# Studies on the Enzyme Immunoassay of Bio-active Constituents in Oriental Medicinal Drugs. VI.[1] Enzyme Immunoassay of Ginsenoside $Rb_1$ from *Panax ginseng*

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**Enzyme immunoassay (EIA) of ginsenoside $Rb_1$ ($GRb_1$), one of the glucosides of protopanaxadiol from *Panax ginseng*, was explored. A carrier protein (bovine serum albumin (BSA)) was coupled to the C-26 position on the unsaturated side chain of the protopanaxadiol moiety to prepare the immunogen. In order to perform bridge heterologous EIA, a label (β-D-galactosidase) was introduced at C-26 of the saturated side chain to obtain labeled antigen. Anti-$GRb_1$ antisera were elicited in rabbits by immunization with $GRb_1$–BSA conjugate (9). The double antibody method (with goat anti-rabbit IgG antiserum) was used to separate the bound and free $GRb_1$–β-Gal. A satisfactory standard curve for EIA of $GRb_1$ was obtained in the range of 0.04—10 ng/tube. In a comparison of the assay results obtained by EIA and HPLC, the linear regression equation and correlation coefficient for the two methods were $y$(EIA) = 9.18$x$(HPLC) − 0.033 and 0.98, respectively. The anti-$GRb_1$ antiserum cross-reacted with $GRb_2$ (21.8%) and GRc (10.6%), which are also constituents of *Panax ginseng*.**

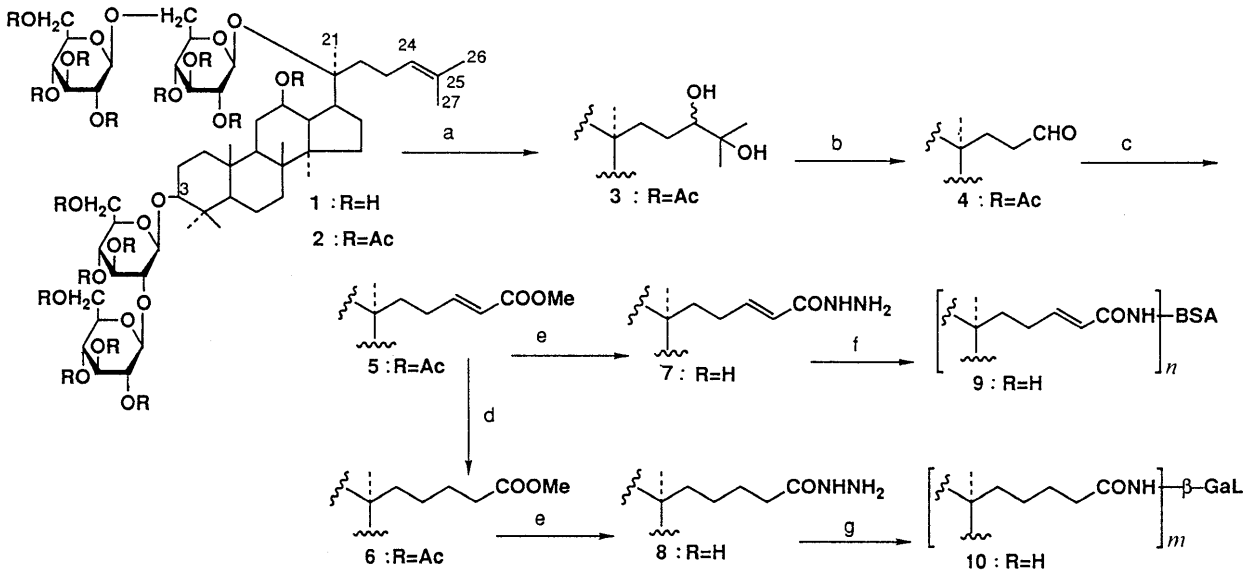
**Keywords** ginsenoside $Rb_1$; enzyme immunoassay; hapten; synthesis; immunogenic conjugate; cross-reaction; HPLC

Crude drugs are widely used, even in modern medicine. However, pharmacokinetic studies of the physiological active principles contained in the crude drugs need to be conducted to assure safety. Tanizawa *et al.*[2] used thin-layer chromatography (TLC) after oral (100 mg/kg) and intravenous (5 mg/kg) administration of ginsenoside $Rb_1$ ($GRb_1$), one of the protopanaxadiol glucosides from *Panax ginseng*, to evaluate the concentration of $GRb_1$ in rat sera. The absorption of $GRb_1$ was less than 0.1% of the dose.

