A New Pentacyclic Triterpenoid Glucoside from *Prunus serrulata* var. *spontanea*

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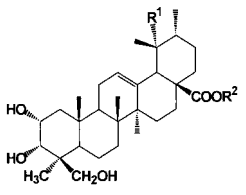
A new triterpenoid, 2α,3α,24-trihydroxyurs-12-en-28-oic acid-28-*O*-β-D-glucopyranosyl ester (4) along with four known triterpenoids, ursolic acid (1), 2α-hydroxyursolic acid (2), 2α,3α,24-trihydroxyurs-12-en-28-oic acid (3), and 2α,3α,19α,24-tetrahydroxyurs-12-en-28-oic acid-28-*O*-β-D-glucopyranosyl ester (5), were isolated from the leaves of *Prunus serrulata* var. *spontanea* (Rosaceae). Compounds 3—5 showed $ONOO^-$ scavenging activity, whereas compounds 1 and 2 were virtually inactive.

Key words *Prunus serrulata* var. *spontanea*; Rosaceae; triterpenoids; 2α,3α,24-trihydroxyurs-12-en-28-oic acid-28-*O*-β-D-glucopyranosyl ester; $ONOO^-$ scavenging activity

Plants of the genus *Prunus* have been used in indigenous medicine such as sedative, anti-inflammatory, anti-hyperlipidemic, and anti-tumor agents.[1] *Prunus serrulata* var. *spontanea* (Rosaceae) is a large sized tree widely distributed throughout Korea and Japan. The red fruits are edible and are used in traditional folk medicine against heart failure from beriberi, dropsy, mastitis, toothache, and as an emmenagogue.[2] Also, the bark of *P. serrulata* var. *sponatenea*, *P. yedoensis*, and *P. sargentii*, so called Pruni Cortex, have been used for detoxification and relaxation, and as an antitussive in traditional Korean medicine.[3] Previously we reported the antioxidant effect of some selected *Prunus* genus in terms of scavenging potential of DPPH radical, total ROS, and $ONOO^-$. Among those, the methanolic extract of the leaves of *P. serrulata* var. *spontanea* exhibited a strong antioxidant activity and afforded antioxidative principles, such as quercetin, kaempferol 3-*O*-α-arabinofuranoside, kaempferol 3-*O*-β-xylopyranoside, kaempferol 3-*O*-β-glucopyranoside, and quercetin 3-*O*-β-glucopyranoside.[4]

There have been some reports on the isolation of triterpenoids from the *Prunus* species such as *P. dulcis*,[5] *P. amygdalus*,[6] *P. persica*,[7] and *P. africana*.[8] No detailed chemical studies as well as biological activities on triterpenoids of *P. serrulata* var. *spontanea*, however, have been reported. Here we report the isolation and structural elucidation of five compounds (**1**—**5**) from the leaves of this plant.

Column chromatography of the EtOAc soluble part of the methanolic extract from the leaves of *P. serrulata* var. *spontanea* yielded compounds **1**—**5** in the order of increasing polarity (Fig. 1). The structures of **1**—**3** were identified by comparison of their spectral data as ursolic acid, 2α-hydroxy ursolic acid and 2α,3α,24-trihydroxyurs-12-en-28-oic acid, respectively.[5,9,10] Further detailed analysis of the ^{1}H- and ^{13}C-NMR spectra (Tables 1, 2), aided by DEPT, HMQC, HMBC, COSY and NOESY experiments, confirmed the structure of **3**. Methylation with CH_2N_2 of **3** gave a **3a**, which was characterized as methyl 2α,3α,24-trihydroxyurs-12-en-28-oic acid, further supported by direct comparison of their spectral data with those reported.[11]



3 : R^1=H, R^2=H
3a : R^1=H, R^2=Me
4: R^1=H, R^2=β-D-glucopyranose
5: R^1=OH, R^2=β-D-glucopyranose

Fig. 1. Chemical Structures of Compounds **3**—**5**

Compound **4** was obtained as white amorphous powder, and showed a positive reaction in Liebermann-Burchard and Molisch tests. The molecular formula of **4** was determined as $C_{36}H_{58}O_{10}$ based on the NMR and high resolution (HR)-FAB-MS data {[M+Na]$^+$, *m/z*: 673.3925 Calcd for $C_{36}H_{58}O_{10}Na$ *m/z*: 673.3928, Δ −0.3 mmu}. The IR spectrum showed a characteristic absorption attributable to an ester carbonyl group at 1735 cm^{-1}, a broad absorption due to hydroxyl group at 3423 cm^{-1}, as well as glycosidic linkage at 1074 cm^{-1}. The ^{13}C-NMR spectrum (Table 2) of compound **4** showed, in addition to 30 signals consistent with a triterpenoid structure of **3**, six peaks in the range at δ 62—96

