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RESEARCH ARTICLE

Inhibitory alkaloids from *Dictamnus dasycarpus* root barks on lipopolysaccharide-induced nitric oxide production in BV2 cells

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Abstract

The methanolic extract of *Dictamnus dasycarpus* root barks afforded one new glycosidic quinoline alkaloid, 3-[1*β*-hydroxy-2-(*β*-D-glucopyranosyloxy)-ethyl)-4-methoxy-2(1*H*)-quinolinone (**1**), together with nine known compounds, preskimmianine (**2**), 8-methoxy-N-methylflindersine (**3**), dictamine (**4**), γ-fagarine (**5**), halopine (**6**), skimmianine (**7**), dictangustine-A (**8**), iso-γ-fagarine (**9**), isomaculosidine (**10**). The isolated alkaloids significantly inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated BV2 cells. Among them, compounds **3** and **7** showed the most potent inhibitory activities on LPS-induced NO production.

Keywords: Dictamnus dasycarpus, alkaloids, nitric oxide, lipopolysaccharide, BV2 microglia

Introduction

Nitric oxide (NO) is a gaseous signalling molecule that regulates various physiological and pathological responses depending on the relative concentration of NO and the surrounding milieu in which NO is produced. NO modulates central nervous system (CNS) functions by regulating blood flow in the brain and acts as a neurotransmitter in nonadrenergic noncholinergic (NANC) neuron[1,2]. However, the excessive NO, which is produced in response to inflammation by the activated microglia in CNS has been reported to be involved in a number of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and ischemia[3–5]. Hence, the inhibition of overproduction of NO has been suggested as an important therapeutic approach for prevention and/or treatment of neurodegenerative diseases[6]. In the course of searching for compounds modulating the overproduction of NO from natural sources using LPS-stimulated BV2 cells as an *in vitro* assay system, it was found that the methanolic extract of *Dictamnus dasycarpus* root barks (Rutaceae) significantly inhibited LPS-induced NO production. *D. dasycarpus* is widely distributed in Asia, and the root bark of this plant has been used for treatment of various ailments such as skin inflammation, eczema, rubella, scabies, acute rheumatoid arthritis, jaundice, cold, and headache in Korean traditional medicine[7]. In the previous study in our lab, the neuroprotective activity of *D. dasycarpus* root bark was first reported through the isolation of neuroprotective limonoids, and identification of their action mechanisms against glutamate-induced neurotoxicity in primary cultures of rat cortical cells[8,9]. Even though quinoline and furoquinoline alkaloids have been reported as constituents of *D. dasycarpus* root bark[10–12], there have been few reports related to neuroprotective effects of these alkaloids. In the present study, we report the isolation and structural elucidation of compounds **1**–**10** and their inhibitory activities on NO production in LPS-stimulated BV2 microglial cells.

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Methods and materials

Plant material

Root barks of *D. dasycarpus* were purchased from Kyungdong Oriental Herbal Market, Seoul, Korea in April, 2006 and identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University. A voucher specimen (SNU-0189) has been deposited in Herbarium of the Medicinal Plant Garden, College of Pharmacy, Seoul National University.

