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A Modified Alkaline Hydrolysis of Total Ginsenosides Yielding Genuine Aglycones and Prosapogenols

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To improve the yield of genuine aglycones from glycosides, the conditions of alkaline hydrolysis were investigated, and a modified method was established. The modified method employed pyridine as an aprotic solvent. To complete the hydrolysis and obtain 20(S)-protopanaxadiol (**1**) and 20(S)-protopanaxatriol (**2**), which are the genuine aglycones of ginsenosides, total ginsenosides were refluxed with sodium methoxide in pyridine. Addition of methanol, a protic polar solvent to the reaction mixture, led partial hydrolysis yielding a mixture of the genuine prosapogenols. Of the prosapogenols compound **3** and **6** characteristically possessed D-glucopyranosyl moiety attached at the sterically hindered C-20 hydroxyl group. **3** and **6** were not obtained by other hydrolysis methods except by the soil bacterial hydrolysis.

Key words: Modified alkaline hydrolysis, Total ginsenosides, 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, Genuine aglycones, Genuine prosapogenols, Aprotic solvent.

INTRODUCTION

The aglycones of ginsenosides (glycosides from Panax ginseng C. A. MEYER) are easily subjected to chemical transformations under acidic conditions. Acid hydrolysis of ginsenosides is always accompanied by various side reactions (Tanaka *et al.*, 1972) such as epimerization at C-20, hydroxylation and cyclization of the side chain. Therefore, it is almost impossible to obtain the genuine aglycones or genuine prosapogenols by acid hydrolysis of ginsenosides. Some devices to avoid the undesirable secondary changes have been reported such as soil bacterial hydrolysis (Yosioka *et al.*, 1966; 1972), enzymatic hydrolysis (Kohda *et al.*, 1975), Smith degradation (Nagai *et al.*, 1972; Ohsawa *et al.*, 1972), but their yields are very poor. Recently a new method, hydrolysis by alkaline condition (Ogihara *et al.*, 1986; Yingjie *et al.*, 1987) has been developed by Ogihara group. They have succeeded in preparing genuine aglycones: 20(S)-protopanaxadiol (**1**) and 20(S)-protopanaxatriol (**2**) and, in addition, some genuine prosapogenols by treating cetrtain gensenosides with alkali in an alcoholic solution.

After the isolation (Kitagawa *et al.*, 1983) of 3-O-β-D-glucopyranosyl derivative of 20(S)-protopanaxadiol (ginsenoside Rh_2) which has antitumor activity (Odashima *et al.*, 1985) from *Ginseng radix rubra*, several attempts to prepare this glycoside have been reported including by hydrolysis of certain glycosides (Koizumi *et al.*, 1982), and by synthesis (Atopkina *et al.*, 1988)). Our group also attempted the synthesis of ginsenoside Rh_2 (Im *et al.*, 1993) by glycosylation reaction of compound **1** and **2** which were obtained by hydrolysis of total ginsenosides in an alkaline condition. We call this method "retro-synthesis" in which degradation of glycoside and reglycosylation steps are followed. The high yield of compound **1** and **2** is essential for this synthesis. Because we found that the yields of aglycones by a method developed by Ogihara also is not satisfactory for this purpose, and reinvestigated the reaction conditions. The hydrolysis reaction of glycosides by alkali is thought to follow S_N2 mechanism. So we modified the hydrolysis method using higher concentration of strong alkali (sodium methoxide) in an aprotic polar solvent (pyridine), and found that the yields were improved. Futhermore, addition of a protic solvent (methyl alcohol) to pyridine conducted partial hydrolysis of glycosides yielding several prosapogenols in good yields which could not be obtained by other known methods. The mixture of monoglucosides thus obtained has been found to show potent cytotoxicities (Im *et al.*, 1995). Some compounds (**3**, **6**) obtained by this method showed characteristic structures by having a glucose moiety linked at sterically crowded C-20 hydroxyl group which is easily removed by an acid hydrolysis.

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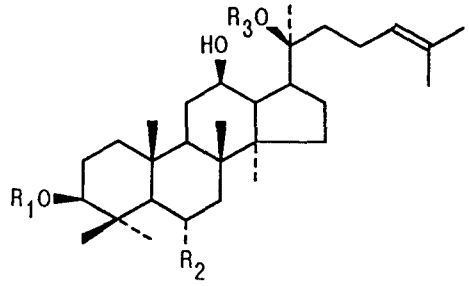

	R_1	R_2	R_3
1:	H	H	H: 20(S)-protopanaxadiol
2:	H	OH	H: 20(S)-protopanaxatriol
3:	H	H	β–glu: compound K
4:	β-glu	H	H: gisenoside Rh_2
5:	H	O-β-glu	H: ginsenoside Rh_1
6:	H	O-β-glu	β-glu: ginsenoside Rg_1

Chart 1.

