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Soil Bacterial Hydrolysis leading to Genuine Aglycone. V.[1] On Ginsenosides-Rb_1, Rb_2, and Rc of the Ginseng Root Saponins

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Through the application of the soil bacterial hydrolysis method on a mixture of ginsenosides Rb_1, Rb_2, and Rc, an additional evidence on the genuineness of 20(*S*)-protopanaxadiol (I) has been provided. In addition, a prosapogenol (termed as compound K) has been isolated as a major hydrolysate and the structure has been assigned as 20-O-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (VI).

Recently, a dammarane-type triterpenoid, 20(*S*)-protopanaxadiol (I), has been elucidated to be a genuine sapogenol of some of the Ginseng root saponins, *i.e.* ginsenosides-Rb_1, Rb_2, and Rc (termed as Rb–c) by Shibata and his co-workers.[3] The elucidation of genuineness has been principally based on the fact that the Smith's degradation[4] of Rb–c afforded 20(*S*)-protopanaxadiol as a sole sapogenol. Together with the previous assignment of a common prosapogenol[5] which was obtained by mild acid hydrolysis of Rb–c, Shibata, *et al.*[3] have presumed the structure of Rb–c to be represented by II.

As a continuation of the study on soil bacterial hydrolysis of saponin leading to genuine sapogenol,[6] we have applied the microbiological method on Rb–c to achieve an additional evidence on the genuineness of 20(*S*)-protopanaxadiol (I), since the latter has been shown to isomerize readily on mild acid treatment to give an equilibrated mixture of C-20 epimers: 20(*S*)- and 20(*R*)-protopanaxadiols (I and III)[7] and it has also been demonstrated to give a mixture of panaxadiol (IV) and 20-epi-panaxadiol (V) under stronger acid conditions.[7] The present paper is concerned with the detail of our investigation which leads to the conclusion that 20(*S*)-protopanaxadiol (I) is a genuine sapogenol of Rb–c and in addition leads to the isolation of another prosapogenol designated as compound K (VI).[8]

The composition of ether extract of hydrolysate obtained by soil bacterial cultivation using a strain (YSB-6) selected on a synthetic medium containing the mixture of Rb–c[9] as a sole carbon source as reported previously[6a] was revealed by thin-layer chromatography (TLC) as depicted in Fig. 1. Of several significant components, two compounds giving spots A and B on TLC were isolated by alumina column chromatography (*vide infra*) as an aglycone

1) Part IV: I. Yosioka, S. Saijoh, and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), **20**, 564 (1972).
2) Location: *Toneyama, Toyonaka, Osaka.*
3) M. Nagai, T. Ando, N. Tanaka, O. Tanaka, and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **20**, 1212 (1972) (Preliminary report: M. Nagai, T. Ando, O. Tanaka, and S. Shibata, *Tetrahedron Letters*, **1967**, 3579), and the iteratures cited ltherein.
4) F. Smith, G.W. Hay, and B.A. Lewis, "Method in Carbohydrate Chemistry," Vol. 5, Academic Press, New York and London, 1965, p. 361.
5) S. Shibata, T. Ando, and O. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **14**, 1157 (1966).
6) *a*) I. Yosioka, M. Fujio, M. Osamura, and I. Kitagawa, *Tetrahedron Letters*, **1966**, 6303; *b*) I. Yosioka, K. Imai, and I. Kitagawa, *ibid.*, **1967**, 2577.
7) O. Tanaka, M. Nagai, T. Ohsawa, N. Tanaka, K. Kawai, and S. Shibata, *Chem. Pharm. Bull.* (Tokyo), **20**, 1204 (1972) (Preliminary report: O. Tanaka, M. Nagai, T. Ohsawa, N. Tanaka, and S. Shibata, *Tetrahed ron Letters*, **1967**, 391).
8) Preliminary report: I. Yosioka, K. Imai, I. Kitagawa, S. Shibata, O. Tanaka, and T. Ando, The 88th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo April 1968, Abstract Papers, p. 255.
9) Kindly provided by Professors S. Shibata and O. Tanaka.

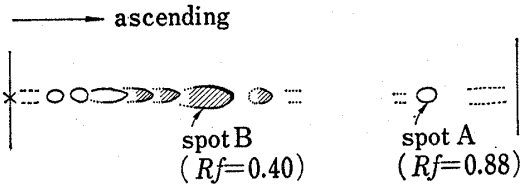
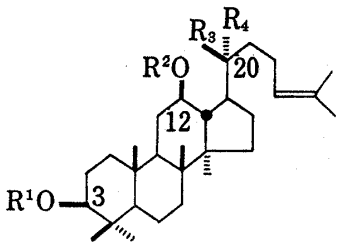


Fig. 1. Thin-Layer Chromatogram ($CHCl_3$: MeOH=6:1, Silica Gel Camag D-5) of the Ether Extract of Soil Bacterial Hydrolysate

(spot A, mp 174—177°) and a prosapogenol (spot B, compound K, amorphous) in 2.2% and 20.5% yields respectively. The aglycone was identified with 20(*S*)-protopanaxadiol (I)[9] by direct comparison and was definitely distinguishable from 20(*R*)-protopanaxadiol (III)[9] by TLC, thus substantiating the genuineness of 20(*S*)-protopanaxadiol. 20(*R*)-Protopanaxadiol was not detected in the total hydrolysate notwithstanding the detailed TLC examination.



