Triterpenoids from the Leaves of *Diospyros kaki* (Persimmon) and Their Inhibitory Effects on Protein Tyrosine Phosphatase 1B

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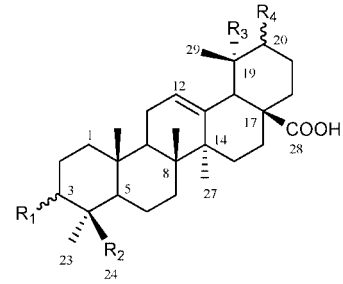
Phytochemical study on a methanol-soluble extract of the leaves of persimmon (*Diospyros kaki*) resulted in the isolation of two new ursane-type triterpenoids, 3α,19α-dihydroxyurs-12,20(30)-dien-24,28-dioic acid (**1**) and 3α,19α-dihydroxyurs-12-en-24,28-dioic acid (**2**), together with 12 known ursane- and oleanane-type triterpenoids (**3**−**14**). Triterpenoids with a 3*β*-hydroxy group were found to inhibit protein tyrosine phosphatase 1B (PTP1B) activity, with IC_{50} values ranging from 3.1 ± 0.2 to 18.8 ± 1.3 *μ*M, whereas those with a 3α-hydroxy moiety were not active.

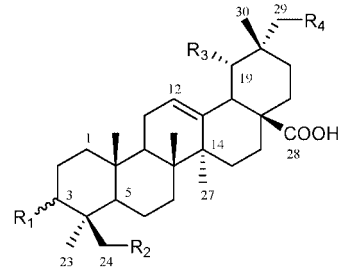
Persimmon (*Diospyros kaki* Thunb., Ebenaceae) is thought to have originated from southern mainland China. This plant is now widely cultivated in Northeast Asia, including the People's Republic of China, Korea, and Japan.[1,2] Persimmon is an abundant source of amino acids, carotenoids, flavonoids, sugars, tannins, terpenoids, and vitamin A.[2−4] It has been found that the extract of the leaves of *D. kaki* showed inhibitory effects on enzymes,[5,6] as well as antioxidant,[7] antigenotoxic,[7] cytotoxic,[1] and neuroprotective activities.[8] The young leaves of persimmon have been used as a tea in Korea and Japan and are thought to be effective against hypertension,[4] while the flavonoid constituents were found to inhibit angiotensin-converting enzyme activity.[5] In the course of our program to search for protein tyrosine phosphatase 1B (PTP1B) inhibitors from plants,[9] it was found that a MeOH extract of young leaves of *D. kaki* exhibited a strong inhibitory activity (>80% inhibition at 30 *μ*g/mL). Since it has been reported that the PTP1B inhibitors may have antidiabetes activity,[10] a phytochemical study was carried out. This paper describes the isolation and characterization of 14 triterpenoids (**1**−**14**) as constituents of the young leaves of *D. kaki*, including two new compounds, **1** and **2**. In addition, the in vitro inhibitory effects on PTP1B of these triterpenoids were determined.

Among the fractions of the MeOH extract of the young leaves of persimmon, the EtOAc-soluble fraction showed a strong inhibitory activity against PTP1B with an inhibition of 93% at 30 *μ*g/mL. Repeated column chromatography of this fraction resulted in the purification of 14 compounds (**1**−**14**). The structures of the known compounds were determined as coussaric acid (**3**),[11] barbinervic acid (**4**), rotungenic acid (**6**), pomolic acid (**7**), 24-hydroxyursolic acid (**8**), ursolic acid (**9**), oleanolic acid (**13**),[12] 24-hydroxy-3-*epi*-ursolic acid (**5**),[13] 24-hydroxy-3-*epi*-oleanolic acid (**11**),[13] 19,24-dihydroxyurs-12-en-3-on-28-oic acid (**10**),[14] and spathodic acid (**14**),[15] by comparing their physicochemical (mp, $[\alpha]_D$) and spectroscopic data (IR, MS, ^{1}H and ^{13}C NMR) with those of reported values.

