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Studies on the Immunoassay of Bioactive Natural Products I.

Synthesis of Ligands for the Immunoassay of Panaxadiol and Panaxtriol

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Abstract—For the immunoassay of ginseng sapogenins, the ligands with which panaxadiol and panaxatriol could be determined together and separately were synthesized. For the total assay of panaxadiol and panaxatriol, panaxatriol-6-hemisuccinate was synthesized. For the separate assay, panaxadiol-3-hemisuccinate and panaxatriol-3-hemisuccinate were also synthesized.

Keywords—*Panax ginseng* · Araliaceae· immunoassay·panaxadiol·panaxatriol·ligands· panaxadiol-3-hemisuccinate · panaxatriol-3-hemisuccinate · panaxatriol-6-hemisuccinate

The root of *Panax ginseng* C.A. Meyer(Araliaceae) is one of the most famous drugs used in Oriental medicine as an elixir. As of now, the saponin components, *e.g.* ginsenosides, contained in this drug are considered as active principles and extensive studies on the chemistry[1], biochemistry[2] and pharmacology[3] of ginsenosides have been performed. However, little has been known concerning the fate of ginsenosides *in vivo*[4]. This is entirely due to the lack of a highly sensitive and specific method for the determination of ginsenosides. Many methods for the determination of ginsenosides such as colorimetry[5], TLC[6], GLC[7], DCC[8] and HPLC[9] are available. These methods are not sensitive and specific for the determination of ginsenosides in biological fluids. Recently, the radioimmunoassay (RIA) method[10] for the determination of one of the ginsenosides, ginsenoside Rg_1, was developed and applied in many fields. Although this RIA method is sensitive, specific and powerful enough to determine the contents of ginsenosides in biological fluids, it is too tedious and time and money consuming work to develop every RIA method for the individual ginsenosides up to thirteen in number. It is, therefore, necessary to develop sensitive and specific immunoassay methods to be applicable to every ginsenosides.

It may be impossible to develop this kind of immunoassay method using the saponins which have a variety of structural characteristics. But the sapogenins, panaxadiol(PD) and panaxatriol (PT) obtained from the treatment of the saponins with acids can be derived from every ginseng saponin and is very typically compared to any other plant constituents and very similar to each other except hydroxyl group attached to C-6 of dammarene skeleton.

It will be possible to develop immunoassay method for two sapogenins if the ligands(*i.e.* antigen determinant) which can be recognized simultaneously or separately are synthesized. And this immunoassay method will be applicable to determine indirectly every saponin.

This short report deals with the synthesis of the ligands for the immunoassay of ginseng sapogenins with which panaxadiol and panaxatriol will be determined together and separately.

For the total assay of PD and PT, panaxatriol-6-hemisuccinate was synthesized and for their separate determination, panaxadiol-3-hemisuccinate and panaxatriol-3-hemisuccinate were also synthesized, respectively.

Experimental

All melting points were measured with Gallenkamp MFB-600-030W and were uncorrected. IR spectra were determined on a Perkin-Elmer Infrared Spectrophotometer Model 783. ^1H NMR spectra were measured on a Bruker Spectrospin 80 with TMS as an internal standard. TLC was performed on Kieselgel GF 254 precoated TLC plate (Merck). For column chromatography, Kieselgel 60 (70-230 mesh, Merck) and benzene-acetone as solvent system were used. All solvents were redistilled and other reagents are all experimental grade.

Preparation of sapogenins

Ginseng sapogenins were prepared by the methods of Shibata *et al.*[11] Panaxadiol (PD) and panaxatriol (PT) were separated on silicagel column chromatography using benzene-acetone (10:1 to 6:1) as solvent system. They were recrystallized from acetone as colorless needles. They are identified by direct comparison with authentic samples of mixed mp, IR and NMR.

