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Antioxidative and anti-inflammatory activity of psiguadial B and its halogenated analogues as potential neuroprotective agents

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ABSTRACT

Psiguadial B (**8**), and its fluoro- (**8a**), chloro- (**8b**), and bromo- (**8c**) derivatives were synthesized using a sodium acetate-catalyzed single step coupling of three components: β-caryophyllene (**5**), diformylphloroglucinol (**11**), and benzaldehyde (**12**). These compounds efficiently and dose-dependently decreased H_2O_2-induced cell death, a quantitative marker of cell death, in primary cultures of mouse cortical neurons. Psiguadial B also decreased neuronal death and accumulation of ROS induced by $FeCl_2$ in cortical cultures. The *in vitro* effects of these compounds in lipopolysaccharide (LPS)-induced expression of nitric oxide (NO), and TNF-α and IL-6 by suppressing the NF-κB pathway in immune cells demonstrated their antioxidative and anti-inflammatory activity. The present findings warrant further research on the development of psiguadial B-based neuroprotective agents for the treatment of neurodegenerative diseases, acute brain injuries and immunological disorders.

1. Introduction

Psidium guajava L., commonly known as guava, is an edible fruit tree cultivated in subtropical and tropical regions has traditionally been used in several conditions such as diarrhea, dysentery, hypertension and hyperglycemia [1,2]. Several researchers have reported that guava contains pharmacologically active constituents including flavonoids [3–5], triterpenoids [6,7], and sesquiterpenoids (Fig. 1A and 1B) [8–10]. A group of Chinese researchers have isolated several new sesquiterpenoid-based meroterpenoids from guava leaves [9,11–15]. Shao et al. first reported isolation and antiproliferative activities of four meroterpenoids (Fig. 1C), psiguadial A (**7**), psiguadial B (**8**), psidial A (**9**), and guajadial (**10**) against doxorubicin-resistant and -sensitive human hepatoma cells, HepG2/ADM and HepG2, respectively [9]. Among them, psiguadial B (**8**) showed the most potent inhibitory activity (IC_{50} = 45 ± 1.41 nM) against HepG2 cells. Despite the potent cytotoxic effect of psiguadial B in preliminary testing, further biological studies of this compound had not been reported due to synthetic challenges of the sample. From a synthetic chemistry point of view, it was challenging to synthesize psiguadial B (**8**), which contains a bicyclo [4.3.1]decane ring, transfused to a highly functionalized chromane and cyclobutane. In 2016, Chapman et al. reported the first 16 steps of the

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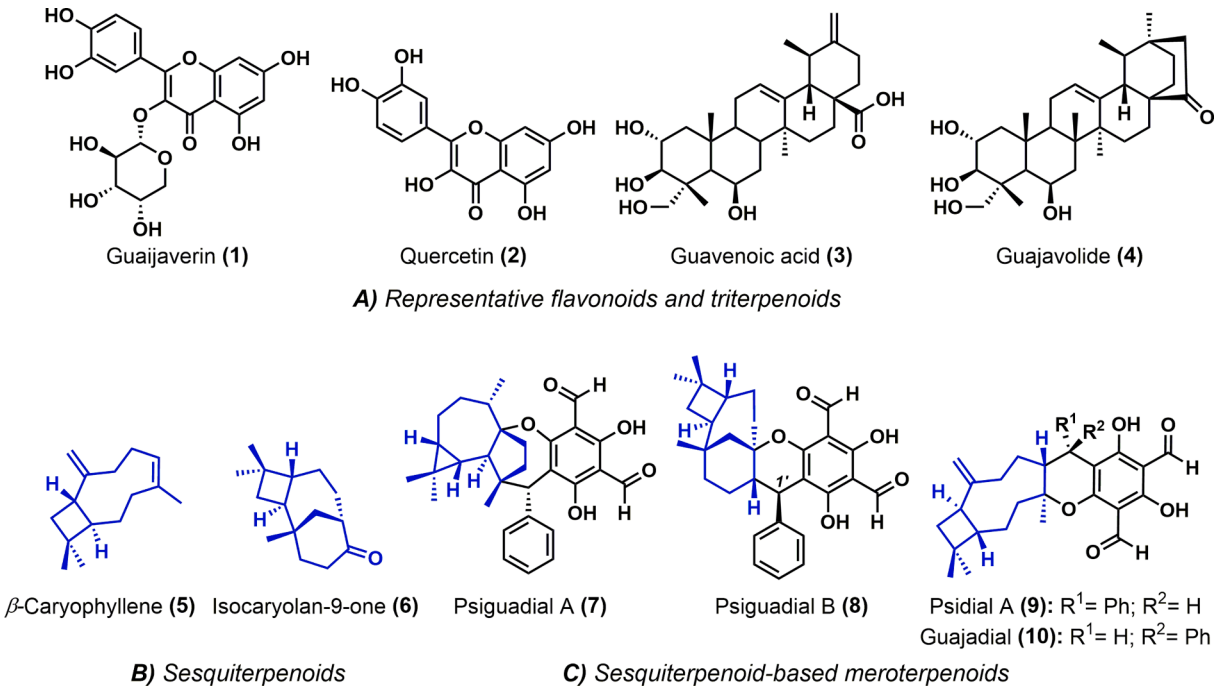
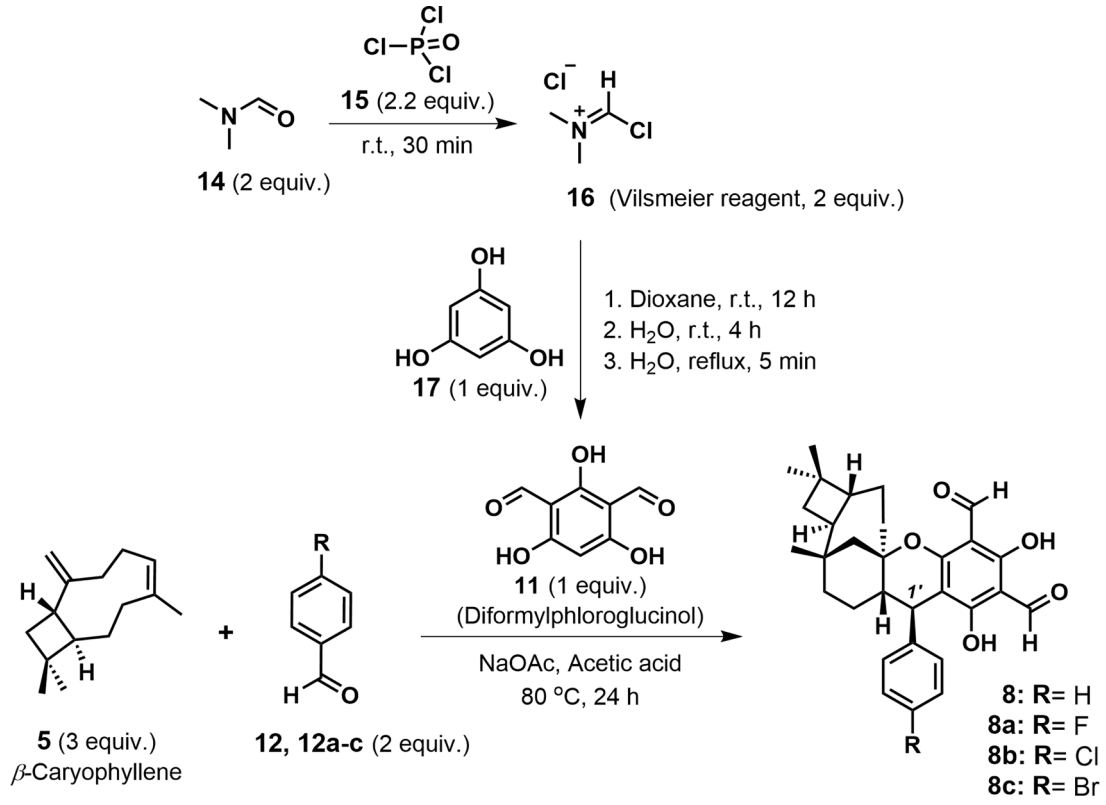


