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Saponins of the Leaves of *Panax ginseng* C.A. Meyer

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From the leaves of *Panax ginseng* C.A. Meyer, six saponins I, II, III, IV, V, and VI were isolated. Saponins IV, V, and VI were proved to be identical, respectively with ginsenosides-Rg_1, -Re, and-Rd, all of which have already been isolated from Ginseng roots. The high contents of these saponins in the leaves indicate significance of the leaves as the source of the dammarane-type saponins and their sapogenins. New saponins I, II, and III designated as ginsenosides-F_1, -F_2, and-F_3 were established to be formulated as 20-O-β-glucopyranosyl-20(*S*)-protopanaxatriol, 3,20-di-O-β-glucopyranosyl-20(*S*)-protopanaxadiol, and 20-O-(α-arabinopyranosyl-(1→6)-β-glucopyranosyl)-20(*S*)-protopanaxatriol, respectively, on the basis of the enzymatic hydrolysis, mass spectra of their acetates, nuclear magnetic resonance, and chemical evidences.

The structures of dammarane-type saponins of Ginseng roots, ginsenosides-Rb_1, -Rb_2, -Rc, -Rd,[2] -Re, -Rf,[3] -Rg_1,[4] and -Rg_2[3] were already established. Physiological activities of the crude saponin fraction as well as purified ginsenosides have also been reported.[5] Although the leaves of *Panax ginseng* C.A. Meyer (Ginseng leaves) have been left unutilized, the presence of dammarane-type saponins in Ginseng leaves has been anticipated by thin-layer chromatography (TLC) and by isolation of modified sapogenins, panaxadiol and panaxatriol from the acid-hydrolysate of the crude extract.[6] Recently, physiological activities of the glycoside-fraction of the leaves were also investigated.[7] For the purpose of finding better sources for production of the dammarane-type saponins, the present authors have undertaken the isolation and the unequivocal identification of saponins of Ginseng leaves.

An aqueous suspension of the methanolic extract of the dried leaves[8] was washed with ether and then extracted with *n*-butanol. The butanolic solution was decolorlized by passing through a column of a mixture of polyamide and alumina and the eluate was concentrated to dryness to give a mixture of crude saponins. An aqueous solution of this mixture was dialyzed and the dialyzed fraction was chromatographed on silica gel to give saponin I, a mixture of saponins II and III, saponin IV and finally saponin V. Separation of the mixture of II and III was achieved by chromatography on polyamide. The non-dialyzed fraction afforded saponin VI after chromatography on silica gel.

1) Location: *a) 1-2-3 Kasumi, Hiroshima-shi*; *b) Maedashi, Fukuoka-shi.*
2) S. Sanada, N. Kondo, J. Shoji, O. Tanaka, and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **22**, 421 (1974).
3) S. Sanada, N. Kondo, J. Shoji, O. Tanaka, and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **22**, 2407 (1974).
4) Y. Nagai, O. Tanaka, and S. Shibata, *Tetrahedron*, **27**, 881 (1971).
5) H. Nabata, H. Saito, and K. Takagi, *Japan J. Pharmacol.*, **23**, 29 (1973); H. Oura, S. Hiai, H. Senoo, R. Takamura, and T. Yokozawa, *J. Japan Biochem.*, **44**, 611 (1972); T. Namba, M. Yoshizaki, T. Tomimori, K. Mitsui, and J. Hase, *Chem. Pharm. Bull.* (Tokyo), **21**, 459 (1973); K. Sakakibara, Y. Shibata, T. Higashi, S. Sanada, and J. Shoji, *ibid.*, **23**, 1009 (1975); H. Saito, *Metabolism and Disease*, **10**, 556 (1973); H. Oura and S. Hiai, *ibid.*, **10**, 564 (1973); M. Yamamoto, *ibid.*, **10**, 581 (1973) and the references cited therein.
6) S. Shibata, T. Ando, O. Tanaka, Y. Megro, K. Soma, and Y. Iida, *Yakugaku Zasshi*, **85**, 753 (1965); M. Komatsu and T. Tomimori, *Shoyakugaku Zasshi*, **20**, 21 (1966); T. Namba, M. Yoshizaki, T. Tomimori, K. Kobashi, K. Mitsui, and J. Hase, *Yakugaku Zasshi*, **94**, 252 (1974).
7) H. Saito, M. Morita, and K. Takagi, *Japan J. Pharmacol.*, **23**, 43 (1973).
8) Cultivated in Daikon-jima, Shimane-ken. Collected in September, 1972.

