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4-Methylthio-butanyl derivatives from the seeds of *Raphanus sativus* and their biological evaluation on anti-inflammatory and antitumor activities

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ABSTRACT

Ethnopharmacological relevance: *Raphanus sativus* seeds (Brassicaceae) known as Raphani Semen have long been used as anti-cancer and/or anti-inflammatory agents in Korean traditional medicine. This study was designed to isolate the bioactive constituents from the seed extracts of *Raphanus sativus* and evaluate their anti-inflammatory and antitumor activities.

Material and methods: Bioassay-guided fractionation and chemical investigation of a methanolic extract of the seeds of *Raphanus sativus* led to the isolation and identification of seven 4-methylthio-butanyl derivatives. Structural elucidation of the isolated compounds was carried out using 1D and 2D nuclear magnetic resonance (NMR) spectroscopy techniques (^{1}H, ^{13}C, COSY, HMQC and HMBC experiments) and mass spectrometry.

Results: The isolated compounds were characterized as in the following: three new 4-methylthio-butanyl derivatives, sinapoyl desulfoglucoraphenin (**1**), (*E*)-5-(methylsulfinyl)pent-4-enoxylimidic acid methyl ester (**2**), and (*S*)-5-((methylsulfinyl)methyl)pyrrolidine-2-thione (**3**), together with four known compounds, 5-(methylsulfinyl)-4-pentenenitrile (**4**), 5-(methylsulfinyl)-pentanenitrile (**5**), sulforaphene (**6**), and sulforaphane (**7**). Full NMR data assignments of the three known compounds **4**–**6** were also reported for the first time. We evaluated the anti-neuroinflammatory effect of **1**–**7** in lipopolysaccharide-stimulated murine microglia BV2 cells. Compound **1** significantly inhibited nitrite oxide production with IC_{50} values of 45.36 μM. Moreover, it also reduced the protein expression of inducible nitric oxide synthase. 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), and all of them showed antiproliferative activity against the HCT-15 cell, with IC_{50} values of 8.49–23.97 μM.

Conclusions: 4-Methylthio-butanyl derivatives were one of the main compositions of *Raphanus sativus* seeds, and activities demonstrated by the isolated compounds support the ethnopharmacological use of *Raphanus sativus* seeds (Brassicaceae) as anti-cancer and/or anti-inflammatory agents.

1. Introduction

The most commonly consumed vegetables or edible plants in the world, well-known Brassica vegetables including broccoli, cabbage, Brussels sprouts, cauliflower, radish, garden cress, salad rocket, watercress, horseradish, and wasabi, belong to Brassicaceae family. Brassica vegetables have aroused great interest for their potential role in helping to maintain human health. They contain carotenoids, vitamin C, fiber, flavonoids, and in particular, a group of health-promoting metabolites known as glucosinolates. This is supported by strong epidemiological evidence for the association

Abbreviations: NMR, nuclear magnetic resonance; SRB, sulforhodamine B; NO, nitric oxide; LPS, lipopolysaccharide; IR, infrared; UV, ultraviolet; HR, high resolution; ESI, electrospray ionization; MS, mass spectrometry; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; HPLC, preparative high performance liquid chromatography; RP, reversed-phase; LPLC, low-pressure liquid chromatography; TLC, thin-layer chromatography; CC, column chromatography; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; NCI, national cancer institute; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; L-NMMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; iNOS, inducible nitric oxide synthase; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence

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of Brassica vegetable consumption with a highly significant cancer risk and inflammatory response reduction, and the protective effect are strongly speculated to be due to the isothiocyanates resulting from myrosinase-catalyzed hydrolysis of glucosinolates in Brassica vegetables (Jeffery and Jarrell, 2001; Holst and Williamson, 2004; Moon and Kim, 2012).

