

Studies on Absorption, Distribution, Excretion and Metabolism of Ginseng Saponins. VIII.¹⁾ Isotope Labeling of Ginsenoside Rb₂

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To clarify the pharmacokinetics of absorption, distribution and excretion of ginsenoside Rb₂ (Rb₂), one of the major saponins of the root of *Panax ginseng*, following oral administration to rats, a tritium (³H) labeling of Rb₂ was examined. The C-12 position of Rb₂ was labeled with ³H-sodium borohydride (³H-NaBH₄) and 12-³H Rb₂ and 12-³H-epi Rb₂ was synthesized. This method of specific position labeling of Rb₂ may be applicable to other ginsenosides. In the near future, the pharmacokinetics of Rb₂ in rats may be clarified with ³H labeled Rb₂.

Keywords isotope labeling; tritium labeling; ginsenoside Rb₂; ¹³C-NMR; FAB-MS; ginseng saponin

We have been studying the pharmacokinetics of ginsenosides regarded as the components principally responsible for the pharmacological activities of the root of *Panax ginseng* C. A. MEYER (Araliaceae). We have found several metabolites of ginsenoside-Rg₁ (Rg₁), -Rb₁ (Rb₁) and -Rb₂ (Rb₂), in the rat gastrointestinal tract after oral administration, and also reported the absorption, distribution and excretion of Rg₁ and Rb₁ in rats. These absorption rates were very low, 0.1% and 1.9%, respectively.²⁾ Sankawa also reported the low absorption rate of Rg₁ of 8.6% in rats using radioimmunoassay method.³⁾ In view of the many important findings on the pharmacological activities of ginsenosides, their low absorption rates seem unconvincing and we, therefore, felt it necessary to determine whether or not these were correct. Tritium (³H) labeling of a ginsenoside was seen as useful to resolve this, because the methods of determining ginsenosides and their metabolites in biological samples using high performance liquid chromatography (HPLC) or thin layer chromatography (TLC)-densitometry are not always perfect and may have inadvertently been missed. The existence of ³H is easily found because of its high detection sensitivity.

Our previous studies revealed that the metabolites of Rg₁, Rb₁ and Rb₂ in rat gastrointestinal tract were mostly prosapogenins derived from hydrolysis of their sugar moieties. Therefore, we considered that the most suitable position for ³H labeling might be at C-12 of dammarene type aglycones. In the present paper, we used Rb₂ as a ginsenoside, and investigated the process of ³H labeling in detail, as there is no report on this.

Experimental

Materials and Equipment Most of the materials and equipment used were the same as described in our previous paper.¹⁾ Sodium borohydride (NaBH₄) and lithium aluminum hydride (LiAlH₄) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and sodium borodeuteride (NaBD₄) from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). ³H-Sodium borohydride (³H-NaBH₄, 185 MBq in 500 μl of 0.01 N NaOH solution) was obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Cation exchange resin (Dowex 50W × 8, 50–100 mesh) was purchased from Muromachi Kagaku Kogyo Co. (Tokyo, Japan). Tetrahydrofuran (THF) was treated with LiAlH₄, and distilled before using.

Partial Acetylation and Oxidation of Rb₂ Rb₂ (2 g) was stirred for 24 h at room temperature in a mixture of acetic anhydride (10 ml) and pyridine (10 ml). After dilution with water (100 ml), the reaction mixture was extracted with ethyl acetate (AcOEt, 500 ml). After washing the AcOEt layer with 10% HCl, saturated aqueous NaHCO₃ and water, the AcOEt solution was dried with MgSO₄, and concentrated to dryness *in vacuo*.

