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Studies on the Constituents of Leaves of *Citrus unshiu* MARCOV.

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Two new glycosides citroside A (**3**) and B (**4**), together with 2-phenylethyl β-D-glucopyranoside (**1**) and 2-phenylethyl D-rutinoside (**2**), were isolated from the methanol extract of leaves of *Citrus unshiu*, in addition to two known terpenoids, limonin (**5**) and friedelin (**6**). On the basis of spectral and chemical evidence the structures of the new glycosides were determined as (5-dehydroxy-grasshopper ketone-5-yl) 5-β-D-glucopyranoside (**3**) and a (5-dehydroxy-allenic ketodiol-5-yl) 5-β-D-glucopyranoside (**4**).

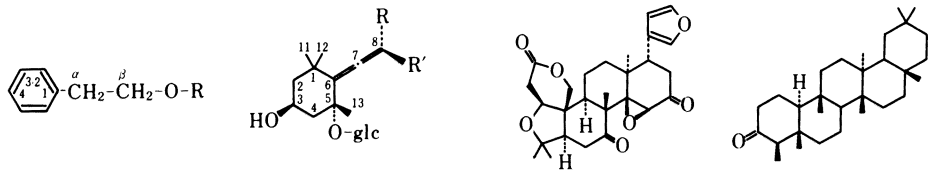
Keywords——*Citrus unshiu*; Rutaceae; citroside A; citroside B; 2-phenylethyl β-D-glucopyranoside; 2-phenylethyl D-rutinoside; limonin; friedelin

Citrus unshiu MARCOV (Rutaceae) is widely distributed in the southern part of Japan. Earlier investigations of the constituents of this plant dealt mainly with the fruit's pericarp (Aurantii Nobilis Pericarpium).[1] This paper describes the isolation and structure elucidation of two new ionone-type glycosides named citrosides A (**3**) and B (**4**), as well as the phenylethyl glycosides 2-phenylethyl β-D-glucopyranoside (**1**) and 2-phenylethyl D-rutinoside (**2**), and the triterpenoids limonin (**5**) and friedelin (**6**) from the leaves of this plant.

2-Phenylethyl β-D-glucopyranoside (**1**), $C_{14}H_{20}O_6 \cdot 5/4H_2O$, $[\alpha]_D$ −28.8° was isolated as a colorless amorphous powder. The infrared (IR) spectrum showed the presence of hydroxyl groups (3500 cm^{-1}). The proton nuclear magnetic resonance (^{1}H-NMR) spectrum exhibited a triplet signal due to benzylic methylene protons at δ 3.00 (2H, J=7 Hz), a doublet signal due to an anomeric proton at δ 4.82 (1H, J=7 Hz) and a singlet signal due to aromatic protons at δ 7.25 (5H, s). The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum exhibited two methylene carbon signals at δ 36.6 and 71.6. The acid hydrolysis of **1** afforded glucose as the sugar moiety and phenylethanol as the aglycone. These data led us to conclude the structure of this compound to be **1**. This is the first report of the isolation of **1** from a natural origin.

2-Phenylethyl D-rutinoside (**2**), $C_{20}H_{30}O_{10} \cdot H_2O$, $[\alpha]_D$ −101.2°, was obtained as a colorless amorphous powder. The ^{1}H-NMR spectrum exhibited a triplet methylene proton at δ 3.00 (2H, J=7 Hz), two doublet signals at δ 1.60 (3H, J=5 Hz) due to C-6 protons of rhamnose, δ 4.81 (1H, J=7 Hz) due to an anomeric proton and an aromatic proton signal at δ 7.25 (5H, s). On acid hydrolysis, **2** afforded D-glucose and L-rhamnose as sugar moieties and phenylethanol as the aglycone. In the ^{13}C-NMR spectrum of **2**, the signal due to C-6 of glucose was shifted downfield by 5.4 ppm and that of C-5 of glucose was shifted upfield by 1.3 ppm in comparison with those of usual glucopyranosides. Thus, rhamnose was attached to C-6 of glucose. From these data, the structure of compound **2** was decided to be **2**.