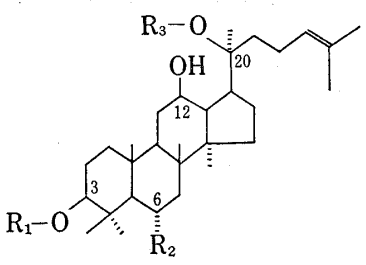
On comparison of TLC with the known Ginseng saponins, saponins IV, V, and VI were suggested to be identical with the root-saponins, ginsenosides-Rg_1, -Re, and -Rd, respectively. The confirmation of the identity was securred by mass spectra (MS) of their acetates,[9] enzymatic hydrolysis,[10] and comparisons of nuclear magnetic resonance (NMR) and optical rotation with those of corresponding authentic samples. It should be noted that the yields of IV, V, and VI from the leaves (*ca.* 1.5% each) are about ten times as much as those from Ginseng roots, indicating significance of the leaves as the source of preparation of these saponins and their sapogenins.

New saponins I, II, and III were designated as ginsenosides-F_1, -F_2, and-F_3, respectively. Ginsenoside-F_1 (I) (yield 0.4%), afforded glucose and 20(*S*)-protopanaxatriol (VII)[4] on hydrolysis with crude hesperidinase.[10] The 20-*tert*-hydroxyl group of dammarane-type triterpenes has been known to remain unacetylated on acetylation under the usual condition. Since this hydroxyl group and its peracetylated glycosyl bonding are readily eliminated by electron impact or by heating at high temperature, MS of acetylated dammarane-type saponins were reported to show no fragment ion having the intact 20-OH or 20-O-(peracetyl-sugar), exhibiting a pair of fragment ions, VIIIa and VIIIb.[9] This phenomenon affords useful information for micro-determination of location of glycosyl linkage in saponins of this type. MS of the acetate of I showed a pair of fragment ions, VIIIaF_1 (*m/e* 585), VIIIaF_1−AcOH(525), VIIIaF_1-2×AcOH(465), VIIIaF_1−3×AcOH(405); VIIIbF_1(584), VIIIbF_1−AcOH(524), VIIIbF_1−2×AcOH(464), VIIIbF_1−3×AcOH(404), indicating that an O-glucosyl linkage of I is limited to C-20 of its sapogenin moiety. Appearance of tetra-O-acetyl-glucosyl ion(IX) (*m/e* 331, base peak) and absence of acetylated oligoglucosyl ions led to formulate I as 20-O-monoglucoside of VII. In NMR of I (in C_5D_5N), the coupling constant of the anomeric proton signal at δ 5.10 ppm (1H doublet, J=8.0 Hz) revealed the β-glucopyranosyl linkage. Further, I was identified as a by-product in the enzymatic hydrolysis of IV along with VII and glucose. It follows that the structure of I must be represented by 20-O-β-glucopyranosyl-20(*S*)-protopanaxatriol.

On hydrolysis with crude hesperidinase,[10] ginsenoside-F_2 (II) (yield 0.2%) afforded glucose and 20-O-β-glucopyranosyl-20-(*S*)-protopanaxadiol (X) which was previously obtained from a mixture of ginsenosides-Rb_1, -Rb_2, and -Rc by the soil bacteria-hydrolysis.[11] MS of the acetate of II exhibited fragment ions, VIIIaF_2(*m/e* 815), VIIIaF_2−AcOH(755), VIIIbF_2−(814), VIIIbF_2−AcOH(754), and tetra-O-acetylglucosyl ion(IX) (331) but no acetylated oligoglucosyl ions. These evidences revealed that II must be represented by 3- or 12-O-monoglucoside of X. The partial hydrolysis of VI with Takadiastase[12] yielded II, establishing the formulation of II as 3,20-di-O-β-glucopyranosyl-20(*S*)-protopanaxadiol.