The residue was purified by column chromatography [silica gel 200 g, benzene–acetone (5 : 1)] to yield trideca-acetyl Rb₂ (I, 2 g, white powder). The proton nuclear magnetic resonance (¹H-NMR) spectral data of I showed 21 methyl signals. To a solution of I (1.5 g) in pyridine (5 ml), a mixture of chromic acid (1.5 g) and pyridine (10 ml) was added dropwise and the mixture was stirred for 5 h at room temperature, then diluted with 2-propanol (2-PrOH, 20 ml) and 10% HCl (100 ml) and extracted with AcOEt (200 ml). After washing the AcOEt layer with 10% HCl, saturated aqueous NaHCO₃ and water, the AcOEt solution was dried with MgSO₄, and concentrated to dryness *in vacuo*. The residue was purified by preparative HPLC (YMC-packed column SH-343-5, 82% aqueous acetonitrile, 5 ml/min, 205 nm) to yield trideca-acetyl 12-keto derivative (II, 1.1 g, white powder). The identification of II was performed by comparing the carbon-13 nuclear magnetic resonance (¹³C-NMR) and ¹H-NMR spectral data of deacetylated II with that of Rb₂ and chikusetsusaponin LN₄.⁴⁾

Deacetylated II: A white powder. ¹H-NMR (pyridine-*d*₅) δ: 0.80, 0.90, 1.10, 1.28, 1.29, 1.56 (3H each, all s, *tert*-CH₃ × 6), 1.68 (6H, s, vinyl. CH₃ × 2), 4.92, 4.96, 5.03, 5.36 (1H each, all d, *J* = 7.7, 5.8, 7.3, 7.6 Hz, respectively, anomeric H × 4). ¹³C-NMR (pyridine-*d*₅) δ: 105.1 (C-3 glc), 106.1 (C-2' glc), 98.5 (C-20 glc), 104.9 (C-6'' arap) (anomeric C × 4), 40.7 (C-1), 26.6 (C-2), 88.6 (C-3), 39.7 (C-4), 56.2 (C-5), 18.5 (C-6), 34.8 (C-7), 40.9 (C-8), 56.4 (C-9), 37.4 (C-10), 40.1 (C-11), 211.3 (C-12), 54.8 (C-13), 56.3 (C-14), 32.3 (C-15), 24.6 (C-16), 42.6 (C-17), 17.0 (C-18), 17.9 (C-19), 81.5 (C-20), 22.3 (C-21), 38.8 (C-22), 24.1 (C-23), 125.9 (C-24), 130.9 (C-25), 25.8 (C-26), 16.5 (C-27), 28.0 (C-28), 16.3 (C-29), 16.0 (C-30).

Reduction of II 1) Isolation of Rb₂ and 12-Epi Rb₂ II (100 mg) was refluxed for 1 h in THF (10 ml) with LiAlH₄ (300 mg). AcOEt (10 ml) and methanol (20 ml) were added to the reaction mixture, and refluxed for 30 min. A clear solution was evaporated to dryness *in vacuo*, the residue, was suspended in water (20 ml) and 10% HCl (50 ml) was added to the suspension, extracted with 1-butanol (100 ml). After washing the 1-butanol layer with water, saturated aqueous NaHCO₃ and water, the 1-butanol solution was concentrated to dryness *in vacuo*. The residue was purified by preparative HPLC (35% aqueous acetonitrile) to yield Rb₂ (8 mg, *t*_R 35 min) and 12-epi Rb₂ (40 mg, *t*_R 60 min). The identification of 12-epi Rb₂ was performed by comparing the ¹H- and ¹³C-NMR spectral data with that of Rb₂.