In our continuing search for bioactive constituents from Korean medicinal plants, we confirmed the potential of the seeds of *Raphanus sativus* L. (Brassicaceae) as an anticancer agent since the methanolic extract from the seeds of *Raphanus sativus* exhibited significant cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15, using a sulforhodamine B (SRB) bioassay in our screening test. Our preliminary study also confirmed that the MeOH extract has a significant inhibitory effect on nitric oxide (NO) production in the lipopolysaccharide (LPS)-stimulated BV-2 microglial cell line. One of the representatives of Brassica vegetables, *Raphanus sativus*, commonly known as radish, has been widely available throughout the world and has been consumed as a vegetable or condiment in human diets. Radish has been used as a traditional Chinese herbal medicine for more than 1400 years, since being recorded in '*Tang Materia Medica*', the first Chinese pharmacopoeia (Duan et al., 2006). Different parts of radish, including the roots, seeds, and leaves, have various medicinal properties (Nadkarni, 1976). The roots have been applied for stimulating the appetite, digestion, and the flow of bile (Chevallier, 1996) and the juice of the fresh leaves have been used as a therapeutic agent for diuretic and laxative (Chopra et al., 1986). The leaves, seeds and old roots have also been used to treat asthma and other chest complaints (Duke and Ayensu, 1985). In particular, the seeds known as Raphani Semen have long been used in Korean traditional medicine as carminative, diuretic, expectorant, laxative and stomachic agents, especially as anti-cancer and/or anti-inflammatory agents (Duke and Ayensu, 1985; Yeung, 1985; Chopra et al., 1986). From the seeds of *Raphanus sativus*, some glucosinolates responsible for above-mentioned cancer-chemoprotective property have been isolated (Daxenbichler et al., 1991; Nastruzzi et al., 1996; Barillari et al., 2005; Duan et al., 2006). Glucosinolates and/or their breakdown products have recently attracted considerable interest because of their anticancer properties. Moreover, previous studies reported that glucosinolates in Brassica genus might exert neuroprotective effect via modulation of inflammatory responses in the central nervous system (Noyan-Ashraf et al., 2005; Cuzzola et al., 2013; Giacoppo et al., 2013). At least 120 different glucosinolates have been identified, and their molecular structures share a common core of a β-D-glucopyrano moiety linked via a sulfur atom to a (*Z*)-*N*-hydroximinosulfate ester group and a variable aglycone side chain derived from α-amino acid biosynthetic precursors (Fahey et al., 2001).

Our interest in the research on new bioactive constituents from the seeds of *Raphanus sativus* led us to investigate the plant source. Using the bioactivity-guided isolation techniques, three new 4-methylthio-butanyl derivatives (**1–3**) were isolated from the most active $CHCl_3$-soluble fraction of the methanolic extract, together with four known compounds (**4–7**). This paper reports the isolation and structural elucidation of compounds **1–7** (Fig. 1) and their antitumor and anti-neuroinflammatory activities.

2. Material and methods

2.1. General

Optical rotations were measured on a Jasco P-1020 polarimeter using methanol or acetone as a solvent. Infrared (IR) spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. Ultraviolet (UV) spectra were recorded with a Shimadzu UV-1601 UV–visible spectrophotometer. High resolution (HR)-electrospray ionization mass spectrometry (ESIMS) and ESIMS spectra were recorded on a Micromass QTOF2-MS. Nuclear magnetic resonance (NMR) spectra, including the spectra in the ^1H–^1H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC) experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer operating at 500 MHz (^1H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Preparative high performance liquid chromatography (HPLC) used a Gilson 306 pump with a Shodex refractive index detector. Silica gel 60 (Merck, 230–400 mesh) and reversed-phase (RP)–C_{18} silica gel (Merck, 230–400 mesh) were used for column chromatography. Low-pressure liquid chromatography (LPLC) was carried out over a LiChroprep Lobar-A Si 60 column (240 mm × 10 mm i.d.; Merck) with a FMI QSY-0 pump. The packing material for molecular sieve column chromatography was Sephadex LH-20 (Pharmacia Co. Ltd.). Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates were used for thin-layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde–sulfuric acid.

2.2. Plant material

The seeds of *Raphanus sativus* were purchased at Kyungdong herbal market, Seoul, Korea, in January 2010, and the plant material was identified by one of the authors (K.R. Lee). A voucher specimen (SKKU-2010-01) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

2.3. Extraction and isolation

The dried seeds of *Raphanus sativus* (3.6 kg) were ground and extracted with 80% aqueous MeOH twice at room temperature and filtered. The filtrate was evaporated in vacuo to obtain a crude extract (325 g), which was then fractionated by sequential liquid–liquid partitioning of H_2O with *n*-hexane, $CHCl_3$, and *n*-BuOH to yield 12, 8, and 30 g of residues, respectively. Each fraction was evaluated for cytotoxicity against A549, SK-OV-3, SK-MEL-2, HCT-15 cells using a SRB assay. The $CHCl_3$-soluble fraction showed the most significant cytotoxic activity against the tested tumor cell lines. The active $CHCl_3$-soluble fraction also strongly inhibited NO production in LPS-stimulated BV-2 microglial cells in our screening test.