Fig. 1. Structures of active constituents isolated from leaves of *Psidium guajava*.



Scheme 1. Preparation of psiguadial B (**8**) and its halogenated analogues **8a-c**.

enantioselective total synthesis of psiguadial B [16]. However, this long synthetic method resulted in overall yield less than 1% of compound **8**, making it quantitatively insufficient for further biological testing. Therefore, developing a new, concise and efficient synthetic method to synthesize psiguadial B was needed. As part of our major program to find a new biological activity of psiguadial B, in May of 2017, we first attempted to synthesize psiguadial B via short biomimetic synthetic route (see Official E-Notebook proof of our work in Supplementary Data) as proposed in Scheme 1 based on report of guajadial and psidial A synthesis by Lawrence et al [17]. Concurrently, Cramer and co-workers [18] previously reported a single step *N,N'*-dimethylethylenediamine (DMEDA)–catalyzed synthesis of psiguadial B, while they reported zero

yield in the presence of NaOAc with different reaction solvent and conditions than our work.

Previous studies have shown that constituents of guava have various therapeutic activities including hepato-protective [19], anti-hyperglycemic [20,21], anti-inflammatory [22], antimicrobial [23] and antioxidant [24]. There are limited studies on effect of guava constituents against oxidative stress and neurodegeneration [25–27]. Neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease are characterized by progressive loss of function and structure of neurons in the brain or spinal cord [28]. The etiology of neurodegenerative diseases are environmental and genetic factors, however, the oxidative stress due to generation of reactive oxygen species such as pathogenic superoxide anion ($O_2^{\cdot-}$) and hydroxyl ($HO^{\cdot}$) free radical are considered to be a major factor of the neurodegenerative process [29,30]. It has been reported that high level of redox-active metal ions such as Fe^{2+} and Cu^{2+} promote neurodegeneration by generating $HO^{\cdot}$ free radicals from hydrogen peroxide (H_2O_2) [31–33]. Recently, studies have shown the interrelation between oxidative stress and inflammation in neurodegeneration [34,35]. Furthermore, several studies have reported that the presence of halogen functionalities in compound is important for improved therapeutic effect by inhibiting reactive oxygen species (ROS) production, which might be due to enhanced physiochemical and pharmacokinetic properties of compounds [36,37]. Based on these studies, we anticipate that psiguadial B and its halogenated analogues would exhibit neuroprotective and anti-inflammatory activity through their antioxidative effects. Here we report, for the first time, sodium acetate (NaOAc)-catalyzed short synthesis psiguadial B and its halogenated analogues **8a-c** as novel and potential neuroprotective agents for the treatment of neurodegenerative diseases and acute brain injuries.

2. Materials and methods

2.1. Chemistry

All commercially available reagent-grade chemicals were used as received without further purification. Reactions were monitored by LC/MS, and thin-layer chromatography (TLC), performed using 0.2 mm silica gel plates (Merck 60 F_{254}) and visualized by UV light (254 nm). Column chromatography was performed using a CombiFlash Rf system with RediSep Rf (Teledyne Isco, Lincoln, NE). Target compounds were purified by preparative HPLC on Kinetex 5 μm biphenyl, 100 Å column (GX-281 HPLC system, Gilson, Middleton, WI, USA; column tube 250 mm × 21.2 mm i.d.), with acetonitrile and water as binary gradient elution. Mobile phase A was water with 0.1% trifluoroacetic acid, and mobile phase B was acetonitrile with 0.1% trifluoroacetic acid. The injection volum was 1 mL and a gradient of 40–70% B was run at a flow rate of 17 mL/min over 60 min. The purity of target compounds was determined to be above 95% by analytical HPLC using dual different wavelength UV detector (254 nm and 280 nm). Bruker Nuclear magnetic resonance (NMR) spectrometer was used to record for ^1H and ^{13}C spectra at 400 MHz and 100 MHz, respectively. Compounds were dissolved in DMSO-d_6 or $CDCl_3$, and the spectra were recorded at 25 °C. Chemical shifts (δ) were reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard and coupling constants were expressed in hertz (Hz). Mass spectra were recorded with a positive electrospray ionization (ESI) mode on LCMS-2020 system (Shimadzu, Tokyo, Japan) and Thermo Scientific Dionex Ultimate 3000 system.

2.2. Synthetic procedures and compound characterization data

2.2.1. Preparation of diformylphloroglucinol (11)

To a stirred solution of N,*N*-dimethylformamide (**14**, 1.23 mL, 15.86 mmol, 2.0 equiv.), phosphorus oxychloride (**15**, 1.62 mL, 17.45 mmol, 2.2 equiv.) was added dropwise at room temperature under a nitrogen atmosphere and the mixture was continued for 30 min to obtain Vilsmeier reagent (**16**). Then to a stirred solution of anhydrous phloroglucinol (1.0 g, 7.93 mmol, 1.0 equiv.) in dioxane (5 mL) reagent **16** (~2 equiv.) was slowly added *via* cannula. This solution was then stirred for 12 h at room temperature, under a nitrogen atmosphere. After 12 h a yellow amorphous solid was formed. This solid mixture was cooled to 0 °C and then added to an ice-water slurry (30 mL). The mixture was stirred for 4 h at room temperature which resulted formation of cream precipitate. Upon filtering and washing with more water, solid was suspended in 8 mL water and refluxed for 5 min. After cooling to 0 °C, a salmon colored solid was formed which was filtered off and washed with cold water (15 mL). The solid was dried under vacuum for 20 h at 90 °C to get diformylphloroglucinol (**11**, 1.062 g, 73.5%) as a salmon pink colored solid. ESI LC/MS: *m/z* calcd. for $C_8H_6O_5$ $[M + H]^+$: 183.03; found 183.25. ^1H NMR (400 MHz, DMSO-d_6) δ 13.51 (br, 1H, OH), 12.49 (br s, 2H, OH), 10.01 (s, 2H, CHO), 5.90 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 191.86 (2C), 169.88 (2C), 169.49, 104.23 (2C), 94.54.