12-Epi Rb₂: A white powder. ¹H-NMR (pyridine-*d*₅) δ: 0.85, 0.96, 1.14, 1.30, 1.41, 1.73 (3H each, all s, *tert*-CH₃ × 6), 1.59, 1.65 (3H each, both s, vinyl. CH₃ × 2), 4.90, 4.99, 5.00, 5.37 (1H each, all d, *J* = 7.6, 6.4, 7.7, 7.6 Hz, respectively, anomeric H × 4), 5.34 (1H, t, *J* = 6.4 Hz, C24-H). ¹³C-NMR (pyridine-*d*₅) δ: 105.1 (C-3 glc), 106.1 (C-2' glc), 98.4 (C-20 glc), 105.0 (C-6'' arap) (anomeric C × 4), 39.3 (C-1), 26.8 (C-2), 89.1 (C-3), 39.8 (C-4), 56.6 (C-5), 18.5 (C-6), 37.1 (C-7), 40.9 (C-8), 45.9 (C-9), 36.8 (C-10), 31.9 (C-11), 67.7 (C-12), 47.1 (C-13), 49.4 (C-14), 30.5 (C-15), 25.3 (C-16), 46.2 (C-17), 20.1 (C-18), 18.0 (C-19), 83.2 (C-20), 21.8 (C-21), 36.0 (C-22), 23.1 (C-23), 126.3 (C-24), 131.0 (C-25), 25.9 (C-26), 16.7 (C-27), 28.1 (C-28), 16.6 (C-29), 15.6 (C-30).

2) Treatment with LiAlH₄ II was refluxed for a predetermined time in THF with LiAlH₄. The reaction mixture was worked up as described above, and the residue was subjected to HPLC analysis.

3) Treatment with NaBH₄ II was refluxed for a definite time in various solvents with NaBH₄. Methanol was added to the reaction mixture, and refluxed for 30 min. A clear solution was neutralized by application of a Dowex 50 W × 8 (H⁺ form), and evaporated to dryness *in vacuo*. The

residue was subjected to HPLC analysis.

4) Deuterium Labeling of Rb₂ with NaBD₄ II (50 mg) was refluxed for 15 h in 2-PrOH (5 ml) with NaBD₄ (240 mg). The reaction mixture was worked up as described above, and the residue was purified by preparative HPLC (35% aqueous acetonitrile) to yield 12-D Rb₂ (2 mg) and 12-D-epi Rb₂ (17 mg). The identification of both D-labeled Rb₂ was performed by fast atom bombardment mass spectrometry (positive FAB-MS) data. 12-D Rb₂: FAB-MS *m/z*: 1080 (M+H)⁺, 1102 (M+Na)⁺. 12-D-epi Rb₂: FAB-MS *m/z*: 1102 (M+Na)⁺.

5) Tritium Labeling of Rb₂ with ³H-NaBH₄ II (20 mg) was refluxed for 24 h in 2-PrOH (10 ml) with NaBH₄ (100 mg) and ³H-NaBH₄ (185 MBq in 500 μl of 0.01 N NaOH solution). The reaction mixture was worked up as described above, and the residue was purified by preparative HPLC (35% aqueous acetonitrile) to yield ³H-labeled Rb₂. The radioactivities of 12-³H Rb₂ and 12-³H-epi Rb₂ were 18.2 and 19.4 kBq/μmol, respectively.

Biological Experiment For intravenous injection, 0.4% solution of 12-D-epi Rb₂ dissolved in 0.9% saline was given *via* the femoral vein at a dose of 10 mg/kg to non-fasted rats. Urine sample was collected after 24 h by the use of a metabolic cage (KN-646, Natsume, Tokyo, Japan), and methanol was added to extract 12-D-epi Rb₂. After centrifugation at 3000 rpm for 15 min, the supernatant was evaporated *in vacuo*. The residue was treated with a SEP-PAK® C₁₈ cartridge in the same manner as in our previous paper,¹⁾ and 12-D-epi Rb₂ (1 mg) was obtained by preparative HPLC as described above. Identification was done by FAB-MS.