The $CHCl_3$-soluble fraction (8 g) was subjected to column chromatography (CC) on reversed phase C18 column using a gradient of increasing MeOH in H_2O from 40% to 100% to give 13 fractions (C1–C13) in consideration of a similar TLC pattern. Fraction C1 (230 mg) was reapplied to a silica gel column with $CHCl_3$–MeOH–H_2O (9:4:0.5) to obtain three subfractions (C11–C13). Subfraction C13 (65 mg) was then subjected to semi-preparative normal-phase HPLC using a 250 mm × 10 mm i.d., 5 μm, Apollo Silica column (Alltech Co. Ltd.) with a solvent system of $CHCl_3$–MeOH–H_2O (9:6:1, flow rate; 2 mL/min) to afford **7** (3 mg, t_R 13.5 min). Further separation of fraction C2 (840 mg) by silica gel CC was performed with $CHCl_3$–MeOH (20:1) to give four subfractions (C21–C24). Subfraction C21 (45 mg) was then applied to semi-preparative reversed-phase HPLC using a 250 mm × 10 mm i.d., 10 μm, Econosil RP-18 column (Alltech Co. Ltd.) with a solvent system of MeOH–H_2O (2:3, flow rate; 2 mL/min) to yield **2** (25 mg, t_R 16.0 min). Subfraction C22 (250 mg) was subjected to semi-preparative reversed-phase HPLC with MeOH–H_2O (2:3, flow rate; 2 mL/min) to furnish **3** (7 mg, t_R 17.5 min) and **4** (50 mg, t_R 15.0 min). Subfraction C23 (40 mg) was purified by semi-preparative reversed-phase HPLC using MeOH–H_2O (2:3, flow rate; 2 mL/min) for elution to afford **5** (6 mg, t_R 14.5 min). Subfraction C24 (450 mg) was passed through a Sephadex LH-20 column with MeOH–H_2O (9:1) as eluent,

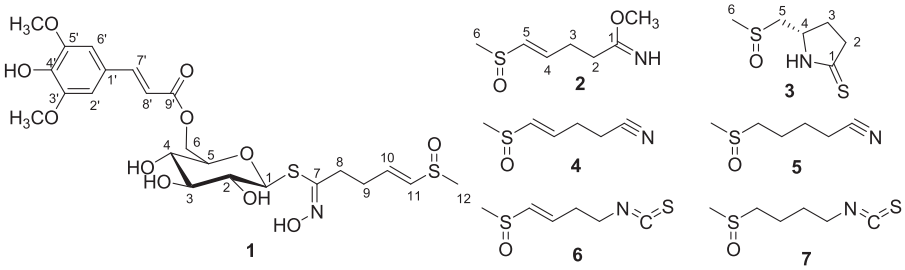

Fig. 1. Structures of compounds **1**–**7**.

Table 1
^{1}H and ^{13}C NMR data of compound **1** in CD_3OD[a].

Position	**1**	
	δ_H	δ_C
1	4.96 d (9.5)	81.9
2	3.30 m	72.5
3	3.38 m	78.3
4	3.41 m	70.3
5	3.62 m	79.1
6	4.50 dd (12.0, 2.0)	64.0
	4.34 dd (12.0, 6.0)	
7		155.8
8	2.72 t (7.0)	31.8
9	2.60 dt (7.0, 7.0)	27.30
10	6.46 dt (15.0, 7.0)	133.9
11	6.67 d (15.0)	135.8
12	2.67 s	39.1
1′		126.2
2′	6.93 s	107.0
3′		148.3
4′		139.0
5′		148.3
6′	6.93 s	107.0
7′	7.63 d (16.0)	147.5
8′	6.46 d (16.0)	116.1
9′		169.0
OCH_3	3.87 s	55.5

[a] ^{1}H and ^{13}C NMR data were recorded at 500 and 125 MHz, respectively. Coupling constants (in Hz) are given in parentheses.

