Anticancer and Anti-Neuroinflammatory Constituents Isolated from the Roots of *Wasabia japonica*

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Abstract: Wasabi (*Wasabia japonica* (Miq.) Matsum.) is a pungent spice commonly consumed with sushi and sashimi. From the roots of this plant, a new 2-butenolide derivative (1) and 17 previously reported compounds (2–18) were isolated and structurally characterized. Their chemical structures were characterized based on the conventional NMR (1H and 13C, COSY, HSQC, and HMBC) and HRESIMS data analysis. All of these phytochemicals (1–18) were evaluated for their antiproliferative effects on the four human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and MKN-1), for their inhibitory activity on nitric oxide (NO) production in lipopolysaccharide (LPS)-activated BV-2 microglia cells, and for their nerve growth factor (NGF)-releasing effect from C6 glioma cells. Among the isolated compounds, compound 15 showed powerful antiproliferative activities against A549 and SK-MEL-2 cell lines with IC50 values of 2.10 and 9.08 μM, respectively. Moreover, the new compound 1 exhibited moderate NO inhibition activity with IC50 value of 45.3 μM.

Keywords: *Wasabia japonica*; Brassicaceae; 2-butenolide; anticancer; anti-neuroinflammation

1. Introduction

*Wasabia japonica* (Miq.) Matsum., commonly known as wasabi, is one of the most well-known species among Brassicaceae plants. This plant is a perennial plant and has been cultivated mainly in Korea and Japan. Because of their tangy taste, the roots of wasabi have long been used as a spice for sushi and sashimi. A wide range of biological studies on *W. japonica* have been investigated so far; however, most of the experiments were focused on its sulfur-containing constituents, isothiocyanates (ITCs). For examples, two major components, 6-(methylsulfinyl)hexyl isothiocyanate (6-MITC) and allyl isothiocyanate (AITC), exhibited anticancer [1–5], antioxidant [6], anti-inflammatory [7,8], neuroprotective [9], and antimicrobial [10] properties. The other minor constituents of *W. japonica* contain antioxidant phenylpropanoid [11], anticancer and anti-inflammatory monogalactosyl diacylglycerides [12], and antifungal indole derivatives [13]. However, minor bioactive components of *W. japonica* with different structural classes other than ITCs remain largely unknown.

In our continuing efforts to discover bioactive constituents from the Korean traditional medicinal plants, we have investigated the roots of *W. japonica* and isolated structurally unique thioglycosides and lignan glycosides with neurotrophic and/or anti-inflammatory
activities [14,15]. To discover anticancer compounds within non-ITCs class from this plant, we have tested antiproliferative effects of hexanes-, chloroform (CHCl₃)-, ethyl acetate (EtOAc)-, and n-butanol (n-BuOH)-soluble fractions of the methanol (MeOH) extract of \textit{W. japonica} roots, and among them, the hexanes-soluble faction displayed the most potent activities with GI₅₀ values of 30.57, 21.71, 16.34, and 50.64 µg/mL against A549, SK-OV-3, SK-MEL-2, and HCT-15 cancer cell lines, respectively (Table 1). Therefore, we then focused mainly on the hexanes-soluble fraction, and herein we report the isolation and structure characterization of anticancer and anti-inflammatory constituents from the \textit{W. japonica} roots. A total 18 compounds were identified (Figure 1), and the structure of the new isolate, wasabolide (1), was elucidated by the conventional spectroscopic (i.e., NMR) and spectrometric (i.e., MS) data analysis.

Figure 1. Chemical structure of compounds 1–18.
Table 1. Antiproliferative activities of hexanes-, CHCl\textsubscript{3}-, EtOAc-, and n-BuOH-soluble fractions of \textit{W. japonica} MeOH extract against four cultured human cancer cell lines in the Sulforhodamine B (SRB) bioassay.

| Fraction   | GI\textsubscript{50} (µg/mL)\textsuperscript{1} | A549 | SK-OV-3 | SK-MEL-2 | HCT-15 |
|------------|-----------------------------------------------|------|---------|----------|--------|
| hexanes    | 30.57                                         | 21.71| 16.34   | 50.64    |        |
| CHCl\textsubscript{3} | 52.32                                         | 57.09| 57.60   | 36.53    |        |
| EtOAc      | >100                                          | >100 | >100    | >100     | >100   |
| n-BuOH     | >100                                          | >100 | 40.01   | >100     | >100   |

\textsuperscript{1}Half-maximal growth inhibitory concentration; the concentration of fraction that caused a 50% inhibition in cell growth.

