

11. Makoto Shirasaka : Microbiological Transformation of Steroids. I.
Hydroxylation of Steroids by *Sclerotinia libertiana*.

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Among the hydroxylation of steroids by microorganism, oxidation of the 2 β -position is a reaction found comparatively recently and is one of rather rare reactions. Only the three strains of *Streptomyces* sp.,¹⁾ *Penicillium* sp.,²⁾ and *Rhizoctonia ferugena*³⁾ are known to effect this reaction, and only two kinds of 2 β -hydroxylated compounds have so far been obtained, from Reichstein's compound S and androstenedione. The 2 β -hydroxyl group in these steroids is comparatively unstable but it is interesting that this group has been introduced easily by a microorganism and this might be termed the distinctive feature of biological reaction.

During the course of examination on oxidative ability of various microorganisms to steroids, it was newly found that *Sclerotinia libertiana* produced 2 β -hydroxylated compound from Reichstein's compound S and this fungus was further tested on various steroids, from which corresponding 2 β -hydroxylated compounds were obtained. Of the steroids tested, results obtained with progesterone, 17 α -hydroxyprogesterone, and compound S have already been described.⁴⁾ In the present paper will be given the isolation and identification of oxidation products from deoxycorticosterone and corticosterone by this microorganism.

Sclerotinia libertiana was shake-cultured in a potato decoction medium with deoxycorticosterone as the substrate, but the growth of the fungus was found to be inhibited and oxidation of the steroid was extremely weak. This was considered to be due to a kind of toxicity of deoxycorticosterone to this fungus. The same culture was carried out with the monoacetate in place of free deoxycorticosterone. In this case, growth of the fungus was normal and oxidation was well effected. The fermentation liquor was then extracted and concentrated extract was examined by paper chromatography, from which one spot with an extremely large polarity was detected.

The concentrated extract was crystallized from acetone and crystals (I) of m.p. 210~219° were obtained. From its elemental analysis, this substance was found to be deoxycorticosterone with two hydroxyls introduced into it and formation of a triacetate by its treatment with acetic anhydride and pyridine showed that the two hydroxyls are both primary or secondary.

Reduction of (I) with zinc dust and acetic acid resulted in elimination of one hydroxyl to form dihydroxysteroid. The constants of this reduction product and its diacetate agreed well with those given for 15 β ,21-dihydroxypregn-4-ene-3,20-dione and its 15 β ,21-diacetate.⁵⁾ It follows, therefore, that one of the newly introduced hydroxyls in (I) is at 15 β -position. The other hydroxyl is easily eliminated by reduction with zinc dust and is primary or secondary, so that its position should be either 2 or 6.⁶⁾ The degree of optical rotation of (I) is -47° and the difference in molecular optical rotation from that of 15 β ,21-dihydroxypregn-4-ene-3,20-dione is -672° , showing a large minus value. Such a fact in-

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1) H. L. Herzog, *et al.* : J. Am. Chem. Soc., **79**, 3921 (1957).