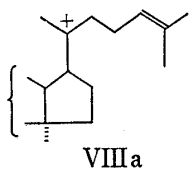
Ginsenoside-F_3 (III) (yield 0.2%) gave glucose, arabinose, 20(*S*)-protopanaxatriol (VII) and a small amount of I on hydrolysis with crude hesperidinase.[10] MS of the acetate of III showed the same series of the fragment ions as those of I (VIIIaF_1-series and VIIIbF_1-series), tri-O-acetylarabinosyl ion(XI) (*m/e* 259), and tri-O-acetylarabinosyl-tri-O-acetylglucosyl ion (XII) (547), indicating that III must be a 20-O-arabinoglucoside of VII. Methylation of III by Hakomori's procedure[13] gave permethyl ether of III which was subjected to methanolysis yielding methyl 2,3,4-tri-O-methylarabinopyranoside and methyl 2,3,4-tri-O-methylglucopyranoside. In the NMR of III (in C_5D_5N),[14] coupling constants of anomeric proton signals at δ 4.85 (1H doublet, J=5.5 Hz, anomeric H of α-arabinopyranoside) and 4.98 ppm (1H doublet, J=7 Hz, anomeric H of β-glucopyranoside) revealed the configurations of both

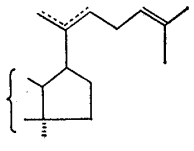
9) T. Komori, O. Tanaka, and Y. Nagai, *Org. Mass Spectrometry*, **9**, 744 (1974).
10) H. Kohda and O. Tanaka, *Yakugaku Zasshi*, **95**, 246 (1975).
11) I. Yosioka, T. Sugawara, K. Imai, and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **20**, 2418 (1972).
12) Crude preparation of hydrolytic enzymes produced from *Aspergillus niger* (Sankyo Co. Ltd.).
13) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
14) Taken at 80° in order to avoid overlapping of hydroxyl proton signals over anomeric proton signals.

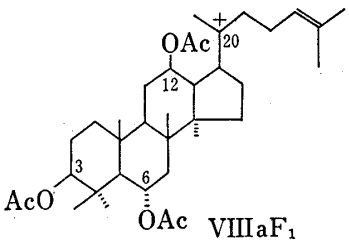


I: R_1=H, R_2=OH, R_3=β-gluc
II: R_1=β-gluc, R_2=H, R_3=β-gluc
III: R_1=H, R_2=OH, R_3=β-gluc^{6}—^{1}α-arabinopyranose
IV: R_1=H, R_2=O-β-gluc, R_3=β-gluc
V: R_1=H, R_2=O-β-gluc^{2}—^{1}α-rhamnopyranose, R_3=β-gluc
VI: R_1=β-gluc^{2}—^{1}β-gluc, R_2=H, R_3=β-gluc
VII: R_1=H, R_2=OH, R_3=H
X: R_1=H, R_2=H, R_3=β-gluc
(β-gluc=β-glucopyranose)

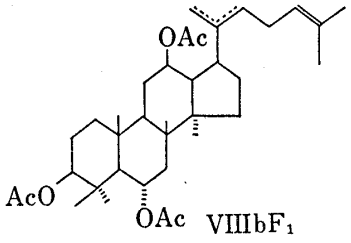
Chart 1


VIIIa


VIIIb



VIIIaF$_1$



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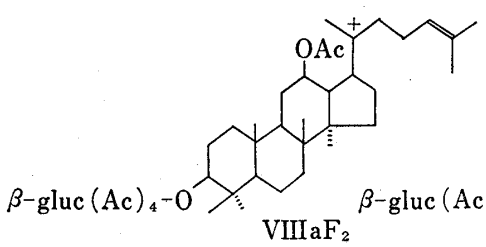
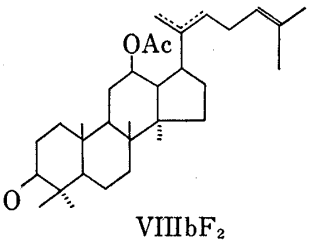
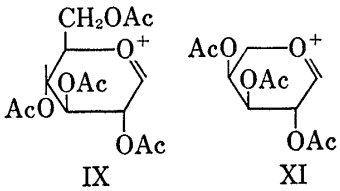


VIIIaF$_2$

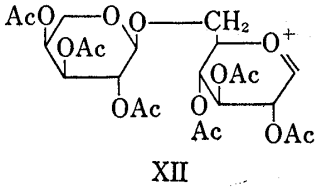


VIIIbF$_2$



IX

XI



XII

Chart 2

glycoside linkages. On the basis of these evidences, the structure of III was assigned as 20-O-[α-arabinopyranosyl-(1→6)-β-glucopyranosyl]-20(*S*)-protopanaxatriol.

The TLC of the methanolic extract of the fresh leaves of Ginseng exhibited the same saponin-pattern as that of the dried leaves, exclucing the comment that I, II, and III might be prosapogenins formed from parent saponins during storage of the leaves.