Results and Discussion

Ginseng saponins, isolated from the root of *Panax ginseng*, have been regarded as the principal components responsible for the pharmacological activities of the drug. Investigations, however, have still not completely elucidated the absorption, distribution, excretion and metabolism of ginseng saponins. The basic reason for this is that there is no satisfactory analytical method for biological samples; the usual analytical methods have problems such as sensitivity, specificity and other complications. The easiest way is to use radioisotopic ginseng saponins, which are labeled with ¹⁴C and/or ³H. However, the synthesis of a radioisotopic ginseng saponin has been thought to be very difficult owing to its natural product. In fact, there is no report on labeling of a specific position in a ginseng saponin using ¹⁴C or ³H, although there are reports describing their overall labeling.⁵⁾

We attempted to obtain ³H labeled Rb₂ as shown in Chart 1. Rb₂ was partially acetylated to I by acetic anhydride and pyridine, that is, the C-12 hydroxyl group

of I was not acetylated. This selective acetylation was previously reported by Tanaka *et al.*⁶⁾ Then, the C-12 hydroxyl group of I was oxidized by chromic acid and pyridine to yield II. The formation of ³H labeled Rb₂ was expected when Rb₂ was reproduced by reduction of II with ³H labeled reductants. Though C-12 epimerization occurs in this reduction, the method for yielding 12β-OH (*i.e.*, Rb₂) predominantly by the reduction with sodium and 2-PrOH was reported by Tanaka *et al.*⁶⁾ On the other hand, ³H-NaBH₄ is widely used because of its high relative radioactivity, and ³H-LiAlH₄ is also occasionally used as a ³H labeled reductant.

Therefore, we began the study by selecting the reductant in this reduction. First, II was refluxed for 24 h in THF with LiAlH₄ or NaBH₄, and the reaction products were determined by HPLC. As shown in Fig. 1, the major product was 12-epi Rb₂ in this reduction with both reductants, but the yield of Rb₂ was better with NaBH₄ than with LiAlH₄.

Then, the suitability of using a protic solvent for the reduction of II with NaBH₄ was examined. II was refluxed for 24 h with NaBH₄ in methanol, ethanol, 1-PrOH, 2-PrOH, 1-butanol and 2-butanol, respectively, and the reaction products in each case were determined by HPLC. Figure 2 shows that the most suitable solvent in this reduction was 2-PrOH.

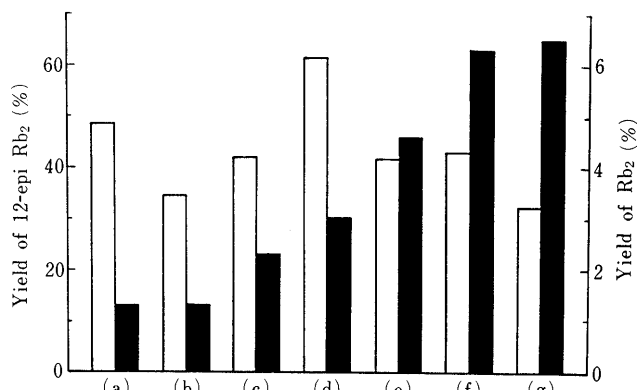


Fig. 1. Reduction of II with LiAlH₄ or NaBH₄ in THF for 24 h

□, 12-epi Rb₂; ■, Rb₂; (a), LiAlH₄ (57 mol ratio to II); (b), LiAlH₄ (117 mol ratio); (c), LiAlH₄ (154 mol ratio); (d), NaBH₄ (53 mol ratio); (e), NaBH₄ (132 mol ratio); (f), NaBH₄ (216 mol ratio); (g), NaBH₄ (305 mol ratio).

