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# Isolation and characterization of new limonoid glycosides from *Citrus unshiu* peels☆

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## Abstract

Three limonoid glycosides were isolated from *Citrus unshiu* peels, and their structures were determined based on MS and NMR spectroscopic data as nomilinic acid 17-*O*-β-D-glucopyranoside (**1**), methyl nomilinate 17-*O*-β-D-glucopyranoside (**2**), and obacunone 17-*O*-β-D-glucopyranoside (**3**). In particular, the location of the sugar moiety was clearly determined by the *B*/*E* constant linked scan FABMS method. No limonoid glycosides obtained here were found to have antitumor activity in NCI-H292 and EL-4 cell lines.

*Keywords:* Limonoid glycosides; *Citrus unshiu*; Methyl nomilinate 17-*O*-β-D-glucopyranoside; *B*/*E* constant linked scan FABMS

## 1. Introduction

Since the early 1980s we have been studying the physiologically active substances obtained from citrus fruit peels for the purpose of exploring their more valuable utilization [1–3]. As a consequence, we found a series of flavonoids [4–6] having a hypotensive effect and phenylpropanoid glycosides [7–9] having an interesting effect on the blood pressure of SHR-SP (spontaneously hypertensive stroke-prone) rats. In addition to these compounds, we also found many limonoid glycosides in a hot-water extract of citrus fruit peels [10,11].

In the limonoids, limonin and nomilin occurs widely in citrus juices [12] as a bitter principle of the citrus. However, limonoid glycosides, which have a glucopyranoside ring, have no bitter taste. Recently, Hasegawa and co-workers [13–15] found 13 limonoid glycosides, limonin 17-*O*-β-D-glucopyranoside, nomilin 17-*O*-β-D-glucopyranoside, deacetylnomilin 17-*O*-β-D-glucopyranoside, obacunone 17-*O*-β-D-glucopyranoside, nomilinic acid 17-*O*-β-D-glucopyranoside, deacetylnomilinic acid 17-*O*-β-D-glucopyranoside, isoobacunoic acid 17-*O*-β-D-glucopyranoside, epiisoobacunoic acid 17-*O*-β-D-glucopyrano-

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☆ Studies on physiologically active substances in citrus fruit peel. Part 23. For Part 22, see Ref. [20].

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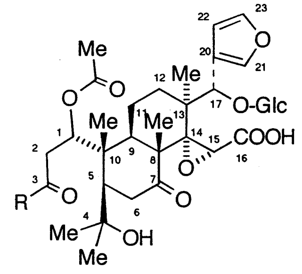
side, obacunoic acid 17-*O*-β-D-glucopyranoside, *trans*-obacunoic acid 17-*O*-β-D-glucopyranoside, calamin 17-*O*-β-D-glucopyranoside, methyl deacetylnomilinic acid 17-*O*-β-D-glucopyranoside, and 6-keto-7β-deacetylnomilol 17-*O*-β-D-glucopyranoside, isolated from grapefruit seeds and calamondin seeds. Among them, limonin 17-*O*-β-D-glucopyranoside has antitumor activity [16,17].

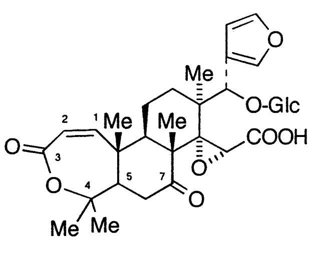
In the limonoid glycosides isolated from peels of *Citrus unshiu*, which is produced abundantly in Japan, we found a new limonoid glycoside. We report here the results of structural characterization of isolated limonoid glycosides by fast-atom bombardment (FAB) mass spectrometry and by NMR spectroscopy, and the test for antitumor activities with NCI-H292 and EL-4 cell lines.

