[Chem. Pharm. Bull. 17(10)2031—2035(1969)]

UDC 547.92.04 : 576.343

Microbial Transformation of Steroid. I. Microbiological Hydroxylation of Diosgenin

Koh Kaneko, Hiroshi Mitsuhashi, and Kohichiro Hirayama

Faculty of Pharmaceutical Sciences, Hokkaido University[1]

(Received January 24, 1969)

Microbial oxidation of diosgenin with *Cunninghamella blakesleeana* gave 7β-hydroxydiosgenin, 7β,12β-dihydroxydiosgenin, and 7β,11α-dihydroxydisogenin. The former two compounds were confirmed by their synthesis from 7-oxodiosgenin and 7,12-dioxodiosgenin (=7-oxogentrogenin)respectively with lithium aluminum hydride.

The microbial hydroxylation of steroids is the most useful technique for the introduction of a hydroxyl group into the steroid molecules, especially for C_{19} and C_{21} steroids and cardiac lactones.

As regards the hydroxylation of steroidal sapogenins, Mininger, *et al.*,[2] attempted the hydroxylation of natural sapogenins with several microörganisms and concluded that the steroidal sapogenins are not readily hydroxylated. Sato, *et al.* extended the microbial technique to the hydroxylation of steroidal alkaloids[3a] and sapogenins.[3b] Kondo[4] observed, using microörganisms, the breaking of carbon chain of diosgenin.

In this paper, we report the oxidation of diosgenin (I) with *Cunninghamella blakesleeana* (+), which resulted in the formation of 7β-hydroxydiosgenin (II), 7β,11α-dihydroxydiosgenin (III), and 7β,12β-dihydroxydiosgenin (IVa).

The product (II) has mp 216—217°, $[\alpha]_D^{15}$ −76°, and was identified as monohydroxydiosgenin, *m/e* 430 (M^+) by mass spectrum.

The result of infrared spectral examination showed the characteristic absorptions of isospirostan derivative, IR cm^{-1}: 866, 900, 922, 986, (isospirostan).

By oxidation with manganese dioxide in chloroform at room temperature, the product (II) readily converted into α,β-unsaturated carbonyl derivative ultraviolet (UV) absorption 235 mμ (log ε 4.2). This spectroscopic datum corresponded with the theoretical wave length and intensity for 5-en-7-one.

Djerassi, *et al.*, previously[5] reported the fragmentation of steroidal sapogenins by mass spectrum.

The fragmentation pattern of the product (II) is shown in Table I. Each fragment ion of the product (II) was in good agreement with each fragment ion of diosgenin plus 16 (*m/e* 430). This result shows that the microbially generated hydroxyl group is probably located on the ring A,B,C, or D. From the result of allylic oxidation with manganese dioxide and mass spectrum of the product (II), it is most reasonably assumed that the new hydroxyl

1) Location: *Kita-12-jo Nishi-15-chome, Sapporo, Hokkaido.*
2) R.F. Mininger, M.E. Wall, R.G. Dworshack, and R.W. Jackson, *Arch. Biochem. Biophys.*, **60**, 427 (1956).
3) *a*) Y. Sato and S. Hayakawa, *J. Org. Chem.*, **28**, 2739 (1963); *idem*, *ibid.*, **29**, 198 (1964); Y. Sato, J.A. Waters, and H. Kaneko, *ibid.*, **29**, 3732 (1964); *b*) Y. Sato and S. Hayakawa, *J. Org. Chem.*, **28**, 2742 (1963).
4) E. Kondo and T. Mitsugi, *J. Am. Chem. Soc.*, **88**, 4737 (1967).
5) H. Budzikiewicz, J.M. Wilson, and C. Djerassi, *Monatsh.*, **93**, 1033 (1962); H. Budzikiewicz, C. Djerassi, and D.H. Williams, "Structure Elucidation of Natural Products by Mass Spectroscopy," Volume II, Holden-Day, San Francisco, 1964.