Table 2
^{1}H and ^{13}C NMR data of compounds **2** and **3** in $CDCl_3$.[a]

Position	**2**		**3**	
	δ_H	δ_C	δ_H	δ_C
1		172.7		206.3
2	2.46 t (6.5)	32.6	2.70 m	41.2
3	2.54 dt (6.5, 6.5)	27.1	2.60 m; 2.14 m	31.2
4	6.44 dt (15.0, 6.5)	135.5	4.36 m	43.7
5	6.32 d (15.0)	137.8	3.17 dd (13.0, 5.5)	59.7
			3.01 dd (13.0, 9.0)	
6	2.57 s	40.9	2.66 s	39.4
OCH_3	3.66 s	51.9		

[a] ^{1}H and ^{13}C NMR data were recorded at 500 and 125 MHz, respectively. Coupling constants (in Hz) are given in parentheses.

Table 3
^{1}H and ^{13}C NMR data of compounds **4** and **5** in $CDCl_3$ and compound **6** in CD_3OD.[a]

Position	**4**		**5**		**6**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		118.5		119.1		132.0
2	2.49 t (6.5)	16.6	2.43 t (7.0)	17.2	3.72 t (6.5)	43.9
3	2.56 dt (6.5, 6.5)	27.5	1.84 m	24.7	2.65 t (6.5)	32.3
4	6.37 dt (16.0, 6.5)	134.3	1.98 m	22.2	6.45 dt (15.0, 6.5)	135.0
5	6.44 d (16.0)	137.2	2.73 t (7.0)	53.6	6.70 d (15.0)	136.9
6	2.58 s	40.7	2.59 s	39.0	2.69 s	39.2

[a] ^{1}H and ^{13}C NMR data were recorded at 500 and 125 MHz, respectively. Coupling constants (in Hz) are given in parentheses.

which gave **1** (16 mg). Fraction C5 (530 mg) was applied to LPLC on a 240 mm × 10 mm i.d., 40–63 μm, LiChroprep Lobar-A Si 60 column (Merck) with a solvent system of *n*-hexane–EtOAc (20:1) to give three subfractions (C51–C53). Subfraction C53 (75 mg) was then separated by semi-preparative normal-phase HPLC using a solvent system of $CHCl_3$–MeOH (30:1, flow rate; 2 mL/min) to give **6** (6 mg, t_R 15.0 min).

*2.3.1. Sinapoyl desulfoglucoraphenin (**1**)*

Yellowish gum; $[\alpha]_D^{25}$-15.6 (*c* 0.70, MeOH); UV (MeOH) λ_{max} nm (log ε): 326 (0.9), 285 (3.1), 232 (1.5), 218 (3.7); IR (KBr) ν_{max} cm^{-1}: 3583, 3395, 2948, 2837, 2507, 2239, 2074, 1663, 1452, 1120, 1037, 980, 674; ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data, see Table 1; ESIMS (positive-ion mode) *m/z* 584 $[M+Na]^+$; HR-ESIMS (positive-ion mode) *m/z* 584.1240 $[M+Na]^+$ (calcd. for $C_{23}H_{31}NNaO_{11}S_2$, 584.1236).

*2.3.2. (E)-5-(Methylsulfinyl)pent-4-enoxylimidic acid methyl ester (**2**)*

Colorless gum; $[\alpha]_D^{25}$-41.8 (*c* 0.85, MeOH); UV (MeOH) λ_{max} nm (log ε) 227 (3.1); IR (KBr) ν_{max} cm^{-1}: 3358, 2946, 2832, 1725, 1665, 1450, 1116, 1031, 674; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 2; ESIMS (positive-ion mode) *m/z* 176 $[M+H]^+$; HR-ESIMS (positive-ion mode) *m/z* 176.0741 $[M+H]^+$ (calcd. for $C_7H_{14}NO_2S$, 176.0745).

*2.3.3. (S)-5-((Methylsulfinyl)methyl)pyrrolidine-2-thione (**3**)*

Colorless gum; $[\alpha]_D^{25}$ + 16.2 (*c* 0.35, acetone); UV (MeOH) λ_{max} nm (log ε) 273 (3.8), 230 (3.4); IR (KBr) ν_{max} cm^{-1}: 3357, 2945, 2832, 2524, 1710, 1451, 1115, 1030, 674; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 2; ESIMS (positive-ion mode) *m/z* 178 $[M+H]^+$; HR-ESIMS (positive-ion mode) *m/z* 178.0362 $[M+H]^+$ (calcd. for $C_6H_{12}NOS_2$, 178.0360).