Compound **3** (citroside A), $[\alpha]_D$ −95.7°, $C_{19}H_{30}O_8 \cdot 1/2H_2O$, was obtained as an amorphous powder. The ultraviolet (UV) spectrum showed an absorption maximum at 232 nm (log ε 4.12) and the IR spectrum showed the presence of hydroxyl groups (3400 cm^{-1}),



1 : R=glucose
2 : R=rutinose

5

6

Chart 1

TABLE I. ^{13}C-NMR Chemical Shifts

	1	2		3	4
Aglycone moiety			Aglycone moiety		
1	139.3	139.4	1	36.4	36.2
2	128.6	128.6	2	50.1	50.1
3	129.3	129.4	3	62.5	62.4
4	126.4	126.4	4	47.5	46.8
			5	78.1	78.6
α	36.6	36.6	6	118.7	118.8
β	71.6	71.7	7	197.6	199.0
			8	100.8	100.9
Sugar moiety			9	211.4	211.2
1′	104.6	104.5	10	26.5[a]	27.1[a]
2′	75.0	74.9	11	27.0[a]	27.6[a]
3′	78.3	78.4	12	29.7[a]	29.6[a]
4′	70.5	70.5	13	32.3[a]	32.5[a]
5′	78.3	77.0			
6′	62.8	68.2	Sugar moiety		
1″		102.3	1′	98.4	98.4
2″		72.1[a]	2′	75.1	75.2
3″		72.7[a]	3′	79.0	79.1
4″		74.0	4′	71.7	71.7
5″		69.7	5′	78.0	78.0
6″		18.6	6′	62.8	62.8

Run at 22.5 MHz in pyridine-d_5 solution. *a*) Interchangeable in each column.

an allenic structure (1945 cm^{-1}) and a conjugated ketone group (1670 cm^{-1}). The ^{1}H-NMR spectrum exhibited four singlet methyl signals at δ 1.17 (3H), 1.62 (6H), 2.20 (3H), the last one being due to a methyl ketone, an anomeric proton signal at δ 5.04 (1H, d, J=7 Hz) and an olefinic proton signal at δ 6.00 (1H, s). The acid hydrolysis of **3** afforded glucose as the sugar moiety and the aglycone **3a**. In the ^{1}H-NMR spectrum of **3a**, a carbinol proton signal was obserbed at δ 4.36 (m, $W_{1/2}$=23 Hz). From these data, **3a** was assumed to be grasshopper ketone, previously isolated from *Romalea microptera*.[2] The identity of **3a** was established by direct comparison with an authentic sample [high-performance liquid chromatography (HPLC), mp, ^{1}H-NMR].[3] In the ^{13}C-NMR spectrum of **3a**, two carbinol carbon signals were observed at δ 63.8 (d) and 72.3 (s). The former was shifted upfield by only 1.3 ppm in the ^{13}C-NMR spectrum of **3**, but the latter was shifted downfield by 5.8 ppm. Therefore, the glucosidation position was decided to be at C-5. These results led us to conclude the structure of citroside A to be **3**.