In order to determine the concentration of $GRb_1$ in sera of patients given red ginseng, we have developed an enzyme immunoassay (EIA) of $GRb_1$. In this paper, we wish to report the preparation of the hapten, the specificity of anti-$GRb_1$ antiserum and the EIA of $GRb_1$.

Bovine serum albumin (BSA), the carrier protein of the immunogen, was introduced at the C-26 position of the unsaturated side chain in the protopanaxadiol moiety according to the method for radioimmunoassay of $GRg_1$ reported by Sankawa *et al.*[3] In order to perform bridge heterologous EIA,[4] β-galactosidase (β-Gal) was introduced at the C-26 position of the saturated side chain to prepare the labeled antigen (Chart 1).

Pentadecaacetyl $GRb_1$ (**2**), obtained by peracetylation of $GRb_1$ (**1**) with acetic anhydride, was oxidized with osmium tetroxide to give pentadecaacetyl 24,25-dihydro$GRb_1$-24,25-diol (**3**), which exhibited a multiplet signal due to the C-24 methine proton at $\delta$ 3.35 in the proton nuclear magnetic resonance ($^1$H-NMR) spectrum, and C-24 and C-25 carbon signals at $\delta$ 78.8 (d) and 71.4 (s) in the carbon-13 NMR ($^{13}$C-NMR) spectrum (Tables I and II). The diol (**3**) was cleaved by periodic acid oxidation to give pentadecaacetyl 24,25,26,27-tetranor$GRb_1$-23-carbaldehyde (**4**), which exhibited a broad singlet signal at $\delta$ 9.74 in the $^1$H-NMR spectrum and a signal at $\delta$ 202.8 in the $^{13}$C-NMR spectrum due to the proton and carbon of the aldehyde group, respectively. The tetranor-aldehyde (**4**) was reacted with methyl (triphenylphosphoranyldiene)




a: $OsO_4$, b: $HIO_4$, c: $(C_6H_5)_3P{=}CH{-}COOMe$, d: Pd–C/$H_2$, e: $NH_2NH_2 \cdot H_2O$, f: $HNO_2$ + bovine serum albumin, g: $HNO_2$ + β-D-galactosidase

Chart 1

TABLE I. $^1$H-NMR Data for $GRb_1$ Derivatives[a]

| Compound | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| $CH_3$ | 0.79, 0.84 | 0.79, 0.84 | 0.79, 0.83 | 0.79, 0.83 | 0.79, 0.84 |
| | 0.91, 0.95 | 0.91, 0.96 | 0.92, 0.96 | 0.91, 0.96 | 0.91, 0.95 |
| | 1.02, 1.18 | 1.02, 1.15 | 1.10, 1.20 | 1.02, 1.19 | 1.02, 1.16 |
| | 1.61, 1.69 | 1.20, 1.26 | | | |
| H-3 | 3.07 (m) | 3.07 (m) | 3.09 (m) | 3.08 (m) | 3.07 (m) |
| H-24 | 5.05 (m) | 3.35 (m) | 9.74 (br s) | 6.93 (m) | — |
| H-25 | — | — | — | 5.83 (d, $J$=16) | 2.32 (t, $J$=8) |
| H-12 | 4.81 (td, $J$=10, 5) | 4.81 (td, $J$=10, 5) | 4.80 (td, $J$=10, 5) | 4.82 (td, $J$=10, 5) | 4.81 (td, $J$=10, 5) |
| glc 3-1′ | 4.45 (d, $J$=7.6) | 4.44 (d, $J$=7.6) | 4.44 (d, $J$=7.6) | 4.45 (d, $J$=7.8) | 4.44 (d, $J$=7.6) |
| glc 20-1′ | 4.55 (d, $J$=7.9) | 4.55 (d, $J$=7.9) | 4.54 (d, $J$=7.6) | 4.62 (d, $J$=8.0) | 4.56 (d, $J$=7.9) |
| glc 20-1 | 4.68 (d, $J$=7.9) | 4.70 (d, $J$=7.9) | 4.69 (d, $J$=7.9) | 4.70 (d, $J$=8.0) | 4.67 (d, $J$=7.9) |
| glc 3-1 | 4.70 (d, $J$=7.9) | 4.74 (d, $J$=7.9) | 4.69 (d, $J$=7.9) | 4.70 (d, $J$=8.0) | 4.70 (d, $J$=7.9) |

a) Measured at 400 MHz in $CDCl_3$. Chemical shifts are in $\delta$ and $J$ values are in Hz.