*2.3.4. 5-(Methylsulfinyl)-4-pentenenitrile (**4**)*

Colorless gum; $[\alpha]_D^{25}$-15.7 (*c* 0.80, MeOH); UV (MeOH) λ_{max} nm (log ε) 229 (3.4); IR (KBr) ν_{max} cm^{-1}: 3382, 2945, 2832, 1661, 1424, 1115, 1031, 771; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 3; ESIMS (positive-ion mode) *m/z* 166 $[M+Na]^+$.

*2.3.5. 5-(Methylsulfinyl)-pentanenitrile (**5**)*

Colorless gum; $[\alpha]_D^{25}$-5.1 (*c* 0.30, MeOH); UV (MeOH) λ_{max} nm (log ε) 260 (3.2); IR (KBr) ν_{max} cm^{-1}: 3357, 2946, 2832, 1660, 1451, 1116, 1031, 674; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Table 3; ESIMS (positive-ion mode) *m/z*: 168 $[M+Na]^+$.

*2.3.6. Sulforaphene (**6**)*

Colorless gum; $[\alpha]_D^{25}$-13.2 (*c* 0.30, MeOH); UV (MeOH) λ_{max} nm (log ε) 246 (3.0); IR (KBr) ν_{max} cm^{-1}: 3357, 2946, 2832, 1661, 1424, 1116, 1031, 674; ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data, see Table 3; ESIMS (positive-ion mode) *m/z*: 198 $[M+Na]^+$.

2.4. Cell cutures

Murine microglia BV2 was maintained in Dulbecco's modified Eagle medium (DMEM), supplemented with 5% fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin. All cells were incubated at 37 °C in a humidified incubator with 5% CO_2. All tumor cell cultures were maintained using RPMI1640 cell growth medium (Gibco, Carlsbad, CA), supplemented with 5% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Human tumor cell lines such as A549 (non-small cell lung carcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) were provided by the National Cancer Institute (NCI).

2.5. Measurement of nitric oxide production and cell viability

BV-2 cells were plated into a 96-well plate (3×10^4 cells/well). After 24 h, cells were pretreated with compounds **1**–**7** for 30 min, and then stimulated with 100 ng/ml of LPS for another 24 h. Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 570 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate the NO_2concentration. Cell viability was assessed by a 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. N^G-monomethyl-L-arginine (L-NMMA, Sigma, St. Louis, MO, USA), a well-known nitric oxide synthase (NOS) inhibitor, was tested as a positive control (Reif and McCreedy, 1995).

2.6. Western blot analysis

BV-2 cells were seeded in a 6-well plate and exposed to LPS (100 ng/ml) in the presence or absence of compounds **1** (20 and 50 μg for 6 h). Protein samples from the cell extracts were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk and incubated with primary antibodies against inducible nitric oxide synthase (iNOS) (BD Transduction Laboratories, San Diego, CA, USA). After washing with Tris-buffered saline-Tween 20, Horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied. The blots were developed using enhanced chemiluminescence (ECL) Western Blotting Detection Reagents.

2.7. Cytotoxicity assessment

The cytotoxicity of the compounds against cultured human tumor cell lines was evaluated by the SRB method (Skehan et al., 1990). Each tumor cell line was inoculated over standard 96-well flat-bottom microplates and then incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The attached cells were then incubated with the serially diluted lignan samples. After continuous exposure to the compounds for 48 h, the culture medium was removed from each well and the cells were fixed with 10% cold trichloroacetic acid at 4 °C for 1 h. After washing with tap water, the cells were stained with 0.4% SRB dye and incubated for 30 min at room temperature. The cells were washed again and then solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance was measured spectrophotometrically at 520 nm with a microtiter plate reader. Doxorubicin was used as a positive control.

3. Results and discussion

The 80% aqueous methanolic extract from the seeds of *Raphanus sativus* was sequentially partitioned from water into *n*-hexane, $CHCl_3$, and *n*-BuOH. Each fraction was evaluated for cytotoxicity against A549, SK-OV-3, SK-MEL-2, HCT-15 cells in a SRB bioassay and for anti-inflammatory activity by measuring NO production in LPS-stimulated BV-2 microglial cells as our screening test. Subsequent chromatographic purification of the most active $CHCl_3$-soluble fraction led to the isolation of three new 4-methylthio-butanyl derivatives (**1–3**), together with four other known compounds. The structures of these compounds were established mainly on the basis of analysis using extensive NMR spectroscopy techniques and mass spectrometry.

