Results and Discussion

As shown above, application of *Botrytis cinerea* to various kinds of substrate steroids revealed that this fungus chiefly effected 6β- and 15β-hydroxylation. Either one or both of these reactions were found to take place according to the structure of substrate steroids and these facts indicate that this fungus has a kind of substrate specificity. These results are listed in Table II.

Table II. Hydroxylation of Various Steroids by *Botrytis cinerea*

Substrate	Position hydroxylated	
	6β	15β
17α-Hydroxyprogesterone	+	−
21-Hydroxypregn-4-ene-3,20-dione	+	+
17α,21-Dihydroxypregn-4-ene-3,20-dione (Compound S)	+	−
11β,21-Dihydroxypregn-4-ene-3,20-dione	−	+

It is seen from this table that 6β-hydroxylation by this fungus is easily effect in all the steroids tested except corticosterone in which this reaction results in 15β-hydroxylation alone. This difference in the reactivity is considered to involve the presence of 11β-hydroxyl group since corticosterone alone among these steroids has 11β-hydroxyl group. In other words, 11β-hydroxyl group must have some kind of steric hindrance against 6β-hydroxylation. On the other hand, 15β-hydroxylation does not occur at all in 17α-hydroxyprogesterone and Reichstein's compound S and this is considered to be due to the effect of 17α-hydroxyl group, which is present only in these two steroids but not in others. This must also be the effect of a kind of steric hindrance due to the comparatively close distance between 17- and 15-positions.

It was also observed that this fungus also effected dehydrogenation of 11β-hydroxyl, besides the above hydroxylation. However, this reaction did not occur in corticosterone which had already been 15β-hydroxylated. Consequently, this reaction also has a kind of substrate specificity.

Experimental

Fermentation and Extraction—Two liters of potato decoction, containing 3% of glucose, was placed in twenty 500-cc. shake flasks, 100 cc. to each flask, and sterilized at 120° for 20 min. After cool, *Botrytis cinerea* was inoculated into each flask and the flasks were shaken at 26° for 48 hr. 2.5% MeOH solution of the substrate steroid was added to the flasks, 2 cc. to each flask, and shake culture was continued for 48~72 hr. After completion of the fermentation, the culture liquid was filtered to separate fungal cells, which was extracted with Me_2CO, and the extract was combined with the filtrate. The combined solution was extracted twice with 2 L. of AcOEt, the combined extract was washed with 2% $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and the solvent was evaporated in reduced pressure.

Paper Chromatography—Same as in the previous work,[1] chromatography was carried out by the descending method, using the modified Zaffaroni procedure with propyleneglycol-toluene system as the solvent.

Hydroxylation of 17α-Hydroxyprogesterone by *Botrytis cinerea*—Fermentation was carried out as described above with 1 g. of 17α-hydroxyprogesterone as the substrate and extraction of the culture liquid afforded 1.7 g. of a concentrate. This concentrate was dissolved in 100 cc. of $C_2H_4Cl_2$ and passed through a column of 80 g. of Florisil. The column was eluted with 100 cc. each of $C_2H_4Cl_2$ and mixed solvent of $C_2H_4Cl_2$ and Me_2CO in 25:1, 15:1, 12:1, 10:1, 8:1, 5:1, 3:1, and 2:1 ratio.

The fraction eluted by $C_2H_4Cl_2$-Me_2CO (15:1) afforded crude crystals of the recovered 17α-hydroxyprogesterone. The fraction eluted by $C_2H_4Cl_2$-Me_2CO (8:1) and (5:1) afforded 300 mg. of crude crystals which were repeatedly recrystallized from MeOH to (I), m.p. 237~240°; $[\alpha]_D$ +15° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 73.00; H, 8.45. UV: λ_{max}^{MeOH} 236 mμ (ε 12,200). IR ν_{max}^{KBr} cm^{-1}: 3371 (OH), 1710 (20-CO), 1665, 1625 (Δ^4-3-CO).

6-Monoacetate of (I): Acetylation of 50 mg. of (I) with 1 cc. each of Ac_2O and pyridine afforded a

monoacetate of m.p. 188~192°; $[\alpha]_D$ +7°($CHCl_3$). *Anal.* Calcd. for $C_{23}H_{32}O_5$: C, 71.10; H, 8.30. Found: C, 70.05; H, 8.21. UV: λ_{max}^{MeOH} 237 mμ (ε 12,300). IR ν_{max}^{KBr} cm^{-1}: 3370 (OH), 1740 (CO in AcO), 1700 (20-CO), 1670, 1623 (Δ^4-3-CO).

