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Anti-inflammatory and antitumor phenylpropanoid sucrosides from the seeds of *Raphanus sativus*

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ABSTRACT

A bioassay-guided fractionation and chemical investigation of the MeOH extract of *Raphanus sativus* seeds resulted in the isolation and identification of eight phenylpropanoid sucrosides (**1**–**8**) including two new compounds, named raphasativuside A and B (**1**–**2**) from the most active $CHCl_3$-soluble fraction. The structures of these new compounds were elucidated through spectral analysis, including extensive 2D-NMR data, and chemical reaction experiments. We evaluated the anti-inflammatory effects of **1**–**8** in lipopolysaccharide (LPS)-stimulated murine microglia BV2 cells. Compounds **2** and **5** exhibited significant inhibitory effect on nitric oxide production in LPS-activated BV-2 cells with IC_{50} values of 21.63 and 26.96 μM, respectively. All isolates were also evaluated for their antiproliferative activities against four human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT-15). Compounds **1**–**7** showed consistent cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines with IC_{50} values of 6.71–27.92 μM. Additionally, the free-radical scavenging activity of **1**–**8** was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay where compounds **1**, **3**, and **4** scavenged DPPH radical strongly with IC_{50} values of 23.05, 27.10, and 29.63 μg/mL, respectively.

Raphanus sativus L. (Brassicaceae), commonly known as radish, is widely consumed as a vegetable or condiment in human diets throughout the world. Radish has been used in traditional Chinese herbal medicine for more than 1400 years, since being recorded in '*Tang Materia Medica*', the first Chinese pharmacopoeia.[1] Different parts of the radish, including the roots, seeds, and leaves, have been known to show various medicinal properties.[2] In particular, the seeds, well-known as Raphani Semen, have long been used in Korean traditional medicine as a carminative, diuretic, expectorant, laxative, and contain stomachic, anti-cancer, and anti-inflammatory agents.[3–5] Preliminary results showed that the MeOH extract of *R. sativus* seeds exhibited significant cytotoxic and anti-inflammatory effects, which led to the isolation and identification of the active 4-methylthio-butanyl derivatives that correlated with the cytotoxic and anti-inflammatory activities in our recent study.[6] In the process of our continuing efforts to study this source, we further isolated eight phenylpropanoid sucrosides (**1**–**8**) including two new compounds, named raphasativuside A and B (**1**–**2**) (Fig. 1) from the most active $CHCl_3$-soluble fraction using a bioassay-guided fractionation method, and evaluated the antitumor and anti-inflammatory activities of all isolates (**1**–**8**).

Compound **1** was isolated as a light yellowish gum. The molecular formula of **1** was determined to be $C_{44}H_{50}O_{22}$ by positive mode HR-ESI-MS data at *m*/*z* 953.2690 $[M+Na]^+$ (calcd for $C_{44}H_{50}O_{22}Na$, 953.2691). The UV and IR spectra displayed absorption bands associated with hydroxyl and α,β-unsaturated carbonyl ester groups, and aromatic ring functionalities. The ^1H NMR spectrum of **1** (Table 1) showed characteristic signals attributable to three pairs of olefinic protons [δ_H 7.71 (d, J = 16.0 Hz, H-7″)/6.46 (d, J = 16.0 Hz, H-8″), 7.48 (d, J = 16.0 Hz, H-7‴)/6.26 (d, J = 16.0 Hz, H-8‴), and 7.57 (d, J = 16.0 Hz, H-7⁗)/6.48 (d, J = 16.0 Hz, H-8⁗)], and a benzyl moiety with an ABX spin system [δ_H 7.21 (d, J = 1.5 Hz, H-2″), 7.09 (dd, J = 8.5, 1.5 Hz, H-6″), and 6.77 (d, J = 8.5 Hz, H-5″)], as well as two 1,3,4,5-tetrasubstituted aromatic rings [δ_H 6.77 (s, H-2‴ and H-6‴) and 6.82 (s, H-2⁗ and H-6⁗)]. Furthermore, the large coupling constants (16.0 Hz) of the three pairs of olefinic protons suggested the *trans*-form of the α,β-unsaturated carbonyl systems in **1**.[7,8] The ^{13}C NMR spectrum of **1** (Table 1) exhibited a total of 44 carbons including 27 sp^2 carbons between