Compound **1** was obtained as a yellowish gum. The molecular formula was established as $C_{23}H_{31}NO_{11}S_2$ by the molecular ion peak $[M+Na]^+$ at *m/z* 584.1240 (calcd. for $C_{23}H_{31}NNaO_{11}S_2$, 584.1236) in the positive-ion HR-ESIMS. The IR absorptions of **1** implied the presence of C=N (1663 cm^{-1}), S=O (1037 cm^{-1}) and OH (3583 and 3395 cm^{-1}) functional groups and its UV maximum absorption at 285 nm suggested the presence of a conjugated phenolic unit in **1**. The ^1H NMR data (Table 1) of **1** showed the presence of (*E*)-sinapoyl moiety in the consideration of the presence of typical signals including a symmetric aromatic proton at δ_H 6.93 (2H, s), a (*E*)-configured double bond proton at δ_H 7.63 (1H, d, *J*=16.0 Hz) and 6.46 (1H, d, *J*=16.0 Hz), and a symmetric methoxyl proton at δ_H 3.87 (6H, s). The ^1H NMR spectrum (Table 1) also showed the presence of another (*E*)-configured C=C bond proton at δ_H 6.67 (1H, dd, *J*=15.0 Hz) and 6.46 (1H, dt, *J*=15.0, 7.0 Hz), two methylene protons at δ_H 2.72 (2H, t, *J*=7.0 Hz) and 2.60 (2H, dt, *J*=7.0, 7.0 Hz), and a methylsulfinyl group at δ_H 2.67 (3H, s). Additionally, the ^1H and ^{13}C NMR spectra displayed the signals for a sugar moiety at δ_H 4.96 (1H, d, *J*=9.5 Hz), 4.50 (1H, dd, *J*=12.0, 2.0 Hz), 4.34 (1H, dd, *J*=12.0, 6.0 Hz), 3.62 (1H, m), 3.41 (1H, m), 3.38 (1H, m), and 3.30 (1H, m) and δ_C 81.9, 79.1, 78.3, 72.5, 70.3, and 64.0. The anomeric signal at δ_C 81.9 in the ^{13}C NMR spectrum indicated that the sugar was linked to an *S*-atom, as in other 1-thioglucosides, and this sugar was finally identified as a 1-thio-β-D-glucoside on the basis of comparing its spectroscopic data with values reported previously (Olsson et al., 1977; Barillari et al., 2005) and the analysis of ^1H–^1H COSY, HMQC, HMBC, and NOESY data. Moreover, inspection of the ^1H and ^{13}C NMR data revealed that the ^1H and ^{13}C NMR spectra of **1** were quite similar to those of desulfo-glucoraphasatin (Barillari et al., 2005), with apparent differences being the additional signals for the (*E*)-sinapoyl group above mentioned and replacement of a methylthiol group with a methylsulfinyl group (δ_H 2.67) in **1**. The structure was confirmed by analysis of the ^1H–^1H COSY, HMQC, and HMBC spectra (Fig. 2). Especially, the correlation of H-6 at δ_H 4.34 with C-9' at δ_C 169.0 in the HMBC spectrum of **1** suggested that the (*E*)-sinapoyl moiety was esterified to the 6-OH function of the sugar (Fig. 2). On the basis of these considerations, compound **1** was determined as sinapoyl desulfoglucoraphenin.

The positive-ion HR-ESIMS mass spectrum of **2** showed a molecular ion peak at *m/z* 176.0741 $[M+H]^+$ ascribable to the molecular formula $C_7H_{13}NO_2S$ (calcd. for $C_7H_{14}NO_2S$, 176.0745). The IR spectrum displayed absorptions at 1665 and 1031 cm^{-1} indicating the presence of C=N and S=O functionalities, respectively, and the UV maximum absorption at 227 nm suggested the

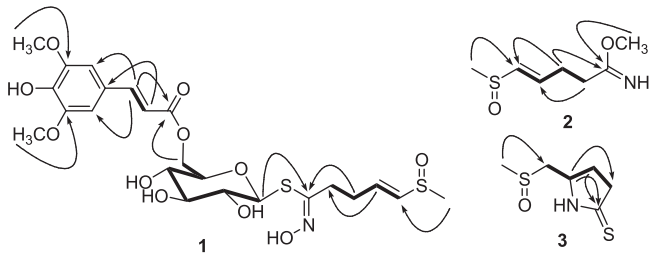

Fig. 2. ^{1}H–^{1}H COSY (bold lines) correlations and key HMBC (arrows) of **1–3**.

