to give any crystalline acetate but paper chromatogram of the reaction mixture showed the presence of a spot with smaller polarity than (IV). Acetylation of (IV) with equivalent of acetic anhydride and pyridine also failed to give any crystalline product and paper chromatogram of this reaction mixture showed a spot intermediate of (IV) and the first acetylation product. Oxidation of this second acetylation product with chromium trioxide in acetic acid gave crystals of m.p. 172~176°, whose m.p. and other constants agreed with those of 11-dehydrocorticosterone 21-acetate, obtained by a similar procedure from corticosterone. Since the constants of (IV) are clearly different from those of corticosterone, it must be its epimer, i.e. epicorticosterone or 11$\alpha$,21-dihydroxypregn-4-ene-3,20-dione.

Finally, the same fermentation of *G. lagenarium* was carried out with Reichstein's compound S as the substrate and paper chromatographic examination of its product indicated one spot with greater polarity than compound S. Repeated recrystallization of the concentrate from acetone gave trihydroxypregn-4-ene-3,20-dione (V), with one hydroxyl newly introduced into the compound S. Acetylation with acetic anhydride and pyridine gave a diacetate, indicating that the newly introduced hydroxyl is primary or secondary. Oxidation of (V) with chromium trioxide in acetic acid gave adrenosterone,[10] m.p. 218~220°; $[\alpha]_D$ +279°($CHCl_3$). The constants and infrared spectra of (V) and its diacetate were in good agreement with the authentic specimen of epihydrocortisone and its diacetate.[11]

Application of *Gl. lagenarium* to corticosterone by the usual procedure failed to afford any oxidized steroid.

Fermentation of both *Gloeosporium kaki* and *Glomerella lagenarium* with each of the foregoing substrate steroids and comparative examination of their oxidizability by paper chromatography revealed that the results were the same as described above. Consequently, the action of these two kinds of fungus to steroids is considered to be entirely the same.

The foregoing experiments seem to indicate that these two kinds of fungus carry out 11$\alpha$-hydroxylation of steroids almost singly and this is endorsed by the fact that no change was found in the case of corticosterone which already has a hydroxyl group in 11$\beta$-position.

9) P. D. Meister, *et al.* : J. Am. Chem. Soc., **75**, 416 (1953).
10) T. Reichstein : Helv. Chim. Acta, **20**, 953 (1937).
11) D. H. Peterson, *et al.* : J. Am. Chem. Soc., **75**, 412 (1953).

*Glomerella lagenarium* (Ascomycetes) and *Gloeosporium kaki* (imperfect fungus) are taxonomically, i.e. morphologically, clearly different but they are of the same system. It is extremely interesting, from the point of taxonomy, that these fungi have the same kind of oxidizability against steroids.

## Experimental

**Fermentation and Extraction**—Two liters of potato decoction, containing 3% of glucose, was poured into twenty 500-cc. shake flasks, 100 cc. to each flask, and sterilized at 120° for 20 min. When cooled to 26°, the fungus was inoculated in each flask and the flasks were shaken at 26° for 48~72 hr. A 2.5% MeOH solution of the substrate steroid was added, 2 cc. to each flask, and fermentaion was continued for 48 hr. The culture liquid was filtered to separate the fungal cells, the cells were extracted with $Me_2CO$, and the extract was combined with the filtrate. The combined extract and filtrate was extracted twice 1.5 L. of AcOEt, AcOEt extract was washed with 200 cc. each of 2% $NaHCO_3$ solution and water, and dried over anhyd. $Na_2SO_4$. The dried extract was evaporated in reduced pressure and the residue was either recrystallized directly from a solvent or purified by adsorption chromatography.

**Paper Partition Chromatography**—Chromatography was carried out as described earlier,[12] by the descending method using propyleneglycol-toluene system as a solvent.

**Hydroxylation of Progesterone by *Gloeosporium kaki***—The fermentation was carried out as described above, with 1 g. of progesterone as the substrate and 1.3 g. of a concentrate was obtained from the extract. This concentrate was dissolved in 100 cc. of benzene, the insoluble matter was collected by filtration, and recrystallized from MeOH to 110 mg. of 6β,11α-dihydroxyprogesterone (I). Repeated recrystallization from MeOH gave crystals of m.p. 238~242°; $[\alpha]_D$ +130° (pyridine). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.74. Found: C, 72.60; H, 8.51. UV: $\lambda_{max}^{MeOH}$ 236 mμ (ε 14,800). IR $\nu_{max}^{Nujol}$ $cm^{-1}$: 3420 (OH), 1695 (20-CO), 1670, 1621 ($\Delta^4$-3-CO).

