

## Bioactive Compounds from the Seeds of *Punica granatum* (Pomegranate)

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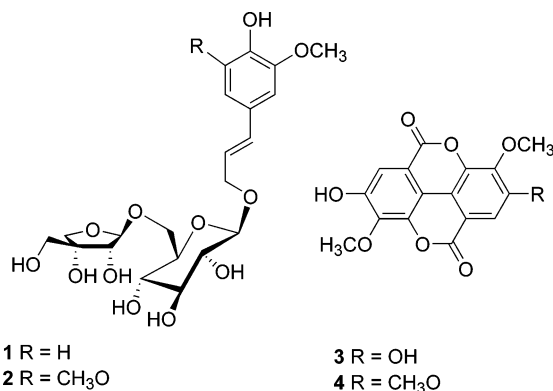
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Two new compounds, coniferyl 9-*O*-[ $\beta$ -D-apiofuranosyl(1 $\rightarrow$ 6)]-*O*- $\beta$ -D-glucopyranoside (**1**) and sinapyl 9-*O*-[ $\beta$ -D-apiofuranosyl(1 $\rightarrow$ 6)]-*O*- $\beta$ -D-glucopyranoside (**2**), were isolated from the seeds of *Punica granatum* (pomegranate), together with five known compounds, 3,3'-di-*O*-methylellagic acid (**3**), 3,3',4'-tri-*O*-methylellagic acid (**4**), phenethyl rutinoside, icariside D<sub>1</sub>, and daucosterol. The structures of **1** and **2** were elucidated by spectroscopic data analysis. Compounds **1**–**4** exhibited antioxidant activity, which was evaluated by measurement of low-density lipoprotein (LDL) susceptibility to oxidation and by determination in vitro of malondialdehyde (MDA) levels in the rat brain.

*Punica granatum* L. (pomegranate) is a shrub belonging to the family Puniceae and is mainly distributed in North America, the Mediterranean region, and the western part of Asia.<sup>1</sup> In the People's Republic of China, this shrub is cultivated and its pericarp is used medicinally for the treatment of colitis, diarrhea, dysentery, leucorrhea, menorrhagia, oxyuriasis, and paralysis.<sup>2</sup> The other parts of this plant such as the roots, leaves, flowers, and seeds are also employed for various therapeutic purposes.<sup>3</sup> The seeds of this topical plant are of high nutritional value and have been reported to have antidiarrheal and antioxidant bioactivity.<sup>4,5</sup> The major constituents of these seeds were found to be monoacylglycerols, glycerides, and sterols,<sup>6–8</sup> in addition to proteins, pectins, and sugars.<sup>9</sup>

The present paper deals with the isolation and the structure elucidation of two new compounds, coniferyl 9-*O*-[ $\beta$ -D-apiofuranosyl(1 $\rightarrow$ 6)]-*O*- $\beta$ -D-glucopyranoside (**1**) and sinapyl 9-*O*-[ $\beta$ -D-apiofuranosyl(1 $\rightarrow$ 6)]-*O*- $\beta$ -D-glucopyranoside (**2**), from the ethanol extract of the dried seeds of *P. granatum*, together with five known compounds, 3,3'-di-*O*-methylellagic acid (**3**), 3,3',4'-tri-*O*-methylellagic acid (**4**), phenethyl rutinoside, icariside D<sub>1</sub>, and daucosterol. All known compounds except daucosterol were isolated from the title plant for the first time.



Compound **1** was obtained as a white powder with a melting point of 276–278 °C. The molecular formula of **1** was calculated as C<sub>21</sub>H<sub>30</sub>O<sub>12</sub>, requiring 7 degrees of unsaturation, on the basis of its negative HRESIMS data. The UV spectrum of **1** exhibited a typical aromatic absorption