Table 1. ^{1}H-NMR Spectral Data of Compounds **3**—**5**

Position	3	4	5
H-2β	4.41 dt (10.0, 3.9)	4.45 dt (4.0, 11.6)	4.44 dt (10.0, 2.0)
H-3β	4.56 d (2.4)	4.57 br ($w_{1/2}$=6)	4.58 d (2.0)
H-12	5.44 s	5.41 s	5.51 s
H-18	2.59 d (11.2)	2.49 d (11.3)	2.89 s
3H-23	1.66 s	1.65 s	1.64 s
H_A and	3.79 d (10.9)	3.80 dd (10.9, 16.0)	3.81 d (10.8)
H_B-24	4.08 d (10.9)	4.10 dd (5.2, 16.0)	4.11 d (11.1)
3H-25	0.98 s	1.03 s	1.05 s
3H-26	1.01 s	1.14 s	1.18 s
3H-27	1.11 s	1.11 s	1.59 s
3H-29	0.91 d (6.4)	0.86 d (5.2)	1.04 d (6.3)
3H-30	0.94 d (6.4)	0.89 d (6.5)	1.36 s
Glu-1′		6.24 d (8.0)	6.26 d (8.1)
2′		4.18 m	4.20 m
3′		4.27 m	4.28 m
4′		4.35 m	4.32 m
5′		4.00 m	4.02 m
6′		4.37 dd (4.6, 12.0)	4.36 dd (4.5, 12.0)
		4.44 dd (4.0, 11.6)	4.45 dd (2.0, 12.0)

^{1}H-NMR data was measured in pyridine-d_5 at 400 MHz, and the coupling constants (*J* values) are presented as Hz except for $w_{1/2}$ values in br.

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Table 2. ^{13}C-NMR Spectral Data of Compounds **3**—**5**

Carbon No.	**3**	**4**	**5**
1	43.2	43.3	43.2
2	66.2	66.2	66.3
3	74.2	74.2	74.2
4	45.1	45.1	45.2
5	49.4	49.4	49.5
6	18.8	18.9	19.1
7	34.0	34.0	34.0
8	40.2	40.4	40.8
9	48.1	48.1	47.9
10	38.5	38.5	38.6
11	23.8	23.9	24.4
12	125.5	126.0	128.4
13	139.2	138.4	139.2
14	42.7	42.5	42.1
15	28.6	28.6	29.2
16	24.9	24.6	26.1
17	48.0	48.3	48.6
18	53.5	53.3	54.4
19	39.4	39.3	72.7
20	39.4	39.1	42.1
21	31.1	30.8	26.7
22	37.4	36.8	37.7
23	23.8	23.8	23.8
24	65.2	65.2	65.2
25	17.2	17.3	17.2
26	17.3	17.6	17.4
27	23.8	23.7	24.5
28	179.9	176.2	177.1
29	21.4	21.2	16.7
30	17.4	17.3	27.0
Glu-1′		95.7	95.8
2′		74.0	74.0
3′		78.9	78.9
4′		71.2	71.2
5′		79.2	79.2
6′		62.3	62.4

^{13}C-NMR data measured in pyridine-d_5 at 100 MHz.

(95.7, 74.0, 78.9, 71.2, 79.2, 62.3) corresponding to the presence of a glucose moiety. Alkaline hydrolysis of **4** afforded 1 mol of D-glucose along with **3** as the aglycon. Thus, compound **4** was shown to be a monoglucoside of **3**. The common D-configuration for glucose was assumed according to this most often encountered among the plant glycosides. The anomeric proton signal at δ 6.24 (J=8.0 Hz) in the ^{1}H-NMR spectrum indicated the β-configuration for the glucopyranosyl moiety. The glycosidic linkage site of β-D-glucose was determined to be C-28 based on the HMBC spectrum. In the HMBC experiment, the anomeric proton peak at δ 6.24 correlated with the carboxyl group of the aglycone at δ 176.2. Cmpound **4** was therefore determined as 2α,3α,24-trihydroxyurs-12-en-28-oic acid-28-*O*-β-D-glucopyranosyl ester. This is the first report of its occurrence in nature.