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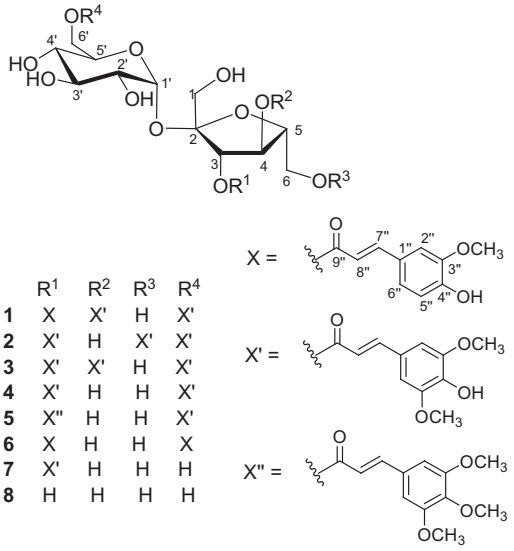

	R^1	R^2	R^3	R^4
1	X	X'	H	X'
2	X'	H	X'	X'
3	X'	X'	H	X'
4	X'	H	H	X'
5	X"	H	H	X'
6	X	H	H	X
7	X'	H	H	H
8	H	H	H	H

Figure 1. Chemical structures of **1**–**8**.

δ_C 167.8 and 105.5, whose ^{13}C NMR chemical shifts were in good agreement with those of two *trans*-sinapoyl and one *trans*-feruloyl moieties.[7,8] The ^1H and ^{13}C NMR spectra of **1** (Table 1) displayed a characteristic α-anomeric resonance at δ_H 5.56 with a small coupling constant (d, J = 4.0 Hz, H-1′) due to a *gauche* conformation, according to the Karplus relation,[9] together with 12 oxygenated carbon signals between δ_C 103.9 and 62.6. These date implied that compound **1** has a disaccharide moiety composed of a pentose and a hexose unit. On the basis of chemical evidence and detailed analysis of ^1H–^1H COSY, HMQC, and HMBC experiments (Fig. 2), as well as comparison of spectroscopic data with authentic samples,[7,8,10] the sugar moiety was confirmed to be sucrose, which were comprised of β-D-fructose and α-D-glucose units connected through a 2→1 linkage (Supplementary data). According to these results, we concluded that compound **1** is a phenylpropanoid sucroside acylated by two *trans*-sinapoyl and one *trans*-feruloyl moieties. Upon inspection of the HMBC spectrum, long range correlations were observed between H-3 (δ_H 5.38)/C-9″ (δ_C 166.5) and H-4 (δ_H 5.69)/C-9‴ (δ_C 166.6) in the fructofuranosyl moiety and between H-6′ (δ_H 4.73 and 4.22)/C-9⁗ (δ_C 167.8) in the glucopyanosyl moiety (Fig. 2). From these data, the positions of the acyl residues were determined as shown in Figure 1. Thus, the structure of **1** was elucidated as (3-*O*-feruloyl-4-*O*-sinapoyl)-β-D-fructofuranosyl-(2→1)-(6-*O*-sinapoyl)-α-D-glucopyranoside and named as raphasativuside A.