Synthesis of panaxadiol-3-hemisuccinate (PDS)

PD (460mg, 1 mmole) and succinic anhydride (200mg, 2 mmole) were dissolved in pyridine (2ml) and the mixture was refluxed under N_2 stream overnight. After reaction, the solution was poured into cooled 10% H_2SO_4 (50ml) and extracted with ether (20ml, 3 times). The ether layer was washed with water (20ml, 3 times), dried over anhydrous sodium sulfate and evaporated to dryness. TLC of the reaction mixture using benzene-acetone (3:1) as solvent system showed main spot at R_f value of 0.40 with some impurities. The residue was purified into PDS by silicagel column chromatography using benzene acetone (7:1) as solvent system. PDS was recrystallized from acetone to give colorless needle (320mg, 57%), mp 241-2°, *Anal.* Cald. for $C_{34}H_{56}O_6$: C, 71.42; H, 9.96. Found: C, 72.82; H, 10.06. IR ν_{max}^{KBr}: 3,450cm^{-1} (hydroxyl), 3,000-2,500cm^{-1} (acid), 1,710cm^{-1} (carbonyl), 1,380 cm^{-1} (gem-dimethyl), 1,120cm^{-1} (ether). ^1H NMR δ: 0.88 (6H), 0.90 (3H), 0.92 (3H), 1.02 (3H) (all singlet, tertiary methyl), 1.22 (3H), 1.25 (3H), 1.29 (3H) (all singlet, tertiary methyl adjacent to oxygen), 2.72 (4H, singlet, succinate), 3.62 (1H, multiplet, oxymethine at C-12), 4.62 (1H, triplet like, oxymethine at C-3).

Synthesis of panaxatriol-3-hemisuccinate (PTS3)

PT (476mg, 1 mmole) and sussinic anhydride (200mg, 2mmole) were dissolved in pyridine (2ml) and the mixture was refluxed under N_2 stream for 5 hours. After reaction, the solution was poured into cooled 10% H_2SO_4 (50ml) and extracted with ether (20ml, 3 times). The ether layer was washed with water (20ml, 3 times), dried over anhydrous sodium sulfate and evaporated to dryness. TLC of the reactoin mixture using benzene-acetone (2:1) as solvent system showed four spots at R_f values of 0.59, 0.43, 0.31 and 0.25, including starting material. The residue was separated into PT, PTS3 and PTS6 on silicagel column. The compound which showed R_f value of 0.25 was not eluted out. The solvent system employed was benzene-acetone (7:1 to 2:1). The elution yield of the latter two compounds was too low for their high polarity and hardly crstallized. Thus, PTS3 which showed R_f value of 0.43 was methylated with diazomethane. PTS3 was dissolved in dried ether and dried etherial diazomethane solution was mixed. The solution was kept in dark place evernight. The solution was evaporated to dryness. TLC of the reaction mixture

using benzene-acetone(4:1) as solvent system showed single spot at R_f value of 0.60. The reaction mixture was purified by silicagel column chromatography using benzene-acetone(10:1) as solvent system. Methyl ester of PTS3(PTS3-Me) was recrysta llized from benzene-hexane to give colorless needle(150mg, 25%), mp 213~4°, *Anal.* Cald. for $C_{35}H_{58}O_7$: C, 71.15; H, 9.90. Found: C, 71.20; H, 9.30. IR ν_{max}^{KBr}: 3,350cm^{-1} (hydroxyl), 1,740cm^{-1}(ester), 1,380cm^{-1}(gem-dimethyl), 1,165cm^{-1}(methyl ester), 1,120cm^{-1} (ether). ^{1}H NMR δ:0.91(3H), 0.96(3H), 1.07 (6H), 1.17(3H)(all singlet, tertiary methyl), 1.17(3H), 1.22 3H), 1.26(3H)(all singlet, tertiary methyl adjacent to oxygen), 2.65(4H, singlet, succinate), 3.54(1H, multiplet, oxymethine at C-12), 3.70(3H, singlet methyl ester), 4.10(1H, multiplet, oxymethine at C-6), 4.48 (1H, oxymethine at C-3).