at 265 nm. In turn, the IR spectrum of this compound showed hydroxyl absorption at 3421 cm<sup>-1</sup>, aromatic bands at 1608, 1516, and 1455 cm<sup>-1</sup>, and a C–O stretching band at 1053 cm<sup>-1</sup>. The <sup>1</sup>H NMR spectrum of **1** exhibited an ABX system comprised of two *ortho*-coupled doublets and one singlet signal assigned to the protons in an aromatic ring, two proton signals assigned to a *trans*-double bond, and other proton signals assigned to a methoxy group, an aliphatic chain, and the sugar moieties (Table 1). The <sup>13</sup>C NMR and DEPT spectra of **1** revealed the presence of one methyl, four methylenes, 12 methines, and four quaternary carbons. Both the <sup>1</sup>H and <sup>13</sup>C NMR spectral data of this compound were similar to those of coniferyl 9-*O*- $\beta$ -D-glucopyranoside (i.e., 2-[3-(4-hydroxy-3-methoxyphenyl)allyloxy]-6-hydroxymethyltetrahydropyran-3,4,5-triol),<sup>10</sup> with **1** having an additional group of <sup>1</sup>H and <sup>13</sup>C NMR signals, assigned to a D-apiosyl group by detailed analysis of its 1D and 2D NMR data.<sup>11</sup> This apiosyl group connected C-6' ( $\delta$  67.7) of the glucosyl group of the coniferyl 9-*O*- $\beta$ -D-glucopyranoside moiety by a 1 $\rightarrow$ 6 *O*-glycosidic linkage, because C-6' of this compound was shifted downfield about 5 ppm and H-1'' [ $\delta$  4.91 (1H, d, *J* = 2.5 Hz)] had a HMBC correlation with C-6' (Figure 1). The apiosyl group was in a  $\beta$ -configuration as suggested by comparing the coupling constant of H-1'' with that published for this compound.<sup>11</sup> The glucosyl group of **1** connected C-9 ( $\delta$  68.7) through an *O*-glycosidic bond, as was confirmed by the downfield chemical shift of C-9 and the HMBC correlations between H-1' [ $\delta$  4.22 (1H, d, *J* = 7.5 Hz)] and C-9 as well as between H-9 [ $\delta$  4.36 (1H, dd, *J* = 12.5, 6.0 Hz); 4.17 (1H, dd, *J* = 12.5, 6.0 Hz)] and C-1' ( $\delta$  101.7). The coupling constant (7.5 Hz) of H-1' verified the  $\beta$ -configuration of the glucosyl group of **1**.<sup>10</sup> The overall structural determination of **1** was based on the detailed analysis of 1D and 2D NMR spectral data including the <sup>1</sup>H NMR, <sup>13</sup>C NMR, DEPT, <sup>1</sup>H–<sup>1</sup>H COSY, HMQC, and HMBC spectra. On the basis of the aforementioned evidence, the structure of compound **1** was assigned as 2-(3,4-dihydroxy-4-hydroxymethyltetrahydrofuran-2-yl-oxymethyl)-6-[3-(4-hydroxy-3-methoxyphenyl)allyloxy]-tetrahydropyran-3,4,5-triol, or coniferyl 9-*O*-[ $\beta$ -D-apiofuranosyl(1 $\rightarrow$ 6)]-*O*- $\beta$ -D-glucopyranoside.

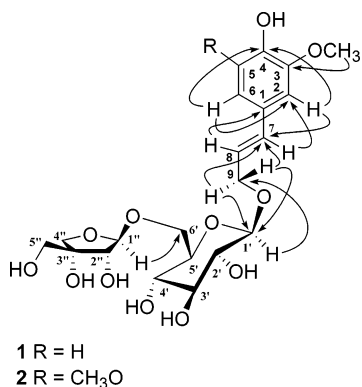
Compound **2** was isolated as an analogue of **1**, with its molecular formula of C<sub>22</sub>H<sub>32</sub>O<sub>13</sub> established by negative HRESIMS. This compound showed UV and IR parameters similar to **1**. The only difference between these substances is the substituent at C-5, which was demonstrated to be a methoxy group in **2**. Comparison of the <sup>1</sup>H NMR spectral

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**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR Spectral Data of **1** and **2** (in  $\text{DMSO-}d_6$ )<sup>a</sup>

position	<b>1</b>		<b>2</b>	
	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (J in Hz)	$\delta_{\text{C}}$
1		128.0		127.0
2	7.02 s	109.8	6.71 s	103.2
3		147.7		148.0
4		146.5		135.5
5	6.74 d (8.5)	115.5		148.0
6	6.84 d (8.5)	119.8	6.71 s	103.9
7	6.56 d (16.0)	132.2	6.55 d (16.0)	132.2
8	6.18 m	122.8	6.22 m	123.2
9	4.36 dd (12.5, 6.0); 4.17 dd (12.5, 6.0)	68.7	4.37 dd (12.5, 6.0); 4.16 dd (12.5, 6.0)	68.6
MeO-3	3.79 s (3H)	55.6	3.34 s (3H)	55.9
MeO-5			3.34 s (3H)	55.9
1'	4.22 d (7.5)	101.7	4.22 d (7.5)	101.7
2'	3.02 t (7.5)	73.4	3.02 t (7.5)	74.4
3'	3.17 t (7.5)	76.7	3.19 t (7.5)	76.3
4'	3.02 t (7.5)	70.3	3.02 t (7.5)	70.0
5'	3.29 m	75.6	3.29 m	75.3
6'	3.89 dd (11.0, 7.0); 3.46 dd (11.0, 7.0)	67.7	3.88 dd (11.0, 7.0); 3.46 dd (11.0, 7.0)	67.8
1''	4.91 d (2.5)	109.3	4.91 d (3.0)	109.2
2''	3.74 d (2.5)	75.9	3.75 d (3.0)	75.9
3''		78.7		78.9
4''	3.87 d (10.5); 3.61 d (10.5)	73.3	3.86 d (10.0); 3.61 d (10.0)	73.2
5''	3.39 d (11.0); 3.36 d (11.0)	63.1	3.34 d (11.0); 3.29 d (11.0)	63.1