6,11-Diacetate of (I): Usual acetylation of 50 mg. of (I) with $Ac_2O$ and pyridine afforded 40 mg. of the diacetete of m.p. 146~147°; $[\alpha]_D$ +68° (MeOH). *Anal.* Calcd. for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96. Found: C, 69.33 H, 7.81.

The foregoing benzene filtrate was passed through a column of 50 g. of alumina and the column was successively eluted with 100 cc. of benzene, benzene-$Et_2O$ (95:5, 90:10, 50:50), $Et_2O$, $Et_2O$-$CHCl_3$ (95:5, 90:10, 50:50), and $CHCl_3$. The fraction eluted by $Et_2O$-$CHCl_3$ (95:5 and 90:10) afforded 360 mg. of crude crystals of 11α-hydroxyprogesterone (II), which were recrystallized from $Me_2CO$ to crystals of m.p. 164~167°; $[\alpha]_D$ +180° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_3$: C, 76.32; H, 9.15. Found: C, 76.20; H, 9.01. UV: $\lambda_{max}^{MeOH}$ 242 mμ (ε 17,100). IR $\nu_{max}^{CHCl_3}$ $cm^{-1}$: 3450 (OH), 1700 (20-CO), 1670, 1610 ($\Delta^4$-3-CO).

11-Acetate of (II): Usual acetylation of 50 mg. of (II) with $Ac_2O$ and pyridine gave 43 mg. of the monoacetate, m.p. 155~156°; $[\alpha]_D$ +140° ($CHCl_3$). *Anal.* Calcd. for $C_{23}H_{32}O_4$: C, 74.16; H, 8.66. Found: C, 74.30; H, 8.43.

11-Oxoprogesterone: To a solution of 60 mg. of (II) dissolved in 1.0 cc. of AcOH, 2 cc. of AcOH containing 10 mg. of $CrO_3$ was added and the mixture was allowed to stand for 2 hr. at room temperature. The reaction mixture was treated by the usual method and recrystallization of the product from $Me_2CO$ gave 31 mg. of the trioxo compound, m.p. 172~175°; $[\alpha]_D$ +268° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 76.79; H, 8.59. Found: C, 76.59; H, 8.55.

**Hydroxylation of 17α-Hydroxyprogesterone by *Gloeosporium kaki***—The same fermentation as above was carried out with 1 g. of 17α-hydroxyprogesterone as the substrate and 1.8 g. of concentrated residue 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 $C_2H_4Cl_2$, $C_2H_4Cl_2$-$Me_2CO$ (25:1, 15:1, 12:1, 10:1, 5:1, 3:1) and the fraction eluted by $C_2H_4Cl_2$-$Me_2CO$ (15:1 and 10:1) afforded some unreacted 17α-hydroxyprogesterone. The fraction eluted by 5:1 and 3:1 mixtures of $C_2H_4Cl_2$-$Me_2CO$ gave 320 mg. of 11α,17α-dihydroxyprogesterone (III) which was recrystallized twice from $Me_2CO$ to crystals of m.p. 219~222°; $[\alpha]_D$ +80° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.11; H, 8.80. UV: $\lambda_{max}^{MeOH}$ 242 mμ (ε 15,800). IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3430 (OH), 1690 (20-CO), 1667, 1615 ($\Delta^4$-3-CO).

11-Acetate of (III): The usual acetylation of 50 mg. of (III) with $Ac_2O$ and pyridine afforded 40 mg. of the monoacetate of m.p. 214~216°; $[\alpha]_D$ +72° ($CHCl_3$). *Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 70.90; H, 8.43. UV: $\lambda_{max}^{MeOH}$ 240 mμ (ε 16,000).

11-Oxo-17α-hydroxyprogesterone: A solution of 100 mg. of (III) dissolved in AcOH was oxidized with $CrO_3$ and 32 mg. of monohydroxytrioxo-steroid of m.p. 234~240°; $[\alpha]_D$ +170° ($CHCl_3$), was obtained.

12) Part I. This Bulletin, **9**, 54 (1961).

*Anal.* Calcd. for $C_{21}H_{28}O_4$ : C, 73.22; H, 8.19. Found : C, 73.10; H, 8.26. UV : $\lambda_{max}^{EtOH}$ 238 mμ ($\varepsilon$ 15,000).

**Hydroxylation of Deoxycorticosterone by *Glomerella lagenarium*** —Fermentation of *Gl. lagenarium* was carried out with 1 L. of the medium as described above, with 0.5 g. of deoxycorticosterone as the substrate and 0.95 g. of concentrated residue was obtained. This residue was dissolved in a small amount of AcOEt with warming, and the crystals that precipitated on cooling, were collected by filtration. Recrystallization from AcOEt afforded 210 mg. of 11$\alpha$,21-dihydroxypregn-4-ene-3,20-dione (IV), m.p. 153~161°; $[\alpha]_D$ +180° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_4$ : C, 72.80; H, 8.74. Found : C, 73.00; H, 8.65. UV : $\lambda_{max}^{MeOH}$ 241 mμ ($\varepsilon$ 15,300). IR $\nu_{max}^{KBr}$ $cm^{-1}$ : 3470 (OH), 1700 (20-CO), 1655, 1615 ($\Delta^4$-3-CO).