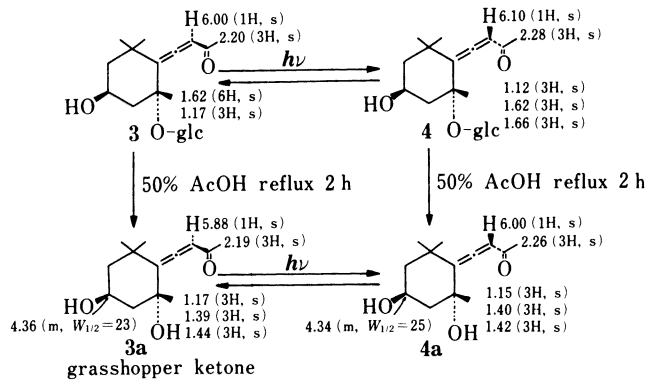


Chart 2

TABLE II. ^{1}H-NMR Chemical Shifts and Coupling Constants

Proton No.	3	3a	4	4a
3	*a)*	4.36 (1H, m, $W_{1/2}=23$ Hz)	*a)*	4.34 (1H, m, $W_{1/2}=25$ Hz)
8	6.00 (1H, s)	5.88 (1H, s)	6.10 (1H, s)	6.00 (1H, s)
10	2.20 (3H, s)	2.19 (3H, s)	2.28 (3H, s)	2.26 (3H, s)
11	1.17 (3H, s)	1.17 (3H, s)	1.12 (3H, s)	1.15 (3H, s)
12	1.62 (6H, s)	1.39 (3H, s)	1.62 (3H, s)	1.40 (3H, s)
13		1.44 (3H, s)	1.66 (3H, s)	1.42 (3H, s)
Anomeric	5.04 (1H, d, $J=7$ Hz)		5.16 (1H, d, $J=7$ Hz)	

Run at 89.55 MHz in pyridine-d_5 solution. *a)* Overlapped with H_2O.

Compound **4** (citroside B), $[\alpha]_D$ −48.2°, $C_{19}H_{30}O_8$ was obtained as a colorless amorphous powder. The IR spectrum showed allenic structure (1945 cm^{-1}) and a conjugated ketone group (1675 cm^{-1}). The ^{1}H-NMR spectrum exhibited four singlet methyl signals at δ 1.12 (3H), 1.62 (3H), 1.66 (3H), 2.28 (3H), the last one being due to a methyl ketone, an anomeric proton signal at δ 5.16 (1H, d, $J=7$ Hz) and an olefinic proton signal at δ 6.10 (1H, s). On comparison of the ^{1}H-NMR spectra of **3** and **4**, the signal of an olefinic proton signal at C-8 was shifted downfield by 0.1 ppm and acetyl methyl signal at C-10 was shifted downfield by *ca.* 0.1 ppm. Acid hydrolysis of **4** with 50% AcOH gave **4a** as the aglycone moiety and glucose as the sugar moiety.[4] Citrosides A and B were indicated to be epimeric at the C-8 position. It was reported by Isoe *et al.* that grasshopper ketone in EtOH could be inverted at C-8 by irradiation with a high-pressure mercury lamp.[5] Compound **3a** gave a product identical with **4a** on irradiation with a high-pressure mercury lamp. Compound **3**, the glucoside of **3a**, gave a product identical with **4** (^{1}H-NMR, HPLC) on irradiation with a high-pressure mercury lamp. These results confirmed that **3** and **4** are C-8 epimers.

Limonin (**5**) was identified by direct comparison (thin layer chromatography (TLC), mp, ^{1}H-NMR and ^{13}C-NMR spectra) with an authentic sample.

Friedelin (**6**) was identified by comparison of various data (mp, ^{1}H-NMR) with reported values.[6]

Experimental

Melting points were taken on a Yanaco MP-500 micromelting point apparatus and are uncorrected. IR spectra

were run on a JASCO A-201 IR spectrometer and UV spectra on a Shimadzu UV-360 recording spectrometer. Mass spectra (MS) were measured on a JEOL LMS-D 100 mass spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL FX-90Q NMR spectrometer (89.55 and 22.5 MHz, respectively). Chemical shifts are given on the δ scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet; br, broad). Gas chromatography (GC) was done on a Hitachi K53 gas chromatograph. HPLC was conducted on a JASCO model 880 PU with UVIDEC 100V systems.

Isolation——Fresh leaves of *C. unshiu* (10 kg) collected in May 1986, in Shizuoka, Japan, were extracted twice with hot methanol. The water-soluble fraction of the extract was passed through a DIAION HP-20 column and the absorbed material was eluted with 50% MeOH, 75% MeOH and MeOH successively. The 50% MeOH eluate (70 g) was chromatographed on a TOYOPEARL HW-40 column with water. After repeated chromatography of the eluate on silica gel with a chloroform–methanol system, four glycosides were isolated. The ethyl acetate-soluble fraction of the methanol extract was repeatedly chromatographed on a silica gel column with a hexane–ethyl acetate and benzene–ethyl acetate to gave two terpenoids.