Extraction and isolation

Root barks of *D. dasycarpus* (21 kg) were extracted with 80% MeOH three times in an ultrasonic apparatus. Upon removal of the solvent under vacuum, the methanolic extract yielded 2.18 kg of material (10.4% by dry weight). The methanolic extract was suspended in H_2O and partitioned successively with *n*-hexane, $CHCl_3$, ethylacetate (EtOAc) and *n*-butanol. *n*-hexane soluble fraction was subjected to column chromatography (CC) over silica gel eluted with *n*-hexane/EtOAc gradient (100:0 to 0:100) to obtain sixteen fractions (hfr.1–hfr.16). Compound **4** (1.0 g) was purified by recrystallization with MeOH from hfr.7. The $CHCl_3$ fraction (220.2 g) was subjected to CC over silica gel eluted with $CHCl_3$/MeOH gradient (100:0 to 0:100) to obtain ten fractions (cfr.1–cfr.10). The cfr.3 was chromatographed on a silica gel column eluting with *n*-hexane-EtOAc mixture to afford five fractions (cfr.3-1–cfr.3-5). The cfr.3-4 was subjected to reverse phase (RP) CC with MeOH–H_2O step gradient (40% MeOH to 100% MeOH) to yield seventeen subfractions (cfr.3-4-1–cfr.3-4-17). The cfr.3-4-7 was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18}, 10 × 250 mm) using CH_3CN:H_2O (60:40, 2 mL/min) as an eluent to give compounds **6** (11.9 mg) and **9** (12.5 mg). Compound **10** (90.0 mg) was purified by recrystallization with MeOH from cfr.3-4-12. The cfr.4 was chromatographed on a silica gel column eluting with *n*-hexane-EtOAc mixture to afford fifteen fractions (cfr.4-1–cfr.4-15). The cfr.4-8 was subjected to reverse phase (RP) CC with MeOH–H_2O step gradient (30% MeOH to 100% MeOH) to yield eight subfractions (cfr.4-8-1–cfr.4-8-8). The cfr.4-8-2 was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18}, 10 × 250 mm) using CH_3CN:H_2O:MeOH (30:65:5, 2 mL/min) as an eluent to give compound **5** (5.8 mg). The cfr.4-8-4 was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18}, 10 × 250 mm) using CH_3CN:H_2O (35:65, 2 mL/min) as an eluent to give compound **7** (2.6 mg). The EtOAc fraction (36 g) was subjected to silica gel CC eluting with a gradient of $CHCl_3$/MeOH (100:0 to 0:100) to yield eight fractions (efr.1–efr.8). The efr.4 was subjected to CC over Sephadex LH-20 using MeOH to yield six subfractions (efr.4-1–efr.4-6). The efr.4-2 was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18}, 10 × 250 mm) using CH_3CN:H_2O (47:53, 2 mL/min) as an eluent to give compound **2** (5.6 mg). The efr.4-6 was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18}, 10 × 250 mm) using CH_3CN:H_2O (40:60, 2 mL/min) as an eluent to give compound **1** (12.9 mg). The efr.5 was subjected to RP CC with MeOH–H_2O step gradient (10% MeOH → 100% MeOH) to yield nineteen subfractions (efr.5-1–efr.5-19). The efr.5-5 was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18}, 10 × 250 mm) using CH_3CN:H_2O (33:67, 2 mL/min) as an eluent to give compound **8** (7.0 mg). The efr.5-6 was subjected to reversed-phase HPLC (YMC-Pack Pro C_{18}, 10 × 250 mm) using CH_3CN:H_2O (47:53, 2 mL/min) as an eluent to give compound **3** (5.8 mg).

3-[1β-hydroxy-2-(β-D-glucopyranosyloxy)-ethyl)-4-methoxy-2(1H)-quinolinone

Yellowish gum; $[\alpha]^{25}_D$ +21.0° (c 0.1, MeOH); UV (MeOH) λ_{max} (logε) 224 (3.19), 270 (2.43), 324 (2.36) nm; IR: νKBr max cm^{-1}: 3355, 2254, 2129, 1652, 1025, 825, 765, 620; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 3.18 (1H, m, H-2′), 3.35 (1H, m, H-3′), 3.39 (1H, dd, *J* = 5.6, 11.6 Hz, H-6′$_a$), 3.28 (1H, m, H-4′), 3.77 (1H, m, H-5′), 3.62 (1H, brd, *J* = 11.6 Hz, H-6′$_b$), 3.95 (3H, s, H-11), 3.97 (2H, m, H-13), 4.23 (1H, d, *J* = 7.8 Hz, H-1′), 5.08 (1H, t, *J* = 6.1 Hz, H-12), 7.23 (1H, t, *J* = 7.8 Hz, H-6), 7.31 (1H, d, *J* = 7.9 Hz, H-8), 7.53 (1H, t, *J* = 7.6 Hz, H-7), 7.73 (1H, d, *J* = 7.4 Hz, H-5); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 60.9 (C-6′), 62.9 (C-11), 65.7 (C-12), 69.5 (C-4′), 72.3 (C-13), 73.5 (C-2′), 76.5 (C-5′), 76.8 (C-3′), 103.4 (C-1′), 115.4 (C-8), 115.9 (C-10), 121.4 (C-3), 122.0 (C-6), 123.0 (C-5), 130.8 (C-7), 138.2 (C-9), 162.4 (C-4), 163.3 (C-2); HRFABMS (positive) *m/z* 398.1456 $[M+H]^+$ (Calcd for $C_{18}H_{23}O_9N$, 398.1450).

Enzymatic Hydrolyses of compound 1

Compound **1** (4.0 mg) was hydrolyzed with 8.0 mg of β-glucosidase (Almonds Lot 1264252, Sigma-Aldrich) in 1.5 mL of H_2O at 37 °C for 12 h. The hydrolyzed product of **1** (compound **1a**) was obtained from the organic phase. The aqueous phase of hydrolyzate of **1** was dried using a stream of N_2 and then subjected to CC over silica gel eluted with $CHCl_3$–MeCN (3:1) to yield glucose (1.5 mg) from **1**, $[\alpha]^{20}_D$ +48.2° (c 0.5, H_2O).