## 2. Results and discussion

A mixture of crude limonoids were obtained from a hot-water extract of *C. unshiu* peels. Three limonoid glycosides (**1**–**3**) in this mixture were purified by column chromatography on silica gel and by gel filtration on TSK HW-60F gel. The yield of each compound was as follows: **1**, 37 mg; **2**, 27 mg; and **3**, 8 mg.



**1**: R = OH
**2**: R = OCH$_3$



**3**

Compound **1** was a colorless amorphous solid and showed a specific rotation of +28.82° (*c* 0.21, MeOH). The molecular weight of **1** was found to be 712 by FABMS; the positive-ion FABMS showed a peak at $m/z$ 735 $[M + Na]^+$, and the negative-ion FABMS showed a peak at $m/z$ 711 $[M - H]^-$. The $^1$H and $^{13}$C NMR spectral data (Tables 1 and 2) were identical with those of nomilinic acid 17-*O*-β-D-glucopyranoside, which had been previously isolated from grapefruit seeds by Bennett and co-workers [14].

Compound **2** was obtained as a colorless amorphous solid and showed a specific rotation of +20.32° (*c* 0.09, MeOH). The molecular weight of **2** was found to be 726 by FABMS; the positive-ion FABMS showed peaks at $m/z$ 727 $[M + H]^+$ and 749 $[M + Na]^+$, and the negative-ion FABMS showed a

Table 1
$^1$H NMR data for limonoid glycosides **1–3**[a]

| H | | 1 | 2 | 3 |
|---|---|---|---|---|
| α-Furan | H-21 | 7.68 br s | 7.67 br s | 7.67 br s |
| | H-23 | 7.34 br s | 7.33 br s | 7.33 br s |
| β-Furan | H-22 | 6.59 br s | 6.60 br s | 6.60 br s |
| | H-17 | 5.53 s | 5.52 s | 5.48 s |
| | H-1 | 3.22 m | 3.24 m | 6.44 d (12.8 Hz) |
| | H-2 | | | 5.95 d (12.8 Hz) |
| | H-15 | 2.67 s | 2.76 s | 2.84 s |
| C–Me | | 1.54 s | 1.53 s | 1.46 s |
| | | 1.26 s | 1.27 s | 1.37 s |
| | | 1.23 s | 1.21 s | 1.09 s |
| | | 0.92 s | 0.90 s | 0.84 s |
| | | 0.82 s | 0.81 s | 0.83 s |
| O–Me | | | 3.66 s | |
| Acetate methyl | | 2.03 s | 2.02 s | |
| Glc-H-1 | | 4.36 d (7.5 Hz) | 4.36 d (7.8 Hz) | 4.35 d (7.8 Hz) |

[a] Solvent: CD$_3$OD.

Table 2
$^{13}$C NMR data for limonoid glycosides **1**–**3**[a]

| Carbon no. | **1** | **2** | **3** |
|---|---|---|---|
| 1 | 78.9 | 77.7 | 152.6 |
| 2 | 38.4 | 36.3 | 119.9 |
| 3 | 175.4 | 175.5 | 169.0 |
| 4 | 75.7 | 75.4 | 85.6 |
| 5 | 50.5 | 50.5 | 51.8 |
| 6 | 40.4 | 40.2 | 41.7 |
| 7 | 217.0 | 217.0 | 213.0 |
| 8 | 53.4 | 53.2 | 53.4 |
| 9 | 42.0 | 42.0 | 50.3 |
| 10 | 46.0 | 54.9 | 46.1 |
| 11 | 19.4 | 19.5 | 20.7 |
| 12 | 29.9 | 28.8 | 28.5 |
| 13 | 45.0 | 45.1 | 46.0 |
| 14 | 71.1 | 70.9 | 70.9 |
| 15 | 62.2 | 62.0 | 61.3 |
| 16 | 174.8 | 174.3 | 171.5 |
| 17 | 79.6 | 79.5 | 79.4 |
| 20 | 128.9 | 127.0 | 127.0 |
| 21 | 143.6 | 143.8 | 143.7 |
| 22 | 113.8 | 113.9 | 113.8 |
| 23 | 141.7 | 141.7 | 141.8 |
| C–Me | 16.3 | 15.9 | 15.7 |
| | 17.6 | 17.8 | 19.0 |
| | 25.8 | 25.6 | 23.3 |
| | 27.5 | 28.2 | 26.2 |
| | 31.9 | 31.1 | 30.1 |
| O–Me | | 52.4 | |
| Acetate carbonyl | 172.7 | 172.3 | |
| Acetate methyl | 21.2 | 21.0 | |
| Glc-1 | 105.2 | 105.5 | 105.6 |
| Glc-2 | 75.7 | 75.8 | 75.7 |
| Glc-3 | 78.2 | 78.3 | 78.2 |
| Glc-4 | 71.6 | 71.7 | 71.6 |
| Glc-5 | 77.3 | 77.2 | 77.2 |
| Glc-6 | 62.8 | 62.8 | 62.8 |

