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Further Studies on Dammarane-Saponins of Ginseng Roots

HIROMICHI MATSUURA,[a] RYOJI KASAI,[b] OSAMU TANAKA,*[,b]
YUH-ICHIRO SARUWATARI,[a] KAZUO KUNIHIRO,[a]
and TOHRU FUWA[a]

Central Research Laboratories, Wakunaga Pharmaceutical Co., Ltd.,[a] Shimokotachi 1624, Koda-cho, Takata-gun, Hiroshima 729-64, Japan, and Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,[b] Kasumi, Minami-ku, Hiroshima 734, Japan

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A new dammarane-saponin named ginsenoside-Ra_3 (**3**) was isolated from both White and Red Ginseng in yields of 0.005%. The structure of **3** was established to be (20*S*)-protopanaxadiol 3-*O*-(β-D-glucopyranosyl(1→2)-β-D-glucopyranosido)-20-*O*-β-D-xylopyranosyl(1→3)-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside. Further, notoginsenoside-R4 (**14**), previously isolated from Sanchi Ginseng, roots of *Panax notoginseng*, was also isolated from Red Ginseng in a yield of 0.002%.

Keywords——Ginseng root; Araliaceae; dammarane-saponin; ginsenoside-Ra_3; notoginsenoside-R4; ^{13}C NMR of oligoglycoside

The isolation and structure determination of two new dammarane-saponins, ginsenosides-Ra_1 (**1**)[1,2] and -Ra_2 (**2**),[1] from both White and Red Ginseng[3] have been reported. The present paper deals with the further isolation and structure determination of two minor saponins.

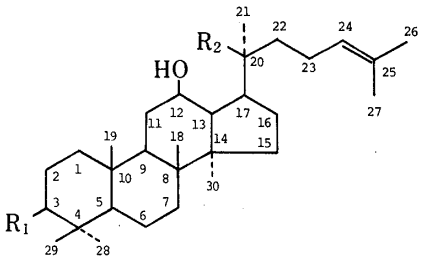
The ginsenoside-Ra fraction reported in previous papers[1,3] was subjected to repeated column chromatography on reversed-phase highly porous polymer and on silica gel to afford a new saponin, named ginsenoside-Ra_3 (**3**), in a yield of 0.005% along with **1** and **2**. On mineral acid hydrolysis, **3** gave glucose and xylose. In the ^{13}C nuclear magnetic resonance (NMR) spectrum of **3**, all of the carbon signals due to the aglycone moiety appeared at almost the same positions as those of ginsenoside-Rb_1 (**4**), indicating that **3** should be a glycoside of 20 (*S*)-protopanaxadiol (**5**) at both the 3- and 20-hydroxyl groups, like **1** and **2**. Inspection of the anomeric carbon signals revealed the presence of five monosaccharide units in this saponin. The field desorption mass spectrum (FD-MS) of **3** exhibited a molecular cluster ion at *m/z* 1263 $(M+Na)^+$ and fragment peaks corresponding to stepwise elimination of the sugar units at *m/z* 1131 $(M+Na-xylosyl)^+$, 1101 $(M+Na-glucosyl)^+$, 969 $(M+Na-xylosyl-glucosyl)^+$, 939 $(M+Na-glucosyl-glucosyl)^+$, and 807 $(M+Na-xylosyl-glucosyl-glucosyl)^+$. It has been reported that a glycosyl linkage at the C-20-*tert*-hydroxyl group of dammarane-saponins is very unstable, and in the electron impact mass spectra (EI-MS) of the acetates or trimethylsilyl (TMSi) ethers, no fragment ions having an intact O-glycosyl group at the C-20 position can be observed.[4] The EI-MS of the acetate of **3** exhibited a pair of ions at *m/z* 1042 and 1043 due to the elimination of the 20-O-glycosyl moiety and ions at *m/z* 259, 331, 547, and 619, characteristic of terminal xylosyl, terminal glucosyl, xylosyl-glucosyl, and glucosyl-glucosyl, respectively.

The mild hydrolysis of **3** with 50% acetic acid gave a prosapogenin mixture and an oligosaccharide (**6**), the former of which, on methylation followed by successive hydrolysis,

reduction, and acetylation, afforded the two partially methylated alditol acetates, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol (**7**) and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol (**8**) detected by gas chromatography-mass spectrometry (GC-MS) (alditol acetate analysis[5]). The oligosaccharide (**6**) was subjected to alditol acetate analysis in the same way as above to afford 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylpentitol (**9**), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol (**10**), and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (**11**). This evidence coupled with the results of MS analysis indicated that **6** should be formulated as either xylosyl(1→3)-glucosyl(1→6)glucose or xylosyl(1→6)glucosyl(1→3)glucose.

