Lipids (2016) 51:989–995
DOI 10.1007/s11745-016-4172-7

COMMUNICATION

Neuroprotective Fatty Acids from the Stem Bark of *Sorbus commixta*

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Received: 10 March 2016 / Accepted: 26 May 2016 / Published online: 7 July 2016

Abstract Phytochemical investigation of the bark from the stems of *Sorbus commixta* led to the isolation and characterization of a new fatty acid, sorcomic acid (**1**), along with nine known analogues (**2**–**10**). The structure of the new compound (**1**) was determined through NMR (^{1}H and ^{13}C NMR, HSQC, HMBC, and NOESY), MS, and specific optical rotation. The known compounds (**2**–**10**) were identified by comparison of their spectroscopic data with those in the literature. The biological activities of all the isolated compounds (**1**–**10**) were evaluated: compounds **1**, **5**, and **7** potently induced NGF secretion from C6 glioma cells (233.40 ± 12.82, 194.40 ± 8.05, and 185.74 ± 10.25 %, respectively) and compound **10** reduced NO levels with an IC_{50} value of 71.25 μM in murine microglia BV2 cells stimulated by LPS.

Keywords *Sorbus commixta* · Rosaceae · Fatty acids · Neuroprotection · Anti-neuroinflammation

Electronic supplementary material The online version of this article (doi:10.1007/s11745-016-4172-7) contains supplementary material, which is available to authorized users.

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Abbreviations

COSY	Correlation spectroscopy
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FAB	Fast atom bombardment
HMBC	Heteronuclear multiple bond correlation
HPLC	High-performance liquid chromatography
HR	High resolution
HSQC	Heteronuclear single quantum coherence
LPS	Lipopolysaccharide
MS	Mass spectrometry
MTPA	Methoxy-α-(trifluoromethyl)phenylacetyl acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NGF	Nerve growth factor
NMR	Nuclear magnetic resonance
L-NMMA	N^{G}-Monomethyl-L-arginine
NO	Nitric oxide
NOESY	Nuclear Overhauser effect spectroscopy
NOS	Nitric oxide synthase
SRB	Sulforhodamine B
TLC	Thin-layer chromatography

Introduction

Sorbus commixta Hedl. (Rosaceae), also known as Mountain ash, is a tree that grows in mountainous regions and is widely distributed throughout Korea. The stem bark of this plant has been used in Korean traditional medicine for the treatment of cough, asthma, bronchial disorders, gastritis, and dropsy [1, 2]. Previous studies have shown that *S. commixta* has antioxidative, anti-atherogenic, anti-inflammatory, anti-sclerotic, and vascular relaxant activities [3]. The water extract of *S. commixta* inhibited the inflammatory

response in LPS-stimulated macrophages [4]. The neuroprotective effect of the ethanolic extract of *S. commixta* was reported to attenuate symptoms of Alzheimer's disease [5]. Additionally, cancer and inflammation have a very close relationship; chronic inflammation is one of the major causes of cancer [6]. The aforementioned research suggests that *S. commixta* and its active constituents may have potential neuroprotective effects. In this study we analyzed the anti-inflammatory effect of those fatty acids via down-regulation of NO release and neuroprotective effect via regulating NGF levels together with anticancer activity of isolated compounds. In the course of our continuing search for bioactive constituents from Korean medicinal plants [7–12], we isolated and identified potential neuroprotective compounds from the stem bark of *S. commixta*. The column chromatographic purification of the $CHCl_3$ fraction of *S. commixta* MeOH extract led to isolation of a new fatty acid, sorcomic acid (**1**), along with nine known analogues (**2–10**) (Fig. 1). The chemical structure of the new compound (**1**) was determined through spectroscopic data (NMR and MS) and specific optical rotation. The neuroprotective and anti-inflammatory activities of all the isolated compounds (**1–10**) were evaluated. Compounds **1**, **5**, and **7** potently induced NGF secretion from C6 glioma cells (233.40 ± 12.82, 194.40 ± 8.05, and 185.74 ± 10.25 %, respectively) and compound **10** reduced NO levels with an IC_{50} value of 71.25 μM in murine microglia BV2 cells stimulated by LPS.