[a] Solvent: $CD_3OD$.

peak at *m*/*z* 725 $[M-H]^-$. The *B*/*E* constant linked scan spectrum of the protonated molecule at *m*/*z* 727 in the positive-ion FABMS of **2** produced fragment ions at *m*/*z* 565, 547, and 469, which correspond to $[M-Glc]^+$, $[MH-GlcOH]^+$, and $[MH-258]^+$, respectively. The *B*/*E* constant linked scan spectrum of $[M+Na]^+$ ion at *m*/*z* 749 in the positive-ion FABMS of **2** also produced fragment ions at *m*/*z* 569 and 491, which correspond to $[M+Na-GlcOH]^+$ and $[M+Na-258]^+$, respectively. Thus, fragment ions of $[MH-258]^+$ and $[M+Na-258]^+$ are assigned to fragmentation A in Fig. 1. This result suggested that a glucopyranosyl moiety of **2** is attached to a hydroxy group of C-17. The $^1$H NMR spectrum of **2** (Table 1) indicated the presence of five methyl groups at $\delta$ 0.81, 0.90, 1.21, 1.27, and 1.53 (3H, s), an acetyl methyl group at $\delta$ 2.02 (3H, s), a methoxy group at $\delta$ 3.66 (3H, s), two methine protons of H-15 at $\delta$ 2.76 and H-17 at $\delta$ 5.52, and three olefinic protons of a furan ring (broadened singlets at $\delta$ 6.60, 7.33, and 7.67). Moreover, the signals of the sugar moiety suggested the presence of a β-glucopyranosyl moiety [an anomeric proton, $\delta$ 4.36 (*J* 7.8 Hz)]. A sugar analysis of **2** gave D-glucose, which was identified by GLC of its acetyl derivative. On the basis of the $^{13}$C–$^1$H COSY analysis of **2**, all the $^{13}$C chemical shifts could be unambiguously determined (Table 2). The location of the sugar moiety was verified by an NOE difference experiment. Irradiation of Glc-H-1 affected H-17, showing that Glc-H-1 and H-17 reside near each other. Upon irradiation of H-17, clear NOEs of the Glc-H-1 and H-21 signals were also observed, thus confirming the connection of a sugar moiety to a hydroxy group of C-17. Based on the foregoing evidence, the structure of **2** was proved to be methyl nomilinate 17-*O*-β-D-glucopyranoside.

Compound **3** was obtained as a mixture with compound **2**. The molecular weight of **3** was found to be 634 by FABMS; the positive-ion FABMS showed a peak at *m*/*z* 657 $[M+Na]^+$, and the negative-ion FABMS showed a peak at *m*/*z* 633 $[M-H]^-$. The *B*/*E* constant linked scan spectrum of $[M+Na]^+$ ion at *m*/*z* 657 in the positive-ion FABMS of **3** produced fragment ions at *m*/*z* 477 and 399, which correspond to $[M+Na-GlcOH]^+$ and $[M+Na-258]^+$, respectively. Thus, a glucopyranosyl moiety of **3** proved to be attached to a hydroxy group of C-17. The $^1$H and $^{13}$C NMR spectral data (Tables 1 and 2) were identical with obacunone 17-*O*-β-D-glucopyranoside, which had been previously isolated from grapefruit seeds by Bennett and coworkers [13].