Experimental

All melting points were determined on Yanagimoto Micro-hot stage and uncorrected. NMR spectra were taken on JEOL PS-100 FT spectrometer at 100MHz at 25° unless otherwise stated. Chemical shifts are given in δ (ppm) with tetramethylsilane as internal standard. All MS were taken as acetates at 75 eV on JEOL-01-SG-2 spectrometer.

Hydrolysis of Saponins with Crude Hesperidinase (General Procedure)[10]——A mixture of a saponin (20 mg), crude hesperidinase (Tanabe Pharm. Ind. Co. Ltd.; the original preparation before addition of diluent was used) (20 mg) and a few drops of toluene in 0.2M phosphate buffer (pH 4.0, 20 ml) was incubated at 37—40° for 2 or 3 days. After dilution with H_2O, the reaction mixture was extracted with ether. From the ether extract, the sapogenin or the prosapogenin was obtained by direct recrystallization of by chromatography on silica gel followed by recrystallization. The aqueous layer was dialyzed against H_2O and the resulted dialyzed fraction was deionized by passing through a column of Amberlite MB-3 and concentrated to dryness. The residue was subjected to detection of monosaccharides by TLC and gas–liquid chromatography (GLC).

Identification of Monosaccharides (General Procedure)——TLC: On silica gel, solvent: $CHCl_3$: MeOH: H_2O (65: 45: 10 homogeneous), detection: aniline hydrogenephthalate. Trimethylsilylation (TMS) for GLC: The dried residue (*ca.* 5 mg) was dissolved in N-trimethylsilylimidazole (0.5 ml) and the solution was subjected to GLC after standing at room temperature for 10 min without concentration. GLC: On a glass column 2 mm×2 m packed with 1.5% OV-1 on Chromosorb W, column temp. 180°, N_2 1.0 kg/cm^2; retention times TMS-glucose 4.9, 6.0, and 8.7 min, TMS-arabinose 1.9 and 2.2 min, TMS-rhamnose 2.0 and 2.8 min.

TLC of Saponins and Sapogenins (General Procedure)——On silica gel. Solvent: 20(*S*)-protopanaxatriol (VII) and other sapogenins–toluene: EtOAc (2: 1) or ether only, ginsenoside-F_1 (I) and 20-O-β-glucosyl-20(S)-protopanaxadiol (XI)–$CHCl_3$: MeOH (8: 1), other saponins–$CHCl_3$: MeOH: H_2O (65: 40: 10 homogeneous) or *n*-BuOH: EtOAc: H_2O (15: 1: 4 upper layer). Detection: H_2SO_4.

Acetylation of Saponins for MS Determination (General Procedure)——To a solution of a saponin (2 mg) in anhyd. C_5H_5N (0.4 ml) was added Ac_2O (0.2 ml) and the solution was allowed to stand at 25° for 2 days. The reagents were removed by blowing N_2 at room temperature and the residue was dried *in vacuo*. Completion of acetylation was substantiated by infrared (IR) and TLC (on silica gel, solvent: $CHCl_3$: ether (9: 1), detection: H_2SO_4).

Extraction and Separation of Saponins——The dried leaves (500 g) were extracted with MeOH at room temperature and the extract was concentrated to dryness. The residue (100 g) was suspended in H_2O and the suspension was washed with ether and then extracted with *n*-BuOH. The BuOH solution was passed through a column of a mixture of alumina and polyamide and the eluate was evaporated to dryness yielding crude glycoside fraction (60 g). A solution of this fraction (10 g) in H_2O (50 ml) was dialyzed through cellophane film against H_2O until most of saponins I, II, III, IV, and V were dialyzed. The dialyzed fraction was concentrated to dryness and the residue was chromatographed on silica gel. Elution with $CHCl_3$: MeOH: H_2O (50: 8: 1) afforded I, a mixture of II and III, IV, and V. The mixture of II and III seemed to be homogeneous by TLC on silica gel but was clearly separated by TLC on polyamide (solvent: H_2O: MeOH (2: 1)). Column chromatography of this mixture on polyamide (gradient elution with MeOH–H_2O (1: 15—5) furnished the separation into II and III. The non-dialyzed fraction was concentrated to dryness and the residue was chromatographed on silica gel. Elution with $CHCl_3$: MeOH: H_2O (300: 70: 7) afforded VI.

