Triterpenoid Saponins from *Pulsatilla cernua* Spreng. I.1

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Four triterpenoid saponins (I—IV), isolated from the root of *Pulsatilla cernua*, were characterized as follows: I, hederagenin (hederag.) 3-O-α-L-rhamnopyranosyl (rham.-pyr)-(1→2)-α-L-arabinopyranoside; II, 3-O-α-L-rham.-pyr-(1→2)-α-L-arabinopyranosyl (ara.-pyr) -hederag. 28-O-α-L-rham.-pyr-(1→4)-β-D-glucopyranosyl (gluc.-pyr)-(1→6)-β-D-glucopyranoside; III, 3-O-α-L-rham.-pyr-(1→2)-[β-D-gluc.-pyr-(1→4)]-α-L-ara.-pyr-hederag. 28-O-α-L-rham.-pyr-(1→4)-β-D-gluc.-pyr-(1→6)-β-D-glucopyranoside; IV, hederag. 3-O-α-L-rham.-pyr-(1→2)-[β-D-gluc.-pyr-(1→4)]-α-L-arabinopyranoside. III and IV are new saponins. II and III were also detected in *Pulsatilla koreana* and *P. chinensis* on TLC.

Keywords—oleanane type saponin; “Haku-to-o”; *Pulsatilla cernua* Spreng.; Ranunculaceae; structure of saponin I, II, III and IV; hederagenin oligoglycoside

The root of *Pulsatilla cernua* Spreng. (Japanese name: ‘Hakutoo’) has been used as a home remedy for antitumor, astringent, antilaxative, diuretic and so on, but the original plants of commercial ‘Hakutoo’ are different in each market. Their anatomical aspects were reported.

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### Table I. Distribution of *Pulsatilla* Species and Their Components

<table>
<thead>
<tr>
<th>Habitat</th>
<th><em>Pulsatilla</em> spp.</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan</td>
<td><em>Pulsatilla cernua</em> Spreng.</td>
<td>Oleanolic acid, hederagenin²b)</td>
</tr>
<tr>
<td>Korea</td>
<td><em>P. Koreana</em> NAKAI</td>
<td>Unknown</td>
</tr>
<tr>
<td>China</td>
<td><em>P. chinensis</em> REGEL</td>
<td>C₉₅H₇₉O₄</td>
</tr>
<tr>
<td></td>
<td><em>P. turczaninovii</em> KRYLOV et SERG.</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td><em>P. davurica</em> Spreng.</td>
<td>Unknown</td>
</tr>
<tr>
<td>China and</td>
<td><em>P. patens</em> MILL.</td>
<td>Unknown</td>
</tr>
<tr>
<td>North America</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Europe</td>
<td><em>P. vulgaris</em> MILL.</td>
<td>Protoanemonin²b)</td>
</tr>
<tr>
<td></td>
<td><em>P. praetens</em> MILL.</td>
<td>Ranunculin²b)</td>
</tr>
<tr>
<td>U.S.S.R</td>
<td><em>P. nemorosa</em> SCHANK</td>
<td>C₉₅H₇₉O₄</td>
</tr>
<tr>
<td></td>
<td><em>P. ucrainica</em> (UGRINSKIY) WISSJUL</td>
<td>Ranunculin²b)</td>
</tr>
<tr>
<td></td>
<td><em>P. montana</em> REICH.</td>
<td>Ranunculin²b)</td>
</tr>
<tr>
<td></td>
<td><em>P. nigricans</em> STOERCK ex DC</td>
<td>Hederagenin²b) ranunculin²b)</td>
</tr>
</tbody>
</table>

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2) Location: a) Sugstani, Toyama; b) Present address; Albus Chemical Industries, Ltd., Takayama, Gifu; c) 3 Ho, Kanagawa-machi, Kanasawa.
by Munesada, Fujita and Higashi. The distribution and the components of Pulsatilla species examined until now are shown in Table I.

With regard to 'Hakuto-bo' cultivated in Japan (Nara prefecture), it has been supposed to contain saponins, but only sapogenins, hederagenin and oleanolic acid, were detected.