Thus, three limonoid glycosides have now been successfully isolated from *C. unshiu* peels and characterized.

Since it is known that limonoid glycosides have antitumor activity [16,17], compounds **1**,

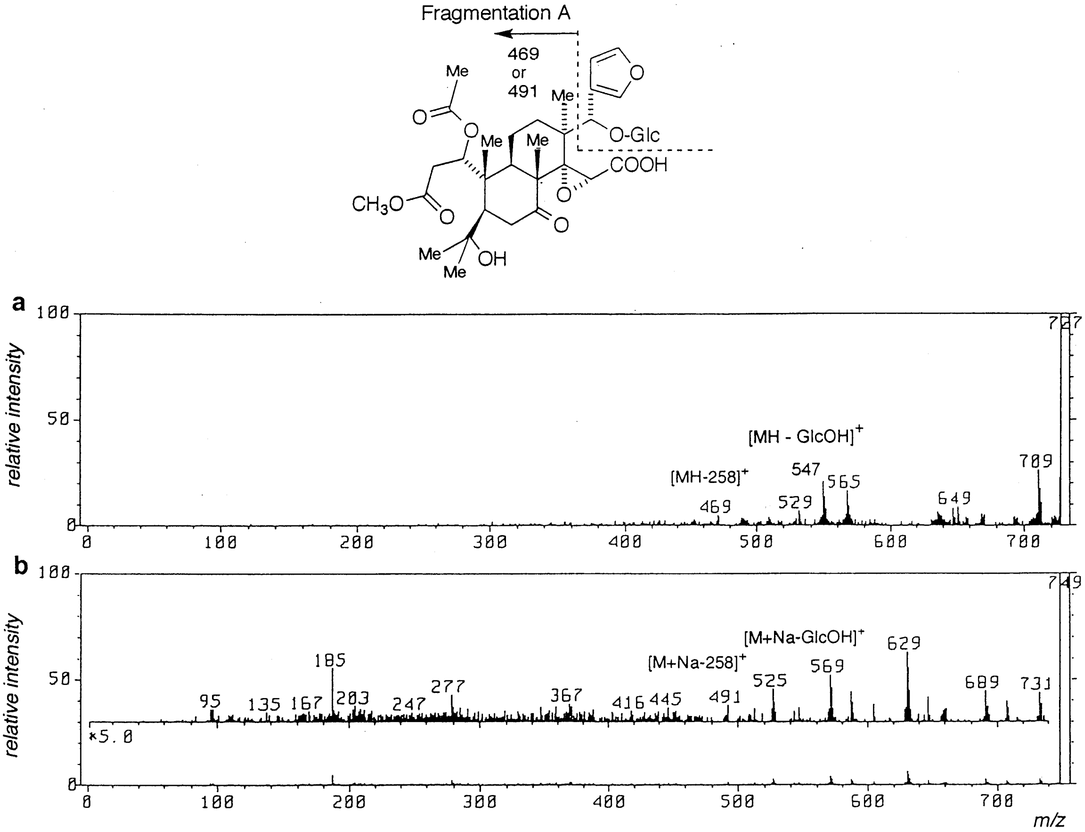


Fig. 1. *B*/*E* constant linked scan spectra of **2**: (a) $[M + H]^+$ ion at *m*/*z* 727 in positive-ion mode FABMS, and (b) $[M + Na]^+$ ion at *m*/*z* 749 in positive-ion mode FABMS.

**2**, and **3** obtained here were evaluated for antitumor activity by using the NCI-H292 human lung mucoepidermoid carcinoma and the EL-4 mouse lymphoma cell lines [18,19]. However, these compounds had no significant antitumoral activity (Table 3).