TABLE II. $^{13}$C-NMR Data for $GRb_1$ Derivatives[a]

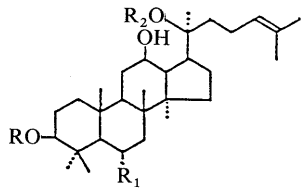
| C No. | Compound | | | | | C No. | Compound | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 | | 2 | 3 | 4 | 5 | 6 |
| 1 | 39.2 | 38.8 | 39.0 | 38.8 | 39.3 | 29 | 16.1 | 16.0 | 16.0 | 16.0 | 16.1 |
| 2 | 26.4 | 26.4 | 26.1 | 26.2 | 26.2 | 30 | 17.8 | 17.8 | 17.5 | 17.7 | 17.9 |
| 3 | 91.0 | 91.0 | 91.0 | 91.0 | 91.1 | glc 3-1 | 100.6 | 100.6 | 100.5 | 100.4 | 100.6 |
| 4 | 39.4 | 39.4 | 39.4 | 39.4 | 39.4 | 2 | 77.2 | 77.0 | 77.0 | 77.0 | 77.2 |
| 5 | 56.2 | 56.2 | 56.1 | 56.1 | 56.2 | 3 | 73.3 | 73.2 | 73.2 | 73.0 | 73.3 |
| 6 | 18.1 | 18.1 | 18.1 | 18.1 | 18.1 | 4 | 68.4 | 68.4 | 68.3 | 68.4 | 68.3 |
| 7 | 34.5 | 34.5 | 34.4 | 34.4 | 34.5 | 5 | 72.9 | 73.2 | 72.9 | 72.9 | 72.9 |
| 8 | 39.5 | 39.5 | 39.5 | 39.5 | 39.5 | 6 | 62.0 | 62.0 | 62.0 | 62.0 | 62.0 |
| 9 | 47.8 | 47.2 | 47.3 | 47.6 | 47.5 | 3-1′ | 103.5 | 103.5 | 103.5 | 103.4 | 103.6 |
| 10 | 36.8 | 36.8 | 36.8 | 36.8 | 36.8 | 2′ | 71.4 | 71.4 | 71.7 | 71.8 | 71.8 |
| 11 | 31.5 | 31.2 | 30.9 | 31.1 | 31.4 | 3′ | 71.8 | 72.8 | 71.8 | 72.0 | 72.0 |
| 12 | 72.0 | 72.0 | 72.0 | 72.0 | 72.0 | 4′ | 68.9 | 69.0 | 69.0 | 69.0 | 68.9 |
| 13 | 45.6 | 45.3 | 45.8 | 45.6 | 45.4 | 5′ | 73.1 | 73.2 | 73.1 | 73.1 | 73.1 |
| 14 | 53.0 | 53.1 | 53.0 | 52.9 | 53.0 | 6′ | 62.0 | 61.9 | 61.9 | 61.9 | 62.0 |
| 15 | 29.0 | 28.9 | 28.7 | 28.8 | 29.0 | glc 20-1 | 94.8 | 94.6 | 94.7 | 94.7 | 94.7 |
| 16 | 26.0 | 25.9 | 25.9 | 25.9 | 26.0 | 2 | 69.5 | 71.1 | 70.9 | 70.8 | 70.9 |
| 17 | 49.9 | 49.7 | 49.6 | 49.7 | 49.9 | 3 | 75.2 | 75.4 | 75.3 | 75.2 | 75.2 |
| 18 | 15.5 | 15.5 | 15.5 | 15.5 | 15.5 | 4 | 68.2 | 68.2 | 68.3 | 68.3 | 68.2 |
| 19 | 15.9 | 15.9 | 15.9 | 15.9 | 15.9 | 5 | 72.6 | 72.8 | 72.7 | 72.7 | 72.7 |
| 20 | 83.3 | 83.3 | 82.9 | 83.0 | 83.1 | 6 | 68.6 | 68.4 | 67.8 | 67.9 | 68.3 |
| 21 | 22.9 | 22.1 | 21.8 | 22.0 | 22.0 | 20-1′ | 100.8 | 100.7 | 100.6 | 100.6 | 100.6 |
| 22 | 38.8 | 37.2 | 38.8 | 37.4 | 38.2 | 2′ | 71.1 | 71.4 | 71.3 | 71.3 | 71.3 |
| 23 | 22.8 | 26.1 | 30.9 | 26.8 | 29.7 | 3′ | 72.5 | 75.2 | 75.2 | 75.2 | 75.2 |
| 24 | 124.7 | 78.8 | 202.8 | 150.0 | 25.7 | 4′ | 69.0 | 69.0 | 69.1 | 69.2 | 69.4 |
| 25 | 131.4 | 71.4 | — | 120.7 | 34.0 | 5′ | 71.3 | 71.9 | 71.4 | 71.4 | 71.4 |
| 26 | 25.7 | 26.1 | — | 167.1 | 174.1 | 6′ | 62.3 | 62.4 | 62.3 | 62.3 | 62.3 |
| 27 | 17.9 | 23.5 | — | — | — | $OCH_3$ | | | | 51.4 | 51.5 |
| 28 | 27.5 | 27.5 | 27.5 | 27.5 | 27.5 | | | | | | |