We have investigated the saponin constituents of Pulsatilla species in various habitats. The methanol extractives of the roots of Pulsatilla cernua cultivated in Kyushu were fractionated successively with ether, ethyl acetate and n-butanol as shown in Chart I.

![Diagram of saponin extraction process]

The mixture of saponin II and saponin III, which was obtained from n-butanol soluble part as precipitates by treatment with methanol and acetone, was separated by chromatography on silica gel column eluting with chloroform–methanol–water (200:70:9). The mixture of saponin I and saponin IV, which was obtained from methanol–acetone soluble part of n-butanol soluble fraction, was separated by chromatography on silica gel column eluting with chloroform–methanol–water (200:53:4) (Chart I). Saponin I was also isolated from ethyl acetate soluble fraction.

Saponin I (I) mp 250–255°C [α]D +17.0°, and saponin II (II), mp 215–216°C, [α]D –8.0°, were respectively identified with hederagenin 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside and 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl-hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside by examination of their acid hydrolysates, by comparisons of their RF values on thin-layer chro-

4) T. Munesada and M. Hayashi, Yakugaku Zasshi, 53, 917 (1933).
7) Authors got a private information that Prof. T. Kawasaki of Kyushu University also isolated the same compounds as I and II from the same plant.
matography (TLC) and infrared (IR) spectra with those of the authentic specimens and by a mixed fusion.

Saponin III (III), mp 219—222 °C, [α]D—9.0 °C, furnished on acid hydrolysis hederagenin, rhamnose, arabinose and glucose. The exhaustively methylated product (V) of III by Kuhn’s method,\(^{10}\) mp 117—120 °C, \(C_{88}H_{142}O_{31}\), was methanolyzed to afford 23-O-methyl-hederagenin, mp 216—217 °C, and methylated sugars, \(S_1, S_2, S_3, S_4\) and \(S_5\). \(S_1, S_2, S_3\) and \(S_4\) were identified by gas-liquid chromatography (GLC) with methyl pyranosides of 2,3,6-tetra-O-methyl-D-glucose, 2,3,4-tri-O-methyl-L-rhamnose, 2,3,6-tri-O-methyl-D-glucose and 2,3,4-tri-O-methyl-D-glucose. \(S_5\) was identified as its acetate by GLC with an authentic sample of methyl 2,4-di-O-acetyl-3-O-methyl-\(\alpha\)-l-arabinopyranoside.\(^{11}\) The lithium aluminium hydride \((LiAlH_4)\) reduction in tetrahydrofuran (THF) of V provided VI, mp 142—144 °C, which showed on a mass spectrum (MS) the fragment ions due to a terminal permethylated methylpentose \((m/e 189)\)\(^{12}\) and hexose \((m/e 213)\)\(^{12}\) residues, and a methylated saccharide which was identified with the authentic sample of 2,3,4-tri-O-methyl-\(\alpha\)-l-rhamnopyranosyl-(1→4)-2,3,6-tri-O-methyl-\(\beta\)-d-glucopyranosyl-(1→6)-2,3,4-tri-O-methyl-D-sorbitol \((S_4)\)\(^{6,9}\) by direct comparison [IR, nuclear magnetic resonance (NMR), MS]. The mode of linkage of esterglycosyl glucose residue was regarded as \(\beta\) on the basis of the NMR spectrum of V showing an anomic proton signal of esterglycosyl glucose at 5.35 ppm as a doublet \((J=8.4 \text{ Hz})\). \(S_5\) was also provided from methylated saponin II through the same procedure. Methanolyis of VI (8% HCl-MeOH) provided 23-O-methyl-olean-12-en-3,23,28-triol,\(^{13}\) which showed an NMR signal at 3.35 ppm (3H, singlet, \(C_3-OCH_3\)), and three kinds of methylated sugars, \(S_1, S_2\) and \(S_5\). III gave on alkaline hydrolysis (0.5 N-KOH) IV, mp 230—242 °C, [α]D+14.9 °C, which furnished on acid hydrolysis hederagenin, rhamnose, glucose and arabinose. IV afforded on partial hydrolysis (0.3% HCl-MeOH) a mixture of hederagenin, hederagenin 3-O-\(\alpha\)-l-arabinopyranoside\(^{10}\) and VII, mp 260—261 °C, [α]D+58.3 °C. Methylation of VII by Kuhn’s method\(^{10}\) afforded VIII, mp 84—87 °C, which exhibited NMR signals for two anomic protons (4.19 ppm, 1H, doublet, \(J=6.5 \text{ Hz}\), arabinose \(C_1\)-H; 4.40 ppm, 1H, doublet, \(J=8.2 \text{ Hz}\), glucose \(C_1\)-H). Methanolyis of VIII afforded 23-O-methyl-hederagenin methyl ester and two kinds of sugars, \(S_1\) and methyl 2,3-di-O-methyl-\(\alpha\)-l-arabinopyranoside \((S_7)\) which was identified by GLC as its monoacetate with authentic methyl 4-O-acetyl-2,3-di-O-methyl-\(\beta\)-l-arabinopyranoside.\(^{11}\) IV is thus considered to be rhamnioside of hederagenin 3-O-\(\beta\)-d-glucopyranosyl-(1→4)-\(\alpha\)-l-arabinopyranoside. The linking of the terminal rhamnose unit to the arabinose residue of IV was regarded as 1 to 2 by the formation of methylated sugar \(S_5\) on the methanolyis of VI. The \(\alpha\)-configuration of the l-rhamnose was suggested by the difference of the molecular rotation between IV and VII.