Compound **2** was isolated as a light yellowish gum. The molecular formula was established as $C_{45}H_{52}O_{23}$ from the $[M+Na]^+$ peak at m/z 983.2793 (calcd for $C_{45}H_{52}O_{23}Na$, 983.2797) in the HR-ESIMS. The UV and IR spectra of **2** were very similar to those of **1**. Likewise, the ^1H and ^{13}C NMR data of **2** (Table 1) were quite similar to those of **1**, with a noticeable difference being the chemical shifts assignable to three *trans*-sinapoyl moieties rather than two *trans*-sinapoyl and one *trans*-feruloyl moieties in **1**.[7,8] Moreover, the carbon signals in the β-D-fructose were different, relative to those of **1**, suggesting a change in the linkage position of the acyl residues. Detailed analysis of the HMBC spectrum of **2** confirmed the presence of two *trans*-sinapoyl moieties at C-4 and C-6 on the fructofuranosyl unit and one *trans*-sinapoyl moiety at C-6′ on the glucopyanosyl unit. Therefore, the structure of **2** was assigned as (3,6-*O*-disinapoyl)-β-D-fructofuranosyl-(2→1)-(6-*O*-sinapoyl)-α-D-glucopyranoside and named as raphasativuside B.

The six known compounds were identified as (3,4-*O*-disinapoyl)-β-D-fructofuranosyl-(2→1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**3**),[7] (3-*O*-sinapoyl)-β-D-fructofuranosyl-(2→1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**4**),[7] tenuifoliside C (**5**),[10] (3-*O*-feruloyl)-β-D-fructofuranosyl-(2→1)-(6-*O*-feruloyl)-α-D-glucopyranoside (**6**),[8] sibiricose A_6 (**7**),[11] and sucrose (**8**),[10] by comparison of their spectroscopic data with reported data.

Table 1
^1H (500 MHz) and ^{13}C NMR (125 MHz) spectral data of **1**–**2** in CD_3OD (δ in ppm)[a]

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1	3.68 d (12.0)	63.8	3.69 d (12.0)	63.8
	3.64 d (12.0)		3.65 d (12.0)	
2		103.9		103.9
3	5.83 d (7.5)	75.5	5.48 d (7.5)	78.2
4	5.69 t (7.5)	75.0	4.52 t (7.5)	74.0
5	4.17 m	81.4	4.20 m	80.0
6	3.98 m	62.6	4.54 m	64.7
	3.92 dd (13.0, 5.0)		4.52 m	
1′	5.56 d (4.0)	91.5	5.51 d (4.0)	91.5
2′	3.53 dd (10.0, 4.0)	71.6	3.52 dd (10.0, 4.0)	71.5
3′	3.73 m	73.5	3.76 m	73.6
4′	3.32 m	70.7	3.32 m	70.5
5′	4.35 dd (11.0, 9.5)	71.4	4.32 dd (11.0, 9.5)	71.3
6′	4.73 br d (11.0)	64.6	4.72 br d (11.0)	64.6
	4.22 m		4.23 m	
1″ X-3 or X′-3		126.3		125.3
2″	7.21 d (1.5)	110.6	6.89 s	105.8
3″		147.9		147.9
4″		149.3		138.4
5″	6.77 d (8.5)	115.0		147.9
6″	7.09 dd (8.5, 1.5)	122.9	6.89 s	105.8
7″	7.71 d (16.0)	146.7	7.71 d (16.0)	146.7
8″	6.46 d (16.0)	113.3	6.47 d (16.0)	113.4
9″		166.5		166.6
OCH_3	3.87 s	55.5	3.87 s	55.5
1‴ X′-4 or X′-6		124.9		125.3
2‴	6.77 s	105.5	6.77 s	105.5
3‴		147.9		147.9
4‴		138.1		138.2
5‴		147.9		147.9
6‴	6.77 s	105.5	6.77 s	105.5
7‴	7.48 d (16.0)	146.8	7.66 d (16.0)	146.5
8‴	6.26 d (16.0)	113.2	6.58 d (16.0)	114.6
9‴		166.6		168.0
OCH_3	3.78 s	55.5	3.83 s	55.5
1⁗ X′-6′		125.2		125.2
2⁗	6.82 s	105.6	6.85 s	105.6
3⁗		147.8		148.0
4⁗		138.3		138.3
5⁗		147.8		148.0
6⁗	6.82 s	105.6	6.85 s	105.6
7⁗	7.57 d (16.0)	145.7	7.57 d (16.0)	145.8
8⁗	6.48 d (16.0)	114.6	6.49 d (16.0)	114.5
9⁗		167.8		167.8
OCH_3	3.85 s	55.5	3.87 s	55.5

[a] Assignments were based on 2D NMR including HMQC and HMBC. Well-resolved couplings are expressed with coupling patterns and coupling constants in Hz in parentheses.

