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Studies on the Constituents of Ophiopogonis Tuber. III.[1] On the Structure of Ophiopogonin D

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The chemical structure of ophiopogonin D, a glycoside isolated from the tuber of *Ophiopogon japonicus* Ker-Gawler var. *genuinus* Maxim. (Liliaceae) was established to be ruscogenin(1)-[α-L-rhamnopyranosyl($1_{rham}\rightarrow 2_{fuc}$)][D-xylopyranosyl($1_{xyl}\rightarrow 3_{fuc}$)]-β-D-fucopyranoside(Ia). Ophiopogonin D is one of spirostane type glycosides whose sugar moiety links to the hydroxyl group other than C_3 hydroxyl group of the aglycone.

As we pointed out in the previous paper,[1] the site of sugar linkage in spirostanol glycoside has been regarded as the hydroxyl group at C-3 of the aglycone, but in the glycoside of a spirostanol with more than two hydroxyl groups, several examples of glycosides whose sugar moiety links to the hydroxyl group other than C-3 hydroxyl group of the aglycone have been reported.

In the previous paper[1] we reported the structure elucidation of ophiopogonin B whose sugar moiety, namely α-L-rhamnopyranosyl($1_{rham}\rightarrow 2_{fuc}$)-β-D-fucopyranoside, linked to 1β-hydroxyl group of ruscogenin and the present paper deals with the structure determination of ophiopogonin D leading to the assignment of the structure Ia.

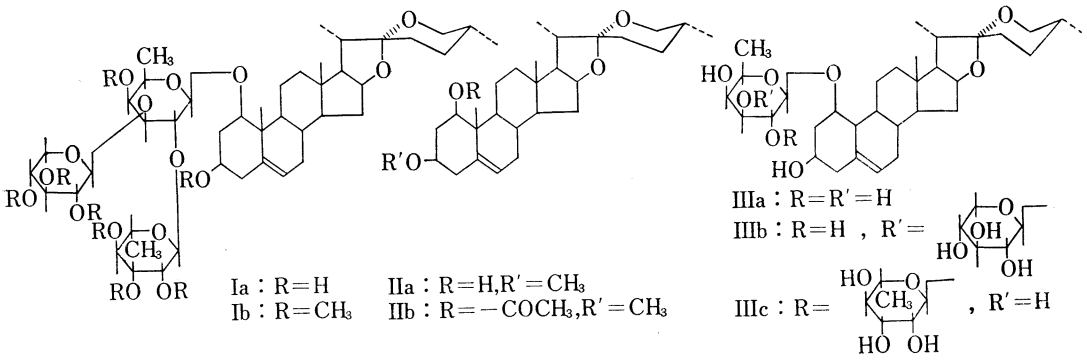

Fig. 1

Ophiopogonin D (Ia), $C_{44}H_{70}O_{16}\cdot 4H_2O$, colorless needles, mp 263—265°, $[\alpha]_D^{14}$ −107.9° (in pyridine), is one of the spirostanol glycosides in the tuber of *Ophiopogon japonicus* Ker-Gawler var. *genuinus* Maxim. (Liliaceae). It is a ruscogenin triglycoside whose sugar moiety is composed of one mole each of D-fucose, L-rhamnose and D-xylose.[3] The infrared (IR) spectum of Ia shows the presence of the isospiroketal ring system[4] and hence the sugar moiety must be attached to the hydroxyl group at C_1 and/or C_3 of ruscogenin.

1) Part II: A. Tada and J. Shoji, *Chem. Pharm. Bull.* (Tokyo), **30**, 1729 (1972).
2) Location: *Hatanodai, Shinagawa-ku, Tokyo.*
3) H. Kato, S. Sakuma, A. Tada, S. Kawanishi, and J. Shoji, *Yakugaku Zasshi*, **88**, 710 (1968).
4) E.S. Rothman, M.E. Wall, and C.R. Eddy, *J. Amer. Chem. Soc.*, **74**, 4013 (1952).

Octa-O-methylophiopogonin D (Ib), $C_{52}H_{86}O_{16}$, colorless needles, mp 210—212°, $[\alpha]_D^{19}$ −57.2° (in chloroform), prepared by the Hakomori's method[5] gives, on methanolysis, an aglycone, $C_{28}H_{44}O_4$ (IIa), colorless needles, mp 220—223°, $[\alpha]_D^{25}$ −108.9° (in chloroform) and a mixture of methylated monosaccharides. The IR spectrum of compound IIa reveals the presence of isospiroketal ring system and the nuclear magnetic resonance (NMR) spectrum indicates the presence of one methoxyl group (δ=3.32 3H(s)). Based on the physical data, compound IIa and its acetate (IIb), $C_{30}H_{46}O_5$, colorless needles, mp 177—178°, $[\alpha]_D^{25}$ −86.0° (in chloroform) are assumed to be ruscogenin-3-O-methyl ether and its 1-acetate,[1] and these products were identfied with authentic samples by the mixed fusion, comparison of IR spectra and thin-layer chromatography (TLC).