The structure of III and IV were thus defined as 3-O-\(\alpha\)-l-rhamnopyranosyl-(1→2)-[\(\beta\)-d-glucopyranosyl-(1→4)]-\(\alpha\)-l-arabinopyranosyl-hederagenin 28-O-\(\alpha\)-l-rhamnopyranosyl-(1→4)-\(\beta\)-d-glucopyranosyl-(1→6)-[\(\beta\)-d-glucopyranosyl-(1→4)]-\(\alpha\)-l-arabinopyranoside\(^{14}\) and hederagenin 3-O-\(\alpha\)-l-rhamnopyranosyl-(1→2)-[\(\beta\)-d-glucopyranosyl-(1→4)]-\(\alpha\)-l-arabinopyranoside, respectively. These two saponins III and IV are new members of the group of hederagenin oligoglycosides. Saponin II and III were also detected in Pulsatilla koreana and P. chinensis on TLC. We also examined other five kinds of materials collected in each prefecture of Iwate, Miyagi, Niigata, Ishikawa

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11) The authentic samples of methyl 2,4-di-O-acetyl-3-O-methyl-\(\alpha\)-l-arabinopyranoside and methyl 4-O-acetyl-2,3-di-O-methyl-\(\beta\)-l-arabinopyranoside were kindly provided by Prof. T. Kawasaki of Kyushu University.
and Nara. The comparative chromatogram of saponin constituents on TLC is partly different, but both saponins II and III were detected in all materials.

Experimental

All melting points were determined on a Yanagimoto micro-meltingpoint apparatus and are uncorrected. Optical rotations were measured with a JASCO-DIP-4 digital polarimeter using pyridine as the solvent. IR spectra were obtained with a JASCO-IRA-2 spectrometer. NMR spectra were taken at 100 MHz with a JEOL-JNM-MH-100 spectrometer in CDCl₃ solution and chemical shifts are given in δ (ppm) scale with tetramethylsilane as the internal standard. MS spectra were recorded on a JMS-OISG-2 mass spectrometer. GLC was run on a Shimazu GC-6-AM with flame ionization detector using glass column (2 m × 4 mm) packed with 15% 1,4-butanediol succinate on chromosorb W (100–120 mesh); column temperature 195°C, H₂ 40 ml/min, He 60 ml/min. TLC was performed on Kieselgel G (Merck) using the solvent system of a) CHCl₃-MeOH-H₂O (25: 11: 2) (for saponin). Detection was made by spraying 10% H₂SO₄ followed by heating. Column chromatography was carried out with Wakogel C-200 using the following eluent systems: b) CHCl₃-MeOH-H₂O (200: 70: 9), c) CHCl₃-MeOH-H₂O (200: 53: 4).

