Studies on the Panaxytriol of *Panax ginseng* C. A. MEYER. Isolation, Determination and Antitumor Activity

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An antitumor-active substance was obtained from the residue of the ethyl acetate extract of red ginseng, a traditional Chinese medicine, by chromatography on a silica gel column. From the proton and carbon-13 nuclear magnetic resonance spectra, it was identified as heptadeca-1-ene-4,6-diyne-3,9,10-triol (panaxytriol). The panaxytriol contents of red ginseng and white ginseng, determined by gas chromatography after solvent extraction and formation of trimethylsilyl derivatives, were 0.38 and 0.25 mg/g, respectively. Panaxytriol showed a growth-inhibitory activity against several tumor cell lines.

Keywords panaxytriol; *Panax ginseng*; red ginseng; 2D-NMR; ^{13}C-NMR; determination; gas chromatography; antitumor activity

Introduction

Red ginseng (the steamed and dried root of *Panax ginseng* C. A. MEYER) is a well-known and very important crude drug in the prescriptions of traditional oriental medicine. Although extensive studies on the pharmacological action and constituents of red ginseng have been reported,[1] our knowledge is not complete. For example, the antitumor effect of *Panax ginseng* is known, but the principles involved have not been identified.[2]

We have isolated a tumor growth-inhibitory substance from red ginseng,[3] and its structure was determined on the basis of instrumental analysis to be heptadeca-1-ene-4,6-diyne-3,9,10-triol (panaxytriol) (I) (Fig. 1). The *in vitro* antitumor effect of panaxytriol was also examined.

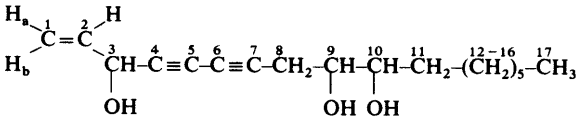

Fig. 1. Chemical Structure of Panaxytriol (I)

Results and Discussion

The Isolation and Structural Elucidation of Panaxytriol (I) Red ginseng was extracted with ethyl acetate (AcOEt). The AcOEt extracts were purified by chromatography on a silica gel column, and crystallization of the product from distilled water gave colorless needles, mp 68—72 °C.

The infrared (IR) spectrum showed absorptions due to a hydroxyl group at 3348 cm^{-1}, a methylene group at 2924 and 2856 cm^{-1} and conjugated triple bonds at 2256 cm^{-1}. The ultraviolet (UV) spectrum of I supported the presence of conjugated triple bonds.[4a]

Figure 2 shows the two-dimensional proton–proton chemical shift correlation (COSY) spectrum of I. The COSY spectrum of I indicated that the signals at δ 5.24 (ddd, J=1.0, 2.0, 9.7 Hz, 1-H$_a$), 5.46 (ddd, J=1.0, 2.0, 16.9 Hz, 1-H$_b$), and 5.90 (ddd, J=5.5, 9.7, 16.9 Hz, 2-H) were assignable to protons of a terminal vinyl group. The signal due to an allylic proton at δ 4.91 (br d, J=*ca.* 5.5 Hz, 3-H) was correlated to the proton signal at δ 5.90 (2-H). The signals due to methine protons at δ 3.69 (br q, J=*ca.* 10.0, 10.0, 5.9 Hz, 9-H) and 3.59 (br q, J=*ca.* 10.0, 10.0, 6.0 Hz, 10-H) were correlated to the signals of methylene