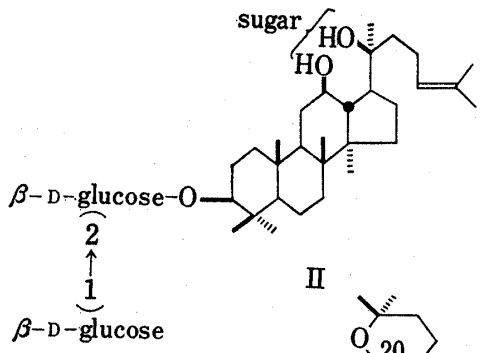
I : $R^1=R^2=H$, $R^3=OH$, $R^4=CH_3$
20(*S*)-protopanaxadiol

III : $R^1=R^2=H$, $R^3=CH_3$, $R^4=OH$
20(*R*)-protopanaxadiol

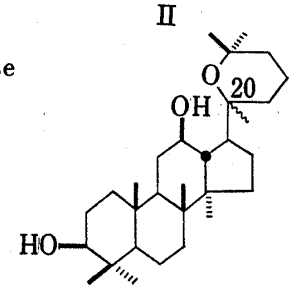
VI : $R^1=R^2=H$, $R^3=$ -O-β-D-glucopyranosyl
$R^4=CH_3$
compound K

VIa : $R^1=R^2=Ac$, $R^3=$-O-2,3,4,6,-tetra-O-acetyl-β-D-glucopyranosyl, $R^4=CH_3$

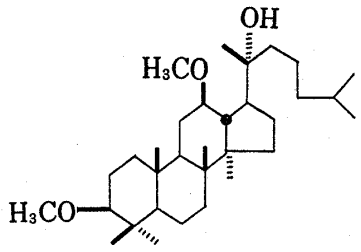
VIb : $R^1=R^2=CH_3$, $R^3=$-O-2,3,4,6-tetra-O-methyl-β-D-glucopyranosyl, $R^4=CH_3$



II



IV : 20β-CH_3
V : 20α-CH_3



VII

Chart 1

Compound K (VI), which was obtained as a major hydrolysate and was presumed to be a monoglycoside derivative on the basis of its mobility on TLC, showed a broad hydroxyl absorption band in its infrared (IR) spectrum. Since it was noticed to decompose partially during silica gel column chromatography (see experimental section), alumina was employed for its isolation as described above. On usual acetylation, compound K gave a crystalline hexaacetate (VIa), mp 177—178°, which showed no hydroxyl absorption band in its IR spectrum. Proton magnetic resonance (PMR) spectrum of the hexaacetate (VIa) shows the presence of five ordinary C-methyl functions, one C-methyl geminal to an oxygen function (8.86 τ, at C-20), two vinylic C-methyl (8.44 and 8.38 τ, at C-25) and one carbinyl proton geminal to O-acetyl function (5.60 τ, at C-3) in addition to six acetyl functions. Therefore, it has been assumed that a glucosyl moiety of compound K is attached to a hydroxyl function at either C-12 or C-20 and not at C-3 of the aglycone.

Methylation of compound K through the Hakomori's procedure[10] afforded a fully methylated product (VIb) whose IR spectrum shows again the absence of hydroxyl function. Catalytic hydrogenation of the permethylate gave a dihydro derivative, which was subsequently subjected to hydrolysis using concentrated hydrochloric acid to give 3,12-di-O-methyl-20(*R*)-dihydroprotopanaxadiol (VII), mp 140—142°, being identified with an authentic sample[9] and 2,3,4,6-tetra-O-methyl-D-glucopyranose. Although TLC of the total hydrolysate showed the minor presence of 20(*S*) derivative as presumed by its close *Rf* value, only VII was isolated in a pure state. The predominant formation of 20(*R*) derivative (VII) is analogously reasoned as has been discussed by Tanaka, *et al.*[7] in the acid catalyzed epimerization of 20(*S*) and 20(*R*)-protopanaxadiols and the related triterpenoids. Therefore, the location of glucosyl moiety in compound K (VI) is now established to be at C-20, which is corroborated by the aforementioned absence of hydroxyl absorption band in the IR spectrum of VIa. In addition, the application of Klyne's rule[11] on 20(*S*)-protopanaxadiol (I) and compound K (VI) disclosed that the glucosyl moiety in the latter attaches with β orientation.