Isolation of Saponins —— The roots of Pulsatilla cornua Spreng. cultivated in Kyushu were extracted with hot MeOH. The MeOH extracts were treated as shown in Chart 1. n-BuOH soluble fraction was evaporated in vacuo and the MeOH solution was added into acetone to precipitate crude saponin showing two main spots (saponin II and III) on TLC using solvent system (a). Saponin II and III were separated by column chromatography over silica gel using eluent system (b). The mother liquor of precipitates (MeOH-acetone soluble part) was evaporated in vacuo and chromatographed over silica gel using eluent system (c) to give saponin I and IV.

Saponin I (I) —— Colorless needles (MeOH), mp 250–255° (dec.), [α]D +17.0°. IR νmax cm⁻¹: 3400 (OH), 1700 (COOH). Anal. Calc. for C₄₃H₃₄O₃·2H₂O: C, 62.57; H, 8.97. Found: C, 62.80; H, 9.25. I was identified with hedgeragenin 3-O-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside by comparison of its Rf value on TLC, IR, and NMR spectra with those of the authentic specimen and by a mixed fusion.

Saponin II (II) —— A white powder (precipitated from MeOH-acetone), mp 215–216° (dec.), [α]D –8.0°. IR νmax cm⁻¹: 3400 (OH), 1730 (COOR). Anal. Calc. for C₄₉H₃₆O₃₃·3H₂O: C, 55.56; H, 8.26. Found: C, 55.21; H, 8.51. II was identified with 3-O-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranosyl-hedgeragenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside by the same way as in the case of identification of saponin K18 isolated from Hedera rhombea and by direct comparison with an authentic sample (TLC, IR, and a mixed fusion).

Saponin III (III) —— A white powder, mp 210–222° (dec.), [α]D –9.0°, IR νmax cm⁻¹: 3350 (OH), 1720 (COOR). Anal. Calc. for C₄₀H₃₄O₃·5H₂O: C, 52.97; H, 7.93. Found: C, 52.71; H, 7.75. III was hydrolyzed by heating with 1 N H₂SO₄ in dioxy-hanane-H₂O (1:3) under reflux for 3 hr. The reaction mixture was diluted with water and the precipitates were recrystallized from MeOH to give colorless prisms, mp >300°, which were identified with hedgeragenin by direct comparison (IR, NMR). The filtrate of the hydrolysate was neutralized with BaCO₃ and concentrated to small volume and examined by TLC and PFC to show the presence of rhamnose, arabinose and glucose.

Permethylate (V) of III (1.48 g) was methylated by the Kuhn’s method (DMF 15 ml, Ag₂O 10 g, CH₃I 10 ml). The reaction mixture was diluted with water and extracted with CHCl₃. The CHCl₃ extract was evaporated and passed through a silica gel column (eluent, benzene-acetone = 85: 15) to give V (800 mg) as a white powder (precipitated from hexane), mp 117–210°. IR: no OH. NMR: 5.35 (1H, doublet, J = 8.4 Hz, ester glucose C₂-H). Anal. Calc. for C₄₉H₃₄O₃·3H₂O: C, 60.94; H, 8.75. Found: C, 61.23; H, 8.67.

Methanolation of V —— V (30 mg) was methanolized with 8% HCl in MeOH (3 ml) for 2 hr to yield 25-O-methyl-hedergenin, mp 216–217°, identified with an authentic sample, and a sugar mixture of S₄, S₅, S₆, S₇, S₈, S₉, S₁₀, S₁₁, S₁₂, S₁₃, S₁₄, S₁₅, and S₁₆ were identified by GLC with methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside (tₚ 9.32, 12.96 min), methyl 2,3,4-tri-O-methyl-α-L-rhamnopyranoside (tₚ 4.56 min), methyl 2,3,6-tri-O-methyl-D-glucopyranoside (tₚ 29.32, 38.48 min) and methyl 2,3,4-tri-O-methyl-D-glucopyranoside (tₚ 22.75, 30.84 min). S₅ was acetylated with Ac₂O-pyridine and the product was identified with authentic methyl 2,4-di-O-acetyl-3-O-methyl-α-L-arabinopyranoside (tₚ 43.36 min) by GLC.