Materials and Methods

General Experimental Procedures

Optical rotations were measured on a Jasco P-1020 polarimeter (Jasco, Easton, MD, USA). IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer (Bruker, Karlsruhe, Germany). HRFABMS spectra were obtained on a JEOL JMS700 mass spectrometer (JEOL, Tokyo, Japan). NMR spectra were recorded on a Varian Unity INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C) and a Bruker AVANCE III 700 NMR spectrometer at 700 MHz (^{1}H) and 175 MHz (^{13}C). Semi-preparative HPLC was performed with a Gilson 306 pump (Gilson, Middleton, WI, USA) with a Shodex refractive index detector (Shodex, New York, NY, USA). Column chromatography was performed with silica gel 60 (230–400 mesh, Merck, Darmstadt, Germany). Merck precoated silica gel F_{254} plates and reversed-phase (RP)-18 F_{254s} plates (Merck, Darmstadt, Germany) were used for TLC. Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde–sulfuric acid.

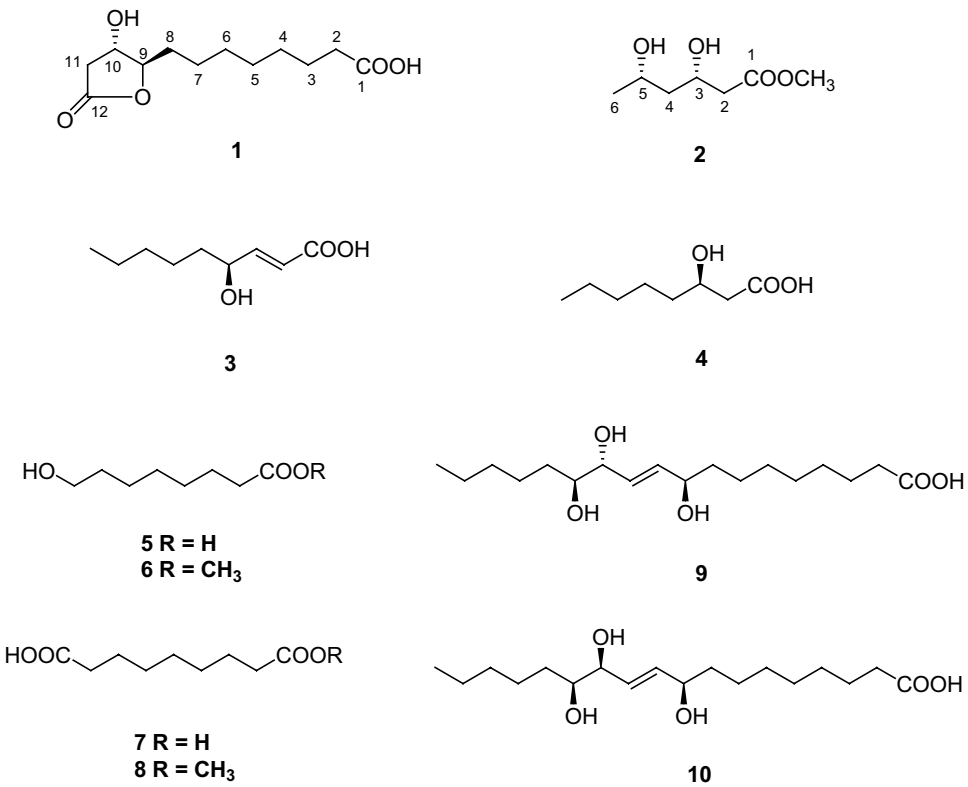

Fig. 1 Chemical structures of compounds **1–10**

Table 1 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **1**

Position	**1**	
	δ_H	δ_C
1		178.1
2	2.28, t (6.9)	35.4
3	1.60, overlap	26.3
4	1.35, overlap	30.4
5	1.35, overlap	30.3
6	1.35, overlap	30.4
7	1.43, overlap	26.5
8	1.62, overlap	34.0
9	4.29, ddd (8.2, 5.5, 2.8)	90.0
10	4.20, ddd (6.5, 3.4, 2.8)	72.3
11α	2.38, dd (17.9, 3.4)	38.2
11β	2.88, dd (17.9, 6.5)	
12		178.3

δ in ppm, *J* values in parentheses

Table 2 ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR data of compound **2**

Position	**2**	
	δ_H	δ_C
1		174.0
2a	2.55, dd (15.2, 4.6)	43.4
2b	2.46, dd (15.2, 8.3)	
3	4.19, m	68.2
4a	1.68, ddd (13.9, 8.6, 7.8)	46.5
4b	1.57, ddd (13.9, 5.2, 4.6)	
5	3.96, m	67.1
6	1.21, d (6.2)	33.7
OCH_3	3.70, s	52.2

δ in ppm, *J* values in parentheses

Plant Material

The bark of *S. commixta* stems was collected in Yangyang, Gangwon-do, Republic of Korea in June 2012, and the plant was identified by one of the authors (K.R.L.). A voucher specimen (SKKU-NPL 1210) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea.