2.2.2. Preparation of (+)-Psiguadial B (8)

To a stirred solution of diformylphloroglucinol (**11**, 100 mg, 0.549 mmol) and sodium acetate (4.5 mg, 0.06 mmol, 0.1 equiv.) in acetic acid (4 mL), benzaldehyde (**12**, 0.11 mL, 1.10 mmol, 2.0 equiv.) and β-Caryophyllene (**5**, 0.37 mL, 1.65 mmol, 3.0 equiv.) were added dropwise at 80 °C, the mixture was continuingly stirred for 24 h at 80 °C. Upon cooling to room temperature, brine (5 mL) and diethyl ether (Et_2O, 20 mL) were added and the layers separated. The aqueous layer was then extracted with Et_2O (10 mL × 3), and the combined organic layers were washed with brine (20 mL) and dried over anhydrous Na_2SO_4. It was filtered and concentrated to give the crude residue, which was purified by preparative HPLC (40–72% ACN in H_2O) to give product (**8**) as a pale cream solid (14 mg, 5.37%). ESI LC/MS: *m/z* calcd. for $C_{30}H_{34}O_5$ $[M + H]^+$: 475.24; found 475.09. ^1H NMR (400 MHz, $CDCl_3$) δ 13.49 (s, 1H, OH), 13.02 (s, 1H, OH), 10.06 (s, 2H, CHO), 7.26–7.18 (m, 3H), 7.17–7.09 (br m, 2H), 3.47 (d, J = 11.48 Hz, 1H), 2.15 – 2.06 (m, 2H), 1.95–1.87 (m, 1H), 1.84–1.77 (m, 1H), 1.73–1.65 (m, 3H), 1.50–1.44 (m, 3H), 1.42–1.29 (m, 3H), 1.26–1.23 (m, 1H), 1.06–1.02 (m, 1H), 0.99 (apparent d, 6H), 0.84 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.36, 191.53, 169.66, 168.53, 163.51, 143.42, 128.18 (3C), 126.23 (2C), 105.73, 104.65, 104.15, 84.14, 50.02, 47.46, 44.04, 40.39, 37.61, 36.93, 35.44, 35.09, 33.46, 30.63, 29.37, 26.09, 23.91, 20.75, 20.11.

2.2.3. Preparation of fluoro (+)-Psiguadial B (8a)

To a stirred solution of diformylphloroglucinol (**11**, 100 mg, 0.549 mmol) and sodium acetate (4.5 mg, 0.06 mmol, 0.1 equiv.) in acetic acid (4 mL), 4-fluorobenzaldehyde (**12a**, 0.12 mL, 1.10 mmol, 2.0 equiv.) and β -Caryophyllene (**5**, 0.37 mL, 1.65 mmol, 3.0 equiv.) were added dropwise at 80 °C, the mixture was continuingly stirred for 24 h at 80 °C. Upon cooling to room temperature, brine (5 mL) and diethyl ether (20 mL) were added and the layers separated. The aqueous layer was then extracted with diethyl ether (10 mL × 3), and the combined organic layers were washed with brine (20 mL) and dried over anhydrous Na_2SO_4. It was filtered and concentrated to give the crude residue, which was purified by Preparative HPLC (40–72% ACN in H_2O) to giveproduct (**8a**) as a white solid (18 mg, 6.66%). ESI LC/MS: *m/z* calcd. for $C_{30}H_{33}FO_5$ $[M + H]^+$: 493.24; found 493.65. ^1H NMR (400 MHz,

$CDCl_3$) δ 13.50 (s, 1H, OH), 13.05 (s, 1H, OH), 10.07 (s, 2H, CHO), 7.04 (br s, 2H), 6.96–6.92 (m, 2H), 3.47 (d, $J = 11.48$ Hz, 1H), 2.15–2.07 (m, 2H), 1.95–1.78 (m, 2H), 1.70–1.57 (m, 3H), 1.51–1.38 (m, 5H), 1.36–1.31 (m, 1H), 1.27–1.24 (m, 1H), 1.08–1.03 (m, 1H), 1.01 (apparent d, 6H), 0.86 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.32, 191.58, 169.58, 168.55, 163.41, 162.56, 160.13, 139.02, 138.99, 128.91, 115.13, 114.92, 105.53, 104.63, 104.17, 84.15, 50.09, 47.44, 44.05, 39.69, 37.59, 36.95, 35.44, 35.08, 33.48, 30.61, 29.37, 26.06, 23.87, 20.73, 20.12.

2.2.4. Preparation of chloro (+)-Psiguadial B (**8b**)

To a stirred solution of diformylphloroglucinol (**11**, 100 mg, 0.55 mmol) and sodium acetate (4.5 mg, 0.06 mmol, 0.1 equiv.) in acetic acid (4 mL), 4-chlorobenzaldehyde (**12b**, 154 mg, 1.10 mmol, 2.0 equiv.) and β -Caryophyllene (**5**, 0.37 mL, 1.65 mmol, 3.0 equiv.) were added dropwise at 80 °C, the mixture was continuingly stirred for 24 h at 80 °C. Upon cooling to room temperature, brine (5 mL) and diethyl ether (20 mL) were added and the layers separated. The aqueous layer was then extracted with diethyl ether (10 mL × 3), and the combined organic layers were washed with brine (20 mL) and dried over anhydrous Na_2SO_4. It was filtered and concentrated to give the crude residue, which was purified by Preparative HPLC (40–72% ACN in H_2O) to give product (**8b**) as a white solid (20 mg, 7.16%). ESI LC/MS: *m/z* calcd. for $C_{30}H_{33}ClO_5$ $[M]^+$: 509.21; found $[M]^+$: 509.05 and $[M + 2]^+$: 511.0. ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.50 (s, 1H, OH), 13.06 (s, 1H, OH), 10.07 (s, 2H, CHO), 7.22 (d, $J = 8.68$ Hz, 2H), 7.03 (d, $J = 6.88$ Hz, 2H), 3.47 (d, $J = 11.44$ Hz, 1H), 2.14–2.07 (m, 2H), 1.94–1.78 (m, 2H), 1.70–1.57 (m, 3H), 1.51–1.38 (m, 5H), 1.36–1.31 (m, 1H), 1.27–1.24 (m, 1H), 1.09–1.04 (m, 1H), 1.01 (apparent d, 6H), 0.86 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.30, 191.58, 169.50, 168.57, 163.42, 141.99, 131.81 (3C), 128.37 (2C), 105.22, 104.64, 104.15, 84.12, 49.94, 47.43, 44.04, 39.87, 37.56, 36.94, 35.44, 35.09, 33.47, 30.61, 29.39, 26.06, 23.87, 20.73, 20.11.