Kochetkov *et al.*[6] reported that the EI-MS of TMSi derivatives of oligosaccharides having a 1,6-linked biosyl unit exhibit characteristic fragment ions, *i.e.*, *m/z* 583 for hexosyl(1→6)hexose and *m/z* 481 for pentosyl(1→6)hexose. The EI-MS of the TMSi ether of **6** showed the ion at *m/z* 859 (**12**), which is characteristic of xylosyl(1→3)glucosyl(1→6)glucose (see Fig. 2). Further, the above sugar sequence of **6** was supported by the fragment ion at *m/z* 513 (**13**) in the EI-MS of the permethyl ether of **6** (see Fig. 2).[7]

A comparison of the ^{13}C NMR spectrum of **3** with that of **4** revealed an additional set of signals due to a terminal β-xylosyl unit in the spectrum of **3**. Further, on going from **4** to **3**, a carbon signal at 78.0 ppm due to C-3 of one of the β-glucopyranosyl units was displaced to low-field by 9.5 ppm,[8] while other signals of the sugar moiety of **3** remained almost unshifted. It follows that **3** can be represented as (20*S*)-protopanaxadiol 3-*O*-(β-D-glucopyranosyl-(1→2)-β-D-glucopyranosido)-20-*O*-β-D-xylopyranosyl(1→3)-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside.



1: R_1 = O-glc$^{2\ 1}$—glc R_2 = O-glc$^{6\ 1}$—ara(py)$^{4\ 1}$—xyl

2: R_1 = O-glc$^{2\ 1}$—glc R_2 = O-glc$^{6\ 1}$—ara(fu)$^{2\ 1}$—xyl

3: R_1 = O-glc$^{2\ 1}$—glc R_2 = O-glc$^{6\ 1}$—glc$^{3\ 1}$—xyl

4: R_1 = O-glc$^{2\ 1}$—glc R_2 = O-glc$^{6\ 1}$—glc

5: R_1 = OH R_2 = OH

14: R_1 = O-glc$^{2\ 1}$—glc R_2 = O-glc$^{6\ 1}$—glc$^{6\ 1}$—glc

glc: β-D-glucopyranosyl ara(py): α-L-arabinopyranosyl
ara(fu): α-L-arabinofuranosyl xyl: β-D-xylopyranosyl

Fig. 1

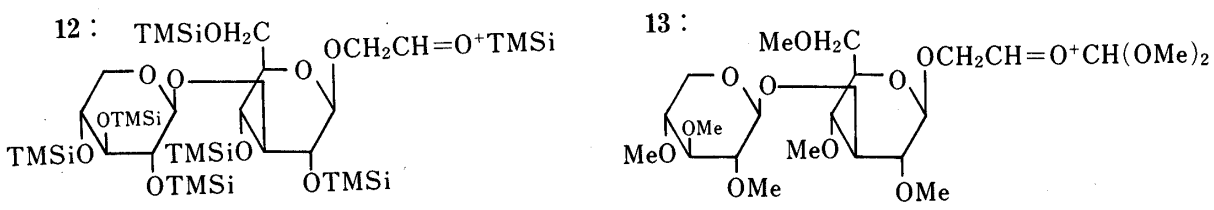


Fig. 2

TABLE I. ^{13}C NMR Chemical Shifts: Aglycone Moiety (in C_5D_5N)

	5	4	3		5	4	3
C- 1	39.5	39.1	39.3	C-16	26.8	26.6	26.6
C- 2	28.2	26.6	26.6	C-17	54.7	51.6	51.6
C- 3	77.9	89.3	89.0	C-18	16.2[a]	16.2[a]	16.2[a]
C- 4	39.5	39.6	39.7	C-19	15.8[a]	15.9[a]	16.0[a]
C- 5	56.3	56.3	56.5	C-20	72.9	83.5	83.5
C- 6	18.7	18.6	18.3	C-21	26.9	22.6	22.8
C- 7	35.2	35.1	35.1	C-22	35.8	36.1	36.1
C- 8	40.0	39.9	40.0	C-23	22.9	23.1	23.2
C- 9	50.4	50.1	50.2	C-24	126.2	125.8	126.0
C-10	37.3	36.8	36.9	C-25	130.6	131.0	130.9
C-11	32.0	30.8	30.8	C-26	25.8	25.8	25.8
C-12	70.9	70.1	70.1	C-27	17.6[a]	17.9[a]	17.9[a]
C-13	48.5	49.3	49.5	C-28	28.6	28.0	28.0
C-14	51.6	51.3	51.4	C-29	16.4[a]	16.5[a]	16.5[a]
C-15	31.8	30.8	30.8	C-30	17.0[a]	17.3[a]	17.4[a]