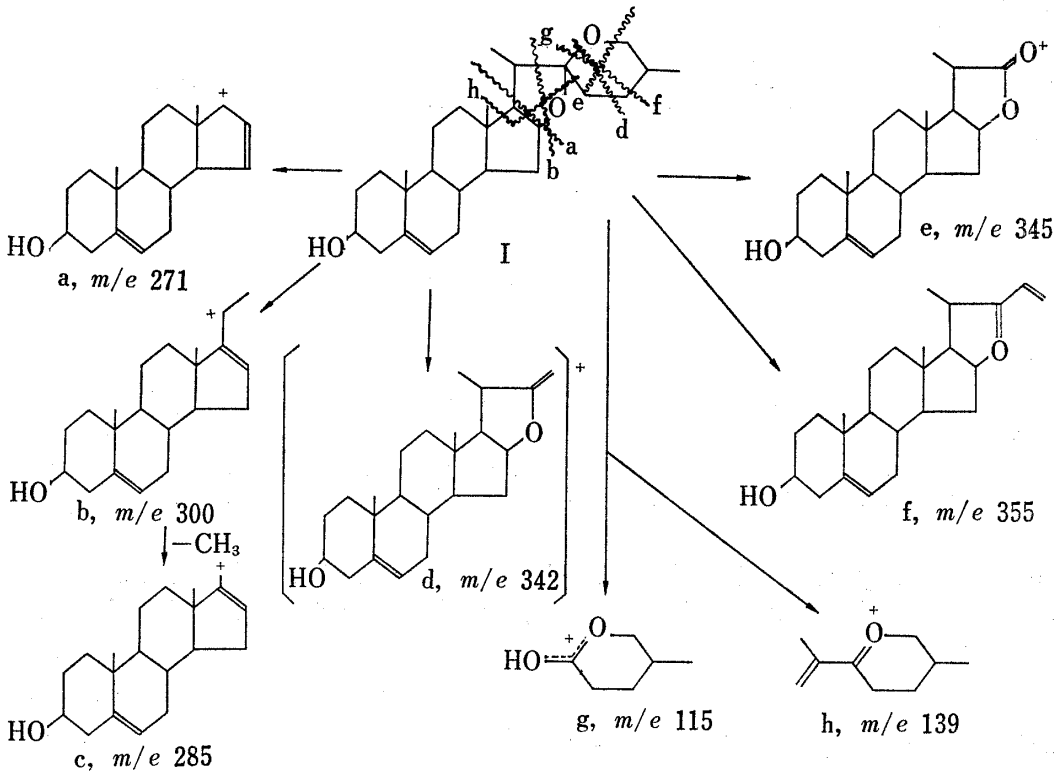


Chart 1

group was introduced into C-7 in diosgenin. 7β-Hydroxydiosgenin was prepared according to the procedure of Djerassi.[6] The infrared (IR) spectra of the product (II) is completely identical with authentic 7β-hydroxydiosgenin. A mixed melting point with the authentic sample and the product (II) showed no depression.

The product (III) has mp 264—266°, $[\alpha]_D^{20}$ −46.0°, and was identified as dihydroxydiosgenin by mass spectrum, *m/e* 446 (M^+). The IR spectra suggested an isospirostan derivative. By oxidation with manganese dioxide in chloroform, the product (III) is readily converted into an α,β-unsaturated carbonyl derivative, UV absorption 235 mμ. The product (III) formed a triacetate with acetic anhydride–pyridine at room temperature. The IR and nuclear magnetic resonance (NMR) spectroscopic data of the acetate showed a triacetate group.

The fragmentation of products (III) and (IVa) by mass spectrum is shown in Table I. The fragment ions of III and IVa are identical with the fragmentation pattern of diosgenin plus two oxygens.

From the result of the fragment ion (a″—h″) Table I shows that the newly introduced hydroxyl groups are located on the rings A, B, C, and D. Also the fragment ion (a″, g″, h″) shows that the hydroxyl groups are located on the ring A, B and C. The fragment ion a″-$3H_2O$ is observed. It is assumed that the C-11 of C-12 on the ring C is the possible position for another hydroxyl group.

7β,11α-Dihydroxydiosgenin (III) was obtained by Sato[3b] from diosgenin with the fungus *Helicostylum piriforme*. The IR spectrum of III is completely identical with that of authentic 7β,11α-dihydroxydiosgenin and mixed melting point of III with the authentic sample was not depressed.