The formation of ruscogenin-3-O-methyl ether from Ib indicates that the three monosaccharides of ophiopogonin D form a trisaccharide which is combined with the hydroxyl group at C_1 of ruscogenin. The monosaccharides of the methanolysate were examined by TLC and gas-liquid chromatography (GLC), and methyl 2,3,4-tri-O-methyl-L-rhamnopyranoside, methyl 2,3,4-tri-O-methyl-D-xylopyranoside and methyl 4-O-methyl-D-fucopyranoside were identified by comparing with authentic samples. Therefore the trisaccharide portion of Ia should have a [L-rhamnopyranosyl($1_{rham}\rightarrow$2 or 3_{fuc})] [D-xylopyranosyl($1_{xyl}\rightarrow$2 or 3_{fuc})]-D-fucopyranoside structure.

Partial hydrolysis of Ia with diluted mineral acid gives three prosapogenins, namely prosapogenin D_1 (IIIa), prosapogenin D_2 (IIIb) and prosapogenin D_3 (IIIc). Prosapogenin D_1, colorless needles, mp 238—240°, $C_{33}H_{52}O_8$, $[\alpha]_D^{25}$ −91.0° (pyridine), gives an acetate, $C_{41}H_{60}O_{12}$, colorless needles, mp 225—230°, $[\alpha]_D^{21}$ −73.0° (in chloroform) by the conventional acetylation. On hydrolysis with 3N hydrogen chloride IIIa gave ruscogenin and D-fucose. The properties of IIIa and its acetate suggest that these compounds will be identical with proophiopogonin B and its acetate[1] and the identity was proved by direct comparison with authentic samples.

Prosapogenin D_2, a minor product of the hydrolysate, was examined by hydrolysis and the constitution of IIIb was deduced to be composed of ruscogenin, fucose and xylose.

The third product, prosapogenin D_3, colorless needles, $C_{39}H_{62}O_{12}$, mp 264—265°, $[\alpha]_D^{25}$ −112.8° (in pyridine), gives an acetate, $C_{51}H_{74}O_{18}$, colorless needles, mp 228—230°, $[\alpha]_D^{23}$ −62.5° (in chloroform) by acetylation, while the methylation by the Hakomori's method gives a per-O-methyl ether, mp 210—212°. On acid hydrolysis with 3N hydrogen chloride, IIIc gives ruscogenin, rhamnose and fucose, while methanolysis of per-O-methyl ether of IIIc with 2N hydrogen chloride in methanol gives ruscogenin-3-O-methyl ether, methyl 3,4-di-O-methyl-D-fucopyranoside and methyl 2,3,4-tri-O-methyl-L-rhamnopyranoside. From these experimental results, IIIc is deduced to be ophiopogonin B. The identification with

Table I. Assignment of the Configuration of D-Xylose

1) Comparison of Molecular Rotation

	$[\alpha]_D$	$[M]_D$
Ophiopogonin D	−107.9°	−922.5°
Prosapogenin D_3	−112.8°	−815.4°
$[M]_D$ (ophiopogonin D) − $[M]_D$ (prosapogenin D_3)	= −107.1°	(β-form)
α-Methyl D-xylopyranoside $[M]_D$	+249°	
β-Methyl D-xylopyranosibe $[M]_D$	−107°	

2) NMR Spectrum

Anomeric H of D-xylose	δ=4.38, J=8 cps	(β-form)

5) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

an authentic sample was carried out by mixed fusion and comparisons of TLC, NMR, and IR spectra.

The foregoing results suggest that the structure of ophiopogonin D might be ruscogenin (1)-[α-L-rhamnopyranosyl($1_{rham}\rightarrow 2_{fuc}$)][D-xylopyranosyl($1_{xyl}\rightarrow 3_{fuc}$)]-β-D-fucopyranoside. The configuration of xylopyranoside is assigned to be β-form by the comparison of chemical shift and the coupling constant of the anomeric proton of xylose and by the application of the Klyne's rule[6] to compare the molecular optical rotation of Ia and IIIc (=ophiopogonin B).

