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Microbiological Transformation of Steroid. III.[1] Hydroxylation of Steroid by *Botrytis cinerea*.

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

6β-Hydroxylation of steroid by microörganisms is a reaction that has been found to occur in comparatively large number of microörganisms but this reaction is rarely effected by itself and is mostly accompanied by other reactions, especially 11α-hydroxylation.[2] The reaction is also known to be accompanied by 14α-[3], 17α-,[4] and 15α-hydroxylation.[5]

During examination of various fungi for their ability to oxidize steroids, it was found that *Botrytis cinerea* effected 6β-hydroxylation of Reichstein's compound S. In this case, 6β-hydroxylation occurs solely and only a trace of 11β-hydroxylation takes place. Application of this fungus to 17α-hydroxyprogesterone, deoxycorticosterone, and corticosterone showed that, whereas 6β-hydroxy compound was obtained from the former two steroids, 6β-hydroxy compound was not formed from corticosterone and 15β-hydroxy compound was obtained in its stead. It was thereby known that the action of *Botrytis cinerea* on steroid was mainly 6β-hydroxylation and that 15β- and 11β-hydroxylation was possible in some cases. Moreover, this fungus was found to have a marked substrate specificity in this reaction.

Botrytis cinerea was cultured by the usual method and applied to 17α-hydroxyprogesterone. The concentrated extract of the oxidation product thereby obtained was submitted to paper chromatography and the chromatogram revealed the presence, besides the spots of some substrate steroid, of a new spot with greater polarity. In order to separate these substances, isolation and purification were carried out with column chromatography using Florisil and the main oxidized steroid was obtained as crystals (I) of m.p. 237~240°. Elemental analysis showed this substance to be dihydroxyprogesterone, with introduction of one hydroxyl group into the original steroid. Acetylation of this product with acetic anhydride and pyridine in the usual manner gave a monoacetate, which showed that the newly introduced hydroxyl is primary or secondary.

Mild reduction of (I) with zinc dust in acetic acid resulted in liberation of the newly introduced hydroxyl group, which suggested that this hydroxyl must be in either 6- or 2-position.[6] The ultraviolet absorption maximum of (I) is at 236 mμ, which is in a shorter wavelength region than that (ca. 242 mμ) of the usual Δ^4-3-oxo-steroids, and the fact suggests that the hydroxyl is in 6β-position.

Oxidation of (I) with chromium trioxide in acetic acid in usual manner afforded a trioxosteroid of m.p. 240~245°, whose constants and other properties agreed with those of the known 17α-hydroxypregn-4-ene-3,6,20-trione.[7] The configuration of this hydroxyl in 6-position was assumed to be 6β since the difference in molecular rotation of ΔM_D −280 between (I) and 17α-hydroxyprogesterone is quite close to that of ΔM_D −252 between pro-

*1 Nishi-Shinagawa, Shinagawa-ku, Tokyo (白坂 亮).

1) Part II : This Bulletin, **9**, 59 (1961).
2) D. H. Peterson, *et al.* : J. Am. Chem. Soc., **74**, 5933 (1952); E. L. Dulany, *et al.* : Mycologia, **47**, 464 (1955); S. H. Epstein, *et al.* : J. Am. Chem. Soc., **75**, 408 (1953).
3) B. Camerino, *et al.* : Gazz. chim. ital., **83**, 684 (1953).
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5) A. Gubler, Ch. Tamm : Helv. Chim. Acta, **41**, 301 (1958).
6) F. Sondheimer, St. Kaufmann, J. Romo, H. Martinez, G. Rosenkranz : J. Am. Chem. Soc., **75**, 4712 (1957).
7) P. D. Meister, *et al.* : *Ibid.*, **75**, 416 (1953).

gesterone and the known 6β-hydroxyprogesterone.[8] The constants of (I) were in good agreement with those of 6β,17α-dihydroxyprogesterone obtained by Meister, *et al.*[7] using *Rhizopus* sp.

The same application of this fungus to deoxycorticosterone and paper chromatography of the concentrated extract of the oxidation product revealed two spots on the chromatogram, one of comparatively large polarity and the other of much larger polarity. The position of the spot with smaller polarity was almost the same as that of 14α,21-dihydroxypregn-4-ene-3,20-dione on paper chromatogram with a solvent system of propyleneglycol-toluene. Separation of these two oxidation products was effected by the above-mentioned column chromatography with Florisil and two crystalline substances were isolated, one of m.p. 198～205°(II) and the other of m.p. 202～209°(III). Both these substances were found to have one hydroxyl newly introduced in the deoxycorticosterone molecule. Since both products formed a diacetate by acetylation with acetic anhydride and pyridine, their hydroxyls are both primary or secondary.