The product IVa melted at 226—229°, $[\alpha]_D^{15}$ −63°, and was identified as dihydroxydiosgenin by mass spectrum, *m/e* 446 (M^+). The IR spectrum suggested an isospirostan deri-

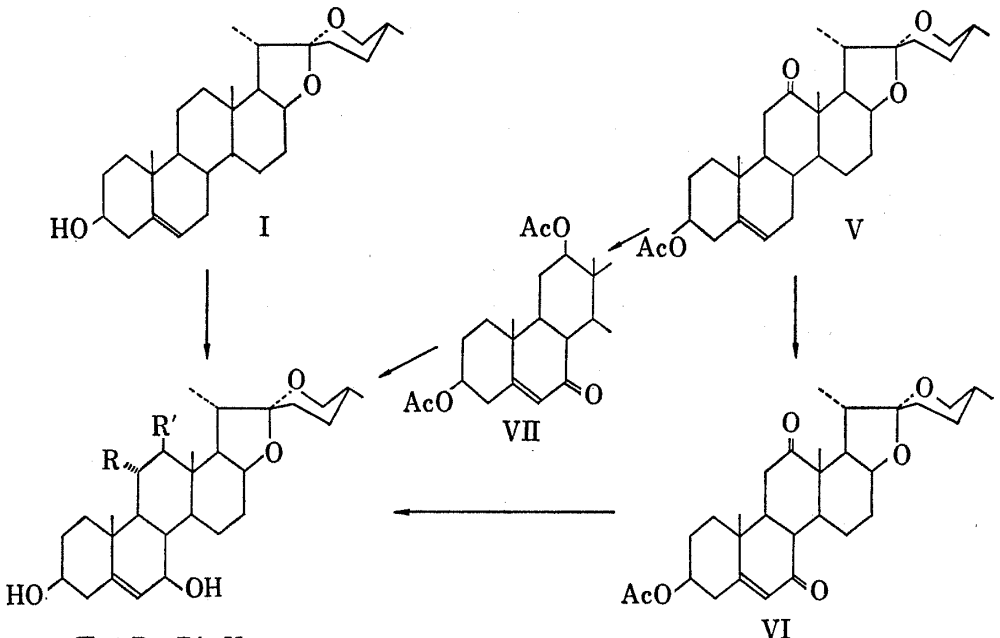
6) H.J. Ringold, G. Rosenkranz, and C. Djerassi, *J. Am. Chem. Soc.*, **74**, 3318 (1952).

Table I

Diosgenin *m/e*	Product $(OH)_n$	Observed fragment ion of the product I, II and III
a, 271	$(OH)_1$	a′,a′–H_2O,a′–$2H_2O$
	$(OH)_2$	a″,a″–$2H_2O$,a″–$3H_2O$
b, 300	$(OH)_1$	b′,b′–H_2O,b′–$2H_2O$
	$(OH)_2$	b″,b″–$2H_2O$,b″–$3H_2O$
c, 285	$(OH)_1$	c′,c′–H_2O,c′–$2H_2O$
	$(OH)_2$	c″,c″–H_2O,c″–$3H_2O$
d, 342	$(OH)_1$	d′,d′–H_2O,d′–$2H_2O$
	$(OH)_2$	d″,d″–H_2O,d″–$2H_2O$,d″–$3H_2O$
e, 345	$(OH)_1$	e′,e′–H_2O,e′–$2H_2O$
	$(OH)_2$	e″,e″–H_2O,e″–$2H_2O$,e″–$3H_2O$
f, 355	$(OH)_1$	f′,f′–H_2O,f′–$2H_2O$
	$(OH)_2$	f″,f″–H_2O,f″–$2H_2O$,f″–$3H_2O$
g, 115	$(OH)_1$	g, 115
	$(OH)_2$	g, 115
h, 139	$(OH)_1$	h, 139
	$(OH)_2$	h, 139
M^+ 414	$(OH)_1$	M^+, 430, M^+–H_2O,M^+–$2H_2O$
	$(OH)_2$	M^+, 446, M^+–H_2O,M^+–$2H_2O$

vative. Oxidation of IVa with manganese dioxide, as described previously, showed the spectra of an α,β-unsaturated carbonyl derivative (235 mμ). The product (IVa) was readily acetylated to a triacetate at room temperature. The IR spectrum of the acetate also showed no hydroxyl group in chloroform. As described above, mass spectrum of III was essentially idential with the product (III) (Table I).