Cell cultures

BV2 mouse microglia cell line originally developed by Dr. Bocchini at University of Perugia (Perugia, Italy) was generously provided by Dr. Sun-yeou Kim at Kyunghee University (Suwon, Korea). The cell line was maintained in DMEM containing 10% FBS with penicillin (100 IU/mL) and streptomycin (10 mg/mL) at 37°C in a humidified atmosphere of 95% air–5% CO_2.

Determination of NO content

Compounds were dissolved in DMSO (final concentration in cultures, 0.1%). To remove any trace of phenol red, the cell cultures were washed and the medium was replaced with phenol red-free DMEM. Then BV2 microglia cells (2×10^5) were treated with samples for 1 h, before exposure to 100 ng/mL of LPS. After 24 h incubation, nitrite in culture media was measured to assess NO production in BV2 cells using Griess reagent. In 96-well plate, 100 μl of sample aliquots was mixed with 100 μl of Griess reagent

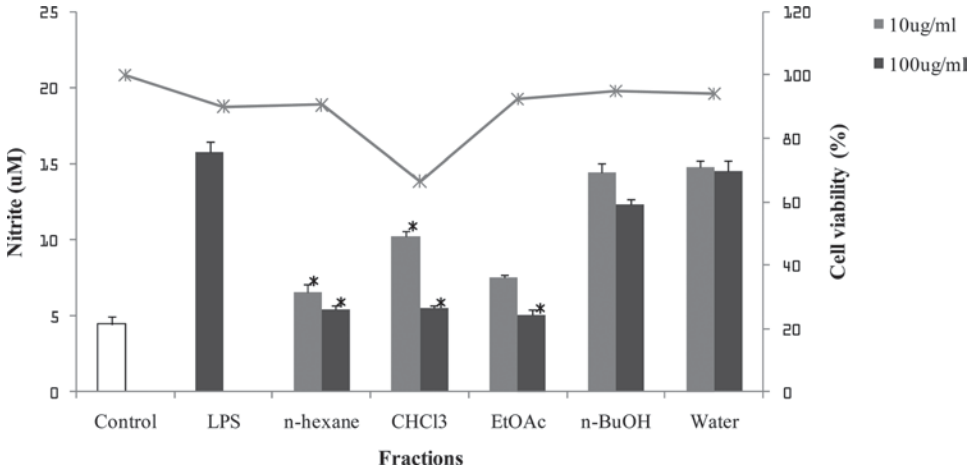

Figure 1. The inhibitory effects of fractions of *D. dasycarpus* on LPS-induced NO production in BV2 cells. BV2 cells were pre-treated with each fraction for 1 h before exposure to LPS for 24 h. The concentration of nitrite in culture medium was measured as described in the methods. The values shown are mean ± SD of data from three independent experiments. Results differ significantly from the LPS-treated, $^{*}p < 0.001$.

(1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2% phosphoric acid) and incubated at room temperature for 15 min. The absorbance (abs) at 550nm was measured on a microplate reader. The concentration was determined using nitrite standard curve.

Estimation of cell viability

After 100 µl of sample aliquot was collected for Griess assay, MTT (0.2 mg/ml) was directly added to cultures, followed by incubation at 37°C for 2 h. The supernatant was then aspirated and 100 µl of DMSO was added to dissolve the formazan. After insoluble crystals were completely dissolved, absorbance at 540 nm was measured using a microplate reader. Data were expressed as percent cell viability relative to control cultures.

Statistical analysis

Data were evaluated for statistical significance using an analysis of variance (ANOVA) with a computerized statistical package. The data were considered to be statistically significant if the probability value was <0.05.

Results and discussion

The methanolic extract of *D. dasycarpus* root barks was suspended in water, and successively partitioned into *n*-Hexane, $CHCl_3$, EtOAc, *n*-BuOH, and water fractions. Each fraction was evaluated for its inhibitory activity on NO production in LPS-stimulated BV2 cells (Figure 1). The *n*-hexane and EtOAc fractions, which showed the potent inhibitory effects on NO production without cytotoxicity, were further subjected to repeated column chromatography to yield nine alkaloids (**1**–**10**) including one new glycosidic quinoline alkaloid (**1**).