Consequently, compound K is expressed as 20-O-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (VI). In connection with the structure elucidation of a common prosapogenol of Rb–c by Shibata, *et al.*,[5] the present work offers an additional evidence on the whole structure of Rb–c, that is, to possess another glycoside linkage at C-20.[12]

It is noteworthy to point out that the present soil bacterial hydrolysis has left the glycosidic linkage at other than C-3 of Rb–c unattacked as has been observed in case of Panax root saponin hydrolysis.[6a] These types of hydrolysis seem to be inaccessible by the other method and the findings suggest that the soil bacterial hydrolysis method could be a useful mean for the structure elucidation of triterpenoid saponin which especially possesses the glycosidic linkage at other than C-3 of the aglycone.

Experimental[13]

Soil Bacterial Hydrolysis of the Mixture of Rb-c——According to the previously reported procedure,[6a] a synthetic medium (two flasks of one liter each) containing the mixture of Rb-c (3 g×2)[9] as an only carbon source was prepared. The concentration of glycosides was 0.3%. After sterilization (120°, 2 Atm, 20 min), a soil bacterial strain (YSB-6, unidentified) selected as before[6a] using the same synthetic medium was cultured stationarily at 32° and the total culture broths were extracted with ether repeatedly. One culture broth was extracted after 16 days cultivation and another after 23 days. TLC (Fig. 1) disclosed no significant difference between two ether extracts. Combined ether extract afforded 1.9 g of a residue, which was chromatographed on neutral alumina (Woelm, grade III, 100 g) eluting with $CHCl_3$ and $CHCl_3$–MeOH mixtures successively. Fractions obtained by $CHCl_3$ and $CHCl_3$–MeOH (98:2) elution gave a compound (spot A on TLC), which, after recrystallization from benzene, afforded colorless needles (42 mg) of mp 174—177°. IR ν_{max}^{KBr} cm^{-1}: 3240 (br.) (OH), 1036. The substance was identified with 20(*S*)-protopanaxadiol (I)[9] by mixed mp, IR (KBr), and TLC. Fractions obtained by $CHCl_3$–MeOH (87:13—85:15) elution gave compound K(VI, 410 mg, amorphous). Although the substance showed a single spot on TLC ($CHCl_3$: MeOH=6:1), the many attempts for crystallization failed. A sample obtained by drying *in vacuo* (at 50°, 4 mmHg, 3 days) gave the following analytical value. *Anal.* Calcd. for $C_{36}H_{62}O_8 \cdot 2H_2O$: C, 65.62; H, 10.10. Found: C, 65.84; H, 9.45. $[\alpha]_D$ +11° (*c*, 1.5 in $CHCl_3$). IR ν_{max}^{Nujol} cm^{-1}: 3450—3150 (br.) (OH), 1025 (br.).

10) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
11) W. Klyne, *Biochem. J.*, **47**, xli (1950).
12) Very recently the structures of ginsenosides Rb_1, Rb_2, and Rc have been established by S. Sanada, N. Kondo, J. Shoji, O. Tanaka, and S. Shibata (The 92th Annual Meeting of the Pharmaceutical Society of Japan, Osaka April 1972, Abstract Papers, II-248), and a further support of the present paper has been provided.
13) The following instruments were used for the physical data. Melting points: Yanagimoto Micro-meltingpoint Apparatus (a hot-stage type); Specific rotations: Rex Photoelectric Polarimeter NEP-2; IR spectra: Hitachi IR Spectrometers EPI-S2 and EPI-2; PMR spectra: Varian HA-100 Spectrometer; Gas–liquid chromatography (GLC): Hitachi Gas Chromatograph Model 063. Silica gel Camag D-5 was employed for TLC.

$\Delta[M]_D = [M]_D$ (in $CHCl_3$) of compound K(VI) $- [M]_D$ (in $CHCl_3$) of 20(*S*)-protopanaxadiol(I) $= (+72°) - (+123°)$[3] $= -51°$. $[M]_D$ of methyl α-D-glucopyranoside $= +309°$; $[M]_D$ of methyl β-D-glucopyranoside $= -66°$.[14]