a) Measured at 100 MHz in $CDCl_3$. The characterization of each signal was made by DEPT.

acetate by the Wittig–Horner method to afford pentadecaacetyl 26,27-dinor$GRb_1$-25-carboxylic acid methyl ester (**5**) which exhibited a doublet signal at $\delta$ 5.83 ($J$=16 Hz, H-25) and a multiplet signal at $\delta$ 6.93 (H-24) due to olefinic protons (*trans*) in the $^1$H-NMR, and olefinic carbon signals of C-24 and C-25 at $\delta$ 150 and 120.7 in the $^{13}$C-NMR spectrum. The dinor-25-acid ester (**5**) was hydrogenated in the presence of palladium carbon to give pentadecaacetyl 24,25-dihydro-26,27-dinor$GRb_1$-25-carboxylic acid methyl ester (**6**), which exhibited a triplet signal at $\delta$ 2.32 ($J$=8 Hz) due to the C-25 methylene protons adjacent to the carbonyl group in the $^1$H-NMR, and carbon signals due to C-25 and C-26 at $\delta$ 34.0 and 174.1 in the $^{13}$C-NMR spectrum. Each acetyl $GRb_1$-carboxylic acid methyl ester (**5** and **6**) was allowed to react with hydrazine hydrate to afford the corresponding 26,27-dinor- (**7**) and 26,27-dinor-24,25-dihydro$GRb_1$-25-carbohydrazide (**8**), which were deacetylated.

These hydrazides were used for the next step without purification because they were unstable. The former hydrazide (**7**) was converted with nitrous acid to the azide, which was immediately coupled with BSA to give 27-nor$GRb_1$–BSA conjugate (**9**). In these reactions the hydrazide and BSA were used in a mole ratio of 30:1. This conjugate was used for immunization after purification by dialysis. The ultraviolet (UV)-spectral method[5] was used to determine the number of hapten molecules linked to one BSA molecule in the hapten–BSA conjugate. However, $GRb_1$ does not absorb UV light, so the number of $GRb_1$ molecules linked to $GRb_1$–BSA conjugate (**9**) was

TABLE III. Cross Reactivity of the Antiserum with Various Ginsenosides



$GRb_1$: R=glc–glc (2, 1), $R_1$=H, $R_2$=glc–glc (6, 1)
$GRb_2$: R=glc–glc (2, 1), $R_1$=H, $R_2$=glc–arap (6, 1)
GRc : R=glc–glc (2, 1), $R_1$=H, $R_2$=glc–araf (6, 1)
GRd : R=glc–glc (2, 1), $R_1$=H, $R_2$=glc
GRe : R=H, $R_1$=O–glc–rah (2, 1), $R_2$=glc
GRf : R=H, $R_1$=O–glc–glc (2, 1), $R_2$=H
$GRg_1$: R=H, $R_1$=O–glc, $R_2$=glc
$GRg_2$: R=H, $R_1$=O–glc–rah (2, 1), $R_2$=H
prosapogenin: R=glc–glc (2, 1), $R_1$=$R_2$=H
protopanaxadiol: R=$R_1$=$R_2$=H