MATERIALS AND METHODS

General experimental procedures

The following instruments were used for obtaining the physical data.

Melting points: Fisher micromelting point apparatus (hot-stage), and recorded uncorrected; Specific rotations: JASCO DIP-181 digital polarimeter (l=1 dm); IR spectra: Shimadzu IR-400 spectrophotometer; Elemental analysis: Carlo Erba CHNS EA 1108 elemental analyzer; NMR spectra: Bruker AC 200 spectrometer (200 MHz for ^{1}H-NMR and 50.3 MHz for ^{13}C-NMR), and recorded δ values using tetramethylsilane(TMS) as an internal standard. For chromatography, Kieselgel 60 (Merck, 70-230 mesh) was used for column, and Kieselgel 60 F_{254} (Merck, Art. 5715, precoated plate) was used for thin-layer chromatography(TLC).

Preparation of total ginsenoside

Air dried leaves (powder, 2 Kg) of *Panax ginseng* C. A. MEYER, collected at Yungpoong, Kyungbuk in october were extracted with MeOH three times under reflux. Evaporation of the combined extracts gave a dark brown residue (600 g). The MeOH ext. was suspended into water and extracted with ether (three times with equal volume for each extraction) and 1-BuOH (three times) successively. The combined 1-BuOH ext. was washed with 5% KOH and water successively, evaporated *in vacuo* to give a 1-BuOH ext. (210 g). The 1-BuOH ext. was dissolved in a small amount of MeOH and added into a large volume of EtOAc dropwise to give precipitate, and repeated the same precedure two more times. The final precipitate thus obtained (150 g) was used as a total ginsenoside.

Complete alkaline hydrolysis of total ginsenosides

To a solution of total ginsenosides(1 g) in dry pyridine (50 ml) was added sodium methoxide powder(1 g), and refluxed for 8 hrs. The reaction mixture was evaporated to dryness under reduced pressure. The residue was suspended into water (100 ml), extracted with ether three times, and the total extract was washed with 5% aq. HCl, saturated aq. $NaHCO_3$, and water, successively, dried over $MgSO_4$ and evaporated to give an ether ext. (350 mg). The residue was adsorbed on silica gel (0.7 g) with the aid of ether, dried *in vacuo*, and put on a culumn of silica gel (18 g) packed with benzene-acetone (5 : 1). Successive elution with the same solvent mixture gave 20(S)-protopanaxadiol (**1**, 98 mg) and 20(S)-protopanaxatriol (**2**, 145 mg). The aqueous layer was then extracted with EtOAc and 1-BuOH, successively to give EtOAc ext. (78 mg) containing prosapogenols and 1-BuOH ext. (45 mg) containing recovered ginsenosides.

20(S)-protopanaxadiol (1): mp 196-198° (colorless needles from ether-n-hexane), $[\alpha]_D$ +26.7° (c=1.03, $CHCl_3$), *Anal.* Calcd. for $C_30H_{52}O_3$: C, 78.19; H, 11.43. Found: C, 77.98; H, 11.29. IR($CHCl_3$, cm^{-1}): 3600 (w), 3530 (OH). ^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.78 (3H, s), 0.88, 0.98 (6H each, both s), 1.16, 1.61, 1.67 (3H each, all s) (8x tert. Me), 5.12 (1H, t-like, 24-H). ^{13}C-NMR: see Table 1.

20(S)-protopanaxatriol (2): mp 197-198° (colorless needles from ether-n-hexane), $[\alpha]_D$ 43° (c=1.0, $CHCl_3$), *Anal.* Calcd. for $C_{30}H_{52}O_4$: C, 75.57; H, 11.21. Found: C, 75.23; H, 11.54. IR($CHCl_3$, cm^{-1}): 3590 (w), 3530 (OH). ^{1}H-NMR(200 MHz, $CDCl_3$) δ: 0.94 (6H, s) 0.97, 1.05, 1.15, 1.18, 1.62, 1.68(3H each, all s) (8 x tert. Me), 5.25 (1H, t-like, 24-H). ^{13}C-NMR: see Table 1.

Partial alkaline hydrolysis of total ginsenosides

To a solution of total ginsenosides (1 g) in dry pyridine-dry MeOH (5 : 1) mixed solvent (50 0ml) was added sodium methoxide powder (1 g), and refluxed for 8 hrs. The reaction mixture was treated as above to give ether ext. (25 mg), EtOAc ext. (650 mg), and 1-BuOH ext. (120 mg). The ether ext. contained compound **1** and **2**, 1-BuOH ext. contained unreacted ginsenosides, and the EtOAc ext. contained prosapogenols (identified by TLC).