<sup>a</sup> Assignments were confirmed by 1D and 2D NMR methods.

**Figure 1.** Key HMBC correlations for **1** and **2**.

data of **2** with those of **1** revealed that **2** had two singlets instead of an ABX system in the aromatic region, which suggested **2** to be a tetrasubstituted aromatic compound with *meso*-protons (Figure 1). Examination of the  $^{13}\text{C}$ ,  $^1\text{H}$ – $^1\text{H}$  COSY, HMQC, and HMBC NMR spectral data of **2** confirmed the above inference and further confirmed that the substituent at C-5 was a methoxy group (Table 1). The secondary glycoside of **2**, sinapyl 9-*O*- $\beta$ -D-glucopyranoside,<sup>10</sup> was isolated previously and its NMR spectra data were in agreement with **2** except for those signals assigned to the terminal apiosyl group. Therefore, the chemical structure of compound **2** was established as 2-(3,4-dihydroxy-4-hydroxymethyltetrahydrofuran-2-yl-oxymethyl)-6-[3-(4-hydroxy-3,5-dimethoxyphenyl)allyloxy]tetrahydropyran-3,4,5-triol, or sinapyl 9-*O*-[ $\beta$ -D-apirofuranosyl(1 $\rightarrow$ 6)]-*O*- $\beta$ -D-glucopyranoside.

Five known compounds were identified from their spectral data by comparison with values reported in the literature as 3,3'-*O*-methylellagic acid (**3**),<sup>12</sup> 3,3',4'-*O*-methylellagic acid (**4**),<sup>12</sup> phenethyl rutinoid, <sup>13,14</sup> icaricidol, <sup>15</sup> and daucosterol.<sup>16</sup>

The antioxidant activities of compounds **1**–**4**, phenethyl rutinoid, and icaricidol were estimated by measure-

**Table 2.** Effect of Compounds **1**–**4** on CD Formation of LDL or MDA in Rat Brain in Vitro<sup>a</sup>

agent	concentration ( $1 \times 10^{-5}$ g/mL)	percentage increase of CD (%) $\pm$ SD <sup>b</sup>	inhibition rate of MDA (%)
control		38.5 $\pm$ 1.8	0
vitamin C	1.25	3.7 $\pm$ 0.8**	50.1
<b>1</b>	5	7.3 $\pm$ 0.7**	35.6
	2.5	10.5 $\pm$ 1.3**	32.4
<b>2</b>	1.25	15.4 $\pm$ 2.3**	28.9
	5	12.2 $\pm$ 2.9**	32.1
<b>3</b>	2.5	14.4 $\pm$ 2.7**	29.8
	1.25	17.6 $\pm$ 3.2**	26.5
<b>4</b>	5	20.4 $\pm$ 0.7**	28.1
	2.5	28.9 $\pm$ 1.7**	20.1
	1.25	27.0 $\pm$ 1.7**	21.8
	5	21.0 $\pm$ 0.5**	31.1
	2.5	27.2 $\pm$ 1.0**	24.6
	1.25	29.8 $\pm$ 1.0**	18.3

<sup>a</sup> For the protocols used, see the Experimental Section. Phenethyl rutinoid and icaricidol were not active, and daucosterol was not tested. <sup>b</sup>  $n = 3$ , \*\*  $P < 0.01$ .

ment of low-density lipoprotein (LDL) susceptibility to oxidation and determination of malondialdehyde (MDA) levels in the rat brain in vitro. The data collected (Table 2) indicated that compounds **1** and **2** moderately decreased conjugated diene (CD) production of LDL and had a significant effect on the inhibition of MDA production in rat brain in vitro in a dose-dependent manner. Compounds **3** and **4** also exhibited appreciable antioxidant activity. The biological test data of phenethyl rutinoid and icaricidol D<sub>1</sub> showed no statistical difference compared with the control. The present results are consistent with the reported antioxidant properties of the seeds of *P. granatum*.<sup>5</sup>