LiAlH₄ Reduction of V —— V (400 mg) in tetrahydrofuran (THF) (20 ml) was treated with LiAlH₄ (350 mg). The reaction mixture was added with water and extracted successively with ether and CHCl₃. The ether extract was evaporated and chromatographed over silica gel (eluent, benzene-acetone = 9: 1) to give VI as a white powder (precipitated with hexane), mp 142–144°, IR: 3450 (OH), no >C=O, MS: m/e 189 (terminal permethylated rhamnose residue, tₚ 19), 219 (terminal permethylated ethylene residue). Anal. Calc. for C₄₀H₃₄O₃·3H₂O: C, 65.60; H, 9.44. Found: C, 65.94; H, 9.68. The CHCl₃ extract was submitted to column chromatography of silica gel (eluent, benzene-acetone = 4: 1) to give a colorless syrup (S₅, [α]D +32.0° (CHCl₃), MS: m/e 616 (M⁺), 189 (terminal permethylated rhamnose residue. S₅ was identified with 2,3,4-tri-O-methyl-α-L-rhamnopyranosyl(1→4)-2,3,6-tri-O-methyl-β-D-glucopyranosyl(1→6)-2,3,4-tri-O-methyl-D-glucopyranoside by direct comparison (IR, NMR, MS).
Methanolation of VI—VI (66 mg) was methanolyzed in the same way as for V to yield 23-O-methyl-
olean-12-en-3,23,28-triol, mp 207—208°, NMR: 3.35 (3H, singlet, C18—OCH3) which was identified with the
authentic sample (IR, NMR), and methylated sugars, S1, S2 and S3 (GLC). S1 was identified with methyl-3-
O-methyl-α-L-arabinopyranoside as in the case of methanolyzed product of V.
Hydrolysis of III with Alkali—III (240 mg) was heated on a water bath with 0.5 x KOH in 30% MeOH
for 30 min. The reaction mixture was diluted with water, neutralized with dil. HCl and evaporated in vacuo
to yield a white powder (IV) (precipitated from MeOH–AcOEt), mp 239—242° (dec.), [α]D +14.9°, which
was identical with IV obtained from MeOH–acetone soluble fraction of η-BuOH extract in Chart 1. Anal.
Calcd. for C47H70O13·2H2O: C, 59.48; H, 8.50. Found: C, 59.40; H, 8.78.
Mild Hydrolysis of IV—IV (150 mg) was heated on a water bath with 0.3% HCl in MeOH for 1 hr to
yield a mixture of hederagenin, 3-O-α-L-arabinopyranosyl-hederagenin9 and compound VII (10 mg). VII:
59.98; H, 8.84. Found: C, 59.71; H, 8.60. Methylation of VII by Kuhn’s method10 as for methylolation of
III gave VIII, a white powder, mp 84—87°, IR: 1720 (COOMe), NMR: 4.19 (1H, doublet, J = 6.5 Hz, arabinose
C1—H), 4.40 (1H, doublet, J = 8.2 Hz, glucose C1—H). Anal. Calcd. for C49H84O12: C, 66.98; H, 9.40. Found:
C, 66.69; H, 9.52.
Methanolation of VIII—VIII was methanolyzed in the same way as for V to give 23-O-methyl-hederagenin
methyl ester, mp 187°, and two kinds of methylated suaras, S1 and S2, S3 was defined as methyl 2,3-
di-O-methyl-L-arabinoside by identification of its acetate on GLC with authentic methyl-4-O-acetyl-2,3-
di-O-methyl-β-L-arabinopyranoside11 (tR 16.09 min).

Acknowledgement The authors are grateful to Prof. T. Kawasaki, Kyushu University for his kind
supply of the authentic samples of methyl pyranosides of 2,4-di-O-acetyl-3-O-methyl-L-arabinose and 4-O-
acetyl-2,3-di-O-methyl-L-arabinose. The authors also wish to thank Dr. J. Higashi of Kanebo Co., Ltd., for
identification of Pulsatilla species and Dr. N. Hiraga of Meiji College of Pharmacy for collecting
plant materials.