presence of a double bond in **2**. The ^{1}H NMR spectrum (Table 2) showed the presence of (*E*)-configured C=C bond protons at δ_H 6.44 (1H, dd, J=15.0, 6.5 Hz) and 6.32 (1H, d, J=15.0 Hz), two methylene protons at δ_H 2.54 (2H, dt, J=6.5, 6.5 Hz) and 2.46 (2H, t, J=6.5 Hz), a methylsulfinyl group at δ_H 2.57 (3H, s) and a methoxyl group at δ_H 3.66 (3H, s). The NMR data (Table 2) of **2** were similar to those of 5-(methylsulfinyl)-4-pentenenitrile (**4**) but with apparent differences in the chemical shift at δ_C 172.7 (C-1), instead of the corresponding one at δ_C 118.5 in **4**, and an additional methoxyl signal at δ_C 51.9. This structure was confirmed by analysis of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra (Fig. 2), and determination of the linkage of the methoxyl group was finally obtained from the HMBC spectrum, which showed the correlation between the methoxyl signal at δ_H 3.66 and the carbon resonance at δ_C 172.7 (C-1). On the basis of this evidence, the structure of compound **2** was established as (*E*)-5-(methylsulfinyl) pent-4-enoxylimidic acid methyl ester.

The positive-ion HR-ESIMS mass spectrum of **3** (*m/z* 178.0362 $[M+H]^+$, calcd. for $C_6H_{12}NOS_2$, 178.0360) supported a molecular formula of $C_6H_{11}NOS_2$. The ^{1}H NMR spectrum (Table 2) showed the presence of a methine proton at δ_H 4.36 (1H, m), three methylene protons at δ_H 3.17 (1H, dd, J=13.0, 5.5 Hz); 3.01 (1H, dd, J=13.0, 9.0 Hz), 2.70 (2H, m) and 2.60 (1H, m); 2.14 (1H, m), and a methylsulfinyl group at δ_H 2.66 (3H, s). The NMR data (Table 2) of **3** were similar to those of (*S*)-4,5-dihydro-5-hydroxymethylpyrrole-2(3*H*)-thione (Busque et al., 2002), with an apparent difference being the additional signal for the methylsulfinyl group [δ_H 2.66 (3 H, s); δ_C 39.4] in **3**. The carbon resonance at δ_C 206.3 to corresponding one of (*S*)-4,5-dihydro-5-hydroxymethylpyrrole-2 (3*H*)-thione allowed us to determine that they shared the same structural framework (Busque et al., 2002). This structure was established by analysis of the ^{1}H–^{1}H COSY, HMQC, and HMBC spectra (Fig. 2), and the assignment of the methylsulfinyl group at C-5 was deduced by the HMBC correlation between the proton signal at δ_H 2.66 and the carbon resonance at δ_C 59.7 (C-5). The configuration of **3** was determined as *S* by the comparison of its specific rotation, $[\alpha]_D^{25}$ +16.2 (*c* 0.35, acetone) with that of (*S*)-4,5-dihydro-5-hydroxymethylpyrrole-2(3*H*)-thione (Busque et al., 2002). Thus, the structure of **3** was assigned as (*S*)-5-((methylsulfinyl)methyl)pyrrolidine-2-thione.

Compounds **4–6** are known compounds reported previously. But, their NMR data have not been reported, although ^{1}H NMR data of **5** obtained from the seeds of broccoli was published (Kore et al., 1993). The full assignments of ^{1}H and ^{13}C NMR chemical shifts (Table 3) for **4–6** were obtained by 2D NMR analysis (including ^{1}H–^{1}H COSY, HMQC, and HMBC) in this study for the first time. The other known compound was identified as sulforaphane (**7**) (Liang et al., 2007) by comparing its spectroscopic data with the values reported previously.

Microglial cells, the immune resident cells of the brain, are principally responsible for immune defense in the central nervous system. However, under pathological conditions, microglia cells are over-activated and then produce a variety of proinflammatory mediators including nitric oxide (NO) (McGeer et al., 1993).

Table 4
Inhibitory effect of compounds **1–7** on NO production in LPS-stimulated BV-2 cells.

Compound	IC_{50} (μM)[a]	Compound	IC_{50} (μM)
1	45.36	**5**	158.79
2	189.54	**6**	> 500
3	463.75	**7**	> 500
4	318.52	**NMMA**[b]	21.04

[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 was used as a positive control.