Among these isolated compounds, compound **2** is a new limonoid glycoside.

## 3. Experimental

*Analytical instruments.*—Optical rotations were measured with a JASCO DIP-1000. IR spectra were recorded on a Perkin–Elmer 1760-X FTIR spectrometer. MS spectra were obtained with JEOL JMS HX 100 and JMA DA 5000 spectrometers. $^1$H NMR (500 MHz or 270 MHz), $^{13}$C NMR (125.7 MHz or 67.5 MHz) and 2D NMR spectra were obtained by JEOL GSX 500 or JEOL EX 270 spectrometers.

*Separation of crude limonoid glycosides.*—The peel (6.4 kg) obtained from 150 *C. unshiu* was air-dried (1.3 kg), chopped by commercial blender, and homogenized after adding 20 L of hot water. The mixture was kept at 96 °C for 30 min and then filtered. The filtrate was deposited on an Amberlite XAD-2 column (Japan Organo Co. Ltd., 5 × 90 cm), washed with 3 L of water and 4 L of 1:4 MeOH–water, and fractionated by successive elution with 7 L of 1:1 MeOH–water and 7 L of

Table 3
Antitumor activities of limonoid glycosides as determined with NCI-H292 and EL-4 cell lines[a]

| Compound | % of intact control absorptions ($n = 3$) | |
|---|---|---|
| | NCI-H292 | EL-4 |
| **1** | 97.3 ± 0.2 | 94.7 ± 1.5 |
| **2** | 99.8 ± 1.3 | 91.1 ± 0.4 |
| **3** | 97.0 ± 2.1 | 91.9 ± 1.8 |
| Actinomycin D | 2.6 ± 0.2 | 4.1 ± 0.5 |
| Mitomycin C | 35.7 ± 2.9 | 35.5 ± 0.9 |

[a] Each datum represents the mean value ± S.E. of these experiments.

MeOH. The 1:1 MeOH–water eluate was concd in vacuo, and then dissolved in 1 L of MeOH. The MeOH solution of 1:1 MeOH–water eluate (34.09 g) was chromatographed over neutral $Al_2O_3$ (70–230 mesh, E. Merck Co. Ltd., 5.5 × 38 cm) with 13 L of 1:1 MeOH–water as an eluent. The eluate was concd to about 200 mL in vacuo and then freeze-dried, giving 9.01 g of a mixture of crude limonoid glycosides.

*Purification of limonoid glycosides.*—The crude glycosides (9.01 g) were chromatographed over silica gel (Wako Gel C-300, Wako Pure Chemical Industries Ltd.) with the lower phase of 65:35:10 $CHCl_3$–MeOH–water as the eluent. Each fraction was concd in vacuo and then freeze-dried. Yield (mg): Fr. 1, 2693; Fr. 2, 1978; Fr. 3, 941; Fr. 4, 404; Fr. 5, 193; Fr. 6, 173, Fr. 7, 199; Fr. 8, 160; Fr. 9, 241; Fr. 10, 205; Fr. 11, 196; Fr. 12, 211; Fr. 13, 302; Fr. 14, 231; Fr. 15, 869. Then these fractions were purified by gel filtration on TSK HW-60F gel.

*Nomilinic acid 17-*O*-β-*D*-glucopyranoside* (**1**).—$[\alpha]_D^{20}$ +28.82° (*c* 0.21, MeOH). FABMS; *m/z* 735 $[M+Na]^+$, 711 $[M-H]^-$. HRFABMS; *m/z* 711.2851 (M − H); calcd for $C_{34}H_{47}O_{16}$: 711.2864. IR (KBr) *ν* max; 3393, 1703, 1596, 1425, 1258, 1079, 1026 $cm^{-1}$. $^1H$ NMR ($CD_3OD$): Table 1. $^{13}C$ NMR ($CD_3OD$): Table 2.