Extraction and Isolation

The stem bark of *S. commixta* (8.0 kg) was extracted with 80 % aqueous MeOH under reflux and filtered. The filtrate was evaporated under reduced pressure to obtain the MeOH extract (1.3 kg), which was suspended in distilled H_2O and successively partitioned with hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding 5, 13, 43, and 160 g, respectively. The $CHCl_3$-soluble fraction (13 g) was separated over a silica gel column ($CHCl_3$/MeOH, 50:1 → 1:1) to yield 12 fractions (C1–C12). Fraction C8 (500 mg) was chromatographed on an RP-C_{18} silica gel column with 50 % aqueous MeOH to give 14 subfractions (C8-1–C8-12). Subfraction C8-1 (15 mg) was purified by semi-preparative HPLC (2 mL/min, 20 % aqueous MeOH) to give compound **2** (4 mg). Subfractions C8-5 (40 mg), C8-6 (30 mg), C8-7 (20 mg), C8-8 (25 mg), and C8-10 (35 mg) were purified by semi-preparative HPLC with 30–60 % aqueous MeOH to yield compounds **5** (8 mg), **1** (4 mg), **7** (15 mg), **6** (3 mg), and **8** (8 mg), respectively. Fraction C11 (900 mg) was separated on an RP-C_{18} silica gel column with 50 % aqueous MeOH to give 12 subfractions (C11-1–C11-12). Compounds **4** (2 mg) and **3** (7 mg) were obtained by purifying subfractions C11-6 (20 mg) and C-11-7 (30 mg) using semi-preparative HPLC with 60 % aqueous MeOH. Subfraction C11-10 (130 mg) was purified by semi-preparative HPLC with 65 % aqueous MeOH to yield compounds **9** (12 mg) and **10** (5 mg).

Sorcomic Acid (1)

Colorless gum, $[\alpha]_D^{25}$ + 28.0 (*c* 0.30, MeOH); IR (KBr) ν_{max} 3343 (OH), 2942, 1717 (C=O), 1263, 1084 cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; HRFABMS *m/z* 245.1385 $[M + H]^+$ (calcd. for $C_{12}H_{21}O_5$, 245.1389).

Methyl (3S,5S)-3,5-dihydroxyhexanoate (2)

Colorless gum, $[\alpha]_D^{25}$ + 20.4 (*c* 0.30, MeOH); ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR data, see Table 2; HRFABMS *m/z* 163.0968 $[M + H]^+$ (calcd. for $C_7H_{15}O_4$, 163.0970).

Preparation of Mosher Ester Derivatives 2r and 2s

Compound **2** (0.5 mg) in deuterated pyridine (0.6 mL) was transferred into a clean NMR tube. (*S*)-(+)-α-MTPA-Cl (10 μL) (Sigma, St. Louis, MO, USA) was added immediately into the NMR tube under a stream of N_2 gas, and then the NMR tube was shaken carefully to mix the sample and MTPA-Cl evenly. The NMR reaction tube was left at room temperature overnight to afford the (*R*)-MTPA ester derivative (**2r**) of **2**. The (*S*)-MTPA ester derivative of **2** (**2s**) was obtained as described for **2r**. The ^{1}H NMR spectra of **2r** and **2s** were measured directly in the NMR reaction tubes.

NGF and Cell Viability Assays

In this study, C6 glioma cells were used to measure the release of NGF into the culture medium. C6 cells were seeded onto 24-well plates at a density of 1×10^5 cells/well. After 24 h, the cells were treated with serum-free DMEM and different concentrations of compound for an additional 24 h. The supernatant was collected from the cultured plates and NGF levels were measured using an ELISA development kit. Cell viability was measured using an MTT assay. The results are expressed as a percentage of the control group (untreated cells). 6-Shogaol was used as the positive control [5, 6].