2. Materials and Methods

2.1. General Experimental Procedures

Bruker AVANCE III 700 NMR spectrometer at 700 MHz (\textsuperscript{1}H) and 175 MHz (\textsuperscript{13}C) was used to measure the NMR spectra (\textsuperscript{1}H, \textsuperscript{13}C, COSY, HSQC, and HMBC) at 700 MHz (\textsuperscript{1}H) and 175 MHz (\textsuperscript{13}C) with chemical shifts given in ppm (\textdelta) (Bruker, Karlsruhe, Germany) and the resultant spectra were processed using MestReNova (Mnova, version 14.1.1-25024). Chloroform-\textit{d} and methanol-\textit{d}_4 (Cambridge Isotope Laboratory, Inc., Tewksbury, MA, USA) were used for NMR analysis of the isolated compounds. High-resolution electrospray ionization mass spectroscopy (HRESIMS) was measured by using an Agilent 1290 Infinity II HPLC instrument (Foster City, CA, USA) coupled to a G6545B quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent Technologies, Foster City, CA, USA) with a Kinetex C\textsubscript{18} 5 µm column (250 mm length × 4.6 mm i.d.; Phenomenex, Torrance, CA, USA). The semipreparative high-performance liquid chromatography (HPLC) furnished with a Gilson 306 pump (Gilson, Middleton, WI, USA), a Luna C18 10 µm column (250 mm length × 10 mm i.d.; Phenomenex, Torrance, CA, USA), an Apollo Silica 5 µm column (250 mm length × 10 mm i.d.; Apollo, Manchester, UK), and a Shodex refractive index detector (Gilion, New York, NY, USA) was used for compounds isolation. Low pressure liquid chromatography (LPLC) was performed with a LiChroprep Lobar-A Si 60 column (Merck, Darmstadt, Germany) and an FMI QSY-0 pump. Column chromatography was performed employing either silica gel 60 (70–230 and 230–400 mesh; Merck, Darmstadt, Germany) or RP-C\textsubscript{18} silica gel (Merck, 230–400 mesh). Merck precoated silica gel F\textsubscript{254} plates and RP-C\textsubscript{18} F\textsubscript{254s} plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC) analysis. Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde–sulfuric acid.

2.2. Plant Material

The roots of \textit{W. japonica} (3.3 kg) were collected in Hanam, Republic of Korea, in October 2014. One of the authors, Kang Ro Lee, identified the plant, and a voucher specimen (SKKU-NPL 1409) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea.

2.3. Extraction and Isolation

The roots of \textit{W. japonica} (3.3 kg) were extracted with 80% aqueous MeOH at room temperature and filtered. The crude extract was concentrated under reduced pressure to yield a MeOH extract (750 g). The extract was suspended in deionized H\textsubscript{2}O, and then it was partitioned with hexanes (1.3 g), CHCl\textsubscript{3} (5.6 g), EtOAc (5.8 g), and n-BuOH (2.7 g). The hexanes-soluble fraction (1.3 g) was chromatographed on silica gel column (hexane-EtOAc, 4:1 → 1:1) to afford 20 fractions (H1-H20). Fraction H1 (100 mg) was applied to Waters Sep-pak silica Vac 6 cc (hexane-EtOAc, 1:0 → 1:1) to yield six sub-fractions (H11-H16), and compound 9 (2 mg, 0.0003%) was acquired by purifying sub-fraction H15 (10 mg), employing semipreparative silica HPLC with an isocratic mixture of hexane-EtOAc (30:1, flow rate of 2.0 mL/min). Compound 13 (3 mg, 0.0004%) was purified from the fraction H2.
(35 mg), using semipreparative silica HPLC eluting with hexane-EtOAc (30:1, 2 mL/min) under isocratic conditions. Fraction H3 (35 mg) was purified by using semipreparative silica HPLC, with an isocratic mixture of hexane-EtOAc (15:1, 2 mL/min), to produce compounds 10 (3 mg, 0.0004%) and 15 (3 mg, 0.0004%). Fraction H4 (33 mg) was divided into four sub-fractions (H41–H44), using C$_{18}$ Waters Sep-pak Vac 6 cc by eluting with 95% aqueous MeOH. Compound 16 (3 mg, 0.0004%) was isolated from sub-fraction H44 (15 mg), using semipreparative silica HPLC eluting with an isocratic mixture of hexane-EtOAc (30:1, 2 mL/min). Fraction H5 (65 mg) was subjected to C$_{18}$ Waters Sep-pak Vac 6 cc with 95% aqueous MeOH as an eluent to acquire three sub-fractions (H51–H53). Compounds 2 (4 mg, 0.0005%) and 5 (2 mg, 0.0003%) were purified from sub-fraction H51 (40 mg). Compound 14 (2 mg, 0.0003%) was isolated by purification, using semipreparative silica HPLC eluting with an isocratic mixture of hexane-EtOAc (8:1, 2 mL/min) from sub-fraction H52 (11 mg). Fraction H6 (100 mg) was separated on a Lobar A Si 60 (240 mm × 10 mm) column (CHCl$_3$-MeOH, 500:1) to yield four sub-fractions (H61–H64). Sub-fraction H64 (19 mg) was further separated by using semipreparative silica HPLC eluting with an isocratic mixture of hexane-EtOAc (4:1, 2 mL/min) to acquire compound 17 (8 mg, 0.001%). Compound 11 (2 mg, 0.0003%) was purified from fraction H7 (13 mg) by employing semipreparative HPLC (85% aqueous MeOH, 2 mL/min) under isocratic conditions. Fraction H9 (40 mg) was separated into two sub-fractions (H91 and H92) through C$_{18}$ Waters sep-pak Vac 6 cc by eluting with 100% MeOH, and sub-fraction H91 (15 mg) was further isolated by semipreparative HPLC (82% aqueous MeOH, 2 mL/min) under isocratic conditions to obtain compound 12 (2 mg, 0.0003%). Fraction H10 (40 mg) was applied to C$_{18}$ Waters Sep-pak Vac 6 cc with 100% MeOH as an eluent to furnish three sub-fractions (H101–H103). Sub-fraction H101 (21 mg) was purified by using semipreparative silica HPLC eluting with an isocratic mixture of hexane-EtOAc (2.5:1, 2 mL/min) to obtain compound 1 (3 mg, 0.0004%). Fraction H11 (28 mg) was isolated into three sub-fractions (H111–H113) using C$_{18}$ Waters Sep-pak Vac 6 cc by eluting with 100% MeOH. Among the sub-fractions, compound 8 (4 mg, 0.0005%) was purified from the sub-fraction H112 through semipreparative silica HPLC eluting with an isocratic mixture of hexane-EtOAc (2.5:1, 2 mL/min). Fraction H13 (45 mg) was divided to three sub-fractions (H131–H133) by C$_{18}$ Waters Sep-pak Vac 6 cc by eluting with 100% MeOH and compound 18 (5 mg, 0.0007%) was obtained by purification of sub-fraction H133 (16 mg), using semipreparative silica HPLC eluting with an isocratic mixture of hexane-EtOAc (1:1, 2 mL/min). The EtOAc-soluble fraction (5.8 g) was fractionated into six fractions (E1-E6) by passage over Diaion HP-20 column with step gradient solvents of MeOH-H$_2$O (0:1, 1:4, 2:3, 3:2, 4:1, and 1:0) as eluents. Fraction E2 (300 mg) was applied to a Lobar A RP-18 (240 mm × 10 mm) column (20% aqueous MeOH) to produce four sub-fractions (E21–E24). Sub-fraction E22 (80 mg) was further isolated into seven sub-fractions (E211–E217) through a Lobar A Si 60 (240 mm × 10 mm) column (CHCl$_3$-MeOH-H$_2$O, 3:1:0.15). Compound 7 (3 mg, 0.0004%) was isolated from sub-fraction E222 (15 mg) by semipreparative silica HPLC eluting with an isocratic mixture of CHCl$_3$-MeOH-H$_2$O (5:1:0.1, 2 mL/min). Sub-fraction E24 (30 mg) was purified by using semipreparative HPLC (10% aqueous CH$_3$CN, 2 mL/min) under isocratic conditions to afford compound 6 (2 mg, 0.0003%). Fraction E5 (160 mg) was chromatographed on silica gel column chromatography (EtOAc-MeOH-H$_2$O, 3:1:0.15 → 1:1:0.1) to furnish five sub-fractions (E51–E55). Compound 3 (4 mg, 0.0005%) was isolated by purification of sub-fraction E51 (10 mg), using semipreparative HPLC (50% aqueous MeOH, 2 mL/min) under isocratic conditions. Through the purification of sub-fraction E52 (17 mg) by using semipreparative HPLC (45% aqueous MeOH, 2 mL/min) under isocratic conditions, compound 4 (3 mg, 0.0004%) was obtained.