*Methyl nomilinate 17-*O*-β-*D*-glucopyranoside* (**2**).—$[\alpha]_D^{20}$ +20.32° (*c* 0.09, MeOH). FABMS; *m/z* 727 $[M+H]^+$, 749 $[M+Na]^+$, 725 $[M-H]^-$. HRFABMS; *m/z* 725.3011 (M − H); calcd for $C_{35}H_{49}O_{16}$: 725.3021. IR (KBr) *ν* max; 3406, 1737, 1596, 1439, 1258, 1079, 1023 $cm^{-1}$. $^1H$ NMR ($CD_3OD$): Table 1. $^{13}C$ NMR ($CD_3OD$): Table 2.

*Obacunone 17-*O*-β-*D*-glucopyranoside* (**3**).—FABMS; *m/z* 657 $[M+Na]^+$, 633 $[M-H]^-$. HRFABMS; *m/z* 633.2524 (M − H); calcd for $C_{32}H_{41}O_{13}$: 633.2547. IR (KBr) *ν* max; 3403, 1687, 1605, 1424, 1079, 1026 $cm^{-1}$. $^1H$ NMR ($CD_3OD$): Table 1. $^{13}C$ NMR ($CD_3OD$): Table 2.

*Sugar analysis.*—A mixture of the sample (5 mg) and 0.5 M HCl (2 mL) was heated at 96 °C for 1 h and concd in vacuo. EtOAc and water were added to the residue. The aq layer was evaporated in vacuo to give a sugar [**1**, $[\alpha]_D^{22}$ +45.8° (*c* 0.12, $H_2O$); **2**, $[\alpha]_D^{22}$ +46.6° (*c* 0.1, $H_2O$); **3**, $[\alpha]_D^{20}$ +46.2° (*c* 0.11, $H_2O$)], which was acetylated with 1:1 $(CH_3CO)_2O$–pyridine at 90 °C for 1 h. The acetyl derivatives obtained by extracting the foregoing reaction mixture with $CHCl_3$ were compared with an authentic sample of glucose pentaacetate by GLC (Shimadzu GC 14B gas chromatograph equipped with FID; column, Dexil 400, 3.0 mm × 3 m; column temperature, 150–300 °C (5 °C/min); carrier gas, nitrogen at 28 mL/min); glucose pentaacetate $t_R$ (min) 14.23.

*Biological activity test of limonoid glycosides*

*Cell line and culture conditions.* NCI-H292 human lung mucoepidermoid carcinoma and EL-4 mouse lymphoma cell line were used [18,19]. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Biowhittaker) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in an atmosphere containing 5% $CO_2$ in air and 100% relative humidity. For subculture, adherent cells were detached using a 0.125% trypsin and 0.01% EDTA mixture.

*Chemicals.* Test compounds, limonoid glycosides, were dissolved in $Me_2SO$ at 0.1 M. Positive antitumor compounds, mitomycin C (MMC, Wako Pure Chemical Industries Ltd.) and actinomycin D (AMD, Wako), were dissolved in distilled water to give 10 mM. All compounds were stored at − 80 °C. The antitumor activity [18,19] was determined using XTT reagents contained in a Cell Proliferation Kit II (Boehringer Mannheim). XTT reagents were freshly prepared before use. This assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by dehydrogenase activity in active mitochondria.

*Antitumor activity.* NCI-H292 and EL-4 cells were inoculated onto a 96-well microculture plate at seeding density of 5000–10,000 cells per well. After inoculation, test compounds, MMC and AMD were added to the well to give a final concentration of 1 μM. Cells were incubated for 72 h, and XTT reagents (final concentration 0.33 mg/mL) were added to each well. At 4 h after addition of XTT reagents, absorbance was measured with a spectrophotometric plate reader at 490/655 nm wavelength. Antitumor activities of test compounds were determined by measuring specific absorbance to quantify cell growth inhibition, and calculated using the following equation: (test compounds OD/intact control OD) × 100.

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