The ultraviolet absorption maximum of (II) is in 236 mμ, which is in a shorter wavelength region than that (ca. 242 mμ) of ordinary Δ^4-3-oxo-steroids. From this and the fact that the newly introduced hydroxyl is removed by mild reduction, the newly introduced hydroxyl was thought to be in 6β-position, which was also endorsed by the similarity of the difference in molecular rotation of ΔM_D −264 between (II) and deoxycorticosterone with that of ΔM_D −252 between 6β-hydroxyprogesterone and progesterone, and of the foregoing ΔM_D −280 between 6β,17α-dihydroxy- and 17α-hydroxy-progesterone. Consequently, the structure of (II) was established as 6β,21-dihydroxypregn-4-ene-3,20-dione. The constants of (II) and its diacetate, as listed in Table I, agreed well with those of the samples obtained by Epstein and others[2] with the use of *Rhizopus* sp.

Table I. Comparison of Constants of 6β,21-Dihydroxyprogesterone and Compound (II)

Constant	6β,21-dihydroxypregn-4-ene-3,20-dione		Compound (II)	
	Free compd.	Diacetate	Free compd.	Diacetate
m.p. (°C)	198～202	127～129	198～205	127～130
$[\alpha]_D$ (in $CHCl_3$)	+101°	+103°	+96°	+97°
UV λ_{max} mμ (ε)	236(13,700)	(13,150)	236(13,900)	

The other crystals (III) of m.p. 202～209°, $[\alpha]_D$ +147°, with greater polarity showed the same constants and infrared spectrum as those of 15β,21-dihydroxypregn-4-ene-3,20-dione obtained by oxidation of deoxycorticosterone with *Sclerotinia libertiana* described in the preceding paper,[9] and their identity was established by mixed melting point. It follows that (III) was formed by hydroxylation of deoxycorticosterone at 15β-position.

The same application of this fungus to Reichstein's compound S (17α,21-dihydroxypregn-4-ene-3,20-dione) and chromatographic examination of its oxidation product revealed the presence of a spot of main oxidation product and another faint spot. Separation of these substances by Florisil column chromatography afforded a large amount of crystals (IV) of m.p. 228～235° and a minute amount of crystals (V) of m.p. 210～219°. Both were found by elemental analysis to have one newly introduced hydroxyl in compound S.

Usual acetylation of (IV) with acetic anhydride and pyridine gave a diacetate, which suggested that the newly introduced hydroxyl is primary or secondary. The ultraviolet absorption maximum of (IV) is at 236.5 mμ (ε 13,600) and reduction of this diacetate with zinc dust and acetic acid gave 21-acetate of compound S, from which the new hydroxyl is thought to be in 6β-position. Oxidation of (IV) with chromium trioxide gave androst-4-ene-3,6,17-trione[10] which showed that this hydroxyl is in 6β-position. Consequently, (IV) was

8) C. P. Balant, E. Ehrenstein : J. Org. Chem., **17**, 1587 (1952).
9) Part I : This Bulletin, **9**, 54 (1961).
10) A. Butenandt, B. Riegel : Ber., **69**, 1163 (1936).