Compound **1** was isolated as a white, amorphous powder, $[\alpha]^{20}_D$ +42.6, and gave a positive red coloration in the Liebermann-Burchard reaction. Its IR spectrum showed absorption bands for hydroxy and carboxylic groups at ν_{max} 3410 and 1715 cm^{-1}, respectively. In addition, the molecular formula was determined as $C_{30}H_{44}O_6$ from a molecular ion peak at *m*/*z* 523.3044 $[M + Na]^+$ (calcd for $C_{30}H_{44}O_6Na$ 523.3036) in the HRFABMS, suggesting that **1** is a triterpenoid. The ^{1}H NMR spectrum of **1** displayed resonances for five singlet methyl groups (δ_H 1.24, 1.27, 1.67, 1.76, and 1.80), an oxygenated methine proton (δ_H 4.73, br s), an olefinic proton (δ_H 5.68, br s), and two exomethylene protons (δ_H 4.82, 5.02, br s). In the ^{13}C NMR spectrum, 30 carbon signals were observed, including five methyls (δ_C 14.2, 17.9, 24.4, 25.8, and



1 R_1 = α-OH, R_2 = COOH, R_3 = OH, R_4 = CH_2
2 R_1 = α-OH, R_2 = COOH, R_3 = OH, R_4 = α-CH_3
3 R_1 = α-OH, R_2 = CH_2OH, R_3 = OH, R_4 = CH_2
4 R_1 = α-OH, R_2 = CH_2OH, R_3 = OH, R_4 = α-CH_3
5 R_1 = α-OH, R_2 = CH_2OH, R_3 = H, R_4 = α-CH_3
6 R_1 = β-OH, R_2 = CH_2OH, R_3 = OH, R_4 = α-CH_3
7 R_1 = β-OH, R_2 = CH_3, R_3 = OH, R_4 = α-CH_3
8 R_1 = β-OH, R_2 = CH_2OH, R_3 = H, R_4 = α-CH_3
9 R_1 = β-OH, R_2 = CH_3, R_3 = H, R_4 = α-CH_3
10 R_1 = (=O), R_2 = CH_2OH, R_3 = OH, R_4 = α-CH_3



11 R_1 = α-OH, R_2 = OH, R_3 = R_4 = H
12 R_1 = α-OH, R_2 = R_4 = OH, R_3 = H
13 R_1 = β-OH, R_2 = R_3 = R_4 = H
14 R_1 = β-OH, R_2 = R_3 = OH, R_4 = H

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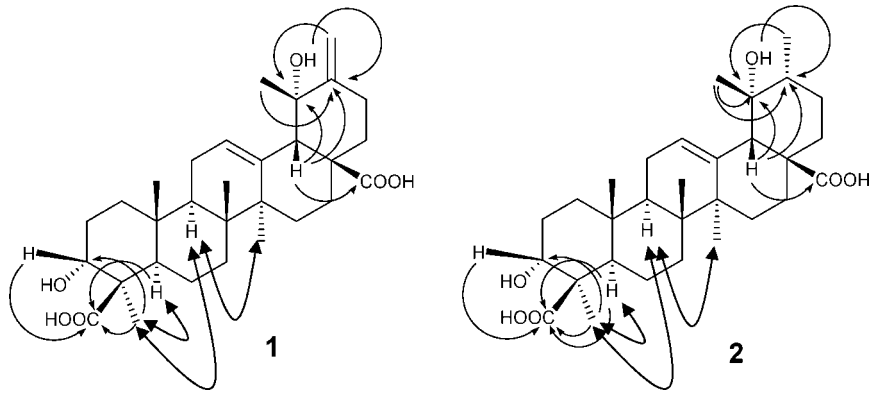

Figure 1. Key HMBC (H → C) and NOESY (H ↔ H) correlations of compounds **1** and **2**.

28.1), an oxygenated methine (δ_C 71.1), an oxygenated quaternary carbon (δ_C 73.4), four olefinic carbons (δ_C 129.0, 140.1 and δ_C 157.1, 105.7), and two carboxylic groups (δ_C 181.2 and 180.6). In the HSQC spectrum, the methine proton resonance at δ_H 4.73 (br s) was assigned as being attached to the carbon that resonated at δ_C 71.1, suggesting that the hydroxyl group at C-3 is α-substituted.[11,16] These spectroscopic data were closely similar to those of coussaric acid, which was also isolated and identified in this study and reported previously from the plant *Coussarea brevicaulis*.[11] However, the ^{13}C NMR and DEPT spectra of **1** showed no resonance for any oxygenated methylene and displayed instead two carbonyl signals at δ_C 181.2 and 180.6. In the HMBC spectrum, the correlations from H-3 (δ_H 4.73) to the carbonyl carbon at δ_C 181.2, and from the methyl protons at δ_H 1.80 to this carbonyl carbon and C-3 (δ_C 71.1), indicated that one carboxylic group is located at C-23 or C-24 of **1**. The downfield chemical shift of the methyl carbon at δ_C 25.8 suggested that this is C-23, and the carboxylic group is located at C-24.[11,16] These assignments were confirmed by NOESY correlations from CH_3-23 to H-5 and H-9 (Figure 1) and the lack of NOESY correlation between H-3 and H-5.[16] Further, the absolute configuration of **1** was considered to be the same as that of coussaric acid, which was exhaustively characterized by Su et al.[11] Therefore, compound **1** was assigned as 3α,19α-dihydroxyurs-12,20(30)-dien-24,28-dioic acid, a new natural product, which has been named diospyric acid A.