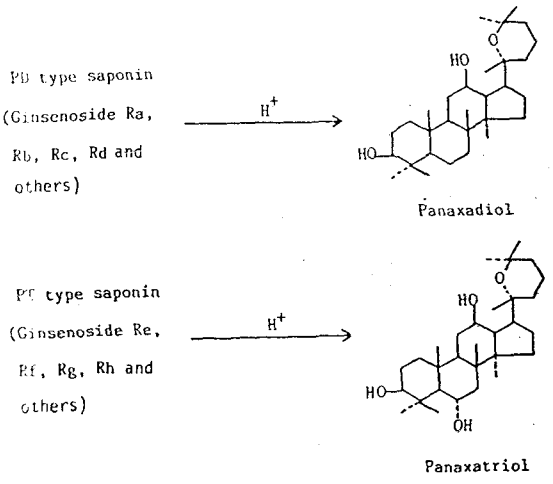
Synthesis of panaxatriol-6-hemisuccinate (PTS6)

PTS6 which was separated from the reaction mixture mentioned above in the column of synthesis of PTS3, showing R_f value of 0.31, was also hardly recrystallized. So that, it was also methylated with diazomethane by the same procedure as that of synthesis of PTS3-Me and purified by column chromatography. Methyl ester of PTS6 (PTS6-Me) was recrystallized from benzene to give colorless needle (70mg, 12%), mp 105~6°, *Anal.* Cald. for $C_{35}H_{58}O_7$: C, 71.15; H, 9.90. Found: C, 72.0; H, 9.80. IR ν_{max}^{KBr}:3, 360cm^{-1}(hydroxyl), 1,730cm^{-1}(ester), 1,380cm^{-1} (gem-dimethyl), 1,160cm^{-1}(methyl ester), 1,120 cm^{-1}(ether). ^{1}H NMR δ:0.86(3H), 0.92(3H), 1.00(3H), 1.12(3H), 1.20(3H)(all singlet, tertiary methyl), 1.20(3H), 1.24(3H), 1.28 (3H) (all singlet, tertiary methyl adjacent to oxygen), 2.64(4H, singlet, succinate), 3.20(1H, triplet like, oxymethine at C-3), 3.54(1H, multiplet, oxymenthine at C-12), 3.70(3H, singlet, methyl ester), 5.40(1H, multiplet, oxymethine at C-6).

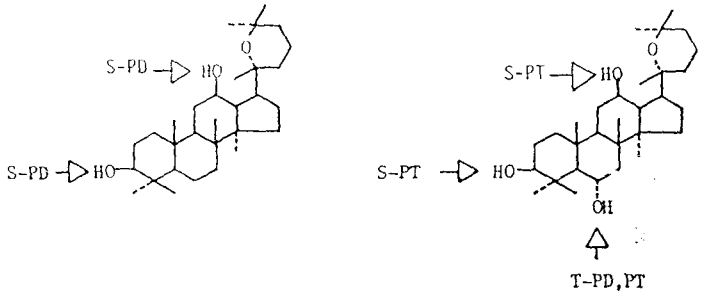
Results and Discussion

Dammarene saponins of ginseng are divided into two groups, panaxadiol group saponins(*e.g.* ginsenosides Ra, Rb, Rc, Rd and others) and panaxatriol group saponins(*e.g.* ginsenosides Re, Rf, Rg, Rh and others), according to their genuine sapogenins, protopanaxadiol and protopanaxatriol. Each of them differs from only its sugar moiety in types, numbers and positions. On acid hydrolysis, each of them produces only two types of sapogenins, PD and PT. (Scheme 1) PD and PT showed typical structural characteristics distinguishable to other plant constituents.

They share same structures except hydroxyl group on C-6 of dammarene skeleton. PD has two secondary hydroxyl groups on C-3 and C-12, and PT has the group on C-6, additionally. When the secondary hydroxyl group is used for the synthesis of ligands for immunogens, there are three possible attaching position of carrier protein as shown in Scheme 2. Hydroxyl group at C-12 is not suitable for the attaching position because antibody produced with immunogen having carrier protein at that position may not react with the total structre of sapogenin fully,



Scheme 1. Ginseng sapogenins.



Scheme 2. Possible attaching position of spacing groups.
S-PD, S-PT: attaching position for separate determination for panaxadiol and panaxatriol
T-PD, PT : attaching position for total determination

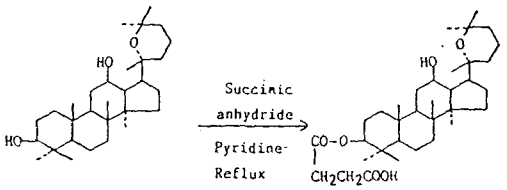
especially with the side ring. On this point of view, hydroxyl group at C-3 or C-6 is fixed for the attaching position. We used succinic anhydride as spacing group. When succinic anhydride is used as spacing group, hydroxyl group at C-12 doesn't react because of its steric hindrance.