Wasabolide (1): colorless gum; $^1$H (700 MHz) and $^{13}$C (175 MHz) NMR data in chloroform-d (see Table 2); HRESIMS (positive-ion mode) $m/z$ 271.1535 [M + H]$^+$ (calcd. for C$_{14}$H$_{22}$O$_5$, 271.1540, error = 1.8 ppm), 293.1348 [M + Na]$^+$ (calcd. for C$_{14}$H$_{22}$NaO$_5$, 293.1365, error = 3.8 ppm).
Table 2. NMR spectroscopic data of compounds 1 and MEO measured in chloroform-\textit{d}.

| Pos. | \(\delta_H\), Multi. (J in Hz) 1 | \(\delta_C\) | \(\delta_H\), Multi. (J in Hz) 1 | \(\delta_C\) |
|------|-----------------|---------|-----------------|---------|
| 1    | 6.22, d (5.7)   | 125.0   | 6.19, d (5.7)   | 124.75  |
| 2    | 7.12, d (5.7)   | 153.7   | 7.10, d (5.7)   | 153.53  |
| 3    | 8.49, m         | 57.1    |                 |         |
| 4    | 1.89, m         | 37.1    | 1.90, m         | 36.98   |
| 5    | 1.37, m         | 23.3    | 1.23, brs; H-6–H-19 | 22.67–31.91; C-6–C-19 |
| 6    | 2.86, t         | 84.4    |                 |         |
| 7    | 1.61, m         | 34.2    |                 |         |
| 8    | 2.29, t (7.5)   | 34.2    |                 |         |
| 9    | 3.22, s         | 51.3    | 3.20, s         | 51.11   |

1 Adapted from the previous research [16]. 2 Exchangeable peaks.

2.4. Sulforhodamine B Colorimetric Assay

The antiproliferative activities of isolated compounds (1–18) were tested against the A549 (non-small-cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), HCT-15 (human colon adenocarcinoma), and MKN-1 (adenosquamous carcinoma), utilizing the SRB method as previously reported (Table 3) [17]. Etoposide (≥98%; Sigma-Aldrich, St. Louis, MO, USA) served as a positive control.

Table 3. Antiproliferative activities of selected compounds against four cultured human cell lines.

| Compound | IC\textsubscript{50} (\textmu M) 1 | A549 | SK-OV-3 | SK-MEL-2 | MKN-1 |
|----------|-------------------------------|------|---------|-----------|--------|
| 10       | 26.03                         | >30.0| >30.0   | >30.0    | >30.0  |
| 11       | 17.95                         | >30.0| 17.43   | >30.0    | >30.0  |
| 14       | 13.28                         | 12.86| 13.04   | 14.17    |        |
| 15       | 13.28                         | 13.23| 9.08    | 10.04    |        |
| 16       | 19.21                         | >30.0| >30.0   | >30.0    |        |
| 18       | 24.64                         | 20.16| 26.72   | 31.49    |        |
| Etoposide 2 | 1.51                         | 1.94 | 1.13    | 3.37     |        |

1 50% inhibitory concentration; the concentration of compound that caused a 50% inhibition in cell growth.
2 Etoposide was used as a positive control.