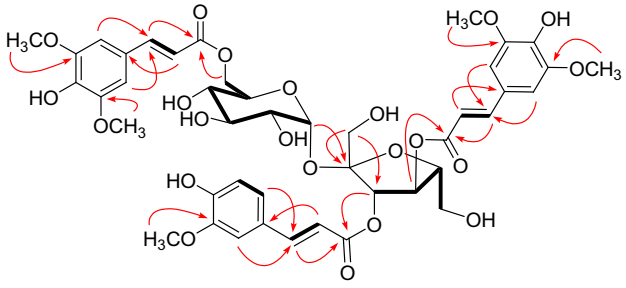

Figure 2. Key ^1H–^1H COSY (bold) and HMBCs (→) of **1**.

Table 2
Inhibitory effects on NO production of compounds **1**–**8** in LPS-activated BV-2 cells

Compound	IC_{50}[a] (μM)	Compound	IC_{50}[a] (μM)
1	34.80	**6**	45.50
2	21.63	**7**	37.40
3	48.92	**8**	>200
4	37.15	NMMA[b]	18.30
5	26.96		

[a] IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.
[b] NMMA as a positive control.

Previously, some studies reported the anti-inflammatory activities of phenylpropanoids from various plant extracts.[12–14] Particularly, among phenylpropanoid glycosides isolated from *Juniperus rigida*, citrusin D and sachaliside I induced approximately 50% inhibition of NO production at the concentration of 100 μM in LPS-stimulated RAW264.7 macrophage cells.[12] Chang et al. also reported the inhibitory effects of phenylpropanoid sucrosides from *Calamus quiquesetinervius* on NO production in LPS-stimulated murine RAW264.7 cell line.[15] However, till now the research about the effect of phenylpropanoid sucrosides from *R. sativus* on inflammation had not been reported yet. In this study, to evaluate the anti-inflammatory activities of the isolated compounds **1**–**8**, we treated them in lipopolysaccharide (LPS)-activated murine microglia BV-2 cells, and then tested for their ability to inhibit the production of the proinflammatory mediator, nitric oxide (NO) for the first time. As shown in Table 2, all compounds, except for **8**, inhibited NO production (IC_{50} values <50 μM). Among them, compounds **2** and **5** significantly inhibited NO levels with IC_{50} values of 21.63 and 26.96 μM, respectively, without affecting viability (Supplementary data). At this point, the active compounds **1**–**7** did not show the big difference in their ability to inhibit the NO production (21.63–48.92 μM) although they have three different acyl residues at C-3, C-4 (or C-6), and C-6′ regions. According to the study of Chang et al., the IC_{50} values of phenylpropanoid sucrosides which contain *trans*-coumaroyl moiety at C-4′ region, were significantly lower than *trans*-feruloyl moiety at same region.[15] However, the fact that the C-4′ position is significant in mediating the anti-inflammatory activity was not identified in default of discovery of any compounds with acyl residue at C-4′ position in this study. On the basis of the result that the sucrose (**8**) was inactive, it was deduced that the presence of acyl residues on the sucrose core in these types of molecules is essential for exerting the anti-inflammatory effect. Structure–activity relationship study applying the related diverse derivatives might be needed to elucidate the key moiety in phenylpropanoid sucrosides for inhibitory activity on NO production.