| Compounds | Cross reaction (%) | Compounds | Cross reaction (%) |
|---|---|---|---|
| $GRb_1$ | 100 | GRe | <0.1 |
| $GRb_2$ | 21.8 | GRf | <0.1 |
| GRc | 10.6 | 20(*R*)-Prosapogenin | <0.1 |
| GRd | 5.8 | 20(*S*)-Prosapogenin | <0.1 |
| $GRg_1$ | 0.39 | 20(*R*)-Protopanaxadiol | <0.1 |
| $GRg_2$ | 0.18 | 20(*S*)-Protopanaxadiol | <0.1 |

estimated from the difference between the number of free amino groups of BSA and that of the BSA–conjugate (**9**). The number of free amino groups was calculated from a calibration curve for absorbance and concentration of trinitrobenzenesulfonyl amino groups which were formed by the reaction of free amino groups in BSA and the BSA-conjugate with trinitrobenzenesulfonic acid.[6] The number of $GRb_1$ molecules bound to $GRb_1$–BSA conjugate (**9**) was twenty-eight.

The other hydrazide (**8**) was similarly converted to an azide and coupled with β-Gal to give 27-nor-24,25-dihydro$GRb_1$–β-Gal conjugate (**10**), which was used as a labeled antigen after purification on a Sepharose 6B column.

Antisera to $GRb_1$ were elicited in two female rabbits immunized with $GRb_1$–BSA conjugate (**9**) in the same manner as previously described.[7] The bound and free $GRb_1$–β-Gal conjugate (**10**) were separated by a double antibody method with a goat anti-rabbit immunoglobulin G (IgG) antiserum, and the enzyme activity of the immune precipitate was determined fluorometrically with 7-β-D-galactopyranosyloxy-4-methylcoumarin as substrate. Optimum dilution of anti-$GRb_1$ antiserum determined by the previously reported method[7] was 20000-fold. A typical standard curve of EIA of $GRb_1$ is shown in Fig. 1. The measurable range was 0.04—10 ng/tube. The cross reactivity of anti-$GRb_1$ antiserum is shown in Table III. The antiserum cross-reacted with $GRb_2$ (21.8%) and GRc (10.6%), which have the same structure as $GRb_1$ except for the terminal sugar at the C-20 position on the protopanaxadiol moiety. This showed that anti-$GRb_1$ antiserum is not highly specific for the C-20 region, which is closer to the C-26 position (coupled with the carrier protein in the immunogen) than other positions bearing sugars.

The accuracy of this EIA was evaluated by comparing

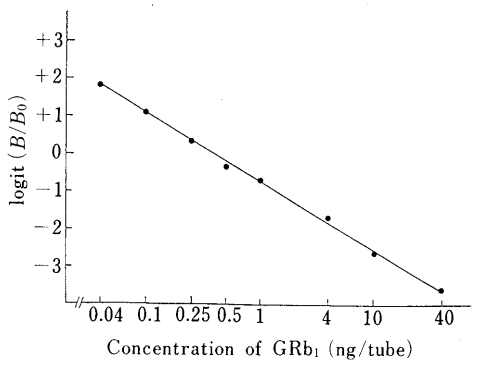



Fig. 1. Standard Curve for EIA of $GRb_1$

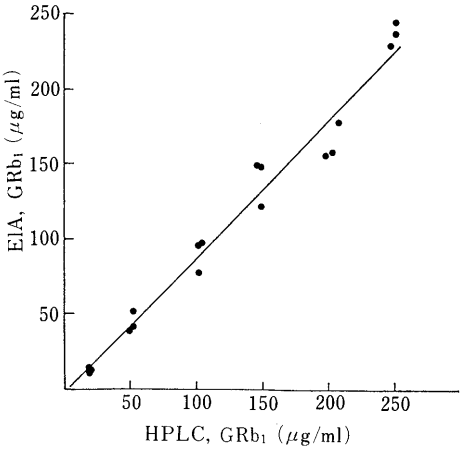



Fig. 2. Correlation between the Values of $GRb_1$ Obtained by EIA and HPLC

the results obtained by EIA and by high-performance liquid chromatography (HPLC). Figure 2 shows that there was a good correlation between the values determined by the two methods. The linear regression equation and correlation coefficient for the two methods were $y$(EIA)=$9.18x$(HPLC)$-0.33$ and 0.98, respectively.