2.5. Nitric Oxide (NO) Assay

The nitric oxide (NO) assay was performed analogously, as described in Reference [18]. The BV-2 cells, which were developed by V. Bocchini at the University of Perugia (Perugia, Italy), were used for this study [19,20]. The cells were seeded in a 96-well plate (4 × 10\textsuperscript{4} cells/well) and incubated in the presence or absence of various doses of tested compounds (1–18). Lipopolysaccharide (LPS) (100 ng/mL) was added to all the pretreated wells, except the control one, and grown for 1 day. The produced levels of nitrite (NO\textsubscript{2}), a soluble oxidized product of NO, was evaluated with 0.1% \(\text{N}^\text{G}\)-monomethyl-L-arginine (L-NMMA), as a nitric oxide synthase (NOS) inhibitor, was used as a positive control. Graded sodium nitrite solution was utilized to determine nitrite concentrations. A 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used for the cell-viability assay (Table 4).
was consistent with HRESIMS data mentioned above, including two ester/carboxylic acid(s) (Figure S2) suggested the presence of two olefinic protons H-2 and H-3, 5.7 Hz, was observed. The similar coupling constant between OCH=OCH and 23.3 (C-6) carbon peaks. To elucidate the planar structure of Wasabolide (1), 2D NMR data of (Figure S3) showed total 14 resonances, which was consistent with HRESIMS data mentioned above, including two ester/carboxylic acid(s) (δC 174.4 (C-12) and 170.1 (C-1)), two double bond [δC 153.7 (C-3) and 125.0 (C-2)], a deoxygenated [δC 111.4 (C-4)], two methoxy [δC 51.6 (OCH3-12) and 51.3 (OCH3-4)], and seven methylene [δC 37.1 (C-5), 34.2 (C-11), 29.4 (C-7), 29.13 (C-9), 29.07 (C-8), 25.0 (C-10), and 23.3 (C-6)] carbon peaks. To elucidate the planar structure of 1, 2D NMR data of 1 including COSY, HSQC, and HMBC (Figures S4–S6) were obtained and analyzed. The presence of an α,β-unsaturated γ-butyrolactone (UBL, = 2-butenolide) moiety in 1 was suggested by observing COSY correlation between H-2 and H-3 and HMBC cross-peaks between H-2/H-3 and C-1 and H-2/H-3/H-5 and C-4 (Figure 2A). The HMBC correlation between OCH3-4 and C-4 indicated a methoxy group located at the γ-position of UBL moiety (Figure 2A). In addition, an unusually smaller coupling constant between the cis-oriented two olefinic protons H-2 and H-3, 5.7 Hz, was observed. The similar J value has been reported from other UBL-containing organic molecules [16,22,23], supporting the presence of UBL functionality in 1. The remaining COSY correlations between H-6 and H-5/H-7 and H-10 and H-9/H-11 and HMBC cross-peaks between H-5 and C-6, H-11 and C-9/C-10/C-12, and OCH3-12 and C-12 indicated the presence of a fully saturated aliphatic chain with a terminal methyl ester group as the other substructure of 1. Therefore, the planar structure of 1 was determined as in Figure 1. The most structurally similar molecule to 1 was found to be 4-methoxy-2-eicosen-4-olide (MEO) isolated from a marine sponge.

### Table 4. Effects of select compounds on NO generation in LPS-stimulated BV-2 cells.

| Compound | IC50 (µM) 1 | Cell Viability (%) 2 |
|----------|-------------|---------------------|
| 1        | 45.3        | 112.07 ± 4.78       |
| 5        | 90.0        | 98.31 ± 11.14       |
| 11       | 59.6        | 67.91 ± 4.06        |
| 15       | 92.4        | 120.77 ± 8.20       |
| L-NMMA 3 | 21.4        | 104.56 ± 4.20       |

1 IC50 value of each compound was defined as the concentration (µM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells. 2 Cell viability after treatment with 20 µM of each compound was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and is expressed in percentage (%). The results are averages of three independent experiments, and the data are expressed as mean ± SD. 3 Nω,Nω-monomethyl-l-arginine (l-NMMA) was used as a positive control.

### 2.6. Nerve Growth Factor (NGF) Assay

The nerve growth factor (NGF) assay was performed analogously, as described in Reference [21]. C6 glioma cell lines were used to measure the NGF of the culture medium containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PS) in 5% CO2 incubator. The cells were seeded in a 24-well culture plate (1 × 10^5 cells/well) and incubated for 24 h. The cells were treated with or without 20 µM of the compounds (1–18), together with serum-free Dulbecco’s modified Eagle’s medium (DMEM) for another 24 h. Released NGF levels from the supernatants from each cell were measured by using an ELISA development kit (R&D System, Minneapolis, MN, USA). Moreover, the cell viability was evaluated by using MTT assay; 6-shogaol used as a positive control, and the results are expressed as percentage of the control group.