Reduction of (I) with Zn and AcOH—A solution of 100 mg. of (I) dissolved in 5 cc. of AcOH, added with 200 mg. of Zn dust, was stirred for 20 min. at room temperature, the reaction mixture was concentrated. The residue was diluted with 10 cc. of water, extracted with CH_2Cl_2, and the extract was evaporated. Recrystallization of the residue from MeOH gave 17α-hydroxyprogesterone, m.p. 115~117°, undepressed on admixture with an authentic specimen.

Oxidation of (I) with Chromium Trioxide—To a solution of 150 mg. of (I) dissolved in 5 cc. of AcOH, 3 cc. of AcOH solution of 35 mg. of CrO_3 was added in small portions with a small quantity of water, the mixture was allowed to stand with occasional shaking for 3 hr., 5 cc. of MeOH was added to it, and the solution was evaporated in reduced pressure. The residue was diluted with 20 cc. of water and extracted with Et_2O. The extract was washed with 5% $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and evaporated in reduced pressure. The residue was recrystallized from Me_2CO and MeOH, and afforded 17α-hydroxypregn-4-ene-3,6,20-trione, m.p. 240~245°; $[\alpha]_D$ −56° ($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{28}O_4$: C, 73.22; H, 8.19. Found: C, 72.88; H, 8.11.

Hydroxylation of Deoxycorticosterone by *Botrytis cinerea*—The same fermentation as above was carried out with 1 g. of deoxycorticosterone as the substrate and extraction of the culture liquid afforded 1.3 g. of a concentrate. This concentrate was dissolved in 100 cc. of $C_2H_4Cl_2$ and chromatographed through a column of Florisil as in the foregoing example.

The fraction eluted by $C_2H_4Cl_2$-Me_2CO (8:1) afforded 300 mg. of 6β,21-dihydroxypregn-4-ene-3,20-dione (II) which, after recrystallization from Me_2CO, melted at 198~205°; $[\alpha]_D$ +96°($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{20}O_4$: C, 72.80; H, 8.73. Found: C, 72.60; H, 8.91. UV: λ_{max}^{MeOH} 236 mμ (ε 13,900). IR ν_{max}^{KBr} cm^{-1}: 3330 (OH), 1710 (20-CO), 1668, 1620 (Δ^4-3-CO).

The fractions eluted by 3:1 and 2:1 mixture of $C_2H_4Cl_2$-Me_2CO afforded 100 mg. of crude crystals of 15β,21-dihydroxypregn-4-ene-3,20-dione (III) which, after recrystallization from Me_2CO, melted at 202~209°; $[\alpha]_D$ +147°($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.30; H, 8.69. UV: λ_{max}^{MeOH} 240 mμ (ε 16,300). IR ν_{max}^{KBr} cm^{-1}: 3430 (OH), 1712 (20-CO), 1650, 1620 (Δ^4-3-CO).

6,21-Diacetate of (II): Acetylation of 50 mg. of (II) with 2 cc. each of Ac_2O and pyridine was carried out in the usual manner. The reaction mixture was diluted with cold water, the precipitate formed was dissolved in AcOEt, Et_2O was added to the solution, and the mixture was allowed to stand over night, from which 40 mg. of the 6,21-diacetate of (II) was obtained as crystals of m.p. 127~130°; $[\alpha]_D$ +97°($CHCl_3$). *Anal.* Calcd. for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96. Found: C, 70.10; H, 7.45.

15,21-Diacetate of (III): (III) was acetylated in the usual manner with Ac_2O and pyridine, and 15,21-diacetate was obtained as crystals of m.p. 182~186°; $[\alpha]_D$ +50°($CHCl_3$). *Anal.* Calcd. for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96. Found: C, 69.78; H, 7.76.

