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## Note

# Urakunoside, a New Tetraglycosyl Kaempferol from Petals of the Wabisuke Camellia cv. Tarokaja

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A new tetraglycosyl flavonol, 3-*O*-[2-*O*-xylosyl-6-*O*-(3-*O*-glucosyl-rhamnosyl) glucosyl] kaempferol was isolated from pale purplish-pink petals of Wabisuke camellia cv. Tarokaja with three known flavonols. It was named urakunoside after the species name of Tarokaja, *Camellia uraku*. Urakunoside was a major flavonol component in the Tarokaja petals, but was not detected in petals of Tarokaja's presumed ancestor species.

**Key words:** *Camellia uraku*; kaempferol tetraglycoside; Tarokaja; urakunoside; Wabisuke camellia

Wabisuke camellias are a group of horticultural cultivars that have been developed and appreciated in Japan. Tarokaja is one of the oldest Wabisuke cultivars, and is thought to be the origin of many other Wabisuke camellia cultivars.[1,2] Kitamura assigned Tarokaja to the species *Camellia uraku* in 1952.[3] Various morphological and physiological characteristics of Tarokaja have raised horticultural interest and prompted us to study the characteristics of the chemical components. In this study, we analyzed the major flavonoid components of Tarokaja petals.

Fresh petals were collected from a tree grown at National Institute of Floricultural Science, frozen in liquid nitrogen, and stored at $-80\,°C$. They were extracted twice with 50% aqueous (aq.) AcOH (2 mL/g fresh weight), and the combined extract was analyzed by high performance liquid chromatography (HPLC). HPLC analysis was conducted using an Inertsil ODS-2 column (4.6 mm $\varphi \times 250$ mm, GL Sciences, Tokyo, Japan), eluted with a linear gradient of 20 to 100% solvent B (1.5% $H_3PO_4$, 20% AcOH, 25% MeCN, and 53.5% $H_2O$, v/v) in solvent A (1.5% aq. $H_3PO_4$) for 40 min at $40\,°C$ at a flow rate of 0.8 mL/min. Chromatograms were obtained by detection at 360 nm using an HP1100 system with a photodiode array detector (220–600 nm, Agilent Technologies, CA, USA). Four major peaks of **1**, **2**, **3**, and **4** were detected at 18.8, 22.3, 23.7, and 27.9 min respectively, and all the compounds were identified as flavonols by typical spectra, with two absorption maxima at 266 and 348 nm (**1** and **2**), 256 and

348 nm (**3**), and 264 and 344 nm (**4**). The contents of the petals were (**1**) $0.27 \pm 0.00$, (**2**) $0.14 \pm 0.00$, (**3**) $0.24 \pm 0.01$, and (**4**) $0.09 \pm 0.00\,\mu$mol/g fresh weight (average $\pm$ SE for five flowers) as quercetin 3-rutinoside equivalent based on the absorbance at 360 nm on HPLC.

