Chem. Pharm. Bull. 26(6)1666—1671(1978)

UDC 547.918.02.05 : 581.192

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

Mineo Shimizu,[2a] Ken-ichi Shingyouchi,[2b] Naokata Morita,[2a] Haruhisa Kizu, and Tsuyoshi Tomimori[2c]

Faculty of Pharmaceutical Sciences, Medical and Pharmaceutical University of Toyama[2a] and School of Pharmacy, Hokuriku University[2c]

(Received October 14, 1977)

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

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

Habitate	*Pulsatilla* spp.	Component
Japan	*Pulsatilla cernua* Spreng.	Oleanolic acid, hederagenin[3]
Korea	*P. Koreana* Nakai	Unknown
China	*P. chinensis* Regel	$C_{30}H_{48}O_4$[a]
	P. turczaninovii Krylov et Serg.	Unknown
	P. davurica Spreng.	Unknown
China and North America	*P. patens* Mill.	Unknown
Europe	*P. vulgaris* Mill.	Protoanemonin[b]
	P. pratensis Mill.	Ranunculin[c]
U.S.S.R	*P. nemorosa* Schrank	$C_{30}H_{48}O_4$[d]
	P. ucrainica (Ugrinsky) Wissjul	Ranunculin[e]
	P. montana Reichb.	Ranunculin[e]
	P. nigricans Stoerck ex DC	Hederagenin[f] ranunculin[g]

a) W.-Y. Huang, W.-K. Chen, Y.-L. Chon, and J.-H. Chu, *Hua Hsueh Hsueh Pao.*, **28**, 126 (1962) [*C.A.*, **59**, 1692 (1963)].
b) H. Baer, M. Halden, and B.C. Seegal, *J. Biol. Chem.*, **162**, 65 (1946).
c) S. Rolski and L. Przyborowski, *Dissertations Pharm.*, **13**, 349 (1961) [*C.A.*, **56**, 8839 (1962)]; U.G. Fil., *Farmatsevt. Zh.*, **17** (5), 47 (1962) [*C.A.*, **59**, 5493 (1963)].
d) A. Bienfait, *Bull. Ordre Pharmaciens*, **15** (42), 167 (1960) [*C.A.*, **61**, 4699 (1964)].
e) U.G. Fil., *Farmatsevt. Zh.*, **19** (1), 59 (1964) [*C.A.*, **61**, 8624 (1964)].
f) M.I. Kurilenko, *Farmatsevt. Zh.*, **23** (6), 75 (1968) [*C.A.*, **70**, 99583 (1969)].
g) M.I. Kurilenko, *Issledovaniya v Oblasti Farm. Zaporozh. Gosudarst. Farm. Inst.*, **1959**, 78 [*C.A.*, **55**, 10597 (1961)].

1) Presented at the 24th Annual Meeting of the Japanese Society of Pharmacognosy, Tokyo, Nov. 1977.
2) Location: *a) Sugitani, Toyama*; *b)* Present address; *Alpus Chemical Industries, Ltd., Takayama, Gifu*; *c) 3 Ho, Kanagawa-machi, Kanazawa.*
3) T. Kikuchi, M. Arimoto, and T. Toyoda, *Yakugaku Zasshi*, **88**, 1078 (1968).

by Munesada,[4] Fujita[5] and Higashi.[6] The distribution and the components of *Pulsatilla* species examined until now are shown in Table I.

With regard to 'Hakutoo' cultivated in Japan (Nara prefecture), it has been supposed to contain saponins,[3] 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 1.