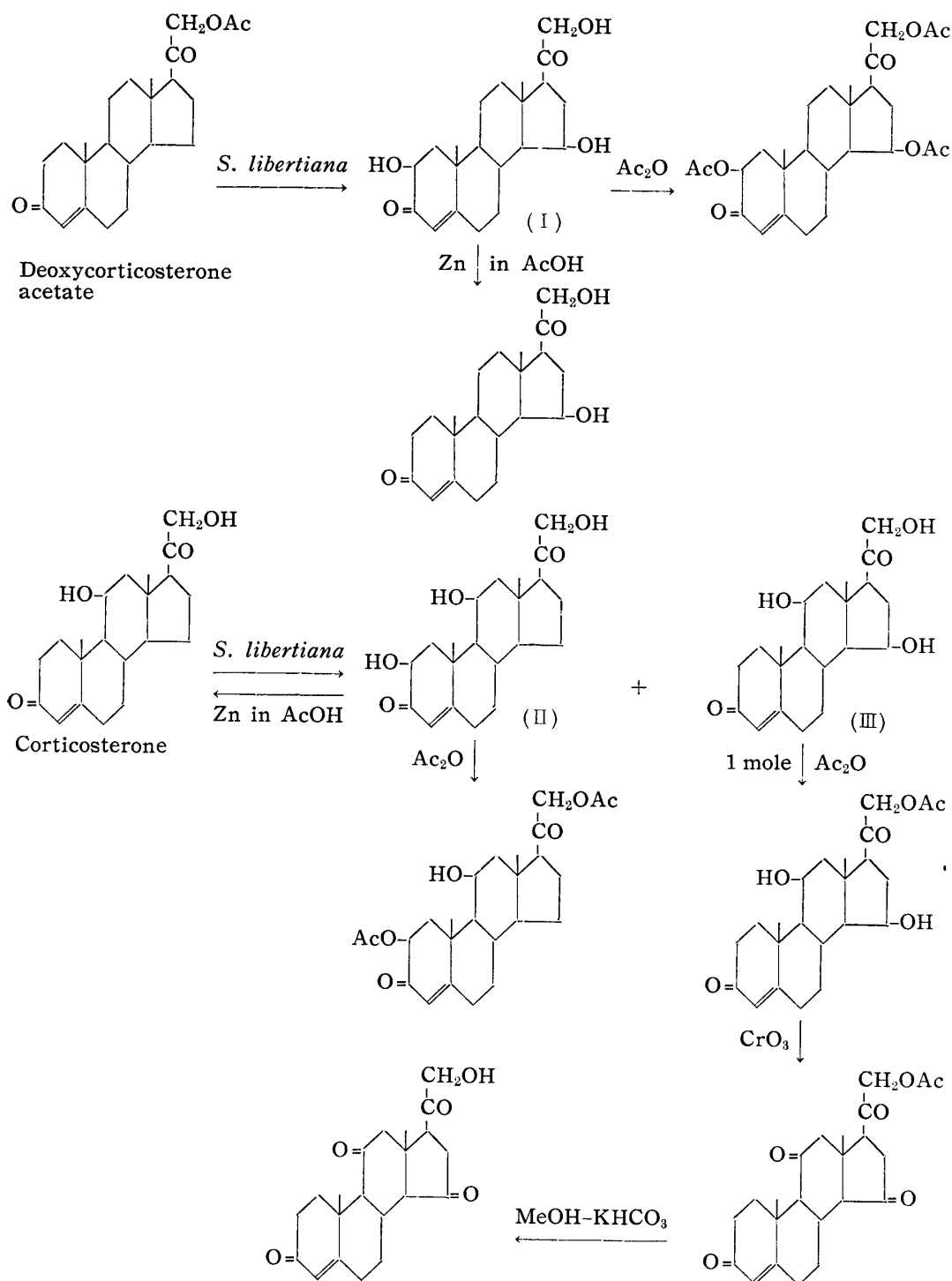
2) R. M. Dodson, *et al.* : *Ibid.*, **79**, 3921 (1957).

3) G. Grunspan, *et al.* : *Ibid.*, **79**, 3922 (1957).

4) H. Tanabe, R. Takahashi, R. Hayashi, M. Shirasaka : This Bulletin, **7**, 804 (1959).

5) A. Wettstein, *et al.* : *Experientia*, **11**, 465 (1955).

6) F. Sonderheimes, *et al.* : J. Am. Chem. Soc., **75**, 4712 (1953).



indicates that this hydroxyl is in 2 β -position and the structure of (I) is established as 2 β ,15 β ,21-trihydroxypregn-4-ene-3,20-dione. This is a new steroidal compound.

The same culture was carried out with corticosterone as the substrate and concentrated extract of the culture filtrate was examined by paper chromatography, from which two spots with greater polarity than corticosterone were detected. Separation of these substances by column chromatography using Florisil afforded crystals (II) of m.p. 183~186° from the initially eluted portion and crystals (III) of m.p. 225~230° from the next-eluted fraction. Analytical values showed that both are corticosterone derivatives with one newly introduced hydroxyl group and both formed a diacetate on acetylation with acetic

anhydride and pyridine, showing the hydroxyl to be primary or secondary in both compounds.

Reduction of (II) with zinc dust resulted in elimination of one hydroxyl to regenerate corticosterone and this new hydroxyl group was considered to be in 2- or 6-position.⁶⁾ The optical rotation of (II) is -7° and the difference in molecular rotation from that of corticosterone is -755° , showing a great negative value, so that the newly introduced hydroxyl in (II) must be in 2 β -position.¹⁾ Consequently, the structure of (II) is determined as 2 β ,11 β ,21-trihydroxypregn-4-ene-3,20-dione.