11-Dehydrocorticosterone Acetate : Acetylation of (IV) with excess of $Ac_2O$ and pyridine by the usual method failed to produce any crystalline product. The evaporation residue of this reaction mixture was submitted to paper chromatography and one spot with extremely small polarity was detected.

Similar acetylation was carried out with 70 mg. of (IV) dissolved in pyridine containing 23 mg. (1 equiv.) of $Ac_2O$ and leaving the mixture to stand over night. After evaporation of the solvent, the residue was diluted with water, extracted with $CH_2Cl_2$, and the extract was washed with 5% $NaHCO_3$ solution and water. The dried ($Na_2SO_4$) extract was evaporated, the residue was dissolved in 3 cc. of AcOH, and 2 cc. of AcOH containing 25 mg. of $CrO_3$ was added to it. After standing for 1 hr. at room temperature, a small amount of water was added and extracted as usual. Recrystallization of the extract residue from $Me_2CO$ gave 35 mg. of crude crystals which were recrystallized from $Me_2CO$-$Et_2O$ to needle crystals, m.p. 172~176°; $[\alpha]_D$ +221° (MeOH). *Anal.* Calcd. for $C_{23}H_{30}O_5$: C, 71.46; H, 7.84. Found : C, 71.20; H, 7.78.

The constants of this product were identical with those of 11-dehydrocorticosterone 21-acetate, obtained by similar $CrO_3$-oxidation of the 21-acetate of corticosterone (11$\beta$,21-dihydroxypregn-4-ene-3,20-dione), and their admixture showed no depression in m.p.

**Hydroxylation of Reichstein's Compound S by *Glomerella lagenarium*** —Fermentation of Gl. lagenarium as above with Reichstein's compound S afforded 1.6 g. of concentrated residue. This residue was directly crystallized from $Me_2CO$ to 510 mg. of epihydrocortisone (11$\alpha$,17$\alpha$,21-trihydroxypregn-4-ene-3,20-dione) (V) which melted, after recrystallization, at 200~209°; $[\alpha]_D$ +110° (MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_5$ : C, 69.58; H, 8.35. Found : C, 69.40; H, 8.37. UV : $\lambda_{max}^{MeOH}$ 242 mμ ($\varepsilon$ 14,200). IR $\nu_{max}^{KBr}$ $cm^{-1}$ : 3500 (OH), 1700 (20-CO), 1655, 1615 ($\Delta^4$-3-CO).

11$\alpha$,21-Diacetate of (V) : Usual acetylation of (V) with $Ac_2O$ and pyridine gave 11$\alpha$,21-diacetate, m.p. 209~217°; $[\alpha]_D$ +125° (MeOH). *Anal.* Calcd. for $C_{25}H_{34}O_7$ : C, 67.24; H, 7.68. Found : C, 67.44; H, 7.53. UV : $\lambda_{max}^{MeOH}$ 240 mμ ($\varepsilon$ 15,000).

Adrenosterone : A solution of 50 mg. of (V) dissolved in 5 cc. of AcOH, added with 9 cc. of AcOH containing 80 mg. of $CrO_3$, was allowed to stand for 2 days at room temperature and the solvent was evaporated in reduced pressure. The residue was diluted with 20 cc. of water, extrated several times with $Et_2O$, and the extract was washed with 5% $NaHCO_3$ solution and water. After drying over anhyd. $Na_2SO_4$, $Et_2O$ was evaporated and the residue was recrystallized from $Me_2CO$ to 31 mg. of crystals, m.p. 218~220°; $[\alpha]_D$ +279° ($CHCl_3$). *Anal.* Calcd. for $C_{19}H_{24}O_3$ : C, 75.96; H, 8.05. Found: C, 76.10; H, 8.08. The constants of this product agreed with those of adrenosterone.[10]

The authors express their gratitude to Prof. K. Tsuda and Prof. T. Asai of The Institute of Applied Microbiology, University of Tokyo, for their unfailing guidance throughout the course of the present work. They are indebted to Mr. M. Matsui, the Director of this Laboratory, and Mr. H. Okazaki, Chief of this Section, both of this company, for their encouragement.

## Summary

11$\alpha$-Hydroxy derivatives of progesterone, 17$\alpha$-hydroxyprogesterone, deoxycorticosterone, and Reichstein's compound S were obtained solely by the use of *Gloeosporium kaki* and *Glomerella lagenarium*. These fungi did not affect corticosterone.

(Received June 6, 1960)