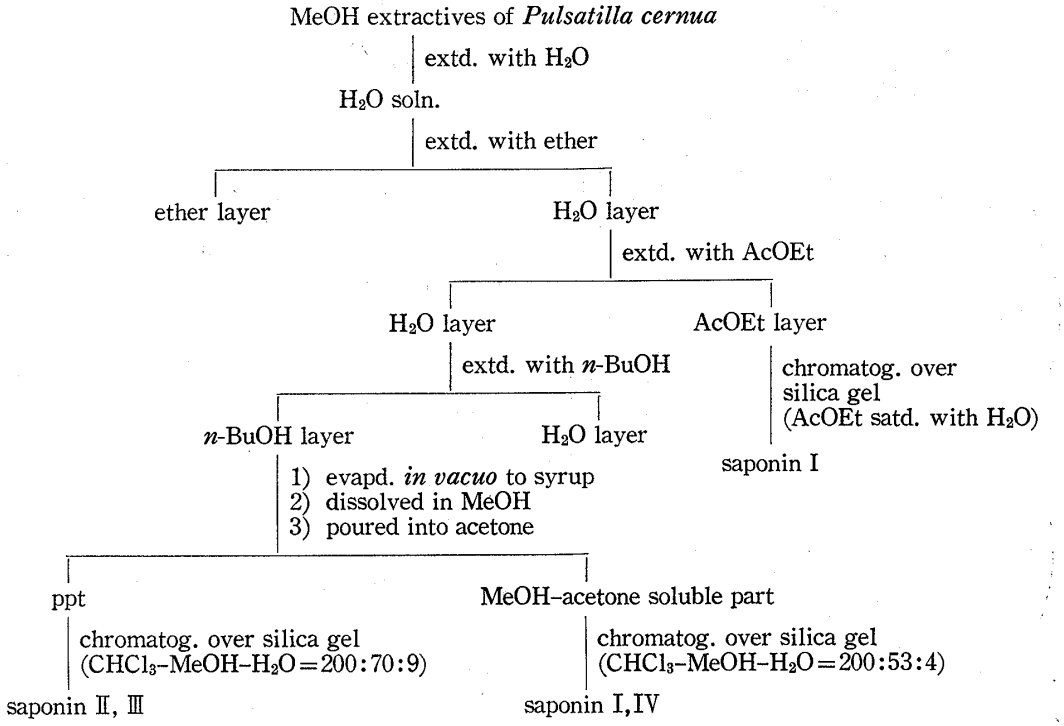

Chart 1

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 1). Saponin I was also isolated from ethyl acetate soluble fraction.

Saponin I[7] (1) mp 250—255° $[\alpha]_D$+17.0°, and saponin II[7] (II), mp 215—216°, $[\alpha]_D$−8.0°, were respectively identified with hederagenin 3-O-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside[8,9] 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[8,9] 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).
5) N. Fujita and Y. Yoshida, *Yakugaku Zasshi*, **57**, 390 (1937).
6) J. Higashi, *Syoyakugaku Zasshi*, **3**, 11 (1949).
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.
8) R. Higuchi and T. Kawasaki, *Chem. Pharm. Bull.* (Tokyo), **24**, 1021 (1976).
9) M. Shimizu, M. Arisawa, N. Morita, H. Kizu, and T. Tomimori, *Chem. Pharm. Bull.* (Tokyo), **26**, 655 (1978).