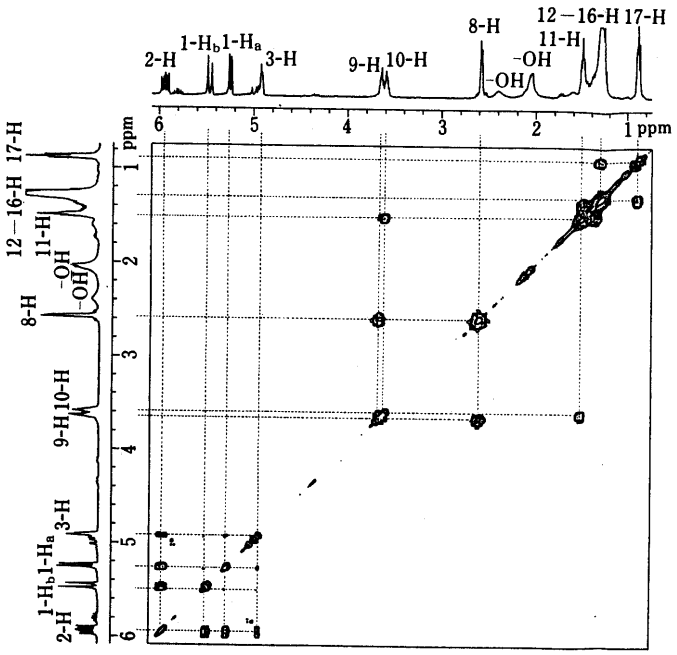

Fig. 2. The Two-Dimensional Proton–Proton Chemical Shift Correlation Spectrum of Panaxytriol

protons at δ 2.58 (br d, J=5.85 Hz, 8-H) and 1.50 (br t, J=*ca.* 5.8 Hz, 11-H), respectively. Further, the proton signals of three secondary hydroxyl groups appeared at δ 2.03 and 2.40 (disappeared on addition of deuterium oxide).

The carbon-13 nuclear magnetic resonance (^{13}C-NMR) ($CDCl_3$) spectrum of I showed methylene carbon signals at δ 22.61, 25.03, 25.55, 29.18, 29.53, 31.79 and 33.63, as well as the signals of terminal methyl carbon of a straight aliphatic chain at δ 14.03, the two carbons of the terminal vinyl group at δ 117.08 and 136.10, and the allylic carbon at δ 63.50 (Fig. 3). The signals of quaternary carbons in the conjugated triple bonds appeared at δ 66.55, 70.92, 74.84 and 78.15, which were identified by means of the insensitive nuclei enhanced by polarization (INEPT) method (Fig. 4). The four quaternary carbon signals disappeared in the INEPT spectrum. The signals of two methine carbons (C-9 and C-10) were seen at δ 73.06 and 72.17.

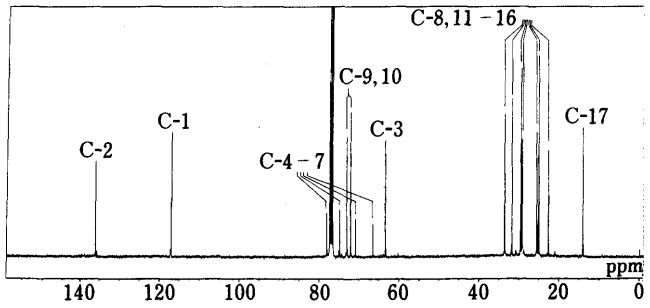

Fig. 3. ^{13}C-NMR of Panaxytriol

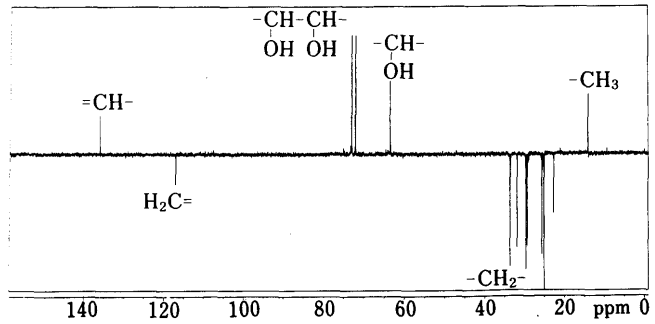

Fig. 4. ^{13}C-NMR of Panaxytriol (INEPT Method)

The high-resolution mass spectrum (HR-MS) of I determined by the chemical ionization (CI) method showed a fragment at m/z 279 ($(M+H)^+$).