Compound **5** was assigned a molecular formula of $C_{36}H_{58}O_{11}$, established by the positive-ion FAB-MS (*m/z* 689 $[M+Na]^+$) as well as ^{13}C-NMR spectral data and DEPT experiments. Analysis of ^{1}H- and ^{13}C-NMR spectra (Tables 1, 2) indicated that its structure is quite similar to those of **4** except that it has a singlet at δ 2.89, the characteristic signal for H-18 of ursane triterpenoid with 19α-hydroxyl substitution. Substitution of the hydroxyl group at C-19 was also deduced by the appearance of signal at δ 72.7 in the ^{13}C-NMR spectrum. The signal of H-2 was observed as a doublet of triplets with the coupling constants of 10.0 and 2.0 Hz, respectively, which indicated a diaxial and two axial-equatorial couplings. Thus, the configurations of both 2 and OH-3 groups were defined as α. The nOe observed between H-2 and CH_3-25 further supported this stereochemical assignment. Thus, compound **5** was determined to be 2α,3α,19α,24-tetrahydroxyurs-12-en-28-oic acid-28-*O*-β-D-glucopyranosyl ester. Although compound **5** was previously isolated from *Rubus xanthocarpus*,[12] their spectral data of H-2β, H-3β, and C-3 at δ 3.83 (m), 3.57 (br s), and 78.9, respectively, were not superimposed on our data at δ 4.44 (dt, J=10.0, 2.0 Hz), 4.58 (d, J=2.0 Hz), and 74.2.

Compounds **3** and **5** showed high $ONOO^-$ scavenging activities with IC_{50} (50% inhibition concentration) 4.90±0.38 μM and 6.88±0.46 μM, respectively (positive control, penicillamine, IC_{50} 5.11±0.23 μM). Compound **4** showed moderate activity with IC_{50} 82.05±2.80 μM, whereas compounds **1** and **2** virtually inactive.

Experimental

General The IR spectrum was taken with a Shimadzu FT-IR spectrometer in KBr disc. The positive-ion LR- and HR-FAB-MS data were collected on a JEOL JMS-HX110/110A spectrometer. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian UNITY-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). All isolated compounds were measured in pyridine-d_5. Chemical shifts were referenced to the respective residual solvent peaks (δ_H 7.19, 7.56, 8.70 and δ_C 123.5, 135.5, 149.5 for pyridine-d_5, δ_H 7.26 and δ_C 77.0 for $CDCl_3$). COSY, NOESY, HMQC, and HMBC spectra were recorded using pulsed field gradients. Column chromatography was performed with Si gel (Merck, 70—230 mesh), Sephadex LH-20 and Sep-Pak PLUS C18. The TLC was performed on a pre-coated Merck Kieselgel 60 F_{254} plate (0.25 mm) and 50% H_2SO_4 was used as spray reagent.

Chemicals The DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid) was purchased from the Sigma Chemical Company (St. Louis, MO, U.S.A.). The DHR 123 (dihydrorhodamine 123), and peroxynitrite were obtained from Molecular Probes (Eugene, Oregon, U.S.A.) and Cayman Chemicals Co. (Ann Arbor, MI, U.S.A.), respectively.

Plant Materials The leaves of *P. serrulata* var. *spontanea* were collected at Gumjung mountain, Busan, Korea in April 1999, and authenticated by Dr. Maeng Ki Kim, Korea Environmental and Ecological Services, Busan, Korea. A voucher specimen has been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Pusan National University, Busan, Korea.

Extraction, Fractionation, and Isolation The dried leaves of *P. serrulata* var. *spontanea* (1.8 kg) were refluxed with methanol (MeOH) for 3 h. The total filtrate was concentrated and dried *in vacuo* at 40 °C to render the MeOH extract (471 g). The extract was suspended in water (H_2O) and sequentially partitioned with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butyl alcohol (BuOH) to afford CH_2Cl_2 extract (144 g), EtOAc extract (77 g), *n*-BuOH extract (105 g), and H_2O layer (145 g). The EtOAc extract (77 g) was first chromatographed over a silica (Si) gel column (12×60, Silica gel 60, Merck, 2 kg) and eluted with EtOAc–MeOH (8 : 1 to 1 : 1) to afford 14 fractions (Fr. 1—Fr. 14). A portion of fraction 1 (430 mg) and fraction 2 (300 mg) were acetylated with acetic anhydride–pyridine reagent and then purified by Si gel column chromatography with petroleum benzin–ether (5 : 1 to 1 : 1, then with pure EtOAc) to give acetates of compounds **1** (**1a**) and **2** (**2a**). Finally, compounds **1** (150 mg) and **2** (170 mg) were obtained from compounds **1a** and **2a** by deacetylation. Fraction 3 (7.53 g) was subjected to column chromatography over a Si gel column with *n*-hexane–EtOAc (1 : 1 to MeOH) to give 9 fractions (Fr. 3-1 to Fr. 3-9). Subfraction 7 (Fr. 3-7, 1 g) was further chromatographied with CH_2Cl_2–MeOH on Si gel column to give 11 fractions (Fr. 3-7-1 to Fr. 3-7-11) and then subfraction 3 (Fr. 3-7-3, 244 mg) was purified by Sephadex LH-20 with MeOH to afford compound **3** (40 mg). Fraction 14 (4.76 g) was chromatographed over Si gel using a EtOAc–MeOH (30 : 1 to pure MeOH) to obtain 9 fractions (Fr. 14-1 to 14-9). Fraction 4 (Fr. 14-4, 2.61 g) was repeatedly fractionated using a Sep-Pak cartridge (Waters, Sep-Pak PLUS C18), eluted with H_2O–MeOH (0, 20%, 40%, 60%, 80%, 100% MeOH) to afford 6 fractions (Fr. 14-4-1 to Fr. 14-4-6). Fraction 4 (Fr. 14-4-4, 0.40 g) was repeatedly subjected to a Sep-Pak PLUS with H_2O–MeOH (0%, 10%, 30%,