From these data, it seems that another hydroxyl group might be located at C-12. To detect the configuration of the hydroxyl group at C-12 of the product (IVa), 7β,12β-dihydroxydiosgenin (IVa) was prepared from gentrogenin (12-oxodiosgenin), as follows: 7-Oxogentro-



II : R=R′=H
III : R=OH, R′=H
IVa : R=H, R′=OH

Chart 2

genin acetate (VI) was prepared from gentrogenin acetate (V) by the *t*-butyl chromate[7] oxidation.

7-Oxogentrogenin acetate was reduced with lithium aluminum hydride in ether without further purification. Four epimers, with the hydroxyl group located on their C-7 and C-12, were separated by thin-layer chromatography. It is commonly recognized that the ketonic group of 7,12-diketo-steroids is predominantly reduced to the equatorial hydroxyl group by lithium aluminum hydride.[6,9]

As shown in Table II, the main product was 7β,12β-dihydroxydiosgenin (IVa).

In order to assign the configuration of four epimers, the data of molecular rotation difference was applied according to the method of Fieser.[8] It had been reported that the molecular rotation difference of 7-hydroxy-diosgenin was +367° of ΔM_D in chloroform[6] [$\Delta M_D = [M]_D$(7β-hydroxydiosgenin)-(7α-hydroxydiosgenin)]. On the other hand the difference of the epimers of 12-hydroxydiosgenin was found to be −116 of ΔM_D in chloroform. The observed ΔM_D of four epimers are listed in Table II.

Table II.

4 Epimers	$[M]_D$	ΔM_D		Assign	Ratio %	$[\alpha]_D$ in $CHCl_3$	mp from MeOH–*n*-hexane
IVa	−280	−82		7β,12β	45	− 63.0°	226—229°
IVb	−198		+273	7β,12α	28	− 44.6°	159—163°
IVc	−553	−74	+281	7α,12β	19	−124°	amorphous
IVd	−479			7α,12α	8	− 71.0°	amorphous

These data show that the experimental values are quite comparable to the previously reported values. Furthermore, the configuration of the hydroxyl group at C-12 was determined by the following experiment. 12β-Hydroxydiosgenin was prepared by the reduction of gentrogenin acetate with lithium aluminum hydride according to the method of Okanishi,[9] and then acetylated with acetic anhydride-pyridine at room temperature. 7-Oxo-12β-hydroxydiosgenin diacetate (VII) was prepared by the oxidation of *t*-butyl chromate, without further purification. The product of this reduction showed a pair of epimers on thin-layer chromatography.

They corresponded to the pair of two epimers at C-7 from the four epimers obtained by the reduction of 7-oxogentrogenin acetate (VI) with lithium aluminum hydride, which were detected by molecular rotation.

The main product as described above is 7β,12β-dihydroxydiosgenin.

The IR spectrum of IVa is essentially identical with that of synthesized 7β,12β-dihydroxydiosgenin (IVa) and their mixed melting point was without depression.

Experimental[10]

Organisms——*Cunninghamella blakesleeana* (+) strain was maintained on Czapek-Dox glucose slant and grown in shaking bottles on liquid medium containing, per liter of water, glucose 50 g, $NaNO_3$ 20 g, K_2HPO_4 5 g, $MgSO_4 \cdot 7H_2O$ 1 g, corn steep liquor 20 g, and malt extract 20 g. After 2 days of shaking culture at 30°, 100 mg of diosgenin in 1.3 ml of EtOH per 100 ml of liquid medium was added. Then the mycelia were allowed to grow for further 7 days.

7) R.V. Oppenauer, H. Oberrauch, *Anales Asoc. Quim. Argentina*, **37**, 246 (1949) [*C.A.*, **44**, 3871 (1950)]; K. Heusler, A. Wettstein, *Helv. Chim. Acta*, **35**, 284 (1952).
8) L.F. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., Chapter 5, New York, 1959.
9) T. Okanishi, A. Akahori, and F. Yasuda, *Chem. Pharm. Bull.* (Tokyo), **10**, 1195 (1962).
10) Melting points were measured on Kofler block and are uncorrected.

Extraction of Products——Five liters of fermentative beer and the cultured mycelia, dried at 110° for 8 hr, were continuously extracted with CH_2Cl_2 from this extract yielded 5.3 g of residue. The extract showed six blue spots with $SbCl_3$ on thin-layer chromatogram.