Phenylethyl β-D-Glucoside (1)——Colorless amorphous powder (5 mg), $[\alpha]_D^{23}$ $-28.8°$ ($c=0.40$, methanol) *Anal.* Calcd for $C_{14}H_{20}O_6$: C, 54.89; H, 6.86. Found: C, 54.80; H, 6.57. IR ν_{max}^{KBr} cm^{-1}: 3400, 2930, 1500, 1450, 1375, 1050. ^{1}H-NMR ($CDCl_3$) δ: 3.00 (2H, t, $J=7$, H-α), 4.82 (1H, d, $J=7$, H-1′), 7.25 (5H, s, H-2, H-3, H-4, H-5, H-6), ^{13}C-NMR: Table I.

Phenylethyl D-Rutinoside (2)——Colorless amorphous powder (20 mg), $[\alpha]_D^{23}$ $-101.2°$ ($c=0.10$, methanol) *Anal.* Calcd for $C_{20}H_{30}O_{10}\cdot H_2O$: C, 53.51; H, 6.92. Found: C, 53.56; H, 6.74. IR ν_{max}^{KBr} cm^{-1}: 3400, 2930, 1500, 1450, 1375, 1050. ^{1}H-NMR ($CDCl_3$) δ: 1.60 (3H, d, $J=5$, H-6″), 3.00 (2H, t, $J=7$, H-α), 4.81 (1H, d, $J=7$, H-1′), 7.25 (5H, s, H-2, H-3, H-4, H-5, H-6). ^{13}C-NMR: Table I.

Citroside A (3)——Colorless amorphous powder (200 mg), $[\alpha]_D^{23}$ $-95.7°$ ($c=0.44$, methanol) *Anal.* Calcd for $C_{19}H_{30}O_8\cdot 1/2H_2O$: C, 57.71; H, 7.90. Found: C, 57.58; H, 7.73. IR ν_{max}^{KBr} cm^{-1}: 3400, 1945, 1670, 1360, 1240. UV λ_{max}^{MeOH} nm (log ε): 230 (4.12). ^{1}H-NMR: Table II and ^{13}C-NMR: Table I.

Citroside B (4)——Colorless amorphous powder (10 mg), $[\alpha]_D^{23}$ $-48.2°$ ($c=0.28$, methanol). *Anal.* Calcd for $C_{19}H_{30}O_8\cdot 1/2H_2O$: C, 57.71; H, 7.90. Found: C, 57.85; H, 7.96. IR ν_{max}^{KBr} cm^{-1}: 3400, 2950, 1945, 1675, 1380, 1070. UV λ_{max}^{MeOH} nm (log δ): 230 (4.17). ^{1}H-NMR: Table II and ^{13}C-NMR: Table I.

Limonin (5)——Colorless needles (85 mg), mp 271—275 °C (AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 1.07 (3H, s, H-30), 1.18 (6H, s, H-18, 29), 1.29 (3H, s, H-28), 4.04 (2H, br s, H-1, 15), 4.45 (1H, d, $J=13$, H-19), 4.79 (1H, d, $J=13$, H-19), 5.46 (1H, s, H-17), 6.36 (1H, m, H-20), 7.40 (1H, s, H-23), 7.43 (1H, s, H-21). ^{13}C-NMR (pyridine-d_5) δ: 17.7 (C-30), 18.7 (C-11), 20.3 (C-18), 21.7 (C-29), 30.0 (C-28), 30.2 (C-12), 36.4 (C-2), 36.8 (C-6), 38.6 (C-13), 46.4 (C-10), 48.1 (C-9), 51.5 (C-8), 54.8 (C-15), 60.0 (C-5), 65.7 (C-19), 67.0 (C-14), 78.2 (C-17), 79.8 (C-1), 80.3 (C-4), 110.5 (C-22), 121.2 (C-20), 141.8 (C-21), 143.6 (C-23), 167.5 (C-16), 170.1 (C-3), 207.7 (C-7).