2.2.5. Preparation of of bromo (+)-Psiguadial B (**8c**)

To a stirred solution of diformylphloroglucinol (**11**, 100 mg, 0.55 mmol) and sodium acetate (4.5 mg, 0.06 mmol, 0.1 equiv.) in acetic acid (4 mL), 4-bromobenzaldehyde (**12c**, 203 mg, 1.10 mmol, 2.0 equiv.) and β -Caryophyllene (**5**, 0.37 mL, 1.65 mmol, 3.0 equiv.) were added dropwise at 80 °C, the mixture was continuingly stirred for 24 h at 80 °C. Upon cooling to room temperature, brine (5 mL) and diethyl ether (20 mL) were added and the layers separated. The aqueous layer was then extracted with diethyl ether (10 mL × 3), and the combined organic layers were washed with brine (20 mL) and dried over anhydrous Na_2SO_4. It was filtered and concentrated to give the crude residue, which was purified by Preparative HPLC (40–72% ACN in H_2O) to give product (**8c**) as a pale cream solid (22 mg, 7.24%). ESI LC/MS: *m/z* calcd. for $C_{30}H_{33}BrO_5$ $[M]^+$: 552.15; found $[M]^+$: 552.95 and $[M + 2]^+$: 554.90. ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.49 (s, 1H, OH), 13.06 (s, 1H, OH), 10.07 (s, 2H, CHO), 7.37 (d, $J = 8.6$ Hz, 2H), 6.97 (d, $J = 6.64$ Hz, 2H), 3.46 (d, $J = 11.44$ Hz, 1H), 2.14–2.06 (m, 2H), 1.93–1.78 (m, 2H), 1.70–1.57 (m, 3H), 1.51–1.38 (m, 5H), 1.36–1.31 (m, 1H), 1.27–1.24 (m, 1H), 1.08–1.04 (m, 1H), 1.01 (apparent d, 6H), 0.86 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.30, 191.58, 169.48, 168.57, 163.43, 142.54, 131.31 (2C), 129.56 (2C), 119.84, 105.14, 104.64, 104.15, 84.12, 49.89, 47.43, 44.04, 39.95, 37.56, 36.94, 35.44, 35.09, 33.47, 29.70, 29.39, 26.06, 23.86, 20.73, 20.11.

2.3. General procedure for the evaluation of biological activity

2.3.1. Cortical cell cultures

Cortical cell cultures were prepared from embryonic day 14 (E14) ICR mice on feeder astrocytic cultures and maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 5% fetal bovine serum (Hyclone), 5% horse serum (Gibco), 2 mM glutamine (Sigma), and 1% penicillin/streptomycin (Cambrex).

2.3.2. Lactate degydrogenase (LDH) assay

Lactate dehydrogenase (LDH) released into culture media was assayed in potassium phosphate buffer containing 23 mM pyruvate and 0.3 mg/ml β-NADH by monitoring the conversion of NADH to NAD + at 340 nm in a spectrophotometer (Molecular Devices, Spectramax). LDH value was scaled to the mean value of sister cultures after 24 h of exposure to 200 μM glutamate, which resulted in nearly complete neuronal death without astrocytic damage (100%).

2.3.3. Measurement of H_2O_2

A ROS-specific probe, CM-H_2DCFDA (1 μM, Invitrogen) and 200 μM H_2O_2 were incubated for 30 min and then without or with indicated concentrations of **8** for additional 1 h. Fluorescent intensity was measured by Envision multiplate reader at a wavelength of 485/535 nm (ex/em).

2.3.4. ROS-staining in cells

Primary cortical cells were incubated with 1 μM CM-H_2DCFDA in growth medium for 30 min and then washed with PBS for three times. ROS-stained cells were observed under EVOS Cell Imaging Systems (Thermo Fisher Scientific) at a wave length of excitation 470/20 nm and emission 510/42 nm.

2.3.5. Cell culture

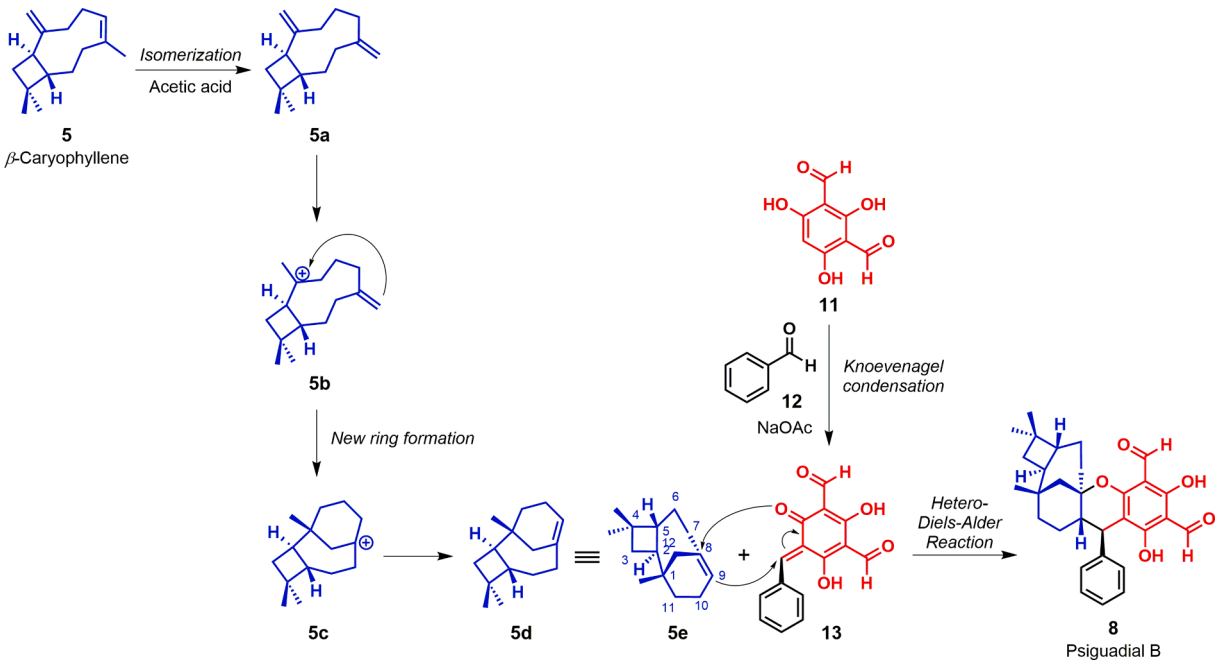
CHO (Chinese hamster ovary), A2780 (Ovary cancer), and BHP (Thyroid cancer) cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured at 37 °C in a 5% CO_2 humidified incubator and maintained in Roswell Park Memorial Institute (RPMI 1640, Coring Inc, New York, USA) medium containing 5 mL penicilin streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). RAW264.7 murine macrophages of immune cell lines were purchased from the Korean Cell Line Bank (KCLB®, Seoul, Korea). Cells were cultured at 37 °C in a 5% CO_2 humidified incubator and maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM, Coring Inc, New York, USA) medium containing 5 mL penicilin streptomycin, and 10% heat-inactivated fetal bovine serum (FBS).