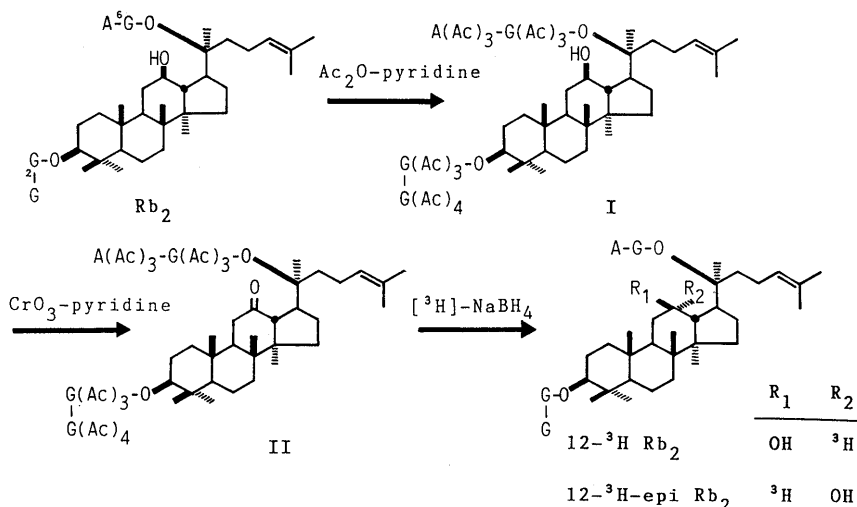


Chart 1. Synthetic Route of ³H-Rb₂

A, α-L-arabinopyranosyl; G, β-D-glucopyranosyl; Ac, acetyl.

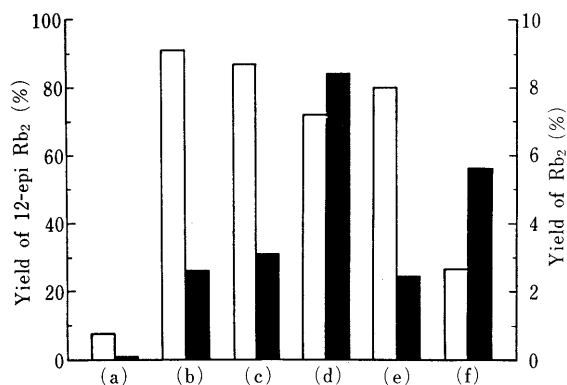


Fig. 2. Reduction of II with NaBH_4 in Several Solvents for 24 h

200 mol ratio of NaBH_4 to II was used in each reaction. $\square$, 12-epi Rb_2 ; $\blacksquare$, Rb_2 ; (a), methanol; (b), ethanol; (c), 1-propanol; (d), 2-propanol; (e), 1-butanol; (f), 2-butanol.

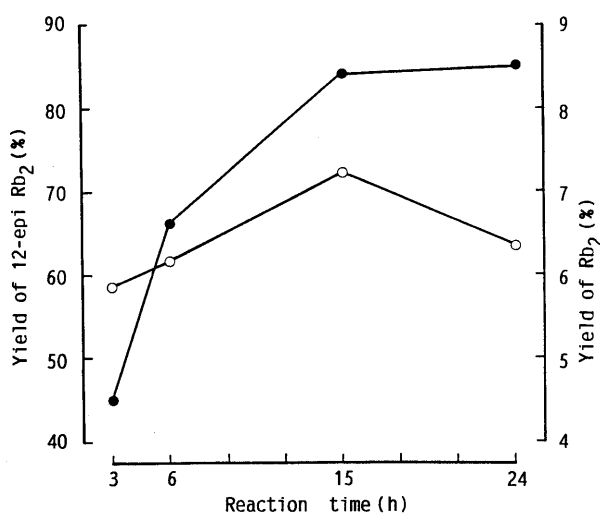


Fig. 3. Time Course of Reduction of II with NaBH_4 in 2-Propanol

200 mol ratio of NaBH_4 to II was used. $\circ$, 12-epi Rb_2 ; $\bullet$, Rb_2 .