Isolation of prosapogenols

The EtOAc ext. (650 mg) obtained by partial alkaline hydrolysis was fractionated on a silica-gel column, eluting with a gradient mixture of $CHCl_3$-MeOH=25 : 1, 10 : 1) to give two fractions: Fr. A (305 mg) and Fr. B (105 mg). The Fr. A was chromatographed on silica-gel, eluting with $CHCl_3$-MeOH-H_2O (10 : 1 : 0.05) to give compounds **3** (109 mg), **4** (123 mg), and 5 (56 mg). The compounds **3**, **4**, and **5** were further purified by HPLC on reversed phase C_{18} column (TSK ODS-120 T, 10

Table 1. ^{13}C-NMR data for compounds **1-6**. Measured at 50.3 MHz in a) $CDCl_3$, b) d_5-pyridine.

	1[a]	2[a]	3[b]	4[b]	5[b]	6[b]
C-1	39.5	39.2	39.4	39.4	39.4	39.5
C-2	28.2	28.0	28.2	27.3	27.9	27.6
C-3	77.9	78.3	78.1	88.9	78.0	78.6
C-4	39.5	40.2	39.5	40.3	40.3	40.1
C-5	56.3	61.7	56.4	56.7	61.4	61.3
C-6	18.7	67.6	18.8	18.7	78.6	77.8
C-7	35.2	47.4	35.2	36.0	45.2	44.9
C-8	40.0	41.1	40.1	37.2	41.1	41.0
C-9	50.4	50.1	50.3	50.7	50.2	49.9
C-10	37.3	39.3	37.4	39.8	39.6	39.5
C-11	32.0	31.9	30.8	32.2	32.0	30.8
C-12	70.9	70.9	70.2	71.1	71.0	70.3
C-13	48.5	48.1	49.5	48.8	48.2	48.9
C-14	51.6	51.6	51.4	51.9	51.6	51.3
C-15	31.3	31.3	30.8	31.5	31.1	30.6
C-16	26.8	26.8	26.7	26.8	27.2	26.4
C-17	54.7	54.6	51.7	54.8	54.7	51.6
C-18	16.2	17.5	16.3	16.8	17.4	17.4
C-19	15.8	17.4	16.0	16.4	17.6	17.4
C-20	72.9	72.9	83.3	73.2	73.0	83.3
C-21	26.9	26.9	22.4	27.0	26.8	22.3
C-22	35.8	35.7	36.1	35.4	35.8	35.9
C-23	22.9	22.9	23.3	23.1	23.0	23.2
C-24	126.2	126.2	125.9	126.4	126.3	125.8
C-25	130.6	130.6	130.9	130.7	130.6	130.9
C-26	25.8	25.8	25.8	25.7	25.8	25.7
C-27	17.6	17.7	17.8	17.7	17.6	17.7
C-28	28.6	31.9	28.7	28.3	31.7	31.6
C-29	16.4	16.4	16.3	16.0	16.4	16.2
C-30	17.0	17.0	17.4	17.3	16.8	17.0
3-O-β-D-gloucosyl						
C-1'				106.7		
C-2'				75.8		
C-3'				78.7		
C-5'				72.2		
C-4'				78.0		
C-6'				63.3		
6-O-β-D-gloucosyl						
C-1'					105.9	105.7
C-2'					75.4	75.3
C-3'					80.0	80.0
C-4'					71.8	71.6
C-5'					79.5	79.3
C-6'					63.1	62.9
20-O-β-D-gloucosyl						
C-1'			98.2			98.1
C-2'			75.1			74.9
C-3'			79.2			78.8
C-4'			71.6			71.3
C-5'			78.2			77.8
C-6'			62.8			62.6

μm) developing with CH_3CN-H_2O (92 : 8 for **3**, 90 : 10 for **4**, and **5**) to afford analytical samples. The Fr. B was subjected to reversed phase HPLC (C_{18} column, TSK ODS-120 T, 10 μm) developing with CH_3CN-H_2O (92 : 8) to give **6** (89 mg).

Compound 3: mp, 254-256° (colorless needles from MeOH-$CHCl_3$), $[\alpha]_D$ +11.5° (c=1.5, $CHCl_3$), *Anal.* Calcd. for $C_{36}H_{62}O_8.2H_2O$: C, 65.62; H, 10.10. Found: C, 65.25; H, 9.56. IR(KBr, cm^{-1}): 3450(OH), ^{13}C-NMR: see Table 1.

Compound 4: mp, 218-221° (colorless needles from MeOH-$CHCl_3$), $[\alpha]_D$ +21.8° (c=1, MeOH), Anal. Calcd. for $C_{36}H_{62}O_8.H_2O$: C, 65.80; H, 9.82. Found: C, 65.46; H, 10.01. IR(KBr, cm^{-1}): 3328(OH), ^{13}C-NMR: see Table 1.