Measurement of NO Production and Cell Viability in LPS-activated BV-2 Cells

The inhibitory effect of the test compounds on LPS-stimulated NO production was studied using BV2 cells. BV2 cells were seeded on a 96-well plate (4×10^4 cells/well) and treated with or without different concentrations of the compounds. These cells were stimulated with LPS (100 ng/mL) and incubated for 24 h. The concentration of nitrite (NO_2), a soluble oxidation product of NO, in the culture medium was measured using Gries reagent (0.1 % *N*-1-napthylethylenediamine dihydrochloride and 1 % sulfanilamide in 5 % phosphoric acid). Fifty microliters of supernatant was mixed with an equal volume of Gries reagent. Absorbance was measured after 10 min using a microplate reader (Emax, Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm. L-NMMA, a well-known NOS inhibitor [13], was used as a positive control. Graded sodium nitrite solution was used as a standard to calculate nitrite concentrations. Cell viability was evaluated by observing the ability of viable cells to reduce the yellow-colored MTT to a purple-colored formazan, using an MTT assay.

Protein Expression of NF-κB in LPS-activated BV-2 Cells

BV-2 cells were pretreated with compound **10** just 30 min before LPS activation. Activated cells were incubated for 1 h. Nuclear extracts from 1 h treated microglia were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Protein level of NF-κB was evaluated by Western blot analysis.

Cytotoxicity Assessment

The cytotoxicity of the compounds against the cultured human tumor cell lines A549, SK-OV-3, SK-MEL-2, and BT549 was evaluated by the SRB method. Cisplatin was used as the positive control. This compound exhibited IC_{50} values of 1.12, 1.82, 1.27, and 1.25 μM against the A549, SK-OV-3, SK-MEL-2, and BT549 cell lines, respectively.

Results and Discussion

Compound **1** was obtained as a colorless gum, and the molecular formula $C_{12}H_{20}O_5$ was determined from the pseudomolecular ion peak $[M + H]^+$ at *m/z* 245.1385 (calcd. for $C_{12}H_{21}O_5$, 245.1389) in the HRFABMS. The IR spectrum of **1** displayed absorptions characteristic of hydroxy (3343 cm^{-1}) and carbonyl (1717 cm^{-1}) groups. The ^1H NMR spectrum of **1** showed the presence of two oxygenated methines [δ_H 4.29 (1H, ddd, $J = 8.2, 5.5, 2.8$ Hz) and 4.20 (1H, ddd, $J = 6.5, 3.4, 2.8$ Hz)] and two methylenes [δ_H 2.88 (1H, dd, $J = 17.9, 6.5$ Hz), 2.38 (1H, dd, $J = 17.9, 3.4$ Hz), and 2.28 (2H, t, $J = 6.9$ Hz)]. The ^{13}C NMR spectrum of **1** contained 12 signals, including two carbonyl groups (δ_C 178.3 and 178.1), two oxygenated carbons (δ_C 90.0 and 72.3), and eight methylenes [δ_C 38.2, 35.4, 34.0, 30.4 (×2), 30.3, 26.5, and 26.3]. These ^1H and ^{13}C NMR data (Table 1) were similar to those of (4*S*,5*R*)-5-butyl-4-hydroxy-4,5-dihydrofuran-2(3*H*)-one [14], but marked differences included the presence of an additional carbonyl carbon signal (δ_C 178.1) and the absence of a terminal methyl group [δ_H 0.93 (3H, t, $J = 6.3$ Hz); δ_C 13.8]. The HSQC data of **1** provided the correlation between each carbon and its attached proton(s) and the ^1H–^1H COSY correlations of **1** from H-2 to H-11 confirmed the linear structure from C-2 to

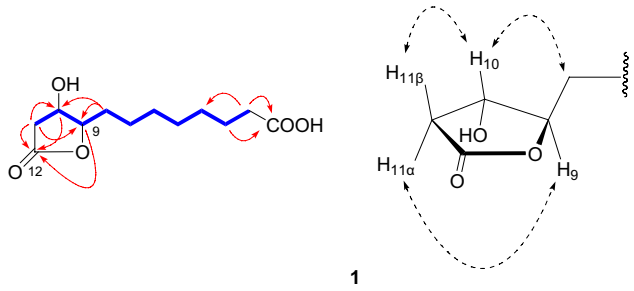

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Fig. 2 ^1H–^1H COSY (*blue bold*), HMBC (*red plain arrow*), and NOESY (*black dashed arrow*) correlations of **1** (color figure online)