In conclusion the complete structure of ophiopogonin D is defined as ruscogenin (1)-[α-L-rhamnopyranosyl($1_{rham}\rightarrow 2_{fuc}$)][β-D-xylopyranosyl($1_{xyl}\rightarrow 3_{fuc}$)]-β-D-fucopyranoside, Ia. It should be noted that both ophiopogonin D and ophiopogonin B are the examples of the spirostane type glycosides, whose sugar moiety links to the hydroxyl group other than C_3 hydroxyl group of the aglycone.

Experimental

All melting points were taken on a Yanagimoto Micro Melting Point apparatus and are uncorrected. IR absorption spectra were obtained with a Hitachi Model EPI-2. NMR spectra were measured with a Japan Electron Co. JNM 4H-100 spectrometer and a Hitachi Model R-20 High Resolution NMR spectrometer with tetramethylsilane as an internal standard. The chemical shifts are reported in δ and the solvents used are indicated. Gas chromatograph used was a Hitachi Model K-53 with hydrogen flame injection detector and the conditions applied are as follows. Column, 5% NPGS on chromosorb W, 3 mm × 2 m; column temperature, 170°; carrier gas, N_2, 0.7 kg/cm^2. Optical rotations were measured with a Yanagimoto automatic polarimeter, Yanaco Model OR-50. The *Rf* values of TLC were determined on silica gel H(Merck) using a mixture of benzene–acetone (4:1) as a solvent and spots were detected by spraying 10% H_2SO_4 followed by heating.

Ophiopogonin D (Ia)——This was obtained from the crude glycoside fraction of the tuber of *Ophiopogon japonicus* KER-GAWLER var. *genuinus* MAXIM. by repeated chromatography on silica gel with 10% MeOH–AcOEt saturated with water and recrystallized from aqueous EtOH as colorless needles, mp 263—265°, $[\alpha]_D^{14}$ −107.9° (*c*=0.66 pyridine). IR ν_{max}^{Nujol} cm^{-1}: 3500—3300 (OH), 983, 920, 900, 862 (intensity 900>920,[7] isospiroketal). *Anal.* Calcd. for $C_{44}H_{70}O_{16}\cdot 4H_2O$: C, 57.00; H, 8.48. Found: C, 56.59; H, 8.67.

Permethylation of Ophiopogonin D (Formation of Ib)——According to the Hakomori's method, NaH (300 mg) was stirred in dimethylsulfoxide (12 ml) at 70° for 1 hr under N_2 gas flow. To this reagent ophiopogonin D(368 mg) in dimethylsulfoxide (12 ml) was added and the mixture was kept at room temperature for 10 min with stirring under N_2 gas flow. Methyl iodide (4 ml) was added and the reaction mixture was allowed to stand at room temperature for 3 hr with stirring. After dilution with water, the mixture was extracted with $CHCl_3$ and the organic layer was washed with water, dried and concentrated to dryness. The syrupy residue (430 mg) was chromatographed on silica gel eluted with AcOEt–hexane (1:1) to give octa-O-methylophiopogonin D, mp 210—212°, colorless needles from MeOH, $[\alpha]_D^{19}$ −57.2° (*c*=0.61 $CHCl_3$). *Anal.* Calcd. for $C_{52}H_{86}O_{16}$: C, 64.57; H, 9.35. Found: C, 64.59; H, 9.07. IR $\nu_{max}^{CCl_4}$ cm^{-1}: OH (nil.), 983, 922, 902, 868 (intensity 902>922, isospiroketal). NMR (in $CDCl_3$) δ: 0.78 (3H(s), $-\overset{|}{\underset{|}{C}}-CH_3$, 3H(d), $-\overset{|}{C}H-CH_3$), 0.96 (3H(d), *J*=7 cps $-\overset{|}{C}H-CH_3$), 1.02 (3H(s), $-\overset{|}{\underset{|}{C}}-CH_3$), 3.34 (3H(s), OCH_3), 3.47—3.58 (7×3H(s), OCH_3), 4.18 (1H(d), *J*=8 cps anomeric H), 4.38 (1H(d), *J*=8 cps anomeric H), 5.25 (1H(broad s.) anomeric H), 5.54 (1H(m) $>C=C<^{H}$). TLC: *Rf* 0.23.