Thus, the antitumor substance (I) was identical as panaxytriol, previously described by several investigators.[4] However, some differences in ^{13}C-NMR chemical shifts (C-9, C-10) of panaxytriol were present between Shim *et al.*'s data[4b] (δ54.0, 56.5) and ours (δ72.17, 73.06). In general, the chemical shifts of secondary carbons bearing a hydroxyl group are observed at *ca.* δ 60—80.[5] Further, our sample was crystalline, whereas theirs was an oil.

Determination of Panaxytriol (I) We developed a gas chromatographic method for the quantitative determination of panaxytriol. A sample containing both panaxytriol and internal standard (1-chloroanthraquinone) was treated with 10 μl of triethylamine and 10 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA). The solution was heated at 60°C for 60 min. After cooling to room temperature, a 1.0 μl aliquot was injected into the column.

A gas chromatograph equipped with flame-ionization detector (FID) and a moving needle solvent cut sample injector (Gasukuro Kogyo) was used for all analyses. The column used was a flexible silica capillary column coated with OV-1701 (50 m × 0.25 mm, i.d., Gasukuro Kogyo). The injection and detector temperatures were set at 250 °C, while the column temperature was kept at 250 °C. Nitrogen was used as the carrier gas and make-up gas at flow rates of 1.1 and 35 ml/min, respectively. The flow rates of air and hydrogen were adjusted to 400 and 40 ml/min, respectively. The split ratio was 68 : 1.

The panaxytriol contents of red ginseng and white ginseng was determined by use of this method; the values obtained were 0.38 and 0.25 mg/g, respectively.

Antitumor Activity of Panaxytriol (I) Panaxytriol was dissolved in RPMI-1640 culture medium containing 10% fetal calf serum (GIBCO Lab., N. Y., U.S.A.) and tested for growth inhibition of various kinds of cultured cells *in vitro*. Nude mouse-transplantable human gastric adenocarcinoma cells (MK-1 cells), human colon adenocarcinoma cells (SW620 cells), human uterus carcinoma cells (HeLa cells), human erythroleukemic cells (K562 cells), mouse melanoma cells (B16 cells) and mouse fibroblast-derived tumor cells (L929 cells) were used as target cells. Human embryo-derived fibroblasts (MRC-5 cells) was used as control cells.

TABLE I. Effect of Panaxytriol on Cell Growth *in Vitro*

Cell line[a]	ED_{50} (μg/ml)[b]
MK-1	0.8±0.3
B16	1.7±0.3
L929	2.2±0.3
SW620	2.3±0.3
HeLa	10.7±1.2
K562	11.7±2.9
MRC-5	>40

Fifty microliters of cell suspension (2×10^5 cells) and 50 μl of panaxytriol solution were plated in flat-bottomed microtiter wells and incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Percent growth inhibition

$$= \left(1 - \frac{\text{no. of viable cells in medium with panaxytriol}}{\text{no. of viable cells in medium without panaxytriol}}\right) \times 100.$$

a) MK-1 (human gastric adenocarcinoma); B16 (mouse melanoma); L929 (mouse fibroblast-derived tumor); SW620 (human colon adenocarcinoma); HeLa (human uterus carcinoma); K562 (human erythroleukemia); MRC-5 (human fibroblast). *b*) ED_{50} is the concentration of panaxytriol required to obtain 50% growth inhibition. Mean ± S.E.D. of three experiments.

Table I shows the effect of panaxytriol on the cell growth *in vitro*. The concentrations of panaxytriol required to give 50% growth inhibition (ED_{50}) were 0.8, 1.7, 2.2, 2.3, 10.7 and 11.7 μg/ml against MK-1 cells, B16 cells, L929 cells, SW620 cells, HeLa calls and K562 cells, respectively. However, panaxytriol did not inhibit the growth of human fibroblasts, MRC-5 cells, by 50% even at concentrations of over 40 μg/ml.