Compound 5: white powder, $[\alpha]_D$ +27.0° (c=1.0, MeOH), Anal. Calcd. for $C_{36}H_{62}O_9.H_2O$: C, 65.81; H, 9.83. Found: C, 65.54; H, 9.98. IR (KBr, cm^{-1}): 3340(OH). ^{13}C-NMR: see Table 1.

Compound 6: white powder, $[\alpha]_D$ +24.8° (c=0.93, MeOH), Anal. Calcd. for $C_{40}H_{72}O_{14}.2H_2O$: C, 60.24; H, 9.62. Found: C, 59.89; H, 10.08. IR (KBr, cm^{-1}): 3400(OH), ^{13}C-NMR: see Table 1.

Alkaline hydrolysis of compound 3, 4, and 5

To a solution of compound **3** (24 mg) in dry pyridine(10 ml) was added sodium methoxide powder (20 mg), and refluxed the solution for 4 hrs. The reaction mixture was treated as above to give an ether ext. (5 mg), which was identified to contain **1**. The same treatment of compound **4** and **5** yielded **1** and **2**, respectively, as their aglycones.

Partial alkaline hydrolysis of compound 6

To a solution of compound **6** (20 mg) in a mixed solvent (10 ml) of dry pyridine and dry MeOH (5 : 1) was added sodium methoxide powder (20 mg), and refluxed the solution for 4 hrs. The reaction mixture was treated as above giving ether ext. (2.5 mg) and EtOAc ext. (8.9 mg).The ether ext. was revealed to contain **2** (ether-n-hexane=5 : 1, benzene-acetone=10 : 1), and EtOAc ext. contained **3** and an unidentified monoglucoside by TLC analysis ($CHCl_3$-MeOH=5 : 1, $CHCl_3$-MeOH-H_2O=7 : 3 : 1, lower layer).

RESULTS AND DISCUSSION

The complete hydrolysis of ginsenosides was carried out with sodium methoxide in dry pyridine solution. The products obtained were 20(S)-protopanaxadiol (**1**) and 20(S)-protopanaxatriol (**2**), and the yields were found to be higher than those obtained by the method originally developed by Ogihar group in which protic n-BuOH was used as solvent. The prolonged treatment with alkali yielded some unidentified artifact sapogenins on TLC, thus reducing yields of genuine aglycones.

To obtain prosapogenols a milder condition is required. So we used a mixed solvent system. Addition of a protic polar solvent (MeOH, 20%) to pyridine slowed down the rate of reaction, and in result, pro-

sapogenols were the major products. The solution of ginsenosides in pyridine-MeOH (5 : 1) mixed solvent was refluxed for the same period as in complete hydrolysis. The EtOAc soluble fraction contained three monoglucosides of **1** (**3**, **4**) or **2** (**5**), and a diglucoside of **2** (**6**). The structures of products have been chracterized on the basis of chemical and spectral evidence. On complete alkaline hydrolysis, compound **3** and **4** furnished **1** as their common aglycone, and compound **5** and **6** furnished **2**, respectively. To consider the facts that all sugar moieties directely attached at their aglycones in ginsenosides are D-glucose with β-configurations, and the number of signals due to anomeric carbon in their ^{13}C-NMR spectra of **3**, **4**, and **5** are shown only one for each, compound **3**, **4**, and **5** are suggested to be monoglucosides of **1** (**3**, **4**) and **2** (**5**). The chemical shifts of anomeric carbons (98.2 ppm in **3**, 106.7 ppm in 4, and 105.9 ppm in 5) and shifts in chemical shifts from those of corresponding carbons in aglycone moiety (+10.4 ppm of C-20 in **3**, +10.0 ppm of C-3 in **4**, and +11.0 ppm of C-6 in **5**) indicate the positions of linkage of D-glucose at respective aglycones. On the above evidence, the structures of three monoglucosides are shown as **3**: 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol, **4**: 3-O-β-D-glucopyranosyl-20(S)-protopanaxadiol, **5**: 6-O-β-D-glucopyranosyl-20(S)-protopanaxatriol, respectively. Finally the structure of diglucoside **6** was established as 6-O-β-D-glucopyranosyl, 20-O-β-D-glucopyranosayl-20(S)-protopanaxatriol on the following evidence. On mild alkaline hydrolysis, it affords two monoglucosides (one of which is **5**), indicating the compound to be a bisdesmoside. The chemical shifts due to anomeric carbons linked at C-20 and C-6 (98.1 ppm, 105.7 ppm) and the changes of chemical shifts of related carbons compared with those in 2 (+10.4 ppm of C-20 and + 10.2 ppm of C-6) support the fact.

The prosapogenols obtained here show characteristic structures having D-glucopyranosyl moiety at sterically hindered C-20 position, which could not be obtained by other methods except by soil bacterial hydrolysis (compound K, Yosioka *et al.*, 1972). The modified method is believed to be available not only for preparative purpose, but also for sturcture elucidation of genuine aglycones of certain glycosides.

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