a) Assignments in any column may be reversed, though those given here are preferred.

TABLE II. ^{13}C NMR Chemical Shifts: Sugar Moiety

		4	3			4	3
3-glc	1	105.0	104.9	20-glc	1	97.9	98.0
(Inner)	2	82.9	83.5	(Inner)	2	74.9	74.8[c]
	3	77.2[a]	78.0[a]		3	78.0[a]	78.0[a]
	4	71.5	71.7[d]		4	71.5	71.7[d]
	5	78.0[a]	78.0[a]		5	76.7	77.0
	6	62.6	62.7		6	71.5	69.6
3-glc	1	105.6	105.9[b]	20-glc	1	105.0	104.9
(Terminal)	2	76.7	77.0		2	74.9	74.1[c]
	3	78.8[a]	79.2[a]		3	78.0[a]	87.5
	4	71.5	71.7[d]		4	71.5	71.3[d]
	5	78.0[a]	78.0[a]		5	78.0[a]	78.0[a]
	6	62.6	62.7		6	62.6	62.4
				20-xyl	1		106.3[b]
					2		75.3[c]
					3		77.0
					4		70.8
					5		67.3

a—*d*) Assignments in any column may be reversed, though those given here are preferred.
glc, β-D-glucopyranosyl; xyl, β-D-xylopyranosyl.

A saponin fraction which showed a lower *Rf* value than **1**, **2**, and **3** on silica gel thin layer chromatography (TLC) was purified by reversed-phase chromatography, affording a minor saponin (**14**) (yield: 0.002%), which was identical with notoginsenoside-R4, previously isolated from Sanchi Ginseng, roots of *Panax notoginseng*.[9]

Experimental

The ^{13}C NMR spectra were taken in pyridine-d_5 on a JEOL PFT-100 spectrometer (25.15 MHz) and the chemical shifts are expressed on the δ scale from an internal standard, tetramethylsilane (TMS). The EI-MS were recorded on a JEOL JMS-DX300 mass spectrometer at 70 eV and the FD-MS were obtained with a JEOL JMS-

DX300 machine with an emitter heating current of 22—30 mA. Identifications of the known saponin and the resulting monosaccharides after hydrolysis, and acetylation and trimethylsilylation for EI-MS were carried out as described in previous papers.[1,10]

Isolation of 3 and 14——The ginsenoside-Ra fraction of White or Red Ginseng (see previous papers)[1,3] was subjected to repeated column chromatography on reversed-phase highly porous polymer (MCI CHP20P, Mitsubishi Chemical Ind., Ltd.) (solvent: 70% aqueous MeOH), affording **1**, **2**, and the saponin mixture. This mixture was purified by silica gel chromatography (solvent: 1-BuOH–AcOEt–H_2O (4 : 1 : 2, upper phase)) to give **3**: yeild, 0.005% each from both White and Red Ginseng.

Ginsenoside-Ra_3 (**3**): white powder (reprecipitated from EtOH–AcOEt), $[\alpha]_D^{17}$ +9.8° (c=0.43, MeOH). *Anal.* Calcd for $C_{59}H_{100}O_{27}\cdot 4H_2O$: C, 53.95; H, 8.29. Found: C, 53.72; H, 8.15. **3**-Acetate: EI-MS % (m/z); 1043 (1), 1042 (2), 619 (1), 547 (1), 331 (24), 259 (20), 169 (100).