Compound **2** was also isolated as a white, amorphous powder with $[\alpha]^{23}_D$ +10.5. The ^{1}H and ^{13}C NMR spectra of **2** were closely similar to those of **1** except for the presence of signals for an additional methyl group (δ_H 1.13, δ_C 17.1) instead of the double bond at C-20 in **1**. The HMBC correlations from two methyl protons (CH_3-19 and CH_3-20) to C-19 (Figure 1) indicated that the methylene group in **1** is replaced by the methyl group in **2**. The molecular formula, $C_{30}H_{46}O_6$, was observed from a molecular ion peak at *m/z* 503.3342 $[M + H]^+$ in the HREIMS and supported this result. Hence, compound **2** was elucidated as the new triterpenoid 3α,19α-dihydroxyurs-12-en-24,28-dioic acid and has been named diospyric acid B.

This study provided the full spectroscopic data of compound **12** (3α,24,29-trihydroxyolean-12-en-28-oic acid), which was first reported from the plant *Akebia quinata*.[17] The identification of this compound was confirmed on the basis of 1D and 2D NMR analysis (see Supporting Information). Although previous studies have revealed the presence of several triterpenoids in *D. kaki*, including **4**, **6**, **7**−**9**, uvaol, and α-amyrin,[18] the known compounds **5** and **10**−**14** were isolated as constituents of persimmon leaf for the first time in the present investigation.

The isolates, **1**−**14**, together with two other triterpenoids, uvaol and oleanonic acid, were examined for their inhibitory effect on PTP1B activity using an in vitro assay,[9] and the results are presented in Table 1. In good agreement with a previous report,[9] triterpenoids with a 3β-hydroxy or a 3-carbonyl group in their molecules (**6**−**10**, **13**, **14**, uvaol, and oleanonic acid) exhibited inhibitory activity against PTP1B with IC_{50} values ranging from 3.1 ± 0.2 to 18.8 ± 1.3 μM. However, those compounds having a 3α-hydroxy group showed no or very weak inhibitory effects on PTP1B activity.

Table 1. ^{1}H and ^{13}C NMR (C_5D_5N, 500 MHz) Spectroscopic Data for Compounds **1** and **2**

	1		**2**	
position	δ_H (*J* in Hz) [a]	δ_C	δ_H (*J* in Hz)	δ_C
1	1.61 m, 1.96 m	35.2 t	1.58 m, 1.93 m	35.1 t
2	1.87 m, 2.76 t (14.1)	28.1 t	1.90 m, 2.75 t (13.0)	28.0 t
3	4.73 br s	71.1 d	4.72 br s	71.0 d
4		48.9 s		48.7 s
5	2.17 m	50.1 d	2.15 m	50.0 d
6	2.02 m, 2.36 m	21.3 t	2.13 m, 2.39 m	21.2 t
7	1.60 m, 1.89 m	34.8 t	1.60 m, 1.81 m	34.5 t
8		40.9 s		40.9 s
9	2.16 m	47.6 d	2.14 m	47.5 d
10		38.8 s		38.7 s
11	1.82 m, 2.10 m	24.7 t	2.14 m, 2.20 m	24.6 t
12	5.68 br s	129.0 d	5.65 br s	128.7 d
13		140.1 s		140.3 s
14		42.9 s		42.7 s
15	1.40 br d (13.2), 2.80 m	29.7 t	1.35 m, 2.38 m	29.6 t
16	2.18 m, 3.24 m	27.4 t	2.06 m, 3.13 td (13.5, 4.0)	26.8 t
17		48.8 s		48.7 s
18	3.27 s	55.9 d	3.08 s	55.1 d
19		73.4 s		73.1 s
20		157.1 s	1.51 m	42.8 d
21	2.30 m, 3.17 td (12.6, 3.9)	29.4 t	2.10 m, 2.13 m	27.3 t
22	2.16 m, 2.38 m	39.8 t	2.08 m, 2.17 m	38.8 t
23	1.80 s	25.8 q	1.80 s	25.7 q
24		181.2 s		181.1 s
25	1.27 s	14.2 q	1.26 s	14.1 q
26	1.24 s	17.9 q	1.25 s	17.7 q
27	1.76 s	24.4 q	1.72 s	24.9 q
28		180.6 s		181.1 s
29	1.67 s	28.1 q	1.45 s	27.5 q
30	5.02 s, 4.82 s	105.7 t	1.13 d (6.5)	17.1 q
19-OH	5.74 s		5.01 s	