**Experimental**

All melting points were taken on a microscopic hot stage (Yanagimoto melting point apparatus) and are uncorrected. Optical rotation was measured with a JASCO polarimeter. Spectra were obtained with the following instruments: $^1$H- and $^{13}$C-NMR on JNM-GX270 and 400 spectrometers [internal standard, tetramethylsilane (TMS); chemical shifts, δ (ppm); abbreviations, s (singlet), d (doublet), t (triplet), m (multiplet), br (broad)]. Fluorometry was performed on a Shimadzu RF-503 recording spectrofluorophotometer. BSA (Cohn fraction V) and γ-globulin (Cohn fractions II, III) were purchased from Sigma Chemical Co. (St. Louis, Mo). β-D-Galactosidase (EC 3.2.1.23) from *E. coli* was purchased from Boehringer Mannheim. Buffer A: 0.02 M phosphate-buffered saline containing 0.1% BSA, 0.1% $NaN_3$, and 0.001% $MgCl_2$. Buffer B: The composition was the same as that of buffer A except for the use of 0.1% γ-globulin instead of 0.1% BSA.

**Pentadecaacetyl $GRb_1$ (2)** $GRb_1$ (**1**, 200 mg) was acetylated with acetic anhydride (1 ml) and pyridine (1 ml) at room temperature for a week. MeOH (10 ml) was added to the reaction mixture and the whole was evaporated to dryness. The MeOH treatment was repeated twice, then the residue was dissolved in $CHCl_3$ (30 ml). The $CHCl_3$ solution was washed with brine, dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The residue was recrystallized from MeOH to give 2 (210 mg 68%) as colorless leaflets, mp 138—140 °C. $[\alpha]_D^{26}$ −10.16° ($c$=1, $CHCl_3$). *Anal.* Calcd for $C_{84}H_{122}O_{38}$: C, 57.99; H, 7.68. Found: C, 57.75; H, 7.43.

**Pentadecaacetyl 24,25-DihydroGRb$_1$-24,25-diol (3)** $OsO_4$ (50 mg) was added to a stirred solution of **2** (200 mg, 0.15 mmol) in pyridine (1 ml) under ice cooling. After 2 h, 40% sodium bisulfite (0.5 ml) was added, and the reaction mixture was stirred at room temperature for 1 h, then

poured into ice water (20 ml). The aqueous solution was extracted with $CHCl_3$ (40 ml) and the $CHCl_3$ extract was washed with 0.5 N HCl (5 ml × 2), brine (5 ml × 2) and 1 N $Na_2CO_3$ (5 ml × 2), dried over anhydrous $MgSO_4$ and evaporated *in vacuo*. The residue was recrystallized from $CH_2Cl_2$–*n*-hexane to give **3** (198 mg, 97%) as colorless needles, mp 128—131 °C. $[\alpha]_D^{25}$ +3.57° ($c$=1, $CHCl_3$). *Anal.* Calcd for $C_{84}H_{124}O_{40}$: C, 56.87; H, 7.05. Found: C, 56.61; H, 7.15.

**Pentadecaacetyl 24,25,26,27-TetranorGRb$_1$-23-carbaldehyde (4)** A 10 % $HIO_4$ solution (500 μl) was added to a solution of **3** (200 mg, 0.11 mmol) in dioxane (2 ml) under ice cooling. After being stirred for 1 h, the reaction mixture was poured into ice water (10 ml). The aqueous solution was extracted with $CHCl_3$ (30 ml), and the $CHCl_3$ extract was washed with brine (5 ml × 2), dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The residue was recrystallized from $CH_2Cl_2$ + *n*-hexane to give **4** (165 mg, 85.5%) as colorless needles, mp 131—134°C. $[\alpha]_D^{25}$ −21.48° ($c$=1, $CHCl_3$). *Anal.* Calcd for $C_{81}H_{117}O_{39}$: C, 56.73; H, 6.88. Found: C, 56.86; H, 6.98.