Large-scale extraction and purification was carried out. Petals of Tarokaja (550 g) were extracted with 2,200 mL of 50% aq. AcOH and 2,200 mL of 10% aq. AcOH, successively. These extracts were diluted to 5% AcOH concentration and applied to an Amberlite XAD-7 column. After the column was washed with 5% aq. AcOH, the flavonols were eluted with 5% AcOH–MeOH. The eluate was evaporated to dryness and the residue was dissolved in an adequate amount of 5% AcOH–EtOH. The solution was added to cellulose powder (about 25 g) and the supernatant was obtained. This procedure was performed repeatedly and the collected supernatant was evaporated to dryness. The residue (13.6 g) was dissolved in 1% aq. AcOH and chromatographed using an ODS LC column (5.2 cm $\varphi \times 20$ cm) and stepwise elutions (1 liter each) of 1% aq. AcOH and 1% AcOH–MeOH at ratios of 100:0, 98:2, 95:5, 90:10, 85:15, 80:20, 70:30, 60:40, and 50:50. A crude sample of **1** was eluted in the 70:30 fraction and those of **2**, **3**, and **4** were eluted in the 60:40 fractions. These crude fractions were further purified by preparative HPLC using a 10 mm $\varphi \times 250$ mm Senshu Pak ODS-4253-D column (Senshu Scientific Co., Tokyo, Japan) at a flow rate of 3.0 mL/min, a temperature of $40\,°C$, and detection at 360 nm. Crude fraction including **1** was purified using solvent C (5% aq. AcOH) and solvent D (5% AcOH, 50% MeOH, and 45% $H_2O$, v/v) at a ratio of 45:55, followed by solvent C and solvent E (5% AcOH, 50% MeCN, and 45% $H_2O$, v/v) at a ratio of 78:22. The result was a pale yellow mass (35 mg) of purified compound **1**. Crude fraction including **2** and **3** was purified using solvents C and D at a ratio of 32.5:67.5, and the purified compounds were a pale brown mass of **2** (15 mg) and a brownish-yellow mass of **3** (19 mg). Crude fraction including **4** was purified using solvents C and D at a ratio of 20:80, and a purified

**Table 1.** UV Spectral Data for Compounds **1** and **2**

| | | | $\lambda_{max}$ (nm) | | | |
|---|---|---|---|---|---|---|
| | MeOH | +NaOMe | +AlCl₃ | +AlCl₃/HCl | +NaOAc | +NaOAc/H₃BO₃ |
| 1 | 266, 348 | 275, 323, 397 (inc.$^z$) | 274, 304, 352, 394 | 275, 302, 350, 393 | 274, 308, 384 | 267, 352 |
| 2 | 266, 346 | 275, 324, 398 (inc.) | 274, 305, 354, 390 | 275, 303, 349, 390 | 274, 307, 384 | 266, 353 |

$^z$inc., increase in intensity relative to the spectrum in MeOH

greenish-yellow mass of **4** (9 mg) was obtained. The structures of **3** and **4** were identified as quercetin 3-rhamnoside and kaempferol 3-rhamnoside respectively based on $^1$H-NMR and ESI-MS spectra (data not shown).

The structures of **1** and **2** were determined by various instrumental analyses and degradation reactions. High-resolution FAB-MS measurements (HCl-glycerol matrix, JMS-700, JEOL, Tokyo, Japan) gave deprotonated molecule peaks at $m/z = 887.2464$ for **1** (calcd. for $C_{38}H_{47}O_{24}$: 887.2457) and $m/z = 755.2032$ for **2** (calcd. for $C_{33}H_{39}O_{20}$: 755.2035). The acid hydrolysis of **1** and **2** at $100\,°C$ for 40 min in a mixture of MeOH and 4 N HCl at a ratio of 1:5 (v/v) gave kaempferol (detected by HPLC analysis). In addition, the hydrolysates of **1** and **2** were analyzed by cellulose TLC ($n$-BuOH:toluene:pyridine:H₂O = 5:1:3:3, v/v). Glucose, rhamnose, and xylose were detected in the hydrolysate of **1**, and glucose and rhamnose were detected in that of **2**. The substitution patterns for kaempferol in **1** and **2** were predicted by UV spectral analysis in MeOH with the addition of NaOMe, AlCl₃, HCl, NaOAc, and H₃BO₃ (Table 1).[4] The results indicated that **1** and **2** have the same substitution pattern of free 5-, 7-, and 4′-hydroxyl and substituted 3-hydroxyl groups as the kaempferol 3-glycosides. Hence it was concluded that **1** was composed of kaempferol with two glucosyl, one rhamnosyl, and one xylosyl residue at the 3-hydroxyl group, and **2** was composed of kaempferol with two glucosyl and one rhamnosyl residue at the 3-hydroxyl group.