Reduction of (II) with Zn and AcOH—A solution of 50 mg. of (II) dissolved in 3 cc. of AcOH, added with 100 mg. of Zn dust, was stirred for 20 min., the mixture was filtered, and the filtrate was evaporated in reduced pressure. The residue was diluted with 20 cc. of water, extracted with CH_2Cl_2, and the extract was washed with $NaHCO_3$ solution and water. After drying over anhyd. Na_2SO_4, the solvent was evaporated from the extract and 40 mg. of a dried residue was obtained. This residue was dissolved in 1 cc. of MeOH and the solution was applied as a band on a filter paper for paper chromatography. After developing for 2 hr., the filter paper was dried in air and a portion of the paper showing smaller polarity than (II) under irradiation of ultraviolet ray was cut out. This was eluted with MeOH and the residue therefrom was repeatedly recrystallized from Me_2CO, affording 20 mg. of crystals of m.p. 140~143°; $[\alpha]_D$ +172°($CHCl_3$). This substance showed no depression of m.p. on admixture with deoxycorticosterone and infrared spectra of the two were identical.

Hydroxylation of Reichstein's Compound S by *Botrytis cinerea*—The same fermentation as above was carried out with 1 g. of the compound S as the substrate and 1.8 g. of the concentrate was obtained. This was chromatographed through Florisil column by the usual procedure and the initial fraction afforded 600 mg. of crude crystals of 6β,17α,21-trihydroxypregn-4-ene-3,20-dione (IV). Repeated recrystallization from Me_2CO gave crystals melting at 228~235°; $[\alpha]_D$ +42°($CHCl_3$). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.50; H, 8.34. Found: C, 69.21; H, 8.00. UV: λ_{max}^{MeOH} 236.5 mμ (ε 13,600). IR ν_{max}^{KBr} cm^{-1}: 3435 (OH), 1710 (20−CO), 1660, 1615 (Δ^4-3-CO).

6,21-Diacetate of (IV): Usual acetylation of 50 mg. of (IV) with Ac_2O and pyridine gave 40 mg. of 6,21-diacetate, m.p. 182~187°; $[\alpha]_D$ +67°($CHCl_3$), after recrystallization from Me_2CO. *Anal.* Calcd. for $C_{25}H_{34}O_7$: C, 67.24; H, 7.68. Found: C, 67.11; H, 8.02.

The second fraction afforded a small amount (20 mg.) of crystals (V) which were recrystallized from Me_2CO, m.p. 201~209°; $[\alpha]_D$ +156°(MeOH). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found: C, 69.20; H, 8.83. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1715 (20-CO), 1640, 1615 (Δ^4-3-CO).

(V) showed on depression of m.p. on admixture with 11β,17α,21-trihydroxypregn-4-ene-3,20-dione (hydrocortisone) and the infrared spectra of the two were identical.

Oxidation of (IV) with CrO_3—To a solution of 100 mg. of (IV) dissolved in 7 cc. of AcOH, 5 cc. of a solution of 95 mg. of CrO_3 dissolved in 10 cc. of AcOH was added and the mixture was allowed to stand at room temperature for 1 hr. with occasional shaking. The remainder of 5 cc. of the CrO_3-AcOH solution was then added and the mixture was again allowed to stand at room temperature over night with occasional shaking. The reaction mixture was evaporated in reduced pressure, the residue was diluted with water, and extracted repeatedly with Et_2O. The Et_2O extract was washed with $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and the solvent was evaporated in reduced pressure. The residue was recrystallized from EtOH and 13 mg. of 4-androstene-3,6,17-trione was obtained as crystals of m.p. 218~222°; $[\alpha]_D$ +41° ($CHCl_3$). *Anal.* Calcd. for $C_{19}H_{24}O_3$: C, 75.96; H, 8.05. Found : C, 76.11; H, 8.01.

Reduction of the Diacetate of (IV) with Zn and AcOH—A solution of 50 mg. of the diacetate of (IV) dissolved in 3 cc. of 90% AcOH, added with 100 mg. of Zn dust, was stirred for 30 min. at room temperature. The usual after treatment and repeated recrystallization of the concentrated residue afforded crystals of 21-acetate of the compound S, which showed no depression of m.p. on admixture with an authentic specimen and identical infrared spectrum.