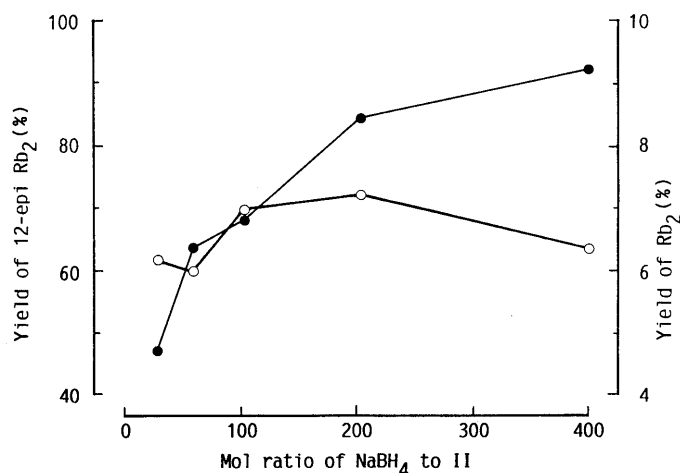


Fig. 4. Reduction of II with NaBH_4 in 2-Propanol for 24 h

$\circ$, 12-epi Rb_2 ; $\bullet$, Rb_2 .

Next, to examine the time-course of this reduction, II was refluxed for 3, 6, 15 and 24 h, respectively. From the result in Fig. 3, we judged that the reaction time of 15 h might be enough to get Rb_2 with good yield.

Finally, to determine the suitable quantity of NaBH_4 , the

reduction was done with various mole ratios of NaBH_4 to II. The result is shown in Fig. 4. From the results, we decided that the suitable mole ratio of NaBH_4 to II might be *ca.* 200. The yield of Rb_2 and 12-epi Rb_2 under the best conditions was 8.4% and 72.0%, respectively, and the ratio of Rb_2 to 12-epi Rb_2 was 8.6.

Thus, we concluded that the most suitable reduction conditions for II to obtain Rb_2 in good yield was 15 h for refluxing, using 2-PrOH as the solvent and 200-fold NaBH_4 to II in mole ratio.

Under these conditions, we synthesized 12-D labeled Rb_2 with NaBD_4 . The yield was 8.8% for 12-D Rb_2 and 56.1% for 12-D-epi Rb_2 . The ratio of Rb_2 to 12-epi Rb_2 was 6.4, almost the same as in the case of NaBH_4 . We therefore went on the synthesize 12- ^3H labeled Rb_2 using our best reduction condition. The yield of 12- ^3H Rb_2 and 12- ^3H -epi Rb_2 was 3.5% and 70.5%, respectively. The ratio of Rb_2 to 12-epi Rb_2 was 20.1. The specific activities of 12- ^3H Rb_2 and 12- ^3H -epi Rb_2 were 18.2 and 19.4 kBq/ μmol , respectively. Total reduction percentage of II by ^3H - NaBH_4 was the same as that by NaBH_4 , but the ratio of Rb_2 to 12-epi Rb_2 was poorer than that of NaBH_4 . The cause of this is not clear, but may be due to "an isotopic effect." The alkali solution used to dissolve ^3H - NaBH_4 did not affect the reduction, and this was confirmed by NaBH_4 . In practice, however, this is not a large problem; we are able to obtain highly radioactive ^3H - NaBH_4 commercially and so can get enough 12- ^3H Rb_2 for animal experiments. We are also able to use 12- ^3H -epi Rb_2 , if the behavior of both 12- ^3H Rb_2 and 12- ^3H -epi Rb_2 in the animal body is proved effective in future.

We also examined the possibility of H-D isotope exchange in the rat body after intravenous injection of 12-D-epi Rb_2 . A comparison of FAB-MS data for 12-D-epi Rb_2 isolated from rat urine with that of 12-D-epi Rb_2 showed that H-D exchange did not occur. Therefore, the pharmacokinetics such as absorption, distribution and excretion of Rb_2 in rats may be clarified using ^3H labeled Rb_2 in the near future. This method of specific position labeling of Rb_2 may also be applicable to other ginsenosides. ^3H labeling of Rb_2 to obtain high radioactivity is now under way.

Acknowledgements The authors are grateful to the Korea Ginseng and Tobacco Research Institute and the Japan-Korea Red Ginseng Co., Ltd., for the supply of pure Rb_2 . They also thank Dr. M. Uchida, of the Central Analytical Laboratory of their university, for measurement of FAB-MS spectra. This work was supported in part by a grant from the Medical Society for Red Ginseng Research.

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