### 3. Results and Discussion

#### 3.1. Structure Elucidation of Compounds 1–18

Wasabolide (1) was obtained as a colorless gum, and its molecular formula was established as C14H22O5 from the protonated and sodiated molecular ions at m/z 271.1535 [M + H]+ (calcd. for C14H23O5+, 271.1540, error = 1.8 ppm) and 293.1348 [M + Na]+ (calcd. for C14H22NaO5+, 293.1359, error = 3.8 ppm), respectively, in the HRESIMS data (Figure S1). The 1H NMR spectrum of 1 (Figure S2) suggested the presence of two olefinic [δH 7.12 (1H, d, J = 5.7 Hz, H-3) and 6.22 (1H, d, J = 5.7 Hz, H-2)], two methoxy [δH 3.66 (3H, s, OCH3-12) and 3.22 (3H, s, OCH3-4)], and seven methylene [δH 2.29 (2H, t, J = 7.5 Hz, H-11), 1.89 (2H, m, H-5), 1.61 (2H, m, H-10), 1.37 (2H, m, H-6), and 1.30 (6H, overlap, H-7–H-9)] protons in 1. The 13C NMR spectrum of 1 (Figure S3) showed total 14 resonances, which was consistent with HRESIMS data mentioned above, including two ester/carboxylic acid(s) (δC 174.4 (C-12) and 170.1 (C-1)), two double bond [δC 153.7 (C-3) and 125.0 (C-2)], a deoxygenated [δC 111.4 (C-4)], two methoxy [δC 51.6 (OCH3-12) and 51.3 (OCH3-4)], and seven methylene [δC 37.1 (C-5), 34.2 (C-11), 29.4 (C-7), 29.13 (C-9), 29.07 (C-8), 25.0 (C-10), and 23.3 (C-6)] carbon peaks.
(Figure 2B, right) [16] by the literature search, using SciFinder. MEO shared most of the NMR features, with 1 especially at the UBL functionality (Table 2 and Figure 2B), thus supporting our structural assignment of 1. Finally, it was concluded that 1 was a racemic mixture based on the almost zero value of specific rotation and no Cotton effect observation in electronic circular dichroism (ECD) spectrum of 1.

Figure 2. Structure elucidation of 1. (A) Key COSY and HMBC correlation of 1. (B) Comparison of the key 1H NMR data around the UBL functionality in 1 (left) and its structurally similar marine metabolite MEO (right).

The 17 known compounds 2–18 were identified as methyl 10-oxodecanoate (2) [24], monomethyl sebacate (3) [25], azelaic acid (4) [26], dimethyl azelate (5) [26], dimethyl succinate (6) [27], 4-isothiocyanatobutanoic acid (7) [28], octadecanoic acid (8) [29], methyl stearate (9) [30], (10E)-12-oxo-10-octadecenoic acid methyl ester (10) [31], methyl 11-oxo-9-dodecenoate (11) [32], (S)-5-hydroxy-3,4-dimethyl-5-pentylfuran-2(SH)-one (12) [33], 6,10,14-Trimethyl-2-pentadecane (13) [34], 5-methyl-5-(4,8,12-trimethyl-tridecyl)-dihydrofuran-2-one (14) [35], 8-tocospiro A (15) [36], stigmast-4-en-3-one (16) [37], β-sitosterol (17) [38], and 7-oxo-β-sitosterol (18) [39] by comparing their spectroscopic data (Figures S7–S24) with those in the literature.

3.2. Biosynthetic Proposal of the New Compound 1

Upon the structural characterization of the new metabolite 1, its biosynthetic pathway was proposed as described below (Figure 3). First, one unit of acetyl-CoA and five units of malonyl-CoA could form the C12-fatty acid 1 with a double bond and a carbonyl group at α, β- and γ-position, respectively, which reaction resembled typical fatty acid biosynthesis. Then, oxidation of the terminal methyl group in i to carboxylic acid could afford dicarboxylic acid ii [40,41]. Geometrical isomerization could occur in ii that transforms the E- to Z-configuration of the double bond to yield iii. A hemi-acetalization reaction on iii could produce iv [22,42], which contains UBL moiety, and, finally, 1 could be formed by O-methylation at both hydroxy and carboxylic groups in iv.
Antioxidants 2022, 11, x FOR PEER REVIEW...IC50 activity on the NO production, with an IC50 value of 90.0, 59.6, and 92.4 μM, respectively. The other compounds were inactive (IC50 > 100 μM).

3.3. Antiproliferative Activity of the Isolated Compounds (1–18)

In line with the potent antiproliferative activity of the hexanes-soluble fraction mentioned above, the isolated compounds (1–18) were evaluated for their antiproliferative activity against four human tumor cell lines, namely A549 (non-small cell lung adenocarcinoma), SK-OV-3 (ovary malignant ascites), SK-MEL-2 (skin melanoma), and MKN-1 (adenosquamous carcinoma), by SRB assay. Compounds 10, 11, 14–16, and 18 isolated from the hexanes-soluble fraction strongly inhibited proliferation in cancer cell lines with different selectivity (Table 3). In detail, α-tocospiro A (15) showed potent activity against A549 cell line with IC50 value of 2.10 μM, which was comparable to that of the positive control substance, etoposide (1.51 μM). While the chemical structures of 13–15 are similar in that these three molecules had the same C15 fully saturated farnesyl unit, we observed no and weaker activity of 13 (IC50 > 20 μM) and 14 (IC50 = 13.28 μM), respectively, on the same cancer cell line. These data suggested that the bulky spirobicyclic moiety in α-tocospiro A (15) would play an important role in exhibiting antiproliferative activities in A549 cell line. Interestingly, Yuan et al. reported that α-tocospiro A (15) had no inhibitory activity on the A549 proliferation (IC50 > 20 μM) [43], but we observed the significant potency of α-tocospiro A (15) in this study. Moreover, α-tocospiro A (15) also displayed a strong antiproliferative effect on SK-OV-2, SK-MEL-2, and MKN-1 cell lines, with IC50 values ranging from 9.08 to 13.23 μM. Compounds 10, 11, 14, 16, and 18 showed antiproliferative activities on several cancer cell lines with IC50 values between 12.86 to 26.72 μM. The other compounds were inactive (IC50 > 30 μM).