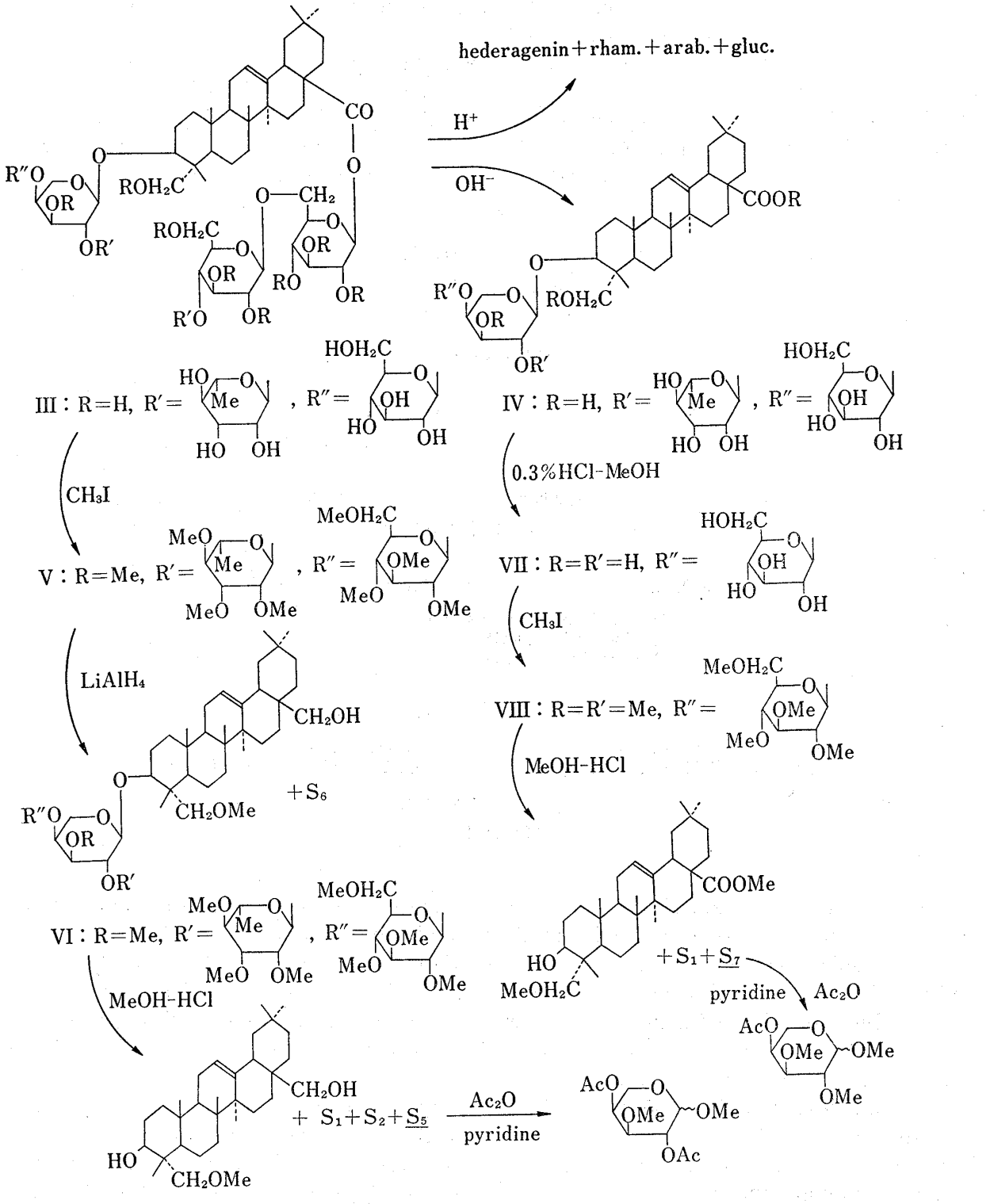

Chart 2

matography (TLC) and infrared (IR) spectra with those of the authentic specimens and by a mixed fusion.

Saponin III (III), mp 219—222°, $[\alpha]_D$—9.0°, furnished on acid hydrolysis hederagenin, rhamnose, arabinose and glucose. The exhaustively methylated product (V) of III by Kuhn's method,[10] mp 117—120°, $C_{83}H_{142}O_{31}$, was methanolyzed to afford 23-O-methyl-hederagenin, mp 216—217°, 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-α-L-arabinopyranoside.[11] The lithium aluminum hydride ($LiAlH_4$) reduction in tetrahydrofuran (THF) of V provided VI, mp 142—144°, which showed on a mass spectrum (MS) the fragment ions due to a terminal permethylated methylpentose (*m/e* 189)[12] and hexose (*m/e* 219)[12] residues, and a methylated saccharide which was identified with the authentic sample of 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-sorbitol (S_6)[8,9] by direct comparison [IR, nuclear magnetic resonance (NMR), MS]. The mode of linkage of esterglycosyl glucose residue was regarded as β on the basis of the NMR spectrum of V showing an anomeric proton signal of esterglycosyl glucose at 5.35 ppm as a doublet (J=8.4 Hz). S_6 was also provided from methylated saponin II through the same procedure. Methanolysis 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_{23}-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 239—242°, $[\alpha]_D$+14.9°, 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-α-L-arabinopyranoside[9] and VII, mp 260—261°, $[\alpha]_D$+58.3°. Methylation of VII by Kuhn's method[10] afforded VIII, mp 84—87°, which exhibited NMR signals for two anomeric protons (4.19 ppm, 1H, doublet, J=6.5 Hz, arabinose C_1-H; 4.40 ppm, 1H, doublet, J=8.2 Hz, glucose C_1-H). Methanolysis of VIII afforded 23-O-methyl-hederagenin methyl ester and two kinds of sugars, S_1 and methyl 2,3-di-O-methyl-L-arabinopyranoside (S_7) which was identified by GLC as its monoacetate with authentic methyl 4-O-acetyl-2,3-di-O-methyl-β-L-arabinopyranoside.[11] IV is thus considered to be rhamnoside of hederagenin 3-O-β-D-glucopyranosyl-(1→4)-α-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 methanolysis of VI. The α-configulation 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-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-L-arabinopyranosyl-hederagenin 28-O-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside[14] and hederagenin 3-O-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-α-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