## Experimental Section

**General Experimental Procedures.** Melting points were determined on an XT-4A micromelting point apparatus without correction. Optical rotations were obtained with an AA-10R polarimeter. The UV spectra were run on a Varian Cary Eclipse 300 spectrometer using  $\text{H}_2\text{O}$  as solvent. The IR spectra were measured on a Thermo Nicolet Nexus 470 FT-IR spectrometer. Both  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed on a Bruker DRX 500 NMR spectrometer using the solvent as internal standard. The HRESIMS were run with a Bruker APEX II mass spectrometer. Preparative HPLC was conducted on a Waters HPLC apparatus equipped with a Waters 996 PAD detector, a Waters 515 pump, and Millennium 32 workstation software. The column used was a RP-18 chromatography column ( $10 \times 250$  mm, Waters). Column chromatography was carried out with silica gel (100–300 mesh) (Tsingtao Marine Chemistry Co. Ltd.), Sephadex LH-20 (18–110  $\mu\text{m}$ ) (Pharmacia Co. Ltd.), and ODS (100–200 mesh) (Fuji Silysia Chemical Co. Ltd.). Laboratory animals were obtained from the Laboratory Animal Institute, Chinese Academy of Medical Science, Beijing, People's Republic of China.

**Plant Material.** The plant material was collected in September 2003 from Zaozhuang City in Shandong Province of China. One of the authors (R.F.W.) authenticated this as the seed of *Punica granatum* L., and a voucher specimen (No. 031011) has been deposited in the herbarium of the Laboratory of Pharmaceutical Sciences, Department of Biological Sciences and Biotechnology, Tsinghua University.

**Extraction and Isolation.** The powdered seeds of *P. granatum* (4 kg) were extracted with 95% ethanol under reflux. After concentration in vacuo, the ethanol crude extract (489 g) was suspended in water and partitioned successively with petroleum ether, ethyl acetate (EtOAc), and *n*-butanol. The *n*-butanol-soluble part (20 g) was subjected to passage over a macroporous resin D<sub>101</sub> column, using water and 30%, 60%, and 95% ethanol solution as eluents to provide four fractions.

The 30% ethanol eluate was concentrated to dryness (3.2 g) and further subjected to ODS column chromatography employing a gradient MeOH–H<sub>2</sub>O mixture (from 10% to 60%) as eluent to provide five fractions (A–E). Fraction B (203 mg) was isolated by preparative HPLC to give compounds **1** (37 mg) and **2** (12 mg). The preparative HPLC conditions were as follows: eluent, MeOH–H<sub>2</sub>O (32:68, v/v) with a flow rate of 2.1 mL/min; detector, Waters 996 PAD,  $\lambda = 254$  nm. Compounds **1** and **2** were detected at  $t_R$  20.19 and 23.17 min, respectively. Fraction C (187 mg) was subjected to ODS column chromatography using the gradient MeOH–H<sub>2</sub>O from 20% to 50% as eluent to give phenethyl rutinoid (20 mg) and icaraside D<sub>1</sub> (8 mg). The EtOAc-soluble part (25 g) was subjected to silica gel column chromatography using a CHCl<sub>3</sub>–MeOH mixture (30:1 → 3:1) as eluent to afford seven fractions (F–L). Fraction G (3.05 g) was chromatographed on a silica gel column eluted with a gradient CHCl<sub>3</sub>–MeOH mixture to give four fractions (G-1–G-4). Fraction G-1 was purified by Sephadex LH-20 column chromatography with MeOH as eluent to yield compound **3** (25 mg). Fraction G-3 was chromatographed on a silica gel column eluted with a gradient CHCl<sub>3</sub>–MeOH mixture (20:1 → 4:1) to provide compound **4** (18 mg). Daucosterol (205 mg) precipitated from the solution of fraction H in MeOH.

**Coniferyl 9-O-[ $\beta$ -D-apiofuranosyl(1→6)]-O- $\beta$ -D-glucopyranoside (1):** white powder, mp 276–278 °C;  $[\alpha]_D^{20} +8.7^\circ$  (*c* 0.50, H<sub>2</sub>O); UV  $\lambda_{max}$  (H<sub>2</sub>O) (log  $\epsilon$ ) 265 (2.29) nm; IR (KBr)  $\nu_{max}$  3421, 2936, 2884, 1608, 1516, 1455, 1428, 1376, 1277, 1223, 1157, 1053, 822, 761, 565 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) and <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>), see Table 1; HRESIMS (negative) *m/z* 473.1667 [M – 1]<sup>-</sup> (calcd for C<sub>21</sub>H<sub>29</sub>O<sub>12</sub>, 473.1664).