3.4. Anti-Neuroinflammatory Activity of the Isolated Compounds (1–18)

The potential anti-neuroinflammatory effects of the isolates (1–18) were tested by measuring the level of NO production in LPS-stimulated BV-2 microglia cells. As shown in Table 4, among the tested phytochemicals, the new compound 1 showed the strongest inhibitory activity on the NO production, with an IC50 value of 45.3 μM and without significant cell toxicity up to 20 μM. Compounds 5, 11, and 15 also exhibited weak activity with IC50 value of 90.0, 59.6, and 92.4 μM, respectively. The other compounds were inactive (IC50 > 100 μM).
3.5. Neurotrophic Activity of the Isolated Compounds (1–18)

Lastly, the neurotrophic effect of compounds 1–18 was also evaluated, and compound 17 displayed a weak effect on NGF release from C6 cells, with a stimulation level of 107.51 ± 5.38%, compared to the positive control substance, 6-shogaol (149.53 ± 5.38%). The other compounds were inactive (stimulation level < 100%).

4. Conclusions

From the roots of W. japonica, a total 18 compounds (1–18), including a new phytochemical (1), were isolated by chromatographic methods and identified by spectroscopic and spectrometric data analysis, including 1D and 2D NMR and HRMS. The structure of the new compound 1 was assigned as a possibly dicarboxylic acid–derived 2-butenolide derivative and its biosynthetic pathway was also proposed based on the assigned structure and related literature search. Compound 1 showed strong anti-neuroinflammatory activity by inhibiting NO production in LPS-stimulated BV-2 cells and α-tocopherol (A (15)) exhibited a potent antiproliferative activity against A549 non-small cell lung adenocarcinoma cells.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antiox11030482/s1. Figure S1: HRESIMS spectrum of 1. Figures S2–S6: 1D and 2D NMR spectra of 1. Figures S7–S34: 1H and/or 13C NMR spectra of 2–18.

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References

1. Manesh, C.; Kuttan, G. Effect of naturally occurring allyl and phenyl isothiocyanates in the inhibition of experimental pulmonary metastasis induced by B16F-10 melanoma cells. Fitoterapia 2003, 74, 355–363. [CrossRef]

2. Lee, M.-J.; Tseng, W.-S.; Lai, J.C.-Y.; Shieh, H.-R.; Chi, C.-W.; Chen, Y.-J. Differential pharmacological activities of oxygen numbers on the sulfoxide moiety of wasabi compound 6-(methylsulfinyl) hexyl isothiocyanate in human oral cancer cells. Molecules 2018, 23, 2427. [CrossRef] [PubMed]

3. Yano, S.; Wu, S.; Sakao, K.; Hou, D.X. Wasabi 6-(methylsulfinyl) hexyl isothiocyanate induces apoptosis in human colorectal cancer cells through p53-independent mitochondrial dysfunction pathway. BioFactors 2018, 44, 361–368. [CrossRef] [PubMed]

4. Fuke, Y.; Hishinuma, M.; Namikawa, M.; Oishi, Y.; Matsuzaki, T. Wasabi-derived 6-(methylsulfinyl) hexyl isothiocyanate induces apoptosis in human breast cancer by possible involvement of the NF-κB pathways. Nutr. Cancer 2014, 66, 879–887. [CrossRef] [PubMed]

5. Chen, Y.-J.; Huang, Y.-C.; Tsai, T.-H.; Liao, H.-F. Effect of wasabi component 6-(methylsulfinyl) hexyl isothiocyanate and derivatives on human pancreatic cancer cells. Evid. Based Complement. Altern. Med. 2014, 2014, 494739. [CrossRef]

6. Mizuno, K.; Kume, T.; Muto, C.; Takada-Takatori, Y.; Izumi, Y.; Sugimoto, H.; Akaike, A. Glutathione biosynthesis via activation of the nuclear factor E2-related factor 2 (Nrf2)–antioxidant-response element (ARE) pathway is essential for neuroprotective effects of sulforaphane and 6-(methylsulfinyl) hexyl isothiocyanate. J. Pharmacol. Sci. 2011, 115, 320–328. [CrossRef]

7. Lohning, A.; Kidachi, Y.; Kamie, K.; Sasaki, K.; Royoama, K.; Yamaguchi, H. 6-(methylsulfinyl) hexyl isothiocyanate (6-MITC) from Wasabia japonica alleviates inflammatory bowel disease (IBD) by potential inhibition of glycogen synthase kinase 3 beta (GSK-3β). Eur. J. Med. Chem. 2021, 216, 113250. [CrossRef]