[a] Multiplicity signals were overlapped in the range 1.0−2.5 ppm; assignments made on the basis of HMBC, HMQC, and ^{1}H−^{1}H COSY spectra.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO P-1020 polarimeter using a 100 mm glass microcell. IR spectra (KBr) were recorded on a Bruker Equinox 55 FT-IR spectrometer. NMR spectra were obtained on a Varian Inova 400 MHz spectrometer with TMS as the internal standard. FABMS and HR-FABMS data were performed on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. For column chromatography,

silica gel (Merck, 63−200 μm particle size) and RP-18 (Merck, 75 μm particle size) were used. TLC was carried out with Merck silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was carried out using a Gilson system with a UV detector and an Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech Korea).

Chemicals and Reagents. Enzyme PTP1B (human, recombinant) was purchased from BIOMOL International LP. Two triterpenoids, uvaol and oleanonic acid, were obtained from Sigma-Aldrich Co., Ltd. All solvents used for extraction and isolation were purchased from DC Chemical Co., Ltd., Seoul, South Korea. Solvents used for bioassay were of analytical grade and obtained from Merck and Sigma-Aldrich.

Plant Material. The dried leaves of *Diospyros kaki* (persimmon) were purchased from a market in Gwangju, South Korea, in May 2007. The plant material was identified by one of us (W.K.O.), and a voucher specimen (CU-KOR-04) has been deposited at the Laboratory of Pharmacognosy, College of Pharmacy, Chosun University.

Extraction and Isolation. The dried leaves of *D. kaki* (5 kg) were extracted with MeOH (10 L × 2 times) at room temperature for 1 week. The MeOH extract was exhaustively concentrated to dryness (1007 g) and then suspended in H_2O (2 L) and partitioned with EtOAc (2 L × 3 times) to give an EtOAc fraction (630 g). This fraction was chromatographed over a silica gel column (2 kg) and eluted with hexane−EtOAc (50:1, 40:1, 30:1, 20:1, 10:1, 5:1, 3:1, 2:1, 1:2, 1:3, 1:5, and 0:1, each 10 L). Selected fractions (from 10:1 to 1:3, 152 g) were repeatedly subjected to passage over a silica gel column (1 kg) using a hexane−EtOAc mixture as solvent, with a stepwise gradient of 3:1, 2:1, and 1:1 (each 20 L), to give three subfractions (hexane−EtOAc 3:1, F.1, 11 g; hexane−EtOAc 2:1, F.2, 5 g; and hexane−EtOAc 1:1, F.3, 51 g). The most potent fraction, F.3, was eluted on a RP18 column (4.5 × 30 cm) with MeOH−H_2O (1:2, 1:1, 2:1, and 1:0, each 1 L) to afford three fractions (F.3.1, 21.6 g; F.3.2, 13.8 g; and F.3.3, 14.5 g). Further separation of F.3.1 by HPLC (Optima Pak C_{18} column, detected at 205 nm), eluted with MeOH in 0.1% formic acid solution (0−25 min: 72% MeOH, 45 min: 90% MeOH, 55 min: 100% MeOH), resulted in the isolation of compounds **12** (t_R 17.5 min, 4.3 mg), **3** (t_R 18.8 min, 94 mg), **1** (t_R 21.2 min, 39 mg), **4** (t_R 25.4 min, 208 mg), and **2** (t_R 27.2 min, 62 mg). F.3.2 was purified by HPLC (Optima Pak C_{18} column, detected at 205 nm), eluted with MeOH in 0.1% formic acid solution (0−40 min: 80% MeOH, 60 min: 90% MeOH, 65 min: 100% MeOH), to yield compounds **10** (t_R 28.3 min, 4.8 mg), **14** (t_R 33.7 min, 7.1 mg), **6** (t_R 35.6 min, 28 mg), **7** (t_R 55.2 min, 62 mg), **11** (t_R 57.0 min, 2.3 mg), and **5** (t_R 58.8 min, 2.1 mg). F.3.3 was separated by HPLC (Optima Pak C_{18} column, detected at 205 nm), eluted with MeOH in 0.1% formic acid solution (0−40 min: 87% MeOH, 60 min: 95% MeOH, 65 min: 100% MeOH), leading to the isolation of compounds **8** (t_R 43.2 min, 46 mg), **13** (t_R 54.9 min, 11 mg), and **9** (t_R 56.3 min, 39 mg).