Hydroxylation of Corticosterone by *Botrytis cinerea*—The same fermentation as above with 1 g. of corticosterone as the substrate afforded 1.7 g. of a concentrate. This concentrate was dissolved in $C_2H_4Cl_2$, the insoluble matter was collected by filtration, and repeatedly recrystallized from MeOH to 210 mg. of 11β,15β,21-trihydroxypregn-4-ene-3,20-dione (VI), m.p. 235~244° (decomp.); $[\alpha]_D$ +180° (pyridine). *Anal.* Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34. Found : C, 69.10; H, 8.55. UV : λ_{max}^{MeOH} 241.5 mμ (ε 16,500). IR ν_{max}^{KBr} cm^{-1} : 3400 (OH), 1705 (20-CO), 1650, 1615 (Δ^4-3-CO).

This substance was found to be identical, both in m.p. and infrared spectrum, with authentic sample of 11β,15β,21-trihydroxypregn-4-ene-3,20-dione, obtained by hydroxylation of a steroid with *Sclerotinia* sp. reported earlier.[9]

The supernatant of the foregoing $C_2H_4Cl_2$ solution was passed through a column of Florisil and fractional elution in the usual manner afforded 230 mg. of crude crystals of unreacted corticosterone from the initial eluate. The second eluate furnished from Me_2CO, 290 mg. of crude crystals of 15β, 21-dihydroxypregn-4-ene-3,11,20-trione (VII), which was repeatedly recrystallized from Me_2CO, m.p. 189~195°; $[\alpha]_D$ +180° (MeOH). *Anal.* Calcd. for $C_{21}H_{28}O_5$: C, 69.97; H, 7.83. Found : C, 70.25; H, 8.59. UV : λ_{max}^{MeOH} 237 mμ (ε 16,500). IR ν_{max}^{KBr} cm^{-1} : 3430 (OH), 1714 (11-CO), 1698 (20-CO), 1665, 1621 (Δ^4-3-CO).

15,21-Diacetate of (VII) : Usual acetylation of 50 mg. of (VII) with Ac_2O and pyridine at room temperature furnished 240 mg. of 15,21-diacetate of m.p. 195~198°; $[\alpha]_D$ +134° (MeOH). *Anal.* Calcd. for $C_{25}H_{32}O_7$: C, 69.56; H, 5.34. Found : C, 69.72; H, 8.21. IR ν_{max}^{KBr} cm^{-1} : 1730 (acetyl CO), 1715 (11,20-CO), 1670, 1615 (Δ^4-3-CO).

Acetylation of 11β,15β,21-Trihydroxypregn-4-ene-3,20-dione (VI) and Oxidation of Acetate of (VI)—The usual acetylation of (VI) with Ac_2O and pyridine afforded 15,21-diacetate of m.p. 200~205°; $[\alpha]_D$ +115° (MeOH). *Anal.* Calcd. for $C_{25}H_{34}O_7$: C, 67.24; H, 7.68. Found : C, 67.11; H, 7.48. IR ν_{max}^{KBr} cm^{-1} : 3430 (OH), 1735 (acetyl CO and 20-CO), 1650, 1620 (Δ^4-3-CO).

A solution of 100 mg. of this diacetate dissolved in 7 cc. of AcOH, added with 3 cc. of 90% AcOH containing 30 mg. of CrO_3, was allowed to stand for 5 hr. at room temperature, a small amount of MeOH was added, and the solution was concentrated. The residue was diluted with water and extracted with Et_2O. The extract was washed with $NaHCO_3$ solution and water, dried over anhyd. Na_2SO_4, and Et_2O was evaporated in reduced pressure. Recrystallization of the residue from Me_2CO afforded 33 mg. of crude crystals which were recrystallized from Me_2CO, m.p. 194~196°; $[\alpha]_D$ +130° (MeOH). IR ν_{max}^{KBr} cm^{-1} : 1730 (acetyl CO), 1715 (11,20-CO), 1670, 1615 (Δ^4-3-CO).

The Rf value of this diacetate on paper chromatogram was identical with that of the diacetate of (VII) and their identity was established through mixed m.p. and infrared spectrum.

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Summary

Application of *Botrytis cinerea* to 17α-hydroxyprogesterone, deoxycorticosterone, Reichstein's compound S, and corticosterone, as the substrate steroids, resulted in the formation of 6β-hydroxy derivative alone from 17α-hydroxyprogesterone and the compound S, 15β-hydroxy derivative alone from corticosterone, and both derivatives from deoxycorticosterone. These results indicated that this fungus possessed a marked substrate specificity. This fungus was also found to dehydrogenate the 11β-hydroxy group in corticosterone.

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