Acetylation of (III) with equivalence of acetic anhydride and pyridine, oxidation of its product with chromium trioxide, and hydrolysis of its product with hydrous methanol containing potassium hydrogencarbonate afforded a tetraoxo compound. Its infrared spectrum showed the absorption at 1750 cm^{-1} for a five-membered ring ketone, so that the newly introduced hydroxyl in (III) must be at 15- or 16-position, but since (III) is negative to the Porter-Silber reaction,⁷⁾ the hydroxyl could not be in 16-position. Configuration of this hydroxyl was assumed to be 15 β from the value of -112° in molecular rotational difference from that of corticosterone, as shown in Table I. These facts suggest that the structure of (III) is 11 β ,15 β ,21-trihydroxy-4-pregnene-3,20-dione. Both (II) and (III) are new steroidal compounds.

TABLE I. Molecular Rotatory Power of 15 α - and 15 β -Hydroxylated Steroids

	M_D	$M_D^{15\beta\text{-OH}}$	$\Delta M_D^{15\beta\text{-OH}}$	$M_D^{15\alpha\text{-OH}}$	$\Delta M_D^{15\alpha\text{-OH}}$
Reichstein's compound S ^{a)}	+395	+348	-47	+529	+130
Deoxycorticosterone ^{b)}	+613	+490	-123	+679	+66
Progesterone ^{c)}	+605	+499	-106	+724	+119

a) S. Bernstein, *et al.*: Chem. & Ind. (London), **1956**, 111.

b) A. Wettstein, *et al.*: *Experientia*, **11**, 465(1955).

c) J. Fried, *et al.*: U.S. Pat. 2,753,290.

Thus, 2 β - and 15 β -hydroxylated compounds were obtained from deoxycorticosterone and corticosterone by the use of *Sclerotinia libertiana* and, considering the results obtained with progesterone, 17 α -hydroxyprogesterone, and compound S as reported previously,⁴⁾ this fungus seems to have the ability to chiefly effect 2 β - and 15 β -hydroxylation, as indicated in Table II. However, in the case of substrate steroids having 17 α -hydroxyl, as

TABLE II. Position of Oxidation of Various Substrate Steroids by *Sclerotinia libertiana*

Substrate steroid	Position of oxidation
Progesterone	2 β , 15 β
17 α -Hydroxyprogesterone	2 β , 11 α , (15 β)
Deoxycorticosterone	2 β , 15 β
Reichstein's compd. S	2 β , (11 α), (11 β)
Corticosterone	2 β , 15 β

Numbers in parentheses indicate that extremely minute amount of oxidation product had been obtained.

17 α -hydroxyprogesterone and compound S, 2 β -hydroxylated compound was chiefly obtained and formation of 15 β -hydroxyl compound was practically nil. On the other hand, 2 β ,15 β -dihydroxyl compound or 2 β - and 15 β -hydroxyl compounds were obtained from steroids not having 17 α -hydroxyl group, such as progesterone, deoxycorticosterone, and corticosterone. It seems, therefore, that 15 β -hydroxylation of this fungus is inhibited by the presence of 17 α -hydroxyl group in the substrate steroids.

When corticosterone is used as the substrate, the amount of 2 β -hydroxylated compound formed is somewhat smaller than that of 15 β -hydroxylated compound and this

7) C. C. Porter, R. H. Silber: *J. Biol. Chem.*, **185**, 201 (1950); **210**, 923 (1951).

suggests that 2β -hydroxylation is inhibited to some extent by the presence of 11β -hydroxyl in corticosterone. In any case, *Sclerotinia libertiana* has a marked substrate specificity in the oxidation of steroids. This fungus was also found to have a kind of esterase activity.

Experimental

Fermentation and Extraction—Potato decoction containing 3% of glucose was poured into 500-cc. flasks, 100 cc. to each flask, and sterilized at 120° for 25 min. After cool, *Sclerotinia libertiana* was inoculated in this medium and shake cultured at 27° for around 48 hr. The substrate steroid was added to the flask as a 2.5% MeOH solution, 2 cc. (50 mg. as the steroid) of this solution to each flask. This was again shake cultured for 48~72 hr. and the culture was filtered to be separated into cells and liquid. The cells were extracted first with Me_2CO and then with AcOEt, and the extracts were combined with the filtrate. The filtrate was extracted twice more with AcOEt. The combined extract was washed twice with $1/5$ volume of 2% NaHCO_3 and water, dried over anhyd. Na_2SO_4 , and evaporated in reduced pressure.