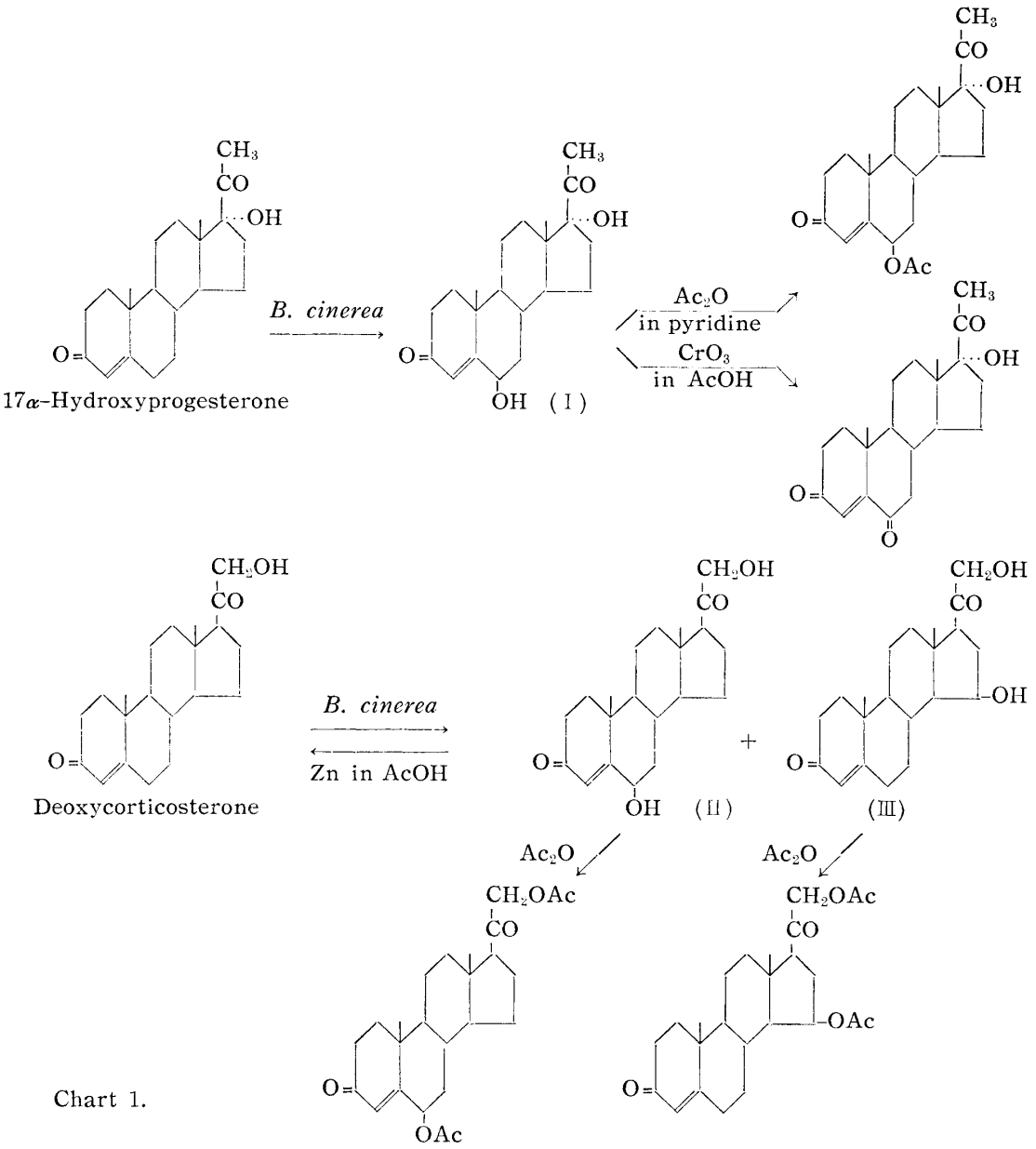

Chart 1.

established as 6β,17α,21-trihydroxypregn-4-ene-3,20-dione.

The usual acetylation of (V) with acetic anhydride and pyridine gave a monoacetate and the constants and infrared absorption of (V) and its monoacetate were in good agreement with those of hydrocortisone and its acetate, and there was no depression of the melting point on their respective admixture. Therefore, (V) was established as hydrocortisone.

These results revealed that this fungus also effected chiefly 6β-hydroxylation with compound S, as with other steroids, except that the formation of a 11β-hydroxy compound, i.e. hydrocortisone, in a minute amount, was observed in this case.

Finally, this fungus was applied to corticosterone and paper chromatographic examination of the concentrated extract showed the presence of two spots with greater polarity than that of corticosterone. This concentrate was dissolved in dichloroethylene, the insoluble matter produced was collected, and recrystallized from methanol to crystals (VI) of m.p. 235~244°(decomp.). This substance was found from elemental analysis to have one newly introduced hydroxyl in corticosterone. Acetylation of (VI) with acetic anhydride and pyridine afforded a diacetate, indicating that the newly introduced hydroxyl is primary or

secondary. The constants and infrared absorption of (VI) and its diacetate were identical with those of 11β,15β,21-trihydroxypregn-4-ene-3,20-dione and its 15,21-diacetate, obtained from corticosterone by similar oxidation with *Sclerotinia libertiana*.[1]

The supernatant of the foregoing dichloroethylene solution was submitted to Florisil column chromatography and unreacted corticosterone and crystals (VII) of m.p. 189~195° were obtained. Analytical values and formation of a diacetate showed that it had one newly introduced primary or secondary hydroxyl in corticosterone. Infrared spectrum of (VII) (in KBr) exhibited absorptions at 3430 (OH), 1714 (CO), 1694 (20-CO), 1665, 1621 (Δ^4-3-CO) cm^{-1}, and its ultraviolet absorption maximum (in methanol) was at 237 mμ, showing the presence of three ketone groups. Since its absorption maximum is in shorter wave-length region than that (ca. 242 mμ) of ordinary Δ^4-3-oxo-steroid, it was assumed that the 11β-hydroxyl had been oxidized to a ketone group.