10) R. Kuhn, *Angew. Chem.*, **67**, 32 (1955).
11) The authentic samples of methyl 2,4-di-O-acetyl-3-O-methyl-α-L-arabinopyranoside and methyl 4-O-acetyl-2,3-di-O-methyl-β-L-arabinopyranoside were kindly provided by Prof. T. Kawasaki of Kyushu University.
12) *a*) T. Komori, Y. Ida, Y. Muto, K. Miyahara, T. Nohara, and T. Kawasaki, *Biomedical Mass Spectrometry*, **2**, 65 (1975); *b*) H. Budzikiewicz, C. Djerassi, and K.H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," Vol. II, Holden-Day, Inc., San Francisco, 1964, p. 203.
13) Y. Kumekawa, H. Itokawa, and M. Fujita, *Chem. Pharm. Bull.* (Tokyo), **22**, 2299 (1974).
14) Saponin III corresponds to the structural isomer of saponin G isolated from Akebia quinata [R. Higuchi and T. Kawasaki, *Chem. Pharm. Bull.* (Tokyo), **20**, 2143 (1972)].

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 $CDCl_3$ 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 mmϕ) packed with 15% 1,4-butanediol succinate on chromosorb W (100—120 mesh); column temperature 195°, H_2 40 ml/min, He 60 ml/min. TLC was performed on Kieselgel G (Merck) using the solvent system of a) $CHCl_3$–MeOH–H_2O (25: 11: 2) (for saponin). Detection was made by spraying 10% H_2SO_4 followed by heating. Column chromatography was carried out with Wakogel C-200 using the following eluent systems: b) $CHCl_3$–MeOH–H_2O (200: 70: 9), c) $CHCl_3$–MeOH–H_2O (200: 53: 4).

Isolation of Saponins——The roots of *Pulsatilla cernua* 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.), $[\alpha]_D$+17.0°. IR ν_{max}^{KBr} cm^{-1}; 3400 (OH), 1700 (COOH). *Anal.* Calcd. for $C_{41}H_{66}O_{12}\cdot 2H_2O$: C, 62.57; H, 8.97. Found: C, 62.80; H, 9.25. I was identified with hederagenin 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–AcOEt), mp 215—216° (dec.), $[\alpha]_D$−8.0°, IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 1730 (COOR). *Anal.* Calcd. for $C_{59}H_{96}O_{26}\cdot 3H_2O$: 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-hederagenin 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 K_{12}[9] 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 219—222° (dec.), $[\alpha]_D$−9.0°, IR ν_{max}^{KBr} cm^{-1}: 3350 (OH), 1720 (COOR). *Anal.* Calcd. for $C_{65}H_{106}O_{31}\cdot 5H_2O$: C, 52.97; H, 7.93. Found: C, 52.71; H, 7.75. III was hydrolyzed by heating with 1 N H_2SO_4 in dioxane–H_2O (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 hederagenin by direct comparison (IR, NMR). The filtrate of the hydrolysate was neutralized with $BaCO_3$ and concentrated to small volume and examined by TLC and PPC to show the presence of rhamnose, arabinose and glucose.