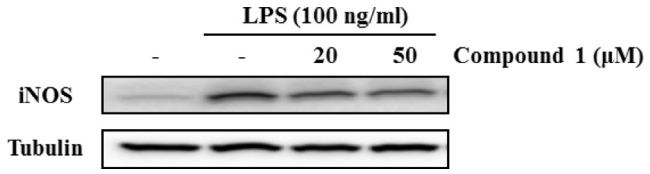

Fig. 3. Inhibitory effect of compounds **1** on iNOS expression in LPS-stimulated BV-2 cells. To investigate the effect of compound **1** on iNOS expression, BV-2 cells were pretreated with compound **1** for 30 min and then stimulated with LPS for 6 h. Then, Western blotting analysis was performed.

Table 5
Cytotoxicity of compounds **1–7** against four cultured human cancer cell lines in the SRB bioassay.

Compound	IC_{50} (μM)[a]			
	A549	SK-OV-3	SK-MEL-2	HCT-15
1	> 30.0	28.32 ± 1.87[b]	13.16 ± 1.16	14.08 ± 2.34
2	> 30.0	29.25 ± 1.78	18.55 ± 0.82	14.06 ± 1.30
3	> 30.0	> 30.0	> 30.0	13.53 ± 0.95
4	> 30.0	> 30.0	19.76 ± 1.20	14.00 ± 2.07
5	> 30.0	> 30.0	> 30.0	18.06 ± 1.58
6	> 30.0	> 30.0	> 30.0	23.97 ± 2.33
7	> 30.0	> 30.0	> 30.0	8.49 ± 0.89
Doxorubicin[c]	0.007 ± 0.002	0.011 ± 0.003	0.001 ± 0.001	0.036 ± 0.007

[a] 50% Inhibitory concentration; the concentration of compound that caused a 50% inhibition in cell growth.
[b] Data are expressed as mean ± SEM of three independent experiments.
[c] Doxorubicin as a positive control.

To investigate the effect of the compounds (**1–7**) isolated from the seeds of *Raphanus sativus* on neuroinflammation, we measured NO levels in murine microglia BV2 cells stimulated by bacterial pathogen, LPS. As shown in Table 4, among compounds **1–7**, compound **1** significantly reduced NO levels in the medium with IC_{50} values of 45.36 μM. Compounds **2** and **5** showed IC_{50} values ranging from 158.79 to 189.54 μM, while IC_{50} values of the rest were more than 300 μM. None of the compounds showed any significant cellular toxicity up to 100 μM (data not shown). In microglia, NO production is regulated primarily by inducible nitric oxide synthase (iNOS). Therefore, the effect of treatment with compound **1** on iNOS expression was examined. As shown in Fig. 3, compound **1** led to a significant decrease in iNOS protein level at 20 and 50 μM. Therefore, compound **1** may reduce NO production via inhibition of iNOS protein expression in LPS-stimulated BV-2 cells. Previous studies showed that glucoraphanin might exert a neuroprotective effect via regulation of inflammation in the brain (Noyan-Ashraf et al., 2005; Cuzzola et al., 2013; Giacoppo et al., 2013). We suggest that the anti-neuroinflammatory activity of compound **1** may be associated with its structural similarity to glucoraphanin.

To evaluate compounds **1–7** as cytotoxic agents, we also performed an evaluation of their antiproliferative activities against

the A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines using the SRB bioassay (Skehan et al., 1990). All compounds tested showed antiproliferative activity against the HCT-15 cell, with IC_{50} values of 8.49–23.97 μM (Table 5), but most of the compounds were inactive ($IC_{50} > 30.0$ μM) against the other human tumor cell lines tested. Nevertheless, compounds **1** and **2** exhibited weak antiproliferative activity against the SK-OV-3 cell with IC_{50} values of 28.32 and 29.25 μM, respectively, whereas compounds **1**, **2** and **4** displayed moderate cytotoxic activity against the SK-MEL-2 cell with IC_{50} values of 13.16, 18.55 and 19.76 μM, respectively.

4. Conclusions

This work shows that the seeds of *Raphanus sativus* are rich in 4-methylthio-butanyl derivatives as the main bioactive constituents with antitumor and anti-inflammatory effects and the observed activity supports the traditional use of the plant. In particular, sinapoyl desulfoglucoraphenin (**1**), which showed good anti-neuroinflammatory activity through inhibition of NO production via suppression of iNOS expression in microglia, could be useful for the development of novel neuroprotective agents.

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