2.3.6. Cell viability assay

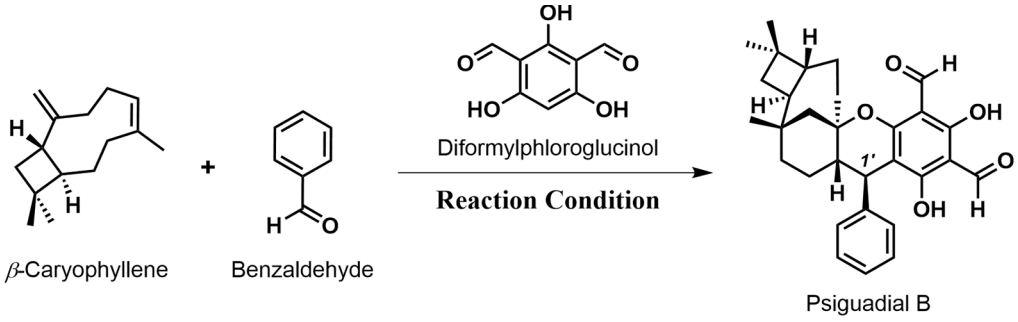
Cells were seeded in a 96-well culture plates and cultured for overnight and then treated with various concentrations of psiguadial B analogues (compound **8**, and **8a-c**) for 24 h. Cell viabilities were evaluated using CellTiter-Glo reagent (Promega. CA, USA) which was added to each well (20 μL) and incubated at 37 °C for 10 min. Absorbance were read using a EnVision Multilabel Reader (Perkin-Elmer, Waltham, MA, USA) at a luminescence signals.

2.3.7. Production of nitric oxide (NO) and cytokines (TNF-α and IL-6)

Immune cells (1×10^5 cells/well) were seeded in 24-well culture plate and cultured for 12 h. Cells were pre-treated with various psiguadial B analogues (compound **8**, and **8a-c**) or dexamethasone of 10 μM for 1 h and then co-incubated with 500 ng/mL of LPS for 24 h. NO concentration in medium were determined using a Griess assay. Griess reagent (50 μL) was added to media supernatant (50 μL) and then incubated at 37 °C for 15 min in the dark. Absorbance was measured at 520 nm. NO concentrations were caculated using 0–100 μM sodium nitrite standard. TNF-α, and IL-6 expression levels in culture medium were quantified using a sandwich-type ELISA kits.



Scheme 2. Our proposed biosynthetic method to psiguadial B using β -caryophyllene.



Reaction Condition	Cramer's work	Our work
Catalyst	NaOAc	NaOAc
Yield	0	5.4%
Solvent	HFIP*	Acetic acid
Temperature	Room Temperature	80 °C
Time	48 h	24 h
β-Caryophyllene	6 equiv.	3 equiv.
Benzaldehyde	6 equiv.	2 equiv.
Diformylphloroglucinol	1 equiv.	1 equiv.

*HFIP: 1,1,1,3,3,3-hexafluoroisopropanol

Fig. 2. Differences in reaction conditions between previously reported work by Cramer and co-workers [18] and our work to synthesize psiguadial B (**8**) using NaOAc.

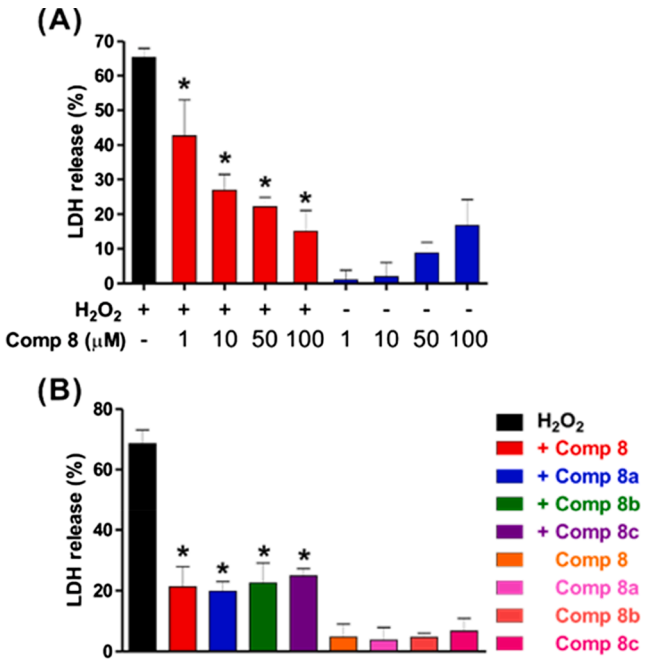


Fig. 3. Protective effect of psiguadial B (**8**) and its halogenated analogues on H_2O_2-induced cell death in mouse cortical cultures. (**A**) Cortical cultures were exposed to 150 μM H_2O_2 in the absence (black bar) or presence of **8** (red bars), and to **8** alone (blue bars) at indicated concentrations (1–100 μM) for 24 h. Bars denote the relative amount of lactate dehydrogenase (LDH) release from cell into medium (*P < 0.001 vs H_2O_2). (**B**) LDH release from cultures exposed to 150 μM H_2O_2 in absence (black bar) or presence of 10 μM of compound **8** (red bar), **8a** (blue bar), **8b** (green bar), and **8c** (purple bar), and LDH release from H_2O_2 untreated cultures by **8** (orange bar), **8a** (pink bar), **8b** (bright red bar), or **8c** (dark pink bar) at indicated concentration for 24 h (*P < 0.001 vs H_2O_2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3.8. NO reporter gene promoter assay

A pNF-κB-Luc plasmid for NF-kB luciferase reporter assay was obtained from Strategene (LaJolla, CA, USA). Expression vectors were obtained as followed: Flag-IKKa, Flag-IKKb, and Flag-p65 (M. Karin, University of California San Diego), Myc-NIK and Flag-TNFR (M. Jung, Georgetown University), HA-TRAF2 andHA-RIP were developed in reference laboratory. Transfections were performed as described previously [38,39]. NF-κB-dependent luciferase activity was measured using IVIS imaging system (perkins elmer, CA, USA).