Permethylate (V) of III——III (1.48 g) was methylated by the Kuhn's method (DMF 15 ml, Ag_2O 10 g, CH_3I 10 ml). The reaction mixture was diluted with water and extracted with $CHCl_3$. The $CHCl_3$ 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—120°. IR: no OH. NMR: 5.35 (1H, doublet, J=8.4 Hz, ester glucose C_1–H). *Anal.* Calcd. for $C_{83}H_{142}O_{31}$: C, 60.94; H, 8.75. Found: C, 61.23; H, 8.67.

Methanolysis of V——V (30 mg) was methanolyzed with 8% HCl in MeOH (3 ml) for 2 hr to yield 23-O-methyl-hederagenin, mp 216—217°, identified with an authentic sample, and a sugar mixture of S_1, S_2, S_3, S_4 and S_5. S_1, S_2, S_3 and S_4 were identified by GLC with methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside (t_R 9.52, 12.96 min), methyl 2,3,4-tri-O-methyl-α-L-rhamnopyranoside (t_R 4.56 min), methyl 2,3,6-tri-O-methyl-D-glucopyranoside (t_R 29.52, 38.48 min) and methyl 2,3,4-tri-O-methyl-D-glucopyranoside (t_R 22.75, 30.84 min). S_5 was acetylated with Ac_2O–pyridine and the product was identified with authentic methyl 2,4-di-O-acetyl-3-O-methyl-α-L-arabinopyranoside[11] (t_R 43.36 min) by GLC.

$LiAlH_4$ Reduction of V——V (400 mg) in tetrahydrofuran (THF) (20 ml) was treated with $LiAlH_4$ (330 mg). The reaction mixture was added with water and extracted successively with ether and $CHCl_3$. 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[12a]), 219 (terminal permethylated glucose residue[12]), *Anal.* Calcd. for $C_{56}H_{96}O_{16}$: C, 65.60; H, 9.44. Found: C, 65.94; H, 9.68. The $CHCl_3$ extract was submitted to column chromatography of silica gel (eluent, benzene–acetone=4: 1) to give a colorless syrup (S_6), $[\alpha]_D$−32.0° ($CHCl_3$), MS: *m/e* 616 (M^+), 189 (terminal permethylated rhamnose residue[12a]). S_6 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-sorbitol[8,9] by direct comparison (IR, NMR, MS).

Methanolysis 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,[13] mp 207—208°, NMR: 3.35 (3H, singlet, C_{23}–OCH_3) which was identified with the authentic sample (IR, NMR), and methylated sugars, S_1, S_2 and S_5 (GLC). S_5 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 N 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.), $[\alpha]_D$+14.9°, which was identical with IV obtained from MeOH–acetone soluble fraction of *n*-BuOH extract in Chart 1. *Anal.* Calcd. for $C_{47}H_{76}O_{17}\cdot 2H_2O$: 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-hederagenin[9] and compound VII (10 mg). VII: mp 260—261° (dec.), $[\alpha]_D$+58.3°, IR: 3400 (OH), 1690 (COOH). *Anal.* Calcd. for $C_{41}H_{66}O_{13}\cdot 3H_2O$: C, 59.98; H, 8.84. Found: C, 59.71; H, 8.60. Methylation of VII by Kuhn's method[10] as for methylation of III gave VIII, a white powder, mp 84—87°, IR: 1720 (COOMe), NMR: 4.19 (1H, doublet, J=6.5 Hz, arabinose C_1–H), 4.40 (1H, doublet J=8.2 Hz, glucose C_1–H). *Anal.* Calcd. for $C_{49}H_{82}O_{13}$: C, 66.98; H, 9.40. Found: C, 66.69; H, 9.52.

Methanolysis 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 suaars, S_1 and S_7 S_7 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-arabinopyranoside[11] (t_R 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.