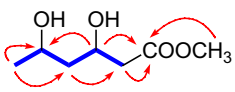

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Fig. 3 ^1H–^1H COSY (*blue bold*) and HMBC (*red plain arrow*) correlations of **2** (color figure online)

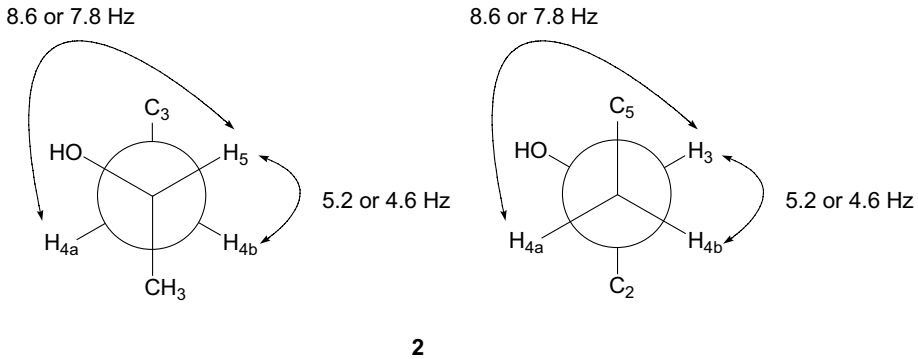

Fig. 4 Newman projections from C-3 to C-5 and *J* values of **2**

C-11 (Fig. 2). The γ-butyrolactone moiety of **1** was corroborated through the distinct HMBC cross-peak of H-9/C-12, as well as the cross-peaks of H-8/C-9 and C-10, H-10/C-12, and H-11/C-10 and C-12 (Fig. 2). The HMBC correlations of H-2 and H-3/C-1 confirmed the existence of a terminal carboxylic acid at C-1 (Fig. 2). The relative configuration of **1** was determined in a NOESY experiment. The cross-peaks of H-10/H-8 and H-11β and H-9/H-11α in the NOESY spectrum of **1** indicated that H-9 and H-10 are in a *trans* configuration (Fig. 2). The absolute configuration of **1** was confirmed as 9*R*,10*S* from the positive specific optical rotation value $\{[\alpha]_D^{25} + 28.0$ (*c* 0.30, MeOH)$\}$ by comparison with that of (4*S*,5*R*)-5-butyl-4-hydroxy-4,5-dihydrofuran-2(3*H*)-one $\{[\alpha]_D^{25} + 35.1$ (*c* 0.94, $CHCl_3$)$\}$ [12]. Thus, the chemical structure of **1** was established as shown in Fig. 1, and it was named sorcomic acid.

Compound **2** was isolated as a colorless gum. The molecular formula was found to be $C_7H_{14}O_4$ based on the pseudomolecular ion peak $[M + H]^+$ at *m/z* 163.0968 (calcd. for $C_7H_{15}O_4$, 163.0970) in the HRFABMS. The ^1H NMR spectrum of **2** displayed the presence of two oxygenated methines [δ_H 4.19 (1H, m) and 3.96 (1H, m)], a methoxy group [δ_H 3.70 (3H, s)], two methylenes [δ_H 2.55 (1H, dd, $J = 15.2$, 4.6 Hz), 2.46 (1H, dd, $J = 15.2$, 8.3 Hz), 1.68 (1H, ddd, $J = 13.9$, 8.6, 7.8 Hz), and 1.57 (1H, ddd, $J = 13.9$, 5.2, 4.6 Hz)], and a terminal methyl group [δ_H 1.21 (3H, d, $J = 6.2$ Hz)] (Table 2). The ^{13}C NMR and HSQC data analysis of **2** revealed the presence of a carbonyl carbon (δ_C 174.0), two oxygenated methine carbons (δ_C 68.2 and 67.1), a methoxy group (δ_C 52.2), two methylene carbons (δ_C 46.5 and 43.4), and a methyl carbon (δ_C 33.7) (Table 2). The ^1H–^1H COSY correlations from H-2 to H-6 and the HMBC cross-peaks of H-2 and H-3/C-1 indicated that compound **2** is a 1-hexanoic acid derivative. The locations of two hydroxy groups and a methoxy group were confirmed to be C-3 and C-5, and C-1, respectively, through full analysis of ^1H–^1H COSY and HMBC data (Fig. 3). The relative configuration of **1** was determined by the coupling constants of H-4a and H-4b. The relatively larger coupling constants of H-4a with H-3 and H-5 (8.6 and 7.8 Hz) compared with those of H-4b with H-3 and H-5 (5.2 and 4.6 Hz) suggest that H-4a is in the *anti* position from H-3 and H-5 whereas H-4b is in a *gauche* position from H-3 and H-5 based on a Karplus curve (Fig. 4).