The saponin fraction of Red Ginseng was chromatographed on a column of silica gel (gradient elution with $CHCl_3$–MeOH–H_2O (50 : 10 : 1 (homogeneous)→7 : 3 : 0.5→13 : 7 : 2 (lower phase))) to provide eight fractions (Fr.), tentatively designated as Frs. I—VIII in increasing order of polarity (see previous paper[3]). Fr. VII was subjected to repeated column chromatography; first on silica gel (solvent: $CHCl_3$–MeOH–H_2O (13 : 7 : 2, lower phase)), then on highly porous polymer (solvent: 70% aqueous MeOH), affording **14** (yield; 0.002%), which was identical with an authentic sample in TLC behavior on Silica gel $100F_{254}$ (Merck) (solvents: 1-BuOH–AcOEt–H_2O (4 : 1 : 2, upper phase) and $CHCl_3$–MeOH–H_2O (13 : 7 : 2, lower phase)) and in HPTLC Rp-$18F_{254s}$ (Merck) (solvent: 80% aqueous MeOH), as well as in EI-MS of the acetate and TMSi ether and ^{13}C NMR spectroscopy.

Partial Hydrolysis of 3 with 50% AcOH——A solution of **3** (10 mg) in 50% AcOH was heated at 70 °C for 4 h. The reaction mixture was diluted with H_2O and extracted with 1-BuOH (saturated with H_2O). The BuOH layers were concentrated to dryness, giving a prosapogenin mixture, while the aqueous layer was deionized on Amberlite IR-45 (OH-form), affording an oligosaccharide (**6**) after freeze-drying.

TMSi Ether of **6**: EI-MS % (m/z); 859 (xyl(TMSi)$_3$–glc(TMSi)$_3$–O–$CH_2CH{=}O^+$TMSi, 0.4), 829 (glc(TMSi)$_4$–glc(TMSi)$_3^+$, 0.2), 727 (xyl(TMSi)$_3$–glc(TMSi)$_3^+$, 0.3), 451 (glc(TMSi)$_4^+$, 3), 349 (xyl(TMSi)$_3^+$, 21), 204 (100).

Permethylation Followed by Alditol Acetate Analysis of Prosapogenin Mixture and 6——According to Hakomori's method,[11] the prosapogenin mixture and **6** were methylated with NaH and dimethylsulfoxide (DMSO), and CH_3I, respectively. Each reaction product was purified by column chromatography on silica gel (solvent: $CHCl_3$–MeOH (70 : 1)) to afford the corresponding permethyl ether of prosapogenin mixture and **6**.

Permethyl Ether of **6**: EI-MS % (m/z); 614 (M^+, weak), 582 ($M^+ - MeOH$, weak), 569 ($M^+ - CH_2OMe$, weak), 513 (xyl(Me)$_3$–glc(Me)$_3$–O–$CH_2CH{=}O^+CH(OMe)_2$, 1), 423 (glc(Me)$_3$–glc(Me)$_4^+$, 0.7), 219 (glc(Me)$_4^+$, 6), 175 (xyl(Me)$_3^+$, 33), 75 (100).

A solution (0.5 ml) of the resulting permethylated prosapogenin mixture and **6** in 93% AcOH containing 0.5 N H_2SO_4 was heated in a sealed tube at 76 °C for 3 h and the reaction mixture was applied to a column (5 mm × 5 cm) of Amberlite IR-45 (AcOH-form). The column was eluted with H_2O (20 ml) and then MeOH (10 ml). The H_2O and MeOH eluates were combined and evaporated *in vacuo*, and the residue was reduced with 1% $NaBH_4$ aqueous solution (1 ml) at room temperature for 2 h. The reaction was stopped by adding a few drops of AcOH and the reaction mixture was concentrated to dryness *in vacuo*. The residue was heated with Ac_2O (0.5 ml) in a sealed microtube at 100 °C for 4 h. After removal of the solvent by evaporation, a solution of the residue in $CHCl_3$ was washed with H_2O, and the $CHCl_3$ layer was concentrated to dryness. The resulting alditol acetates were subjected to GC-MS. GC-MS conditions: a) 3% OV-225 on Gas Chrom Q; glass column, 2 mm × 2 m; column temperature, 200 °C; injection temperature, 240 °C; carrier gas, He (40 ml/min). t_R (min): **7** (8.1), **8** (13.7), **9** (5.7), **10** (15.7), **11** (18.8). b) 2% OV-17 on Gas Chrom Q; glass column, 2 mm × 1 m; column temperature, 180 °C; injection temperature, 240 °C; carrier gas, He (40 ml/min). t_R (min): **7** (5.0), **8** (8.3), **9** (3.2), **10** (9.9), **11** (11.0).

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