We examined the absorption of panaxytriol by MK-1 cells in RPMI-1640 medium, by use of the gas chromatographic method. Panaxytriol was absorbed by MK-1 cells to the extents of at 28, 53, 66 and 84% at 1, 2, 3, and 6 h, respectively. When MK-1 cells were incubated with a high concentration (100 μg/ml) of panaxytriol for 10 h, 50% of the panaxytriol was absorbed by MK-1 cells, but only 5% was detected intracellularly. The reason why only 5% of panaxytriol could be recovered from MK-1 cells is still unknown.

Conclusion

In this study, a tumor growth-inhibitory substance was isolated from *Panax ginseng* and identified as panaxytriol. A method for determination of panaxytriol by gas chromatography was developed. Further studies relating to growth inhibition by panaxytriol will be presented in the following paper.

Experimental

The melting point was taken on a Yanagimoto micromelting point apparatus and is uncorrected. The IR spectrum was recorded with a Hitachi 270-30 spectrometer, the UV spectrum with a Shimadzu UV-240 spectrometer, ^{1}H- and ^{13}C-NMR spectra with a JEOL JNM-GX400 spec-

trometer (with tetramethylsilane as an internal standard, $CDCl_3$ solvent) and HR-MS with a JEOL JMS-D300. Gas chromatography was done with a Hitachi 663-30 gas chromatograph. Column chromatography was carried out on Silica gel 60 (100—200 mesh, Nakarai). Thin-layer chromatography (TLC) was performed on Kiesel gel 60 plates (Merck). The spots were detected by spraying the plates with concentrated H_2SO_4 and by heating.

Extraction and Isolation of Panaxytriol (I) Korean red ginseng powder (Nikkan Korai Ninjin Co., Ltd., Kobe, 300 g) was extracted with AcOEt (1000 ml) for 24 h at room temperature. The AcOEt extract was evaporated and the residue (1.7 g) was fractionated by column chromatography (SiO_2, 45 g) using AcOEt–*n*-hexane (1 : 1, v/v) as the eluant. Fractions containing panaxytriol (74 mg) were further fractionated by column chromatography (SiO_2, 7 g) using chloroform and AcOEt–*n*-hexane (1 : 1) to give crude panaxytriol. Finally, a part (1 mg) of the crude panaxytriol was crystallized from distilled water, giving colorless needles (0.5 mg).

Panaxytriol (I): $UV_{max}^{H_2O}$ nm (ε): 256 (376), 243 (627), 231 (711). MS *m/z* (%): 279 ($(M+H)^+$, 28), 261 (100), 243 (42), 205 (10), 159 (30), 145 (21), 129 (13), 102 (18), 93 (8), 81 (9), 69 (6). HR-MS *m/z*: $((M+H)^+ - H_2O)$ Calcd for $C_{17}H_{25}O_2$ 261.1870. Found: *m/z* 261.1855. IR, ^1H- and ^{13}C-NMR spectral data were described above.

Preparation of Red Ginseng and White Ginseng for Gas Chromatography Red ginseng or white ginseng (0.1 g) were added to distilled water (1 ml) and incubated at 40 °C for 30 min. After cooling, the suspension was added to AcOEt (5 ml). The mixture was vigorously shaken for 10 min at room temperature and centrifuged for 10 min at 1500 *g*. The organic phase (0.25 ml) was transferred to a glass tube and 40 μl (100 ng) of internal standard, 1-chloroanthraquinone, was added. The solution was evaporated to dryness under nitrogen. The dry residue was subjected to gas chromatography.

Preparation of Culture Medium for Gas Chromatography Panaxytriol-containing culture medium (1.0 ml) was added to AcOEt (5 ml) with 40 μl (100 ng) of internal standard. The mixture was vigorously shaken for 10 min at room temperature and centrifuged for 10 min at 1500 *g*. The organic phase was transferred to a glass tube and evaporated to dryness under nitrogen. The dry residue was subjected to gas chromatography.

Antitumor Activity Fifty microliters of cell suspension (2×10^5 cells) and 50 μl of panaxytriol solution were plated in flat-bottomed microtiter wells and incubated for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 in air.

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