Paper Chromatography—A modification of Zaffroni's method⁸⁾ was used. A mixture of propylene glycol and MeOH (1:2) was used as the stationary phase and a mixture of toluene and dioxane (78:22) as the developing solvent, using the descending method. With these conditions, the developing solvent front reached the tip of the filter paper (ca. 30 cm. from the original point) in about 2 hr. The development was therefore allowed to proceed for ca. 2~4 hr. Detection of steroidal spots on the filter paper was made by ultraviolet absorption for Δ^4 -3-keto-steroids. For other keto-steroids, 0.15% EtOH solution of 2,4-dinitrophenylhydrazine was sprayed to detect colored spots. For steroids possessing α -ketol, a 2:1 mixture of 0.5% aqueous solution of triphenyltetrazolium chloride and 10% NaOH solution was sprayed to detect the colored spot (pink).

Hydroxylation of Deoxycorticosterone Acetate—*Sclerotinia libertiana* was cultured as described above, with 5 g. of deoxycorticosterone 21-acetate as the substrate. The resulting 4 g. of the concentrate was dissolved in Me_2CO and 1.1 g. of crude crystals was obtained. Recrystallization from Me_2CO -Et₂O afforded the crystals of $2\beta,15\beta,21$ -trihydroxypregn-4-ene-3,20-dione (I), m.p. $210\sim 219^\circ$; $[\alpha]_D -47^\circ$ (MeOH). Anal. Calcd. for $\text{C}_{21}\text{H}_{30}\text{O}_5$: C, 69.58; H, 8.34. Found: C, 70.14; H, 8.73. UV: $\lambda_{\text{max}}^{\text{MeOH}}$ 242.5 μ (ϵ 15,000). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3470 (OH), 1690 (20-CO), 1673, 1620 (Δ^4 -3-CO). $2,15,21$ -Triacetate of (I): Acetylation of (I) with Ac_2O and pyridine afforded the triacetate of m.p. $153\sim 156^\circ$; $[\alpha]_D -16^\circ$ (MeOH). Anal. Calcd. for $\text{C}_{27}\text{H}_{36}\text{O}_8$: C, 66.37; H, 7.40. Found: C, 66.40; H, 7.43.

Reduction of (I) with Zinc and Acetic Acid—A solution of 200 mg. of (I) dissolved in 6 cc. of AcOH, added with 500 mg. of Zn dust and 1 cc. of H_2O , was stirred for 30 min. at room temperature, Zn dust was filtered off, and the filtrate was concentrated to a small volume. The residue was diluted with H_2O , extracted with CH_2Cl_2 , the extract was washed with H_2O , dried over anhyd. Na_2SO_4 , and the solvent was evaporated. The residue was dissolved in a small amount of $\text{C}_2\text{H}_4\text{Cl}_2$ and purified through a column chromatography over Florisil, from which 170 mg. of crystals, m.p. $200\sim 215^\circ$, was obtained. $[\alpha]_D +145^\circ$ (MeOH). Anal. Calcd. for $\text{C}_{11}\text{H}_{20}\text{O}_4$: C, 72.80; H, 8.72. Found: C, 72.92; H, 8.68. UV: $\lambda_{\text{max}}^{\text{EtOH}}$ 242 μ (ϵ 17,000). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3430 (OH), 1715 (20-CO), 1650, 1620 (Δ^4 -3-CO).

Acetylation of this substance with Ac_2O and pyridine in the usual manner gave a diacetate, m.p. $183\sim 187^\circ$, $[\alpha]_D +53^\circ$ (MeOH). Anal. Calcd. for $\text{C}_{25}\text{H}_{34}\text{O}_6$: C, 69.74; H, 7.96. Found: C, 69.66; H, 7.92.

These constants agreed with the constants of the known $15\beta,21$ -dihydroxypregn-4-ene-3,20-dione and its $15,21$ -diacetate.