Methanolysis of Ophiopogonin D per-O-Methyl Ether(Ib)——The above permethylate (250 mg) was refluxed with 2N HCl in MeOH (25 ml) for 4 hr. The solution was diluted with water and MeOH was removed *in vacuo*. After cooling, the resulting precipitate (130 mg) was filtered off and the aqueous filtrate was neutralized with Ag_2CO_3 and evaporated to give a syrup (80 mg).

Ruscogenin Monomethyl Ether (IIa)——The above precipitate was recrystallized from MeOH to provide pure aglycone (IIa), colorless needles, mp 220—223°, $[\alpha]_D^{25}$ −108.9° (*c*=0.45 $CHCl_3$). *Anal.* Calcd. for $C_{28}H_{44}O_4$: C, 75.63; H, 9.97. Found: C, 75.78; H, 10.16. IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 980, 920, 865 (intensity 900>920, isospiroketal). NMR (in $CDCl_3$) δ: 0.78 (3H(s), $-\overset{|}{\underset{|}{C}}-CH_3$, 3H(d), $-\overset{|}{C}H-CH_3$), 0.98 (3H(d), *J*=7 cps, $-\overset{|}{C}H-CH_3$), 1.02 (3H(s), $-\overset{|}{\underset{|}{C}}-CH_3$), 3.32 (3H(s), $-OCH_3$), 5.52 (1H(m), $>C=C<^{H}$). It was identified with an authentic sample of ruscogenin 3-O-methyl ether by mixed mp, TLC and IR comparison. TLC: *Rf* 0.35.

Acetylation of Aglycone (IIa) to Monoacetate (IIb)——Compound IIa (50 mg) was left standing with

6) W. Klyne, *Biochem. J.*, **47**, xli (1950).

7) "900>920" means the absorbance of the 900 cm^{-1} band is stronger than that of 920 cm^{-1}.

pyridine (1 ml) and Ac_2O (1 ml) at room temperature for 48 hr. The reaction product (54 mg) was recrystallized from MeOH to provide monoacetate IIb as colorless needles, mp 177—178°, $[\alpha]_D^{25}$ −86.0° (c=0.50 $CHCl_3$). *Anal.* Calcd. for $C_{30}H_{46}O_5$: C, 74.03; H, 9.53. Found: C, 74.12; H, 9.40. IR ν_{max}^{KBr} cm^{-1}: 1740 (ester), 982, 918, 900, 864 (intensity 900>918, isospiroketal). NMR (in $CDCl_3$) δ: 0.78 (3H(s), $-\overset{|}{C}-CH_3$, 3H(d), $-\overset{|}{C}H-CH_3$), 0.95 (3H(d), J=6 cps $-\overset{|}{C}H-CH_3$), 1.13 (3H(s), $-\overset{|}{C}-CH_3$), 2.01 (3H(s), $-OCOCH_3$), 3.30 (3H(s), OCH_3), 5.54 (1H(m), $>C=C<^{H}$). It was identified with an authentic sample of ruscogenin 1-acetate 3-methyl ether (IIb) by mixed mp, TLC and IR comparison. TLC: *Rf* 0.70.

Detection of Methylated Sugars——The sugar portion of the methanolysate was examined by TLC and GLC comparing with authentic samples. Methyl 2,3,4-tri-O-methyl-L-rhamonopyranoside: *Rf* 0.46 (α), 0.37(β); t_R(min) 3.4(α), 4.5(β). Methyl 2,3,4-tri-O-methyl-D-xylopyranoside: *Rf* 0.48(α), 0.34(β); t_R 3.4(α), 3.9(β). Methyl 4-O-methyl-D-fucopyranoside: *Rf* 0.04(α), 0.00(β); t_R 15.0(α), 24.5(β).

Methyl 4-O-methyl-D-fucopyranoside was isolated from the methanolysate of ophiopogonin D permethyl ether by chromatography on silica gel eluted with AcOEt as colorless plates from acetone–petroleum ether, mp 135—136°, $[\alpha]_D^{25}$ +125° (c=1.00 in H_2O). *Anal.* Calcd. for $C_9H_{16}O_5$: C, 49.99; H, 8.39. Found: C, 50.09; H, 8.41. NMR (in $CDCl_3$) δ: 1.28 (3H(d), J=11 cps, $-\overset{|}{C}H-CH_3$), 3.38 (3H(s), $-OCH_3$), 3.58 (3H(s), $-OCH_3$), 4.85 (1H(d), J=4 cps, anomeric H). The isolation of methyl 2,3,4-tri-O-methyl-L-rhamnopyranoside and methyl 2,3,4-tri-O-methyl-D xylopyranoside was not successful.