Diospyric acid A (1): white, amorphous powder; mp >300 °C; $[\alpha]^{20}_D$ +42.6 (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3410, 2990, 1715, 1660, 1460, 1100 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 523.3044 $[M + Na]^+$ (calcd for $C_{30}H_{44}O_6Na$, 523.3036).

Diospyric acid B (2): white, amorphous powder; mp >300 °C; $[\alpha]^{23}_D$ +10.5 (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3415, 2990, 1720, 1670, 1460, 1090 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRFABMS *m/z* 503.3342 $[M + H]^+$ (calcd for $C_{30}H_{46}O_6H$, 503.3373).

3α,24,29-Trihydroxyolean-12-en-28-oic acid (12): white, amorphous powder; mp 278−280 °C; $[\alpha]^{20}_D$ −26.3 (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3410, 2990, 1710, 1650, 1450, 1100 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) δ 5.56 (1H, br s, H-12), 4.45 (1H, br s, H-3), 4.10 and 3.85 (each 1H, d, J = 10.5 Hz, H-24), 3.59 (2H, s, H-29), 3.44 (1H, br d, J = 10.5 Hz, H-18), 2.02 (1H, m, H-11) 1.95 (1H, m, H-9), 1.90 (1H, m, H-5), 1.64 (3H, s, H-23), 1.238 (3H, s, H-27), 1.236 (3H, s, H-30), 1.07 (3H, s, H-26), 1.00 (3H, s, H-25); ^{13}C NMR (C_5D_5N, 125 MHz) δ 180.7 (C, C-28), 145.4 (C, C-13), 123.0 (CH, C-12), 74.3 (CH_2, C-29), 70.3 (CH, C-3), 66.1 (CH_2, C-24), 50.5 (CH, C-5), 48.5 (CH, C-9), 47.5 (C, C-17), 44.3 (C, C-4), 42.6 (C, C-14), 41.8 (CH, C-18), 41.7 (CH_2, C-19), 40.4 (C, C-8), 37.9 (C, C-10), 37.0 (C, C-20), 34.2 (CH_2, C-1), 34.1 (CH_2, C-7), 33.1 (CH_2, C-22), 29.5 (CH_2, C-21), 28.7 (CH_2, C-15), 26.8 (CH_2, C-2), 26.5 (CH_3, C-27), 24.4 (CH_2, C-11), 24.2 (CH_2, C-16), 24.0 (CH_3, C-23), 20.1 (CH_3, C-30), 19.4 (CH_2, C-6), 17.8 (CH_3, C-26), 16.3 (CH_3, C-25); HRFABMS *m/z* 511.3418 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_5Na$, 511.3399).

Determination of PTP1B Activity. The inhibitory effect of isolated compounds on enzyme activity was measured using a method described previously.[9] Briefly, to each of 96 wells (final volume: 200 μL) was added 2 mM *p*NPP and PTP1B (0.05−0.1 μg) in a buffer containing 50 mM citrate (pH 6.0), 0.1 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT) with or without test compounds. Following incubation at 37 °C for 30 min, the reaction was terminated with 10 N NaOH. The amount of produced *p*-nitrophenol was estimated by measuring the absorbance at 405 nm. The nonenzymatic hydrolysis of 2 mM *p*NPP was corrected by measuring the increase in absorbance at 405 nm obtained in the absence of PTP1B enzyme.

Table 2. Inhibitory Activity of Triterpenoids **1**−**14** against PTP1B

compound	inhibitory activity (IC_{50}, μM)[a,b]
rotungenic acid (**6**)	10.9 ± 1.0
pomolic acid (**7**)	3.9 ± 0.8
24-hydroxyursolic acid (**8**)	12.8 ± 1.3
ursolic acid (**9**)	3.1 ± 0.2
19α,24-dihydroxyurs-12-en-3-on-28-oic acid (**10**)	8.1 ± 0.2
oleanolic acid (**13**)	7.6 ± 0.3
spathodic acid (**14**)	18.8 ± 1.3
uvaol[c]	15.6 ± 2.1
oleanonic acid[c]	3.5 ± 0.1
RK-682[d]	4.1 ± 0.2

[a] Compounds **1**−**5**, **11**, and **12** showed no or very weak activity (IC_{50} >30 μM). [b] IC_{50} values were determined by regression analysis and expressed as means ± SD of three replicates. [c] Compounds obtained from a commercial source. [d] Reference compound.

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Supporting Information Available: ^1H and ^{13}C NMR, HSQC, HMBC, and NOESY spectra for compounds **1**−**3** and **12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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