8. Kim, M.W.; Choi, S.; Kim, S.Y.; Yoon, Y.S.; Kang, J.-H.; Oh, S.H. Allyl isothiocyanate ameliorates dextran sodium sulfate-induced colitis in mouse by enhancing tight junction and mucus expression. Int. J. Mol. Sci. 2018, 19, 2025. [CrossRef] [PubMed]

9. Subedi, L.; Venkatesan, R.; Kim, S.Y. Neuropeptidic and anti-inflammatory activities of allyl isothiocyanate through attenuation of JNK/NF-κB/TNF-α signaling. Int. J. Mol. Sci. 2017, 18, 1423. [CrossRef] [PubMed]
10. Ko, M.-O.; Kim, M.-B.; Lim, S.-B. Relationship between chemical structure and antimicrobial activities of isothiocyanates from cruciferous vegetables against oral pathogens. J. Microbiol. Biotechnol. 2016, 26, 2036–2042. [CrossRef]

11. Hosoya, T.; Yun, Y.S.; Kunugi, A. Antioxidant phenylpropanoid glycosides from the leaves of Wasabia japonica. Phytochemistry 2008, 69, 827–832. [CrossRef]

12. Weil, M.J.; Zhang, Y.; Nair, M.G. Tumor cell proliferation and cyclooxygenase inhibitory constituents in horseradish (Armoracia rusticana) and Wasabi (Wasabia japonica). J. Agric. Food. Chem. 2005, 53, 1440–1444. [CrossRef]

13. Pedras, M.S.C.; Sorensen, J.L.; Ókanga, F.I.; Zaharia, I.L. Wasalexins A and B, new phytoalexins from wasabi: Isolation, synthesis, and antifungal activity. Biorg. Med. Chem. Lett. 1999, 9, 3015–3020. [CrossRef]

14. Kim, C.S.; Oh, J.; Subedi, L.; Kim, S.Y.; Choi, S.U.; Lee, K.R. Rare thioglycosides from the roots of Wasabia japonica. J. Nat. Prod. 2018, 81, 2129–2133. [CrossRef] [PubMed]

15. Kim, C.S.; Subedi, L.; Kwon, O.W.; Park, H.B.; Kim, S.Y.; Choi, S.U.; Lee, K.R. Wasabisides A–E, lignan glycosides from the roots of Wasabia japonica. J. Nat. Prod. 2016, 79, 2652–2657. [CrossRef] [PubMed]

16. Miller, M.; Hegedus, L.S. Synthesis of optically active butenolides via chromium alkoxycarbene complexes: Total synthesis of (+)-tetrahydrocerulenin and two butenolides from the marine sponge Plakortis lita. J. Org. Chem. 1993, 58, 6779–6785. [CrossRef]

17. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J.T.; Bokesch, H.; Kenney, S.; Boyd, M.R. New colorimetric cytotoxicity assay for anticancer-drug screening. [NCl]. Nat. Cancer Inst. 1990, 82, 1107–1112. [CrossRef]

18. Kim, C.S.; Oh, J.; Suh, W.S.; Jang, S.W.; Subedi, L.; Kim, S.Y.; Choi, S.U.; Lee, K.R. Investigation of chemical constituents from Spiraea prunifolia var. simplicifolia and their biological activities. Phytochem. Lett. 2017, 22, 255–260. [CrossRef]

19. Blasi, E.; Barluzzi, R.; Bocchini, V.; Mazzolla, R.; Bistoni, F. Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. J. Neuroimmunol. 1990, 27, 229–237. [CrossRef]

20. Choi, Y.; Lee, M.K.; Lim, S.Y.; Sung, S.H.; Kim, Y.C. Inhibition of inducible NO synthase, cyclooxygenase-2 and interleukin-1β by torilin is mediated by mitogen-activated protein kinases in microglial BV2 cells. Br. J. Pharmacol. 2009, 156, 933–940. [CrossRef] [PubMed]

21. Kim, C.S.; Subedi, L.; Oh, J.; Kim, S.Y.; Choi, S.U.; Lee, K.R. Bioactive triterpenoids from the twigs of Chaenomeles sinensis. J. Nat. Prod. 2017, 80, 1134–1140. [CrossRef] [PubMed]

22. Mansoor, T.A.; Hong, J.; Lee, C.-O.; Sim, C.J.; Im, K.S.; Lee, D.S.; Jung, J.H. New cytototoxic metabolites from a marine sponge Homaxinella sp. J. Nat. Prod. 2004, 67, 721–724. [CrossRef] [PubMed]

23. De Guzman, F.S.; Schmitz, F.J. Peroxy aliphatic esters from the sponge Plakortis lita. J. Nat. Prod. 1990, 53, 926–931. [CrossRef]

24. Cravotto, G.; Calcio Gaudino, E.; Barge, A.; Binello, A.; Albertino, A.; Aghemo, C. Synthesis of 1-octacosanol and GC-C-IRMS discrimination of samples from different origin. Nat. Prod. Res. 2010, 24, 428–439. [CrossRef]

25. Khlebnikova, T.B.; Pai, Z.P.; Fedoseeva, L.A.; Mattsat, Y.V. Catalytic oxidation of fatty acids. II. Epoxidation and oxidative cleavage of unsaturated fatty acid esters containing additional functional groups. React. Kinet. Catal. Lett. 2009, 98, 9–17. [CrossRef]