The linkage patterns of the sugar residues in **1** and **2** were determined using various 1D and 2D NMR techniques, including $^1$H-NMR, $^{13}$C-NMR, HOHAHA, COSY, HMQC, and HMBC (in DMSO-$d_6$ or CD₃OD at $25\,°C$, JEOL ECA-500, $^1$H: 500 MHz, $^{13}$C: 125 MHz). The signal assignments for the $^1$H- and $^{13}$C-NMR spectra are shown in Table 2.

For compound **2**, the signals at $\delta$ 7.96 (2H, d, $J = 8.9$ Hz), 6.84 (2H, d, $J = 8.9$ Hz), 6.37 (1H, d, $J = 1.5$ Hz), and 6.17 (1H, d, $J = 1.5$ Hz) in the $^1$H-NMR spectrum indicated a kaempferol skeleton. Three anomeric protons at $\delta$ 5.23 (H-1, glucose-1), 4.38 (H-1, rhamnose), and 4.24 (H-1, glucose-2), along with the assignment of sugar signals by 1D-HOHAHA measurements by irradiation at the anomeric protons and COSY data confirmed the presence of the three sugar residues. HMBC correlations were observed between the signals at 5.23 ppm (H-1, glucose-1) and 133.9 ppm (C-3, kaempferol); 4.38 ppm (H-1, rhamnose) and 68.0 ppm (C-6, glucose-1); and 4.24 ppm (H-1, glucose-2) and 82.2 ppm (C-3, rhamnose). The $^1$H-NMR coupling constants of the anomeric protons indicated that the linkages of glucose-1 ($J = 7.5$) and glucose-2 ($J = 7.5$) were $\beta$-conjugation and that of rhamnose (singlet peak) was $\alpha$-conjugation. Thus the attachment positions and

**Table 2.** $^1$H and $^{13}$C NMR Spectral Data for Compounds **1** and **2** in DMSO-$d_6$ ($^1$H: 500 MHz, $^{13}$C: 125 MHz, 25 °C)

| | **1** | | **2** | |
|---|---|---|---|---|
| | C | H | C | H |
| **Kaempferol** | | | | |
| 2 | 156.5 | | 157.1$^a$ | |
| 3 | 133.4 | | 133.9 | |
| 4 | 177.9 | | 177.9 | |
| 5 | 161.7 | | 161.6 | |
| 6 | 99.2 | 6.15 (d, 2.0) | 99.2 | 6.17 (d, 1.5) |
| 7 | 164.5 | | 164.5 | |
| 8 | 94.2 | 6.35 (d, 2.0) | 94.3 | 6.37 (d, 1.5) |
| 9 | 157.0 | | 157.3$^a$ | |
| 10 | 104.5 | | 104.5 | |
| 1′ | 121.4 | | 121.4 | |
| 2′6′ | 131.5 | 7.98 (d, 8.9) | 131.4 | 7.96 (d, 8.9) |
| 3′5′ | 115.7 | 6.86 (d, 8.9) | 115.6 | 6.84 (d, 8.9) |
| 4′ | 160.4 | | 160.4 | |
| **Glucose-1** | | | | |
| 1 | 98.9 | 5.51 (d, 7.5) | 102.2 | 5.23 (d, 7.5) |
| 2 | 82.0 | 3.41 m | 74.7 | 3.16 m |
| 3 | 77.2 | 3.40 m | 76.9$^b$ | 3.16 m |
| 4 | 69.9 | 3.09 (t, 8.8) | 70.5 | 2.99 (t, 8.9) |
| 5 | 76.1 | 3.24 m | 76.1 | 3.25 m |
| 6 | 66.8 | 3.59 (brd, 11.5) | 68.0 | 3.65 (brd, 11.5) |
| | | 3.32 (dd, 11.5, 5.9) | | 3.32 (dd, 11.5, 5.9) |
| **Rhamnose** | | | | |
| 1 | 101.0 | 4.36 (s) | 101.4 | 4.38 (s) |
| 2 | 69.7 | 3.65 (bs) | 69.8 | 3.66 (bs) |
| 3 | 82.3 | 3.25 m | 82.2 | 3.29 m |
| 4 | 71.2 | 3.24 m | 71.2 | 3.24 m |
| 5 | 68.1 | 3.24 m | 68.2 | 3.24 m |
| 6 | 18.0 | 0.86 (d, 6.0) | 18.1 | 0.91 (d, 6.0) |
| **Glucose-2** | | | | |
| 1 | 104.8 | 4.22 (d, 7.5) | 104.9 | 4.24 (d, 7.5) |
| 2 | 74.6 | 2.99 (brt, 8.8) | 74.5 | 2.99 (brt, 8.9) |
| 3 | 76.5 | 3.17 (t, 8.8) | 76.5$^b$ | 3.15 (t, 8.9) |
| 4 | 70.1 | 3.04 m | 70.0 | 3.05 m |
| 5 | 76.9 | 3.05 m | 76.9 | 3.06 m |
| 6 | 61.3 | 3.54 (dd, 11.5, 1.5) | 61.2 | 3.54 (brd, 11.5) |
| | | 3.41 (dd, 11.5, 4.0) | | 3.41 (dd, 11.5, 4.5) |
| **Xylose** | | | | |
| 1 | 104.8 | 4.55 (d, 7.5) | | |
| 2 | 74.2 | 3.03 (brt, 8.8) | | |
| 3 | 76.5 | 3.10 (t, 8.8) | | |
| 4 | 69.9 | 3.25 (td, 8.8, 5.0) | | |
| 5 | 66.1 | 3.69 (dd, 11.2, 5.0) | | |
| | | 3.01 (dd, 11.2, 8.8) | | |