If antibody is produced with the ligand of PT S6, PD and PT are recognized by immune cells in the same, as the rest part of PT share the same structural characteristics as PD including their hydroxyl group at C-3 and C-12, and their side ring. With this antibody it will be possible to determine PD and PT together. While, when antibody is produced with the ligand having 3-succinate of both sapogenins, PD and PT are recognized separately, as their structure are different from each other in hydroxyl group at C-6. Using these antibodies will make it possible to determine PD and PT separately. Neswender and Medgley[12] studied RIA of steroid hormone extensively. They reported the relative activity of several steroids with the RIA system of progesterone using anti-progesterone-20-BSA. 11-Hydroxy-progesterone cross reacted only 7.1%, which differs from progesterone only in hydroxyl group at C-11.

Ligands for separate determination

1) **Panaxadiol-3-hemisuccinate:** Reaction of secondary hydroxyl group and succinic anhydride requires rather vigorous conditions.[13~15] There is only one hydroxyl group which can react with succinic anhydride, i.e. hydroxyl group at C-3. PD refluxed with succinic anhydride in pyridine under N_2 stream overnight showed single spot on TLC. (Scheme 3 and Fig. 1) As mentioned above, hydroxyl group at C-12 didn't react for its steric hindrance. In IR spectra, PDS showed absorption of carbonyl group of carboxylic acid at 1710cm^{-1} and typical absorption of carboxylic acid at 3000~2500cm^{-1}. Oxymethine group at C-3 and C-12 of PD showed chemical shift at δ3.24(triplet like) and δ3.62(multiplet), respectively. In ^{1}H NMR spectra of PDS, the peak at δ3.24 was disappeared and the peak at δ4.62(1H, triplet like) was appeared newly, which indicated that the peak of oxymethine at C-3 of PD was shifted from δ3.24 to δ4.62 by the influence of succinyl group. The peak of succinyl group was observed at δ2.72(4H, singlet). These spectral data indicated that PDS was synthesized.

2) **Panaxatriol-3-hemisuccinate:** In the case of synthesis of PTS, the situation is rather different from that of PDS. There are two possible



Panaxadiol

Scheme 3. Synthesis of panaxadiol-3-hemisuccinate.

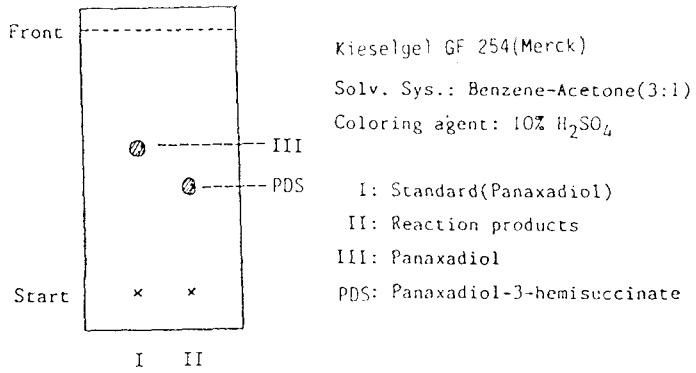
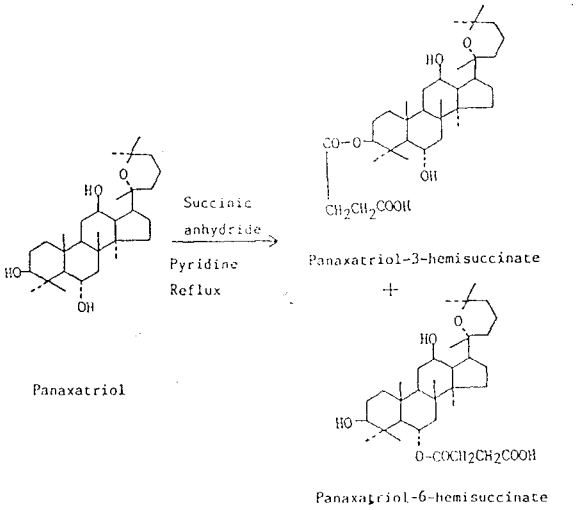


Fig. 1. Chromatogram of reaction mixture of PD and succinic anhydride.