26. Zimmermann, F.; Meux, E.; Mielszynski, J.-L.; Lecuire, J.-M.; Ogé, N. Ruthenium catalysed oxidation without CCl4 of oleic acid, other monoenic fatty acids and alklenes. Tetrahedron Lett. 2005, 46, 3201–3205. [CrossRef]

27. Dawar, P.; Raju, M.B.; Ramakrishna, R.A. One-pot esterification and Ritter reaction: Chemo-and regioselectivity from tert-butyl methyl ether. Tetrahedron Lett. 2011, 52, 4262–4265. [CrossRef]

28. Radulovič, N.; Dekić, M.; Stojanović-Radić, Z. A new antimicrobial glucosinolate autolysis product, 4-isothiocyanatoobutan-2: From the diffuse wallflower (Erysimum diffusum): Methyl 4-isothiocyanatobutanoate, a long unrecognized artifact of the Allylic hydroperoxides from the autoxidation of methyl oleate. J. Chem. Soc. Perkin Trans. 2005, 125–130. [CrossRef]

29. Liu, X.Q.; Baek, W.-S.; Ahn, D.-K.; Choi, H.-Y.; Yook, C.-S. The constituents of the aerial part of Gastrodia elata Blume. Nat. Prod. Sci. 2002, 8, 137–140. [CrossRef]

30. Crosignani, S.; White, P.D.; Linclau, B. Polymer-supported O-alkylsioxare: Useful reagents for the O-alkylation of carboxylic acids. J. Org. Chem. 2004, 69, 5897–5905. [CrossRef]

31. Frankel, E.N.; Garwood, R.F.; Khabay, B.P.; Moss, G.P.; Weedon, B.C. Stereochemiistry of olefin and fatty acid oxidation. Part 3. The allylic hydroperoxides from the autoxidation of methyl oleate. J. Chem. Soc. Perkin Trans. 1984, 2233–2240. [CrossRef]

32. Lin, D.; Zhang, J.; Sayre, L.M. Synthesis of six epoxyketooctadecanoic acid (EKODe) isomers, their generation from nonenzymatic oxidation of linoleic acid, and their reactivity with imidazole nucleophiles. J. Org. Chem. 2007, 72, 9471–9480. [CrossRef] [PubMed]

33. Zhang, J.; Liang, Y.; Liao, X.-J.; Deng, Z.; Xu, S.-H. Isolation of a new butenolide from the South China Sea gorgonian coral Subergorgia suberosa. Nat. Prod. Res. 2014, 28, 150–155. [CrossRef] [PubMed]

34. Yildizhan, S.; van Loon, J.; Sramkova, A.; Ayasse, M.; Arsene, C.; ten Broeke, C.; Schulz, S. Aphrodisiac pheromones from the wings of the small cabbage white and large cabbage white butterflies, Pieris rapae and Pieris brassicae. ChemBioChem 2009, 10, 1666–1677. [CrossRef] [PubMed]

35. De Shan, M.; An, T.Y.; Hu, L.H.; Chen, Z.L. Diterpene derivative and chromone from Hypericum perforatum. Nat. Prod. Res. 2004, 18, 15–19. [CrossRef]

36. Chang, Y.-M.; Kuo, Y.-H. Two novel α-tocopheroids from the aerial roots of Ficus microcarpa. Tetrahedron Lett. 2003, 44, 5125–5128. [CrossRef]

37. Della Greca, M.; Monaco, P.; Previtera, L. Stigmasters from Typha latifolia. J. Nat. Prod. 1990, 53, 1430–1435. [CrossRef]

38. Chang, Y.C.; Chang, F.R.; Wu, Y.C. The constituents of Lindera glauca. J. Chin. Chem. Soc. 2000, 47, 373–380. [CrossRef]
39. Pettit, G.R.; Numata, A.; Cragg, G.M.; Herald, D.L.; Takada, T.; Iwamoto, C.; Riesen, R.; Schmidt, J.M.; Doubek, D.L.; Goswami, A. Isolation and structures of schleicherastatins 1−7 and schleicheols 1 and 2 from the teak forest medicinal tree Schleichera oleosa. *J. Nat. Prod.* 2000, 63, 72−78. [CrossRef]

40. Liu, L.; Zhou, S.; Deng, Y. The 3-ketoacyl-CoA thiolase: An engineered enzyme for carbon chain elongation of chemical compounds. *Appl. Microbiol. Biotechnol.* 2020, 104, 8117−8129. [CrossRef]

41. Han, L.; Peng, Y.; Zhang, Y.; Chen, W.; Lin, Y.; Wang, Q. Designing and creating a synthetic omega oxidation pathway in *Saccharomyces cerevisiae* enables production of medium-chain α, ω-dicarboxylic acids. *Front. Microbiol.* 2017, 8, 2184. [CrossRef] [PubMed]

42. Teichert, A.; Lübken, T.; Schmidt, J.; Porzel, A.; Arnold, N.; Wessjohann, L. Unusual bioactive 4-oxo-2-alkenoic fatty acids from *Hygrophorus eburneus*. *Z. Naturforsch.* 2005, 60b, 25−32. [CrossRef]

43. Yuan, Z.; Duan, H.; Xu, Y.; Wang, A.; Gan, L.; Li, J.; Liu, M.; Shang, X. α-Tocospiro C, a novel cytotoxic α-tocopheroid from *Cirsium setosum*. *Phytochem. Lett.* 2014, 8, 116−120. [CrossRef]