**Pentadecaacetyl 26,27-DinorGRb$_1$-25-carboxylic Acid Methyl Ester (5)** A mixture of **4** (74 mg, 0.43 mmol) and methyl (triphenylphosphanyldiene) acetate (15 mg, 0.44 mmol) and dimethyl sulfoxide (DMSO) (1 ml) was heated at 110 °C for 6 h and then allowed to stand at room temperature. The reaction mixture was poured into ice water (30 ml) and extracted with $CHCl_3$ (40 ml). The $CHCl_3$ extract was washed with brine (5 ml × 3), dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The residue was purified by preparative TLC using 5% acetone–$CHCl_3$ as the developing solvent. The zone with *Rf* 0.3 gave **5** (70 mg, 91.6%) colorless leaflets, mp 138—140 °C. $[\alpha]_D^{25}$ −12.0 ($c$=1, $CHCl_3$). *Anal.* Calcd for $C_{84}H_{121}O_{40}$: C, 56.97; H, 6.89. Found: C, 57.16; H, 7.17.

**Pentadecaacetyl 26,27-Dinor-24,25-dihydroGRb$_1$-25-carboxylic Acid Methyl Ester (6)** A stirred solution of **5** (50 mg, 0.028 mmol) in AcOEt (5 ml) was catalytically hydrogenated over 5% Pd–carbon (50 mg) at atmospheric pressure overnight. The catalyst was filtered off and the filtrate was concentrated *in vacuo*. The residue was recrystallized from $CH_2Cl_2$–isopropyl ether to give **6** (33 mg, 66.0%) as colorless needles, mp 131—133 °C. $[\alpha]_D^{25}$ −2.42° ($c$=1, $CHCl_3$). *Anal.* Calcd for $C_{84}H_{123}O_{40}$: C, 56.91; H, 6.99. Found: C, 56.79; H, 7.14.

**Preparation of GRb$_1$–BSA Conjugate (9)** (i) 26,27-DinorGRb$_1$-25-carbohydrazide (**7**): A mixture of carboxylic acid methyl ester (**5**) (34 mg, 0.019 mmol), hydrazine hydrate (0.4 ml) and MeOH (0.4 ml) was stirred at 70 °C for 1 h and allowed to stand at room temperature for 4 h. The reaction mixture was concentrated *in vacuo*, and the residue was washed with 30% MeOH–$CH_2Cl_2$ (3 ml × 2) to give **7** (12 mg, 54.5%). This crude product was used for the next step without purification.

(ii) BSA Conjugate (**9**): A stirred solution of the carbohydrazide (**7**) (30 mg, $2.6 \times 10^{-2}$ mmol) in dimethylformamide (DMF) (0.3 ml) and $H_2O$ (3 ml) was treated with 10% $NaNO_2$ (30 μl) and kept at pH 2 by adding 1 N HCl at 0 °C. After 15 min, BSA (58 mg, $8.6 \times 10^{-4}$ mmol) was added to the reaction mixture, which was kept at pH 9.0 by adding 10% $K_2CO_3$ at 5 °C overnight. The resulting solution was dialyzed against distilled water with two changes a day for 3 d. The dialysate was lyophilized to afford BSA-conjugate (43.8 mg).

**Preparation of GRb$_1$–β-D-Galactosidase Conjugate (10)** (i) 26,27-Dinor-24,25-dihydroGRb$_1$-25-carbohydrazide (**8**): A mixture of dihydrocarboxylic acid methyl ester (**6**) (27 mg, 0.015 mmol), hydrazine hydrate (0.3 ml) and MeOH (0.3 ml) was treated in the same manner as described above to give the dihydro-carbohydrazide (**8**) (15 mg, 86%).

(ii) β-Gal Conjugate (**10**): A stirred solution of the carbohydrazide (**8**) (2 mg, $1.75 \times 10^{-3}$ mmol) in DMF (50 μl) and $H_2O$ (550 μl) was treated with 0.1% $NaNO_2$ (125 μl) held at pH 2 by adding 0.1% HCl (65 μl) at 0 °C. After 15 min, 37 μl (equivalent of $8.19 \times 10^{-5}$ mmol of **8**) of the reaction mixture was pipetted off, and added to a solution of β-D-galactosidase (4.2 mg, $8.12 \times 10^{-6}$ mmol) in 0.05 M phosphate buffer (pH 7.3, 1 ml). The mixture was stirred at 0 °C overnight. The reaction mixture was applied to a Sepharose 6B column (1.5 × 30 cm), using buffer A as the eluent. The fraction of eluate containing the peak of enzyme activity were collected, pooled and stored at 4 °C until use. The amount of enzyme conjugate was expressed as units of enzyme activity, one unit of enzyme activity being defined as the amount that hydrolyzed 1 μmol of 7-β-D-galactopyranosyloxy-4-methylcoumarin per min.