reactive hydroxyl group at C-3 and C-6. Although both hydroxyl group have equatorial conformation, the steric environmental condition is greatly different from each other. Thus it can be predicted that the reactivity of hydroxyl group at C-3 is greater than that at C-6. Actually the ratio of reactivity was about 2:1 by their yield. By our privious experiment of synthesis that, as reaction time proceeds, the compound which showed R_f value of 0.25, predicted as 3,6-dihemisuccinate of PT, was increased, the reaction was stopped in 5 hours. PT was refluxed with succinic anhydride in pyridine under N_2 stream for 5 hours. (Scheme 4) TLC of the reaction mixture showed 4 spots at R_f values of 0.59, 0.43, 0.31 and 0.25, which corresponds to PT, PTS3,PTS6 and PT-3,6-dihemisuccinate (PTS36, not identified). (Fig. 2) PTS3 was separated by silicagel column chromatography. The recovery of PTS3 was too low from the column using silicagel as adsorbant for its high polarity, which caused the bad yields(25%).



Scheme 4. Synthesis of panaxatriol-3-hemisuccinate and panaxatriol-6-hemisuccinate.

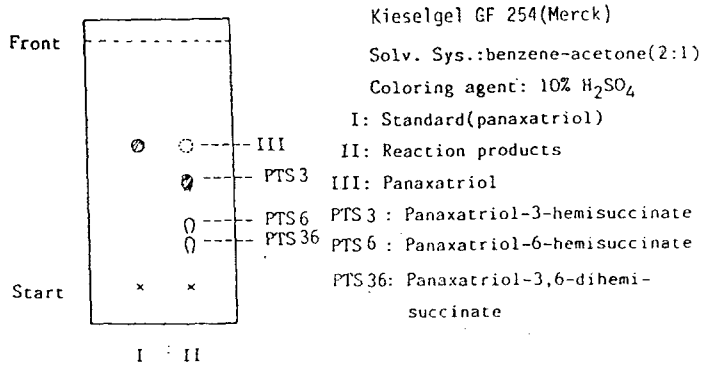


Fig. 2. Chromatogram of reaction mixture of PT and succinic anhydride.

Isolated PTS3 was hardly recrystallized, so that recrystallization and structure elucidation was performed by its methyl ester(PTS3-Me). In IR spectra of PTS3-Me, the band at 1,740cm^{-1} and 1,165cm^{-1} indicated the presence of methyl ester. Oxymethine group at C-3, C-6 and C-12 of PT was appeared at δ3.24(triplet like), δ4.20 (multiplet) and δ3.60(multiplet), respectively. In ^{1}H NMR spectra of PTS3-Me, the peak at δ3.24 of PT was disappeared and the peak at δ4.48(1H, triplet like) was appeared newly, which indicated that the peak of oxymethine at C-3 of PT was shifted from δ3.24 to δ4.48 by the influence of succinyl group. The peak of succinyl group was appeared at δ2.65 (4H, singlet). And the methyl group of methyl ester was appeared at δ3.70(3H, singlet). These spectral data indicated that PTS3-Me was synthesized.

Ligand for total determination(Panaxatriol-6-hemisuccinate)

PTS6 which showed R_f value of 0.31 was spearated from the reaction mixure of PT and succinic anhydride as metioned at the experimental section. The low recovery of PTS from the sicagel column also caused the bad yields(12%). Isolated PTS6 was also hardly recrystallized. Thus recrystallization and structure elucidation were also performed by its methyl ester(PTS6-Me). In IR spectra of PTS6-Me, the bands at 1,730cm^{-1} and 1,160cm^{-1} indicated the presence of methyl ester. In ^{1}H NMR spectra of PTS6-Me, the peak at δ3.60 of PT was disappeared and the peak at δ5.40(1H, multiplet) was newly appeared. This indicated that the peak of oxymethine group at C-6 of PT was shifted from δ3.60 to δ5.40 by the influence of succinyl group. The peak of succinyl group was appeared at δ2.64(4H, singlet) and the methyl peak of methyl ester was appeared at δ3.70(3H, singlet). These spectral data indicated that PTS6-Me was synthesized.

Synthesis of immunogen with these three ligands, PDS, PTS3 and PTS6, is in progress.

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