Acetylation of Compound K(VI)——To a solution of VI (77 mg) in pyridine (2.5 ml) was added Ac_2O (1 ml) and the total solution was allowed to stand at 28° for 5 days. After working up in a usual way, the product was crystallized from EtOH to give colorless needles (35 mg) of hexaacetate (VIa), mp 177—178°, $[\alpha]_D$ +9.6° (*c*, 0.94 in $CHCl_3$). *Anal.* Calcd. for $C_{48}H_{78}O_{14}$: C, 65.58; H, 8.94. Found: C, 65.94; H, 8.49. IR $\nu_{max}^{CCl_4}$ cm^{-1}: no OH, 1760, 1735, 1250, 1225 (OAc). PMR ($CDCl_3$, 100 MHz) τ: 9.18, 9.15, 9.09, 9.06 (totally 15H, each s, $C\underline{H}_3 \times 5$), 8.86 (3H, s, $C_{(20)}$-$C\underline{H}_3$), 8.44, 8.38 (3H each, s, $=C_{(25)}(C\underline{H}_3)_2$), 8.06, 8.03, 8.01, 7.99 (totally 18H, each s, OAc × 6), 6.42 (1H, m, $C_{(5')}\underline{H}$), 5.96 (2H, d, $J=2$ Hz, $C_{(6')}\underline{H}_2$), 5.74—4.90 (6H, complex signal), 5.60 (1H, t-like, $C_{(3)}\underline{H}$).

Treatment of Compound K (VI) with Silica Gel——Compound K (VI, 18 mg) was developed on TLC (Silica gel Merck) using $CHCl_3$: MeOH = 5:1 mixture and the plates were left standing at room temperature for 72 hr. The parts of TLC plates adsorbing compound K (detected by I_2 vapor) were collected and extracted with MeOH to give a product (13 mg) which was subjected again to preparative TLC (silica gel Camag D-5) developing with $CHCl_3$: MeOH = 7:1 mixture (detected by I_2 vapor) to give a substance (2 mg, presumably a sapogenol as judged from its *Rf* value), recovered compound K (5 mg), and sugar (2 mg). The sugar portion was identified with glucose by paper partition chromatography (Toyo Roshi No. 50, iso-PrOH:*n*-BuOH:water = 7:1:2, ascending for 20 hr, *Rf* = 0.38). The sapogenol portion showed a *Rf* value close to 20(*S*)- or 20(*R*)-protopanaxadiol, but could not be identified.

3,12-Di-O-methyl-20(*R*)-dihydroprotopanaxadiol (VII) from Compound K (VI)——A mixture of NaH (50%, 50 mg, washed with petr. ether 3 times beforehand) in DMSO (10 ml) was stirred at 75° for one hour. To this solution was added a solution of VI (110 mg) in DMSO (5 ml) and the total mixture was stirred further for one hour at room temperature. After addition of CH_3I (10 ml) under ice-cooling, the reaction mixture was left standing for 24 hr, poured into a large quantity of ice-water, and extracted with ether. The ether solution was then washed with aq. 5% $Na_2S_2O_3$, water, and dried. Evaporation of the solvent afforded an oily methylated product (85 mg), which was purified by preparative TLC (benzene: EtOAc = 4:1, detected by I_2 vapor) to give a pure fully methylated derivative of compound K (VIb, 26 mg, oily), $[\alpha]_D$ +12° (*c*, 0.76 in $CHCl_3$). IR $\nu_{max}^{CCl_4}$ cm^{-1}: no OH, 1100 (C-O-C).

A solution of the fully methylated derivative (VIb, 26 mg) in EtOAc (20 ml) containing 3 drops of AcOH was hydrogenated over Adams' catalyst (50 mg) for 24 hr. After working up in a usual way, a hydrogenated product (a single spot on TLC) was obtained as an oil, $[\alpha]_D$ +12.8° (*c*, 0.8 in $CHCl_3$). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: no OH, 1094 (C-O-C).

The hydrogenated product (8 mg) was then treated with conc. HCl (2 drops) at room temperature for 4 hr, diluted with water, and extracted with ether. Working up of the ether extract in a usual way furnished a product (3 mg), which was subjected to preparative TLC (benzene: EtOH = 10:1, detected by I_2 vapor) followed by recrystallization from MeOH to give colorless needles of mp 140—142°. IR ν_{max}^{KBr} cm^{-1}: 3386 (OH), 1102, 1068. The substance was identified with authentic 3,12-di-O-methyl-20(*R*)-dihydroprotopanaxadiol (VII)[9] by mixed mp, IR, and TLC. Fraction obtained from $Rf \fallingdotseq 0$ in the above preparative TLC afforded a methylated sugar, which was identified with 2,3,4,6-tetra-O-methyl-D-glucopyranose (prepared from methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside by conc. HCl treatment) by GLC (column: 2% SE-52, 3 mm × 1 m; column temp: 225°; detect. temp.: 220°; inject. temp.: 215°; flow rate: N_2 3 kg/cm^2, air 1.2 kg/cm^2, H_2 0.6 kg/cm^2).

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14) T. Kawasaki and T. Yamauchi, *Chem. Pharm. Bull.* (Tokyo), **10**, 703 (1962).