Next, the cytotoxic activities of the isolates (**1**–**8**) were evaluated by determining their inhibitory effects on human tumor cell lines including A549 (non-small cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) using the SRB bioassay.[16] Some phenylpropanoid sucrosides have been also reported to possess anti-tumor activity in recent studies.[17–20] However, no reports are available on the cytotoxic activities of the phenylpropanoid sucrosides (**1**–**7**) isolated from *R. sativus* in this study against A549, SK-OV-3, SK-MEL-2, and HCT-15 cells. The results (Table 3) showed that phenylpropanoid sucrosides **1**–**7** had consistent cytotoxicity against all of the cell lines tested, with IC_{50} values ranging from 6.71–27.92 μM. The other compound, sucrose (**8**) was inactive (IC_{50} > 30.0 μM). Recently, it was reported that substitution on phenyl ring of phenylpropanoid sucrosides did not influence the cytotoxic activity.[18,19] This hypothesis was concluded by the variable activity data for the compounds containing various

Table 3
Cytotoxicity of compounds **1**–**8** against four cultured human tumor cell lines using the SRB bioassay in vitro

Compound	IC_{50}[a] (μM)			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	26.94	23.79	16.70	18.85
2	20.82	6.71	9.50	12.37
3	25.87	19.25	18.55	14.06
4	21.05	16.69	14.15	17.20
5	26.00	24.64	21.55	18.30
6	>30.0	20.03	27.92	17.75
7	23.95	20.50	23.88	26.99
8	>30.0	>30.0	>30.0	>30.0
Doxorubicin[b]	0.002	0.012	0.001	0.129

[a] IC_{50} value of compounds against each tumor cell line, which was defined as the concentration (μM) that caused 50% inhibition of cell growth in vitro.
[b] Doxorubicin served as a positive control.

phenolic and acetyl groups on the phenyl ring.[18,19] This was also identified in this study where compound **1** and its analog compound **3** showed similar cytotoxicity against the tested cell lines and compounds **4**–**6** with different acyl residues at the same position had similar potency. However, the stronger cytotoxic properties of **1**–**7** compared to **8** clearly suggested that acyl residues on the sucrose core in phenylpropanoid sucrosides are essential for enhancing the cytotoxicity. Interestingly, among the tested compounds, compound **2** had significantly better cytotoxicity against SK-OV-3 and SK-MEL-2 cells (IC_{50} values of 6.71 and 9.50 μM, respectively) than other tested compounds. The fact that only compound **2** has the acyl residue at C-6 position encouraged us to suggest that the acyl group attached to C-6 position of the sucrose core plays a positive role in mediating the cytotoxicity against SK-OV-3 and SK-MEL-2 cells.

On the other hand, it was also reported that phenylpropanoid sucrosides showed significantly free radical-scavenging activities.[15] Thus compounds **1**–**8** were tested for radical-scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals.[21] Compounds **1**–**7** displayed free-radical scavenging activity (antioxidant activity) in the DPPH assay, and compounds **1**, **3**, and **4** had better potencies (IC_{50} < 30 μg/mL) than other active compounds. The IC_{50} values of **1**–**7** were found to be 23.05, 44.59, 27.10, 29.63, 54.98, 35.33, and 78.20 μg/mL, respectively, compared to 7.63 μg/mL for ascorbic acid, a well-known standard antioxidant. However, sucrose (**8**) was inactive. The free-radical scavenging activity of the tested compounds may be possibly due to a hydrogen-donating ability (to DPPH) provided by the hydroxy groups of acyl moieties in the molecule.

In conclusion, this is the first study investigating the anti-inflammatory, anti-tumor, and antioxidant activities of phenylpropanoid sucrosides isolated from *R. sativus*. In this study, compound **2** showed both the most significant anti-inflammatory properties in murine BV-2 microglia cells, and the highest cytotoxicity against the human tumor cell lines, SK-OV-3, SK-MEL-2 and HCT-15. Therefore, there is a possibility that it could be applicable in developing treatments for activated microglia-mediated brain diseases and tumors. The biological test results also indicated that group of phenylpropanoid sucrosides might be potentially valuable source for new potent drug candidates.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.11.001.

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