2.3.9. Statistical analysis

All data are expressed as the mean ± standard deviation from at least three representative experiments, and statistical significance was determined using unpaired Student's *t* test. *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Short synthetic route of psiguadial B (8) and its halogenated analogues (8a-c)

As indicated in Scheme 1, our initial aim was to synthesize psiguadial B (**8**) using concise synthetic procedure. For this, diformylphloroglucinol (**11**) was first prepared with previously reported methods [17,40], by treating phloroglucinol (**17**) with two equivalent amounts of Vilsmeier reagent (**16**) that was obtained from N,*N*-dimethylformamide (**14**) and phosphorus oxychloride (**15**). Diformylphloroglucinol (**11**) was mixed with three equivalents of β -caryophyllene (**5**), and two equivalents of benzaldehyde (**12**) in a solution of sodium acetate (NaOAc) and acetic acid at 80 °C for 24 h to produce a mixture of psiguadial B (**8**) and guajadial (**10**). As proposed in Scheme 2, we believe that *ortho*-quinone methide (**13**) obtained from NaOAc-catalyzed Knoevenagel condensation of **11** and **12**, undergoes hetero-Diels-Alder reaction with tricyclododec-8-ene (**5e**) derived from acetic acid-catalyzed isomerization, and subsequent cyclization of β -caryophyllene (**5**) to form psiguadial B (**8**).

Compound **8** was separated from the mixture of **10** using preparative HPLC. We confirmed the structure of prepared psiguadial B (**8**) by comparing NMR spectra with previously reported data of natural psiguadial B [9] and the synthetic sample [16] (Table S1). Furthermore, we synthesized fluorine-, chlorine-, and bromine-containing analogues (**8a**, **8b**, and **8c**) of psiguadial B by substituting 1′-position phenyl group with different *para*-halo-phenyls following the same short synthetic procedure (Scheme 1). The compounds were successfully purified by auto-preparative HPLC (GX-281 HPLC, Gilson, USA). The structures of the synthesized compounds **8** and **8a-c** were confirmed by NMR spectra (for details of their synthesis and LC-MS, HPLC data, see the Supplementary Data). The yields of these compounds were comparable (5.4–7.2%) to the method reported by Cramer and co-workers [18]. Most importantly, both our work and Cramer et al.'s work reported reaction using NaOAc to synthesize psiguadial B. It is important to note that they reported zero yield, while here we report above 5% yield, and this finding could be explained as such difference in reaction solvent and condition (Fig. 2). This synthetic route involved a simple and one-step reaction with inexpensive starting compounds and reagents allowing to produce target compounds in large scale for further bioassay and development.

3.2. Neuroprotective effect on mouse cortical cells by chelating ROS

As one of the major aims of this study was to evaluate neuroprotective effect of psiguadial B and its halogenated analogues, mouse cortical cultures were exposed to 150 μM H_2O_2 in the absence or presence of psiguadial B (**8**) at indicated concentrations (1, 10, 50 or 100 μM) for 24 h. Psiguadial B (**8**) efficiently reduced H_2O_2-induced cell death in a dose-dependent manner, when it was measured by release of lactate dehydrogenase (LDH), a quantitative marker for cell death, in primary cultures of mouse cortex (Fig. 3A). Even compound **8** exhibited the neuroprotective effect in a concentration-dependent manner, however, it started to show toxicity from 50 μM. The MTS and CellTiter-Glo assay to evaluate cytotoxicity of psiguadial B (compound **8**) in HepG2 and HCT116 cancer cell lines is shown in Supplementary Figure S1. We used 10 μM psiguadial B in further experiments. Moreover, as shown in Fig. 3B, other halogenated psiguadial B analogues (**8a**, **8b** and **8c**) also showed similar neuroprotective effect against H_2O_2-induced cell death at 10 μM.

To determine the ROS-chelating effect of compound **8**, we monitored changes in fluorescence of CM-H_2DCFDA, H_2O_2-specific indicator. As shown in Fig. 4A, compound **8** reduced fluorescent intensity increased by H_2O_2 in a concentration-dependent manner. In accordance with these results, compound **8** dramatically decreased cell death and the level of ROS in cortical cultures exposed to $FeCl_2$, which is well known as stimulus for H_2O_2 generation (Fig. 4B and 4C). These results suggest that psiguadial B analogues have ability to protect neurons from oxidative stress in neurodegenerative diseases.