Friedelin (6)——Colorless needles (40 mg). mp 261—262 °C (AcOEt). ^{1}H-NMR ($CDCl_3$) δ: 0.76 (3H, s), 0.88 (3H, d, $J=7.2$), 0.87 (3H, s), 0.96 (3H, s), 1.02 (6H, s), 1.06 (3H, s), 1.20 (3H, s).

Acid Hydrolysis of Phenylethyl β-D-Glucoside (1) and Phenylethyl D-Rutinoside (2)——A solution of glycoside (5 mg) in 10% H_2SO_4 (1—2 drops) was well stirred in a boiling water bath for an hour. The reaction mixture was diluted with water and extracted with ethyl acetate 3 times. Ethyl acetate was evaporated off and the residue was purified by HPLC (YMC-ODS, 4.6 × 250 mm; H_2O–CH_3CN (85 : 15)) to give the aglycone as a colorless oil (2 mg). ^{13}C-NMR (C_5D_5N) δ: 40.5 (C-α), 63.6 (C-β), 126.6 (C-4), 128.8 (C-2), 129.8 (C-3), 140.4 (C-1). The water-soluble fraction was passed through an Amberlite IRA-45 column and the eluate was concentrated to give a residue, which was reduced with sodium borohydride (*ca.* 2 mg) for 1h at room temperature. The reaction mixture was passed through an Amberlite IR-120 column and the eluate was concentrated to dryness. Boric acid was removed by co-distillation with methanol and the residue was acetylated with acetic anhydride and pyridine (1 drop each) at room temperature overnight. The reagents were evaporated off *in vacuo*. From **1**, glucityl acetate was detected by GC. From **2**, glucityl acetate and rhamnityl acetate were detected by GC. Conditions: column, SUPELCO capillary column SPB-35; carrier gas, N_2 10 ml/min; column temperature, 170 °C; t_R 10.4 min (rhamnityl acetate); column temperature, 200 °C; t_R 10.8 min (glucityl acetate).

Acid Hydrolysis of Citrosides A (3) and B (4)——A solution of citroside A (5 mg) in 50% AcOH (2—3 drops) was heated at 70 °C for 2 h, then the solvent was evaporated off, and the residue was partitionated in water–ethyl acetate. From the water-soluble fraction, glucose was detected as the glucityl acetate by GC. The ethyl acetate fraction was worked up in the same way as described for **1** to give the aglycone (**3a**) (2 mg) as colorless needles (acetone–benzene), mp 134–136 °C.[3] ^{1}H-NMR: Table II. From **4** (5 mg), **4a** (1 mg) was obtained in the same manner as colorless needles (ethyl acetate–hexane), mp 150—152 °C.[8] ^{1}H-NMR: Table II. GC conditions: column; SUPELCO capillary column SPB-35; carrier gas, N_2; column temperature, 200 °C; t_R 10.8 min (glucityl acetate).

Photo-epimerization of Citrosides A (3) and B (4)——An ethanolic solution (2 ml) of citroside B (**4**) (5 mg) was irradiated with a high- pressure mercury lamp (400 W) at room temperature through a Pyrex glass filter under a 1 : 1 nitrogen–air mixture for 30 min. The reaction mixture was purified by HPLC to give citroside A (2.5 mg) and B (1 mg). ^{1}H-NMR: Table II. From citroside A (**3**), citroside B (**4**) was obtained in the same manner. ^{1}H-NMR: Table II. HPLC conditions: column, YMC-ODS (4.6 mm × 25 cm); H_2O–CH_3CN (85 : 15); flow rate, 1 ml/min; t_R 11 min

(citroside A), 16 min (citroside B).

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