Compound **1** was isolated as a yellowish gum. The molecular formula was determined to be $C_{18}H_{23}O_9N$ from the HRFABMS at *m/z* 398.1456 $[M+H]^+$ (calcd *m/z* 398.1450). The ^1H NMR spectrum of **1** showed the

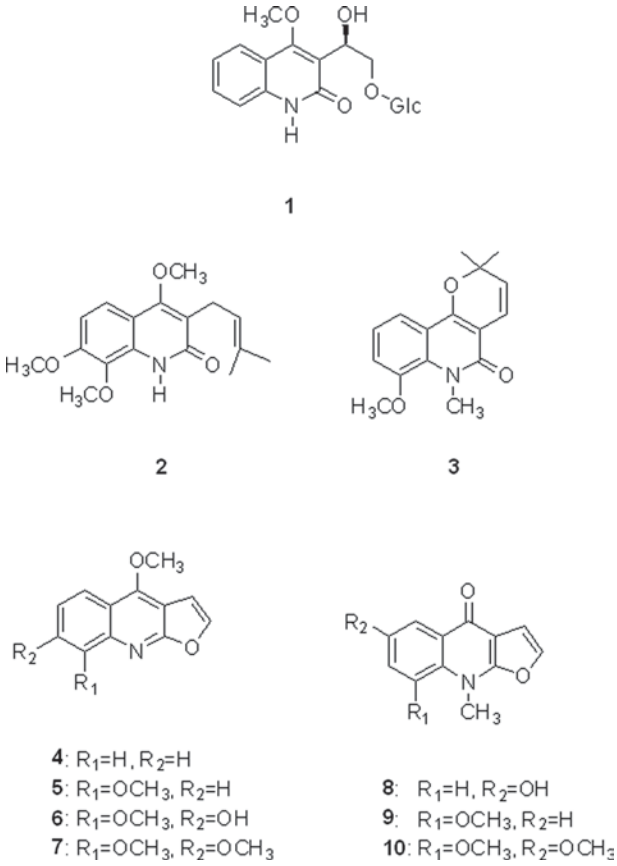

Figure 2. The structures of compounds **1**–**10.**

presence of one methoxy group [δ_H 3.95 (3H, s)], and 1, 2-substituted aromatic ring [δ_H 7.23 (1H, t, J=7.8 Hz), 7.31 (1H, d, J=7.9 Hz), 7.53 (1H, t, J=7.6 Hz), and 7.73 (1H, d, J=7.4 Hz)]. In addition, the ^{13}C spectrum had signals indicating one carbonyl group [δ_C 163.3 (C-2)], two quaternary carbon [δ_C 115.9 (C-10), and 138.2 (C-9)], together with signals characteristic for a glucose unit. The NMR spectra indicated that **1** has a 2(1*H*)-quinolinone

alkaloid derivative by comparing its spectroscopic data with previously reported information[13,14]. Hydrolysis of **1** with β-glucosidase yielded D-glucose ($[\alpha]^{20}_{D}$ +48.2°)[15]. The glucose was determined to be at C-13 from the HMBC correlation between H-1′ and C-13 (Figure 3). The ^{13}C and DEPT spectra had signals indicating one oxymethine carbon [δ_C 65.7 (C-12)], and one oxymethylene carbon [δ_C 72.3 (C-13)]. HMBC correlations from oxymethine proton [δ_H 5.08 (1H, t, J=6.1 Hz)] to C-3, C-2, C-4 and C-13 indicated that oxymethine was at C-3. Thus, compound **1** was determined as 3-[1β-hydroxy-2-(β-D-glucopyranosyloxy)-ethyl)-4-methoxy-2(1*H*)-quinolinone.

Nine known compounds were identified from their spectroscopic data by comparison with values reported in the literature as preskimmianine (**2**)[16], 8-methoxy-N-methylflindersine (**3**)[17], dictamine (**4**)[18,19], γ-fagarine (**5**)[19], halopine (**6**)[20], skimmianine (**7**)[16], dictangustine-A (**8**)[21], iso-γ-fagarine (**9**)[21], isomaculosidine (**10**)[22]. Among these compounds, compounds **3**, **6** and **8** were isolated for the first time from this plant (Figure 2).

Inhibitory effects of compounds **1**–**10** on LPS-induced NO production in BV2 microglia cells were evaluated using the Griess reaction described in Methods (Table 1). The treatment of LPS resulted in a significant increment of nitrite concentration in the medium as compared to control. The NO production induced by LPS was significantly inhibited by pretreatment of compounds **1**–**10**. Among them, compound