3.3. Anti-inflammatory effects of compounds via inhibition of expression of nitric oxide (NO), TNF-α and IL-6

The cytotoxicities of compounds **8** and **8a-c** on the viability of

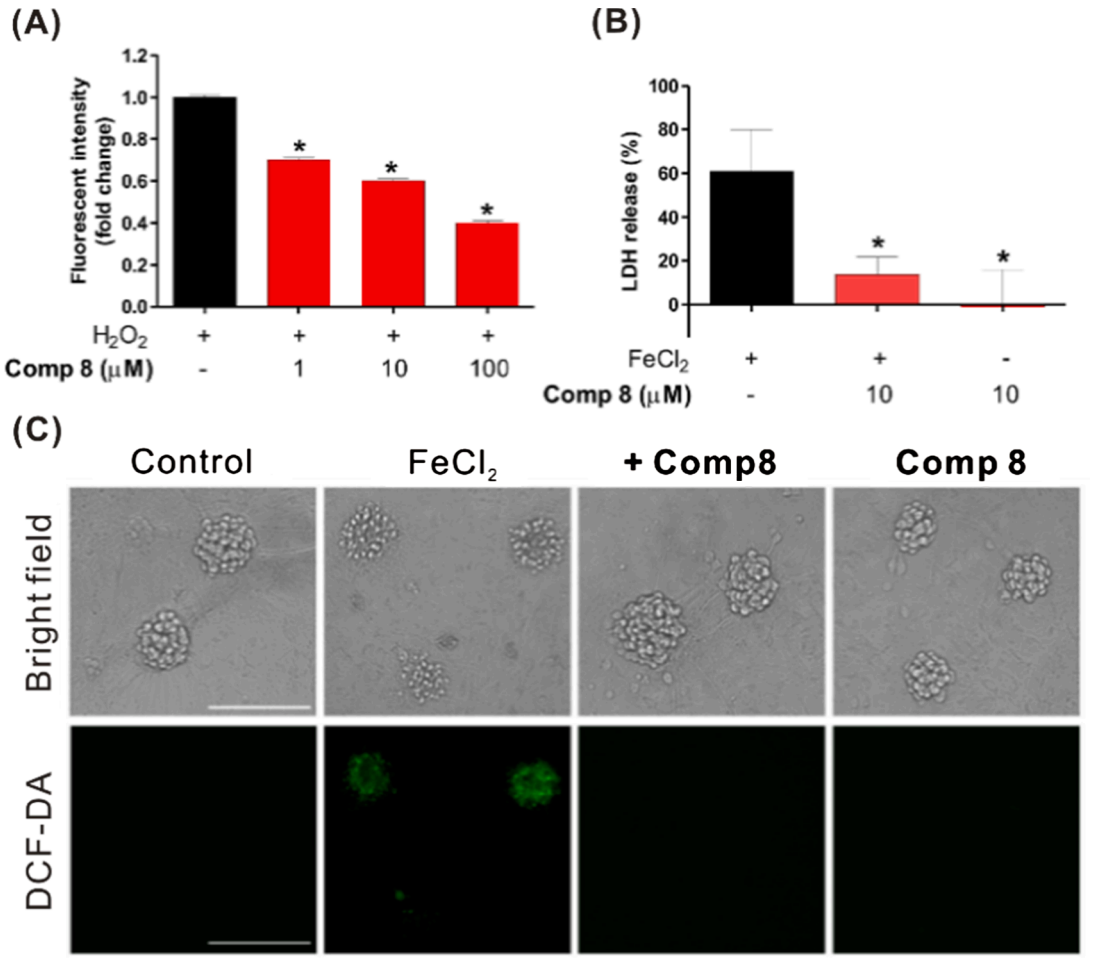


Fig. 4. Inhibitory activity of psiguadial B (**8**) against reactive oxygen species (ROS)-induced cell death in mouse cortical cultures. (**A**) To assess H_2O_2-chelating efficacy, 1 μM CM-H_2DCFDA was mixed with 200 μM H_2O_2 for 30 min and then treated without (black bar) or with indicated concentrations of **8** (red bars). The fluorescent intensity of CM-H_2DCFDA was measured by microplate reader at 485/535 nm (ex/em). (**B**) lactate dehydrogenase (LDH) release from cultures exposed to 10 μM $FeCl_2$ in absence (black bar) or presence of 10 μM of compound **8** (+Comp 8, red bar), and to 10 μM of compound **8** alone (Comp **8**, blue bar) for 24 h (*$P < 0.001$ vs $FeCl_2$). (**C**) Cultures were exposed to 10 μM $FeCl_2$ in absence or presence of 10 μM of **8**, and to $FeCl_2$ non-treated cells with same concentration of **8** for 24 h. Phase contrast images were obtained under light microscope (upper panels). Cells were stained with 1 μM CM-H_2DCFDA for 30 min and observed under fluorescent microscope at excitation 470/22 and emission 510/42 nm (lower panels). Scale bar, 125 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

various cell lines were evaluated in range from 0.7 to 50 μM (Fig. 5) using normal cells (CHO), immune cells (RAW 264.7), and cancer cells (A2780 and BHT). Since all the tested compounds were non-toxic at given concentrations, we used 10 μM in further experiments. Several studies have reported that the inflammation and oxidative stress are interlinked to cause neurodegeneration [34,35]. On the basis of the promising results of neuroprotective effects of compound **8**, and **8a-c** as antioxidant in cortical cells, we were interested to further investigate whether these compounds have ability to protect inflammation. NF-κB, an important transcriptional activator for inflammatory diseases, is activated by oxidative stress and pro-inflammatory cytokines such as TNF-α and IL-6. Thus, we investigated anti-inflammatory activity of these compounds via the production of nitric oxide (NO), TNF-α and IL-6 by suppressing the NF-κB pathway in lipopolysaccharide (LPS)-induced immune cells. We performed NO assay, cytokine assays, and NF-κB promoter assay in CHO cells, with dexamethasone (DEX) as a reference compound (Fig. 6).

Fig. 6A illustrates that addition of compounds (DEX, **8**, and **8a-c**) significantly decreased nitric oxide in immune cells exposed to LPS, expression of nitric oxide was markedly increased upon exposure to LPS. However, cotreatment of compounds (DEX, **8**, and **8a-c**) and LPS significantly inhibited overnight cotreatment of nitric oxide production. Similarly, to determine whether these compounds affect the expressions of pro-inflammatory cytokines, levels of TNF-α and IL-6 were assessed in LPS-induced immune cells. When CHO cells were treated with 10 μM LPS, levels of TNF-α and IL-6 were highly increased. However, compounds **8** and **8a-c** significantly inhibited the expressions of both TNF-α and IL-6 in LPS-induced immune cells (Fig. 6B and 6C).

3.4. *Effect of compounds on NF-κB activation*

NF-κB is an important transcriptional activator for inflammatory diseases. Luciferase assay with bioluminescence imaging was performed to understand the mechanism underlying the inhibitory activity of compounds on LPS-induced expressions of NO, TNF-α and IL-6 in immune cells. Compound **8**, and **8a-c** inhibited the level of luciferase