**Determination of the Number of GRb$_1$ Molecules Linked to One BSA Molecule in GRb$_1$–BSA Conjugate** Aliquots of 50, 75, 100, 200, and 300 μl of BSA solution and GRb$_1$–BSA conjugate solution (1 mg/ml), respectively, were used in the following tests. (i) The amount of BSA ($W_L$) in the GRb$_1$–BSA conjugate sample was determined by the Folin–Lowry method.[8] (ii) From the relationships between variation of absorbance and amount of BSA in the reaction of BSA standard solution with trinitrobenzenesulfonic acid (TNBS) according to Habeeb,[6] the absorbance ($A_L$) of $W_L$ was calculated. (iii) The absorbance ($A_c$) of the GRb$_1$–BSA conjugate sample in the reaction of GRb$_1$–BSA conjugate with TNBS was determined. (iv) The number of free amino groups ($N_f$) in BSA of the GRb$_1$–BSA conjugate sample was given by $N_f = 60 \times A_c / A_L$. (v) The number of amino groups ($N_o$) occupied by GRb$_1$ of the GRb$_1$–BSA conjugate sample was given by $N_o = 60 - N_f$.

**Preparation of Antiserum of GRb$_1$** Two domestic strain female albino rabbits weighing 2.0 kg were used for immunization. The GRb$_1$–BSA conjugate (**9**, 1.2 mg) was dissolved in sterile isotonic saline (2 ml) and emulsified with the same amount of complete Freund's adjuvant (Difco, Detroit, Mich., U.S.A.). The emulsion was injected into the rabbits subcutaneously and intramuscularly at multiple sites on the back and legs. Half the initial dose of immunogen was used as a booster once every 2 weeks for two months and then once a month for 2 months. The rabbits were bled 3 weeks after the last booster injection. The sera were separated by centrifugation at 3000 rpm for 15 min and stored at −20 °C until use.

**Procedure of Enzyme Immunoassay** Unless otherwise stated, dilution was carried out with Buffer B. A sample or standard solution of GRb$_1$ (100 μl) was added to 20000-fold-diluted anti-GRb$_1$ antiserum (100 μl) and GRb$_1$–β-Gal conjugate (50 μl), and the mixture was incubated at room temperature for 2 h. Then 10-fold diluted goat anti-rabbit IgG antiserum (50 μl) and 100-fold diluted normal rabbit serum (20 μl) were added to the incubation mixture, and the solution was vortexed, then allowed to stand at 4 °C overnight. After addition of buffer A (1 ml), the mixture was centrifuged at 2500 rpm for 15 min, and the supernatant was removed. The immune precipitate was washed twice with buffer A (1 ml) and used for measurement of enzyme activity.

**Measurement of β-D-Galactosidase Activity** The immune precipitate was incubated with $1 \times 10^{-4}$ M 7β-D-galactopyranosyloxy-4-methylcoumarin (150 μl) at 30 °C for 30 min. After incubation, 0.1 M glycine–NaOH (pH 10.3, 2 ml) was added to the reaction mixture, and the fluorescence intensity of 7-hydroxy-4-methylcoumarin was measured on 365 and 448 nm (excitation and emission, respectively).

**Specificity of the Antiserum** The cross reaction of anti-GRb$_1$ antiserum with aglycons and glucosides of panaxadiol and panaxatriol, constituents of *Panax ginseng*, were examined by using GRb$_1$–β-D-Gal conjugate according to the assay procedure described above. The results are summarized in Table III.

**HPLC Conditions** A Shimadzu LC-6A liquid chromatograph equipped with a Shimadzu SPD-6A UV spectrophotometric detector for peak detection (203 nm) and a Shimadzu C-R3A Chromatopac for calculation of peak area was used. The column (Nucleosil 5$C_{18}$, 150 mm × 4.6 mm i.d.) was maintained at 40 °C with a column oven. The mobile phase was 31.5% $CH_3CN$–$H_2O$ and the flow rate was 1.0 ml/min. The retention time of GRb$_1$ was 12.5 min.

Each sample (20, 50, 100, 150, 200, or 250 μl/ml) of GRb$_1$ was assayed by HPLC three times. The same samples diluted 10000 fold were used for EIA.

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