**Sinapyl 9-O-[ $\beta$ -D-apiofuranosyl(1→6)]-O- $\beta$ -D-glucopyranoside (2):** white powder, mp 284–286 °C;  $[\alpha]_D^{20} +13.2^\circ$  (*c* 0.50, H<sub>2</sub>O); UV  $\lambda_{max}$  (H<sub>2</sub>O) (log  $\epsilon$ ) 275 (2.42) nm; IR (KBr)  $\nu_{max}$  3400, 2936, 1600, 1510, 1450, 1123, 1056, 874, 749, 602 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) and <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>), see Table 1; HRESIMS (negative) *m/z* 503.1774 [M – 1]<sup>-</sup> (calcd for C<sub>22</sub>H<sub>31</sub>O<sub>13</sub>, 503.1769).

**Measurement of LDL Susceptibility to Oxidation.** Isolation and analysis of LDL from the plasma of New Zealand White rabbits were performed as prescribed previously,<sup>17</sup> with a slight modification. The dialysis of LDL was conducted against a buffer (NaCl, 140 mmol/L; KCl 3 mmol/L; Na<sub>2</sub>HPO<sub>4</sub> 8 mmol/L; NaH<sub>2</sub>PO<sub>4</sub> 2 mmol/L; pH 7.4) for 4 h at 4 °C in the dark (four changes). Protein content was measured by the method of Lowry et al.,<sup>18</sup> using bovine serum albumin as the standard. LDL was diluted in dialysis buffer to a final concentration of 0.25 mg LDL/mL. Compounds **1–4**, phenethyl rutinoid, icaraside D<sub>1</sub> (each for three different final concentrations: 1.25, 2.5, and 5.0 × 10<sup>-5</sup> g/mL), and positive (freshly prepared vitamin C, final concentration 1.25 × 10<sup>-5</sup> g/mL) were added to buffer containing LDL for incubation about 30 min at 20 °C, respectively. The control was treated with the same volume of the vehicle solution of the solvent, saline. Subsequently, oxidation was initiated by addition of a freshly prepared aqueous CuSO<sub>4</sub> solution (final concentration, 5.0 μmol/L). Incubation was performed at 37 °C for 4 h. The LDL oxidation was monitored by the change in the 234 nm absorbance at 37 °C for 4 h in a spectrophotometer. The percentage increased of conjugated diene between 0 and 4 h was determined by the formula (Absorbance<sub>4h</sub> – Absorbance<sub>0h</sub>)/Absorbance<sub>0h</sub> × 100%.

**Determination of MDA Levels in Brain in Vitro.** MDA levels in rat brains were determined through the thiobarbituric acid (TBA) method,<sup>19</sup> with a slight modification. In brief, rat brains were obtained from male Wistar rats, and fresh homogenates (10% w/v) were made with a Potter-Elvehjem tissue grinder in 50 mM phosphate buffer, pH 7.4. Protein content was measured by the method of Lowry et al.,<sup>18</sup> using bovine serum albumin as a standard. Aliquots (0.2 mL) were then oxidized by incubating for 30 min at 37 °C with compounds **1–4**, phenethyl rutinoid, icaraside D<sub>1</sub> (each at three different final concentrations: 1.25, 2.5, and 5.0 × 10<sup>-5</sup> g/mL), and vitamin C (final concentration: 1.25 × 10<sup>-5</sup> g/mL), respectively. The control was treated with the same volume of the vehicle solution of the solvent, saline. Reactions were stopped by addition of 0.1 mL SDS (10% w/v), prior to analysis of the concentration of TBA-reactive material, MDA, as an index of lipid peroxidation. Then, 2.0 mL of HCl (0.1 M) and 1.0 mL of acetic acid solution (50% v/v) containing TBA (1% w/v) were added to the reaction samples. The samples and MDA standards (1.25–20 mM) were heated in a boiling water bath for 15 min and then cooled to 25 °C. Next, 1 mL of water and 5 mL of *n*-butanol–pyridine (15:1, v/v) were added, with the samples shaken vigorously and centrifuged at 1000 rpm for 10 min. Absorbance of the organic phase was measured at 532 nm and compared with MDA standards to determine the TBA-reactive material of each sample. MDA was expressed as nmol/mg protein. The inhibition rate was calculated by the formula (MDA<sub>control</sub> – MDA<sub>agent</sub>)/MDA<sub>control</sub> × 100%.

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