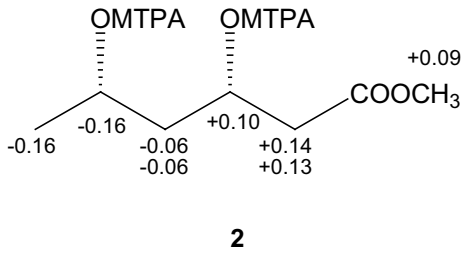

Fig. 5 Δδ values ($\delta_S - \delta_R$) in ppm of the two MTPA esters derived from **2**

Table 3 Effects of compounds **1–10** on NGF secretion in C6 cells

Compound	NGF secretion (%)[a]	Cell viability (%)[b]
1	233.40 ± 12.82	98.98 ± 2.04
2	141.99 ± 9.96	101.01 ± 1.88
3	166.63 ± 12.60	97.33 ± 1.81
4	139.63 ± 11.46	103.46 ± 2.28
5	194.40 ± 8.05	115.12 ± 4.37
6	167.65 ± 10.85	99.74 ± 1.92
7	185.74 ± 10.25	98.56 ± 1.03
8	154.09 ± 2.84	103.29 ± 4.76
9	163.78 ± 10.36	98.46 ± 0.45
10	149.54 ± 15.84	98.53 ± 12.24
6-Shogaol[c]	168.58 ± 7.16	125.80 ± 0.93

[a] C6 cells were treated with 20 μM of each compound. After 24 h, the content of NGF secreted in the C6-conditioned medium was measured by ELISA. The level of secreted NGF is expressed as the percentage of the untreated control (set as 100 %). Data are mean ± SD of three independent experiments performed in triplicate

[b] Cell viability after treatment with 20 μM of each compound was determined by the MTT assay and is expressed as a percentage (%). Results are means of three independent experiments, and the data are expressed as mean ± SD

[c] Positive control substance

The absolute configuration of **2** was confirmed as 3*S*,5*S* through the modified Mosher ester method using (*R*)- and (*S*)-MTPA-Cl (Fig. 5). Therefore, the structure of **2** was established as methyl (3*S*,5*S*)-3,5-dihydroxyhexanoate. Although this compound (**2**) was previously reported by Tschesche *et al*., herein, the full NMR assignment of this compound (**2**) is presented for the first time [15].

The eight known compounds (**3–10**) were identified as (*S*)-(*E*)-4-hydroxy-2-nonenoic acid (**3**) [16, 17], 3(*R*)-hydroxyoctanoic acid (**4**) [18], 9-hydroxynonanoic acid (**5**) [19], methyl 9-hydroxynonanoate (**6**) [20], azelaric acid (**7**) [21], monomethyl azelate (**8**) [22], (9*S*,12*S*,13*R*)-(*E*)-9,12,13-trihydroxy-10-octadecaenoic acid (**9**) [23], and (9*S*,12*R*,13*R*)-(*E*)-9,12,13-trihydroxy-10-octadecaenoic acid (**10**) [23] by comparison with spectroscopic and physical data reported previously. To the best of our knowledge, compound **3** was isolated for the first time from nature.

Table 4 Inhibitory effect of compounds **1–10** on NO production in LPS-activated BV-2 cells

Compound	IC_{50} (μM)[a]	Cell viability (%)[b]
1	180.12	119.02 ± 8.05
2	265.03	109.53 ± 12.84
3	>500	119.17 ± 2.50
4	>500	92.60 ± 6.40
5	187.87	116.74 ± 3.59
6	196.67	101.38 ± 7.24
7	150.22	101.27 ± 14.34
8	>500	92.12 ± 9.98
9	186.67	117.86 ± 10.73
10	71.25	113.16 ± 6.42
L-NMMA[c]	21.82	114.08 ± 2.96