50%, 100% MeOH) to give 5 fractions (Fr. 14-4-4-1 to Fr. 14-4-4-5). Compound **4** (33.7 mg) was isolated from subfraction 4 (Fr. 14-4-4-4, 50% MeOH, 0.24 g) by column chromatography on Si using CH_2Cl_2–MeOH (7 : 1 to MeOH) as mobile phase. Fraction 8 (Fr. 14-8, 0.20 g) was separated by Sephadex LH-20 with MeOH to afford 4 fractions (Fr. 14-8-1 to Fr. 14-8-4). Fraction 2 (Fr. 14-8-2, 160 mg) was consecutively purified, using a Sep-Pak cartridge, eluted with H_2O–MeOH (0%, 10%, 30%, 50%, 100% MeOH) to afford compound **5** (34.1 mg) from the H_2O fraction (Fr. 14-8-2-1).

2α,3α,24-Trihydroxyurs-12-en-28-oic Acid (**3**): Amorphous white powder, HR-FAB-MS *m*/*z* 511.3396 $[C_{30}H_{48}O_5+Na]^+$ (Calcd for 511.3399), ^{1}H-NMR data (pyridine-d_5) see Table 1. ^{13}C-NMR data (pyridine-d_5) see Table 2.

2α,3α,24-Trihydroxyurs-12-en-28-oic Acid-28-*O*-β-D-glucopyranosyl Ester (**4**): Amorphous white powder, HR-FAB-MS *m*/*z* 673.3925 $[C_{30}H_{48}O_5+Na]^+$ (Calcd for 673.3928), $[\alpha]_D^{20}$ −25° (*c*=0.08, MeOH), IR (KBr) cm^{-1}: 3423, 1735, 1459, 1074, 1030, ^{1}H-NMR data (pyridine-d_5) see Table 1. ^{13}C-NMR data (pyridine-d_5) see Table 2.

2α,3α,19α,24-Tetrahydroxyurs-12-en-28-oic Acid-28-*O*-δ-D-glucopyranosyl (**5**): Amorphous white powder, FAB-MS *m*/*z* 689 $[C_{36}H_{58}O_{11}+Na]^+$, ^{1}H-NMR data (pyridine-d_5) see Table 1. ^{13}C-NMR data (pyridine-d_5) see Table 2.

Measurement of the ONOO$^-$ Scavenging Activity The ONOO$^-$ scavenging activity was measured by monitoring the oxidation of DHR 123, by modifying the method of Kooy *et al.*[13] The DHR 123 (5 mM), in dimethylformamide, was purged with nitrogen, stored at −80 °C and used as a stock solution. This solution was then placed on ice, and kept from exposure to light, prior to the study. The buffer used consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride, at pH 7.4, and 100 μM diethylenetriaminepentaacetic acid, each of which were prepared with high quality deionized water, and purged with nitrogen. The final concentration of the DHR 123 was 5 μM. The background and final fluorescent intensities were measured 5 min after treatment, both with and without the addition of authentic ONOO$^-$. The DHR 123 was oxidized rapidly by authentic ONOO$^-$, and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of the oxidized DHR 123 was measured using a microplate fluorescence reader FL 500 (Bio-Tek Instruments Inc.), with excitation and emission wavelengths of 480 and 530 nm, respectively. The results were expressed as the mean±standard error (n=3 or 5) of the final fluorescence intensity minus the background fluorescence. The effects were expressed as the percentage of inhibition of the DHR 123 oxidation.

Statistical Analysis All values were expressed as the mean±standard error of three or five replicate experiments.

Acknowledgments The FAB-MS spectra were provided by the Korea Basic Science Institute. This research was supported by a grant (PF0320801-00) from Plant Diversity Reasearch Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government.

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