$^{a,b}$Assignments may be interchanged in each column.

the stereochemistry of these sugars were determined and the structure of **2** was identified as 3-O-[6-O-(3-O-$\beta$-glucopyranosyl-$\alpha$-rhamnopyranosyl)-$\beta$-glucopyranosyl] kaempferol (Fig. 1). This triglycosyl kaempferol has also been identified in black tea, which is made from processed leaves of *C. sinensis*.[5]

The $^1$H-NMR spectrum of compound **1** was very similar to that of **2** except for additional signals due to the xylosyl residue and downfield shifts for H-1, H-2,
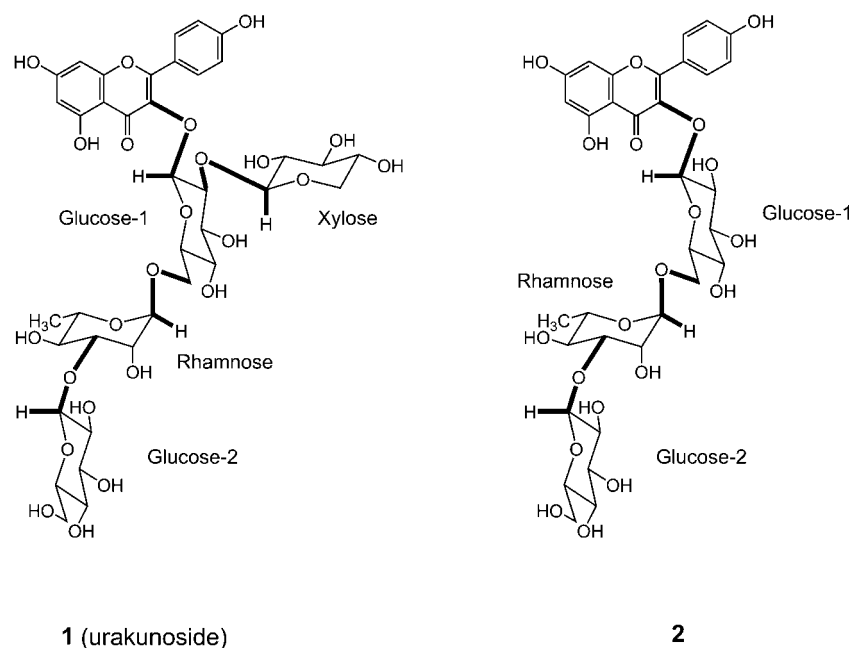
**1 (urakunoside)**                                          **2**

**Fig. 1.** Structures of Compounds **1** and **2** and Their HMBC Correlations (bold lines).
Correlations were observed in DMSO-$d_6$, except for the correlation between glucose-1 and kaempferol in **1**, which was detected in CD$_3$OD.