Identification of the Known Saponins——Ginsenoside-Rg_1 (IV): White powder, yield 1.5%. On hydrolysis with crude hesperidinase, IV yielded glucose, VII, and a small amount of its prosapogenin, I, latter two of which were proved to be identical with authentic samples by TLC, IR, and NMR (in C_5D_5N), respectively. The identity of IV with an authentic sample of ginsenoside-Rg_1 was confirmed by comparison of TLC, optical rotation, MS, and NMR (in C_5D_5N).

Ginsenoside-Re(V): Colourless needles from MeOH–H_2O, mp 196—198°, yield 1.5%. On hydrolysis with crude hesperidinase, V afforded glucose, rhamnose, and VII. Direct comparison of V with an authentic sample of ginsenoside-Re was achieved by TLC, optical rotation, MS, and NMR (in C_5D_5N).

Ginsenoside-Rd (VI): White powder, yield 1.5%. On hydrolysis with crude hesperidinase, VI yielded glucose and X, latter of which was proved to be identical with an authentic sample by TLC and NMR. Direct comparison of VI with an authentic sample of ginsenoside-Rd was performed by comparison of TLC, optical rotation, MS, and NMR (in C_5D_5N).

Ginsenoside-F_1 (I)——White powder. *Anal.* Calcd. for $C_{36}H_{62}O_9 \cdot H_2O$: C, 65.82; H, 9.82. Found: C, 65.26; H, 9.48. $[\alpha]_D^{25}$ +36.6° (c=1.12, MeOH), yield 0.4%. On hydrolysis with crude hesperidinase, I afforded glucose and VII, latter of which was proved to be identical by TLC, IR, and NMR with an authentic sample of 20(*S*)-protopanaxatriol (VII).

Ginsenoside-F_2(II)——White powder. *Anal.* Calcd. for $C_{42}H_{72}O_{13} \cdot 2\ 1/2H_2O$: C, 60.77; H, 9.35. Found: C, 60.89; H, 9.26. $[\alpha]_D^{25}$ +21.1° (c=1.14, MeOH), yield 0.2%. Hydrolysis of II with crude hesperidinase gave glucose and X, latter of which was proved to be identical with an authentic sample by TLC, IR, and NMR.

Preparation of II from VI——A mixture of VI (50 mg), Takadiastase (100 mg)[12] and a few drops of toluene in 0.2 M phosphate buffer (pH 4.0, 40 ml) was incubated at 37° for 60 hr. The mixture was extracted with ether and the ether-extract was concentrated to dryness. The resulted residue was chromatographed on silica gel eluting with $CHCl_3$: MeOH (8: 1) to give II (yield 10 mg) along with X and unchanged starting material. The identity of II with an authentic sample was confirmed by comparison of TLC, MS, and NMR (in C_5D_5N).

Ginsenoside-F_3 (III)——White powder. *Anal.* Calcd. for $C_{41}H_{70}O_{13} \cdot 3H_2O$: C, 59.68; H, 9.27. Found: C, 59.76; H, 8.78. $[\alpha]_D^{25}$ +26.5° (c=0.98, MeOH), yield 0.2%. Hydrolysis of III with crude hesperidinase

yielded glucose, arabinose, VII, and a small amount of I, latter two of which were identified with authentic samples of VII and I, respectively, by TLC, IR, MS, and NMR (in $CDCl_3$ for VII and in C_5D_5N for I).

A mixture of NaH (7 mg) and dimethyl sulfoxide (DMSO) (1 ml) was heated at 70° for 1 hr under N_2 and to this mixture was added a solution of III (5 mg) in DMSO (1 ml). After stirring at room temperature for 1 hr, MeI (0.5 ml) was added to this mixture and the solution was stirred at room temperature for 18 hr. After dilution with H_2O, the reaction mixture was extracted with $CHCl_3$. The $CHCl_3$-layer was washed with H_2O and evaporated to dryness. Chromatography of the residue on silica gel (elution with $CHCl_3$: MeOH (70: 1) gave permethyl ether of III, IR no OH band. This permethyl ether, which was homogeneous by TLC, was dissolved in 5% HCl–MeOH (1 ml) and the solution was heated at 90—95° for 6 hr in a sealed tube. The reaction mixture was deionized by passing through a column of Amberlite MB-3 and then concentrated to dryness. The residue was subjected to GLC: On a glass column 2 mm × 2 m packed with 5% DPGS on Chromosorb W, column temp. 170°, N_2 1.0 kg/cm^2; retention times: Methyl 2,3,4-tri-O-methylarabinopyranoside 3.2 min, methyl 2,3,4-tri-O-methylglucopyranoside 7.4 and 10.0 min.

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