Oxidation of the diacetate of (VI) with chromium trioxide in acetic acid gave an oxidation product whose constants and infrared spectrum were identical with those of the diacetate of (VII). It follows, therefore, that (VII) is 15β,21-dihydroxypregn-4-ene-3,11,20-trione.

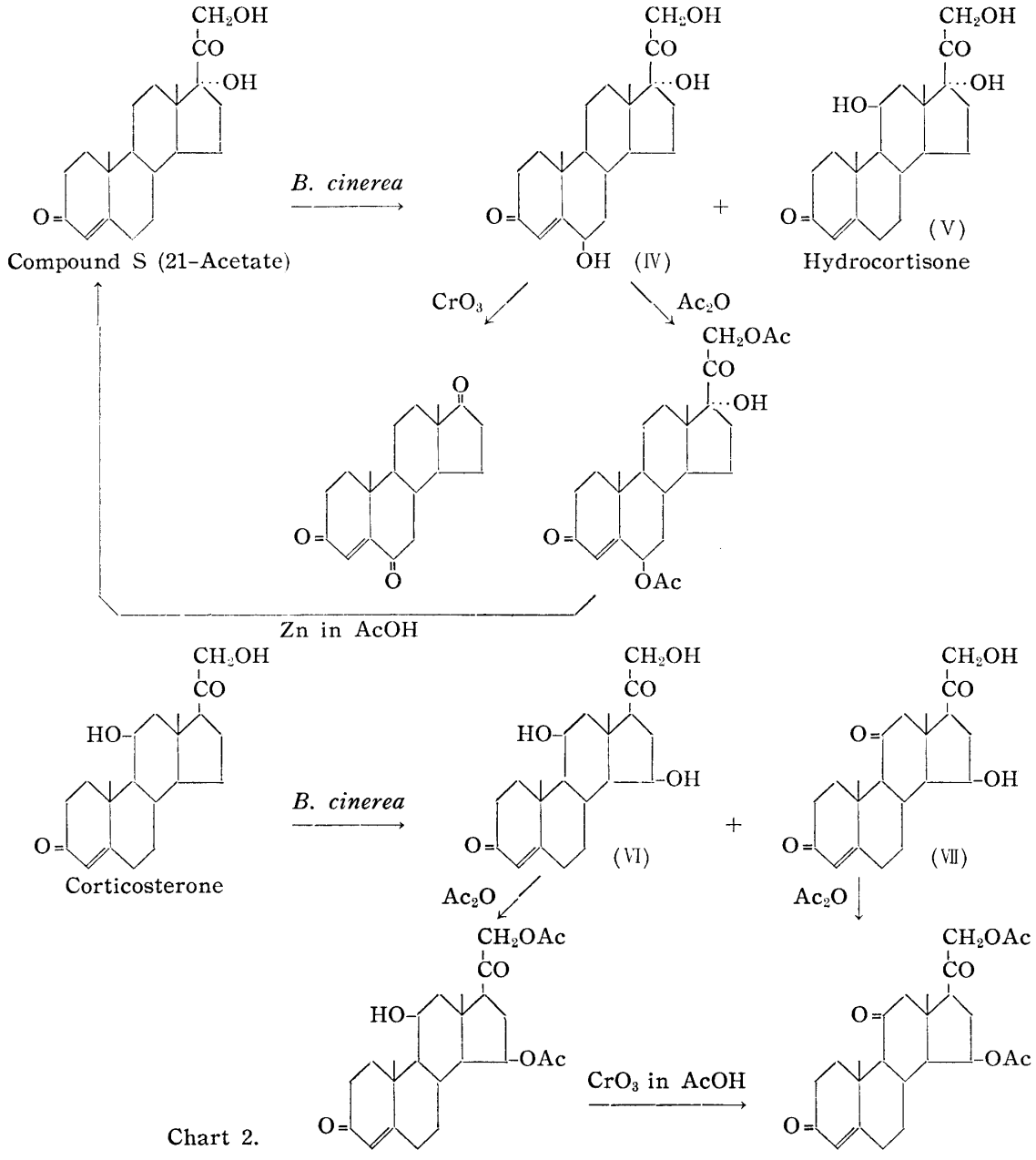

Chart 2.