Hydroxylation of Corticosterone—*Sclerotinia libertiana* was cultured as described above, with 1 g. of corticosterone as the substrate, and 1.8 g. of a concentrate was obtained. This concentrate was dissolved in $\text{C}_2\text{H}_4\text{Cl}_2$ and passed through a column of 80 g. of Florisil. The column was eluted consecutively with various mixtures of $\text{C}_2\text{H}_4\text{Cl}_2$ and Me_2CO . Some unreacted corticosterone was recovered from the initial eluate. The solvent was evaporated from the following effluent and the residue was recrystallized from Me_2CO to 200 mg. of crude crystals of $2\beta,11\beta,21$ -trihydroxypregn-4-ene-3,20-dione (II). Further purification by recrystallization from MeOH raised the m.p. to $183\sim 186^\circ$; $[\alpha]_D -7^\circ$ (MeOH). Anal. Calcd. for $\text{C}_{21}\text{H}_{30}\text{O}_5$: C, 67.24; H, 7.68. Found: C, 69.51; H, 8.30. UV: $\lambda_{\text{max}}^{\text{MeOH}}$ 243.5 μ (ϵ 15,100). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3500, 3300 (OH), 1690 (3,20-dione), 1625 (Δ^4).

$2,21$ -Diacetate of (II): Acetylation of (II) with Ac_2O and pyridine in the usual manner afforded a diacetate, m.p. $146\sim 148^\circ$; $[\alpha]_D -2^\circ$ (MeOH). Anal. Calcd. for $\text{C}_{25}\text{H}_{36}\text{O}_7$: C, 67.24; H, 7.68. Found: C, 67.30; H, 7.72. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3480 (OH), 1740 (AcO-CO), 1690 (3,20-dione), 1625 (Δ^4).

8) A. Zaffaroni, *et al.*: J. Biol. Chem., **193**, 749 (1951).

From the final eluate, recrystallization from Me_2CO gave $11\beta,15\beta,21$ -trihydroxypregn-4-ene-3,20-dione (III), m.p. $225\sim 230^\circ$; $[\alpha]_D +180^\circ$ (pyridine). *Anal.* Calcd. for $\text{C}_{21}\text{H}_{30}\text{O}_5$: C, 69.58; H, 8.34. Found: C, 69.80; H, 8.98. UV: $\lambda_{\text{max}}^{\text{MeOH}}$ 241.5 $\text{m}\mu$ (ϵ 17,500). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3350 (OH), 1702 (20-one), 1650, 1615 (Δ^4 -3-CO).

15,21-Diacetate of (III): Acetylation of (III) with Ac_2O and pyridine in the usual manner gave a diacetate, m.p. $202\sim 210^\circ$; $[\alpha]_D +115^\circ$ (MeOH). *Anal.* Calcd. for $\text{C}_{25}\text{H}_{34}\text{O}_7$: C, 67.24; H, 7.68. Found: C, 67.82; H, 8.32.

21-Hydroxypregn-4-ene-3,11,15,20-tetraone from (III)—(III) was acetylated in the usual manner with Ac_2O and pyridine, and 100 mg. of crude crystals of 21-monoacetate so obtained was dissolved in 2 cc. of AcOH. To this solution, 2.0 cc. of AcOH containing 20 mg. of CrO_3 was added and the mixture was allowed to stand for 8 hr. at room temperature. The solution was concentrated, diluted with H_2O , and extracted with CH_2Cl_2 . The extract was washed with NaHCO_3 solution and H_2O , dried over anhyd. Na_2SO_4 , and the solvent was evaporated. The residue was washed with a small quantity of Me_2CO , dissolved in hydr. MeOH containing 170 mg. of KHCO_3 with application of heat, and the solution was allowed to stand over night. MeOH was evaporated from this solution, the residue was extracted with CH_2Cl_2 , and the extract was evaporated. The residue was recrystallized from Me_2CO to 20 mg. of the tetraketo-steroid which was found to be 21-hydroxypregn-4-ene-3,11,15,20-tetraone, m.p. $220\sim 225^\circ$. *Anal.* Calcd. for $\text{C}_{21}\text{H}_{26}\text{O}_5$: C, 70.37; H, 7.31. Found: C, 70.11; H, 7.52. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3400 (OH), 1750 (five-membered ring ketone), 1730 (11-CO), 1715 (20-CO), 1645, 1620 (Δ^4 -3-CO).

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Summary

Hydroxylation products of deoxycorticosterone and corticosterone were obtained by the fermentation with *Sclerotinia libertiana*, $2\beta,15\beta$ -dihydroxy derivative was obtained from deoxycorticosterone acetate, and 2β - and 15β -monohydroxy derivatives were obtained from corticosterone. These products are all new steroidal compounds.

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