Partial Hydrolysis of Ophiopogonin D——Ophiopogonin D (540 mg) was refluxed with 2N HCl (90 ml), dioxane (30 ml) and benzene (90 ml) for 1.5 hr and water was added to the reaction mixture. The solution was extracted with benzene and the aqueous layer was extracted with BuOH saturated with water. The BuOH solution was washed with water and then evaporated *in vacuo*. The white powder (105 mg) was examined by TLC (plate: silica gel; solvent: $CHCl_3$–MeOH–H_2O (7:3:1, the lower phase) to reveal the presence of three prosapogenins (*Rf* 0.63 (prosapogenin D_1), 0.46 (prosapogenin D_2) and 0.40 (prosapogenin D_3)). The BuOH extract was chromatographed on a silica gel column eluted successively with $CHCl_3$–MeOH–H_2O (7:3:1, the lower phase). Prosapogenin D_1 (12 mg), prosapogenin D_2 (trace) and prosapogenin D_3 (25 mg) were fractionated.

Acetylation of Prosapogenin D_1 to Tetraacetate——Prosapogenin D_1 (6 mg) was treated by the same procedure applied to the acetylation of II. The acetate (6 mg) was recrystallized from EtOH containing water to give colorless needles, mp 225—230°, $[\alpha]_D^{21}$ −73.0° (c=0.40 $CHCl_3$), IR ν_{max}^{KBr} cm^{-1}: 1750 (ester), 985, 918, 901, 865 (intensity 901>918, isospiroketal), TLC: *Rf* 0.66, which was identified with an authentic sample of proophiopogonin B tetraacetate (mixed mp, IR and TLC).

Hydrolysis of Prosapogenin D_1——Prosapogenin D_1 (5 mg) was refluxed with 3N HCl (3 ml), dioxane (1 ml) and benzene (3 ml) for 4 hr. The reaction mixture was treated as usual and ruscogenin was found in the benzene layer (TLC: *Rf* 0.14) and fucose in the aqueous layer (PPC: paper, Toyo Roshi No. 50; solvent, BuOH: AcOH: H_2O=4:1:5 the upper layer; spray reagent, aniline hydrogen phthalate, *Rf* 0.25).

Hydrolysis of Prosapogenin D_2 (IIIb)——Compound IIIb was hydrolyzed by the same method as that described above, and ruscogenin, fucose and xylose were detected by TLC and PPC.

Prosapogenin D_3 (IIIc)——Prosapogenin D_3 (IIIc) was crystallized from EtOH containing water to give colorless needles, mp 264—265°, $[\alpha]_D^{25}$ −112.8° (c=0.54 pyridine). *Anal.* Calcd. for $C_{39}H_{62}O_{12}\cdot 2H_2O$: C, 61.72; H, 8.76. Found: C, 61.68; H, 8.77. IR ν_{max}^{KBr} cm^{-1}: 3500—3300 (OH), 985, 920, 900, 864 (intensity 900>920, isospiroketal). It was identified with an authentic sample of ophiopogonin B(mixed mp, IR and TLC).

Acetylation of Prosapogenin D_3 to Hexaacetate——Prosapogenin D_3 (10 mg) was acetylated with pyridine and Ac_2O as usual. The crude acetate (11 mg) was recrystallized from aqueous EtOH to give colorless needles, mp 228—230°, $[\alpha]_D^{28}$ −62.5° (c=0.60 $CHCl_3$). *Anal.* Calcd. for $C_{51}H_{74}O_{18}$: C, 62.82; H, 7.65. Found: C, 62.74; H, 7.64. IR ν_{max}^{Nujol} cm^{-1}: 1745 (ester), 984, 926, 903, 866 (intensity 903>926, isospiroketal). It was identified with an authentic sample of ophiopogonin B hexaacetate (mixed mp, IR and TLC).

Hydrolysis of Prosapogenin D_3 (IIIc)——Prosapogenin D_3 (5 mg) was refluxed with 3N HCl (3 ml), dioxane (1 ml) and benzene (3 ml) for 4 hr. The reaction mixture was treated as usual and ruscogenin, rhamnose and fucose were characterized by TLC and PPC.

Permethylation of Prosapogenin D_3 (IIIc) and Methanolysis of its Per-O-methyl Ether——Prosapogenin D_3 (10 mg) was methylated by the Hakomori's method to afford 10 mg of per-O-methylprosapogenin D_3, colorless needles from MeOH, mp 210—212° which was identified with an authentic sample of ophiopogonin B per-O-methyl ether by mixed mp and TLC.

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