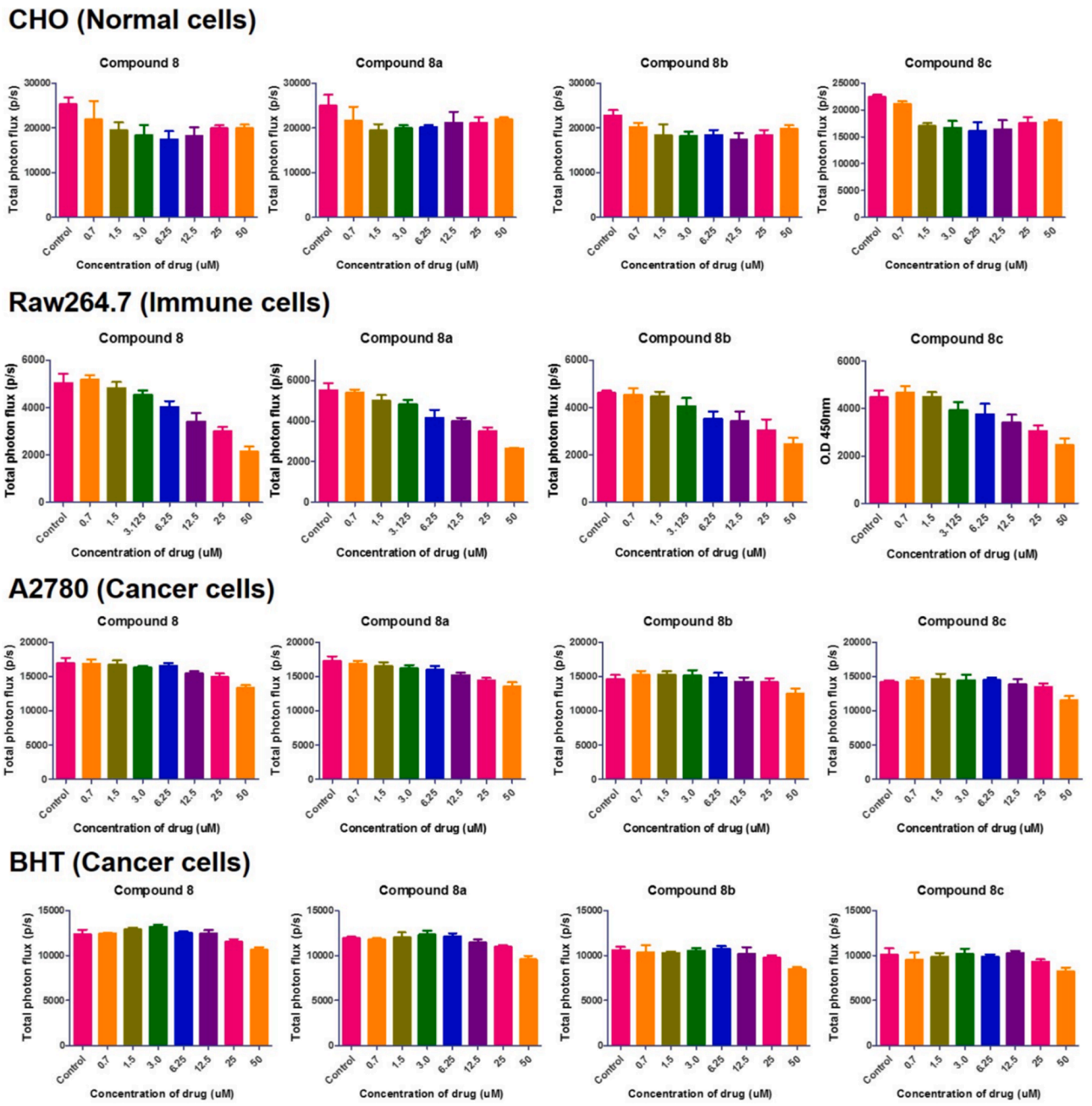


Fig. 5. Effect of psiguadial B (**8**) and its halogenated analogues (**8a-c**) on cell viability in various cell lines. Cell proliferation in CHO: normal cells, RAW 264.7: immune cells, A2780 and BHT: cancer cells with or without addition of compounds **8** and **8a-c**.

signal, indicating that these compounds downregulate NF-κB activity in LPS-induced immune cells (Fig. 6D). These results suggested that psiguadial B and its halogenated analogues have potential to be used as neuroprotective agents by inhibiting production of nitric oxide and expression of pro-inflammatory mediators via suppression of NF-κB activity.

4. Conclusions

In conclusion, we developed a short synthetic route of psiguadial B (**8**) and its halogenated analogues (**8a-c**) by coupling β -caryophyllene, benzaldehyde and diformylphloroglucinol using a non–toxic and cheaper sodium acetate catalyzed single step approach. The preliminary *in vitro* study in H_2O_2-induced cell death using primary cultures of mouse cortical neurons demonstrates psiguadial B and its halogenated analogues as novel and effective neuroprotective agents by chelating ROS. Furthermore, inhibition of LPS-induced expressions of NO, TNF-α and IL-6 in immune cell by suppression of NF-κB pathway suggested that these compounds have anti-inflammatory activity. These findings provide evidence for the development of psiguadial B-based neuroprotective and anti-inflammatory drugs against brain diseases and immunological disorders. Further research on synthesis and neuroprotective effects of psiguadial B derivatives with a modified diformylphloroglucinol group to identify lead compounds is ongoing and planned to be reported in near future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

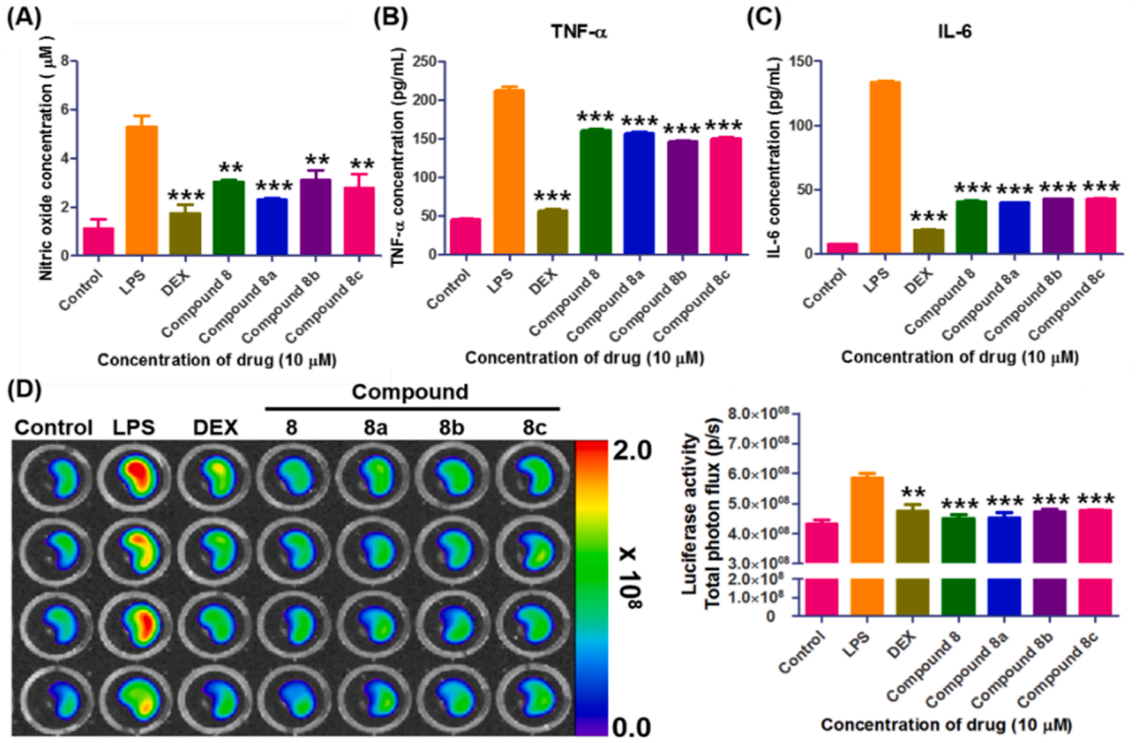


Fig. 6. Effect of psiguadial B and its halogenated analogues in immune cells. (**A**) Effect of compounds on LPS-induced NO production in immune cells. Immune cells were exposed to 1 μg/mL LPS and combination of 10 μM dexamethasone (DEX), compound **8**, and **8a-c**. (**B-C**) Effect of compounds on expressions of TNF-α (B) and IL-6 (**C**) in medium as determined by enzyme-linked immunosorbent assay (ELISA) in LPS-induced immune cells. Immune cells were pre-treated with indicated concentration of DEX, compound **8**, and **8a-c** for 1 h and then with LPS (500 ng/mL, 24 h). (**D**) Effect of compounds on NF-κB activation in LPS-induced immune cells as determined by luciferase assay with IVIS imaging system (**$P < 0.01$, ***$P < 0.001$).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.105027.

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