[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] Cell viability following treatment with 20 μM each compound was determined by the MTT assay and is expressed as a percentage (%). Data are expressed as the mean ± SD of three independent experiments

[c] Positive control substance

Neuroprotective activities of the isolated compounds (**1–10**) were evaluated using an ELISA development kit that measured NGF release from C6 glioma cells into the medium. Cell viability was tested with the MTT assay. All the compounds (**1–10**) caused induction of NGF secretion by 139.63 ± 11.46–233.40 ± 12.82 %. Significantly, the new compound **1** displayed strong activity (233.40 ± 12.82 %) which was more potent than that of a positive control, 6-shogaol (168.58 ± 7.16 %), and caused no cellular toxicity at 20 μM (Table 3). Compounds **5** and **7** also induced NGF secretion potently, by 194.40 ± 8.05 and 185.74 ± 10.25 %, respectively, but, interestingly, their methyl esters were less effective [**6** (167.65 ± 10.85 %) and **8** (154.09 ± 2.84 %), respectively].

We also evaluated the anti-neuroinflammatory activities of the isolated compounds (**1–10**) by measuring NO inhibitory effect in LPS-stimulated murine microglia BV2 cells (Table 4). Compound **10** exhibited moderate inhibition of NO production with an IC_{50} value of 71.25 μM, while the other compounds (**1–9**) showed weak or no activity (IC_{50} 150.22 to >500 μM). There were no cytotoxic effects of any of these compounds, as assessed by a cell viability evaluation, at concentrations up to 20 μM.

The antiproliferative activities of compounds **1–10** were tested by determining their inhibitory effects on four human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and BT549), using an SRB bioassay, but they were inactive (IC_{50} > 10 μM).

Note that this study is just the primary screening for the probable anti-inflammatory and anticancer activity of the isolated compounds. Compounds inhibited the NO production in LPS-activated microglia and induced the NGF production in astrocyte cells, thereby giving an indication of anti-inflammatory or neuroprotective effects; whereas, the cytotoxicity assay is the most important indicator of the anticancer activity of those compounds. From these basic screening results, we concluded that sorcomic acid and its analogues possess anti-inflammatory as well as anticancer activity. Also, we checked the NF-κB experiment in LPS-induced microglia with compound **10** pretreatment. As we expected, compound **10** inhibited the NF-κB protein

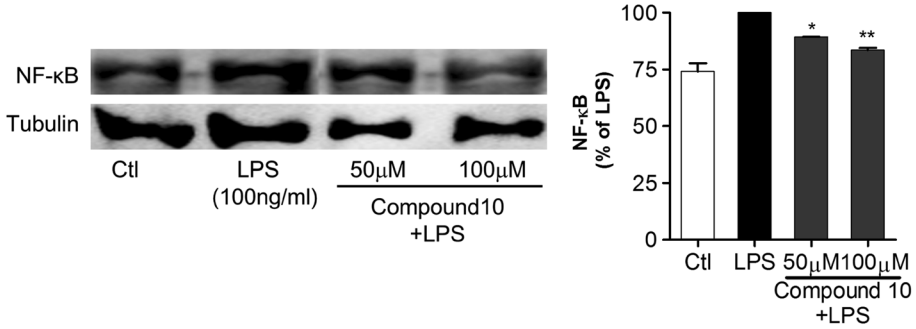

Fig. 6 Effect of compound **10** on protein expression of NF-κB in LPS-activated BV-2 cells

expression in LPS-activated BV-2 cells (Fig. 6). This result proves that fatty acids from *S. commixta* possess potential anti-inflammatory activity.

In conclusion, a new fatty acid (**1**, sorcomic acid) was isolated from the stem bark of *S. commixta*, along with nine known ones (**2**–**10**). Among them, compounds **1**, **5**, and **7** potently induced NGF secretion from C6 glioma cells and compound **10** reduced NO levels with an IC_{50} value of 71.25 μM in murine microglia BV2 cells stimulated by LPS.

Acknowledgments This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2013R1A1A2A10005315) and partly by the High Value-added Food Technology Development Program, Ministry of Agriculture, Food and Rural Affairs (114006041HD020). We are thankful to the Korea Basic Science Institute (KBSI) for the measurements of NMR and mass spectra.

Compliance with Ethical Standards

Conflict of Interest All authors declare that there are no conflicts of interest.

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