Isolation of the Oxidative Products——The extract was submitted to column chromatography on alumina (150 g, neutral) and eluted successively with benzene, 5%, 10%, and 20% ether in benzene. The fraction eluted with benzene gave 1.5 g of a residue which was composed of diosgenin. The fractions eluted with 5% ether in benzene, gave 132 mg of a product (II) and showed blue color with $SbCl_3$ on thin-layer chromatography. After repeated chromatography involving column and thin-layer chromatography, it showed mp 216—217° from MeOH, $[\alpha]_D^{15°}$ −76° (c=1.0, $CHCl_3$). The second fraction of 5% ether in benzene, gave 68 mg of the product (III) blue color with $SbCl_3$ on thin-layer chromatogram. The fraction was applied to repeated thin-layer chromatography and yielded 10 mg of III crystals mp 263—266° from MeOH, $[\alpha]_D^{15°}$ −46.0° (c=1.0, $CHCl_3$).

The product (IVa) from the fractions of 20% ether in benzene was purified by repeated thin-layer chromatography, and crystallized from MeOH to afford 7 mg of IVa mp 226—229°, $[\alpha]_D^{15°}$ −63° (c=1.0, $CHCl_3$).

Manganese Dioxide Oxidation of the Products (II), (III), and (IVa)——One mg of each product was submitted to the following experiments by the method of Sato.[3b] 7β-Hydroxydiosgenin (II)[6]: 7-Bromodiosgenin acetate was obtained from diosgenin acetate with N-bromosuccinimide. 7-Bromo derivative without purification was oxidized to 7-oxodiosgenin acetate by CrO_3 in aqueous AcOH at room temperature. 7-Oxodiosgenin acetate was purified by silica gel column chromatography and recrystallized from MeOH; mp 194—197°, $[\alpha]_D^{20°}$ −164° (c=1.0, $CHCl_3$); UV λ_{max}^{EtOH} 237 mμ (log e 4.23). *Anal.* Calcd. for $C_{29}H_{42}O_5$: C, 74.01; H, 9.00. Found: C, 74.03; H, 9.00. On treatment with $LiAlH_4$ in ether, a mixture of 7-hydroxydiosgenin was obtained. As usual reduction with $LiAlH_4$ in ether 7-oxodiosgenin was converted to 7β-hydroxydiosgenin, mp 216—219°, $[\alpha]_D^{15°}$ −75° (c=1.0, $CHCl_3$). *Anal.* Calcd. for $C_{27}H_{42}O_4$: C, 75.30; H, 9.83. Found: C, 75.34; H, 9.75.

7-Oxogentrogenin Acetate (VI)——*t*-Butyl chromate was prepared by the method of Wettstein.[7]

To 150 mg of gentrogenin acetate (V) suspended in 0.7 ml of anhyd. CCl_4 and warmed to 80°, a mixture of 0.93 ml of *t*-butyl chromate, 0.23 ml of AcOH, and 0.12 ml of Ac_2O were added dropwise during 30 min with stirring, and the stirring was continued for further 10 hr at 80°. Then the mixture was cooled in an ice bath, 200 mg of oxalic acid in 1 ml of water was added, stirred for 15 min, 134 mg of crystalline oxalic acid, was further added, and again stirred for 2 hr.

The reaction mixture was poured on ice and extracted with CCl_4. The extract was washed with H_2O dried on Na_2SO_4, concentrated under reduced pressure, and 130 mg of crude 7-oxogentrogenin acetate was obtained.

Reduction of 7-Oxogentrogenin Acetate (VI)——To 130 mg of crude 7-oxogentrogenin acetate dissolved in 6.6 ml of ether, 18 mg of $LiAlH_4$ in 1 ml of ether was added dropwise during 30 min and the mixture was refluxed for 3 hr. The reaction mixture was extracted with ether, and the extract was washed successively with 5% $NaHCO_3$, H_2O and dried on Na_2SO_4. Concentration of this extract gave 96 mg of a mixture of four epimers which were separated by thin-layer chromatography, the results have shown in Table II.

Acknowledgement We wish to thank Dr. Y. Sato of NIH, Bethesda, U.S.A., for the gift of 7β,11α-dihydroxydiosgenin and Dr. E. S. Rothoman for the gift of gentrogenin acetate. We also thank Hitachi Co., Ltd., for the service of mass spectrometer, Miss Y. Kishio for NMR service, and Mrs. T. Thoma and Miss A. Maeda for elementaly analysis.