H-3, and C-2 and an upfield shift for C-1 of glucose-1 (Table 2). Using the same procedures as those used for **2**, the presence of the kaempferol skeleton and the four sugar residues (glucose-1, rhamnose, glucose-2, and xylose) was confirmed. The attachment sites for the rhamnose, glucose-2, and xylose residues were determined by the existence of HMBC correlations between the signals at 4.36 ppm (H-1, rhamnose) and 66.8 ppm (C-6, glucose-1); 4.22 ppm (H-1, glucose-2) and 82.3 ppm (C-3, rhamnose); and 4.55 ppm (H-1, xylose) and 82.0 ppm (C-2, glucose-1) (Fig. 1). Because no correlation between glucose-1 and kaempferol was observed in DMSO-$d_6$, the HMBC measurements were carried out in CD$_3$OD, and a cross peak was observed between the signals at 5.35 ppm (H-1, glucose-1) and 133.4 ppm (C-3, kaempferol). The $^1$H-NMR coupling constants of the anomeric protons indicated that the linkages of glucose-1 ($J = 7.5$), glucose-2 ($J = 7.5$), and xylose ($J = 7.5$) were β-conjugation, and that of rhamnose (singlet peak) was α-conjugation. Therefore, the structure of **1** was identified as 3-O-[2-O-β-xylopyranosyl-6-O-(3-O-β-glucopyranosyl-α-rhamnopyranosyl)-β-glucopyranosyl] kaempferol (Fig. 1). This is a new tetraglycosyl kaempferol. We named the compound urakunoside after *C. uraku*.

Based on morphological characteristics, Tarokaja is now thought to be an interspecific hybrid between the Japanese native species *C. japonica* and a Chinese native species.[1] Based on chloroplast DNA polymorphisms, we identified *C. pitardii* var. *pitardii* as the most likely maternal ancestor species.[6] Flavonoids in the petals of *C. pitardii* var. *pitardii* and *C. japonica* were analyzed by HPLC. In the *C. pitardii* var. *pitardii* petals, **3** and **4** were detected as major flavonols, but **1** and **2** were not detected. In the *C. japonica* petals, no flavonols in significant amounts were detected. While the occurrence of **3** and **4** in Tarokaja suggests the relationship with *C. pitardii* var. *pitardii*, the origins of **1** and **2** are not clear.

It appears that the 3-O-(6-O-rhamnosyl-glucosyl) flavonol attached glycoside(s) at the 2-position of glucose and/or the 3-position of rhamnose is a core structure of the glycosyl flavonol components in *Camellia* species. Camelliaside A and B, in which the galactosyl or xylosyl residue is attached at the 2-position of glucose in 3-O-(6-O-rhamnosyl-glucosyl) kaempferol, have been reported in seeds of *C. sinensis*,[7] and Camellianoside, in which the xylosyl residue is attached at the 3-position of rhamnose in 3-O-(6-O-rhamnosyl-glucosyl) quercetin, have been reported in leaves of *C. japonica*.[8] Since **1** and **2** also have the same core structure, interspecific hybridization in *Camellia* spp., including *C. japonica*, might trigger metabolism leading to **1** and **2** in the petals of Tarokaja. Another possibility is that a variation of *C. pitardii* var. *pitardii* that produces **1** and **2** became the parent of Tarokaja. These compounds are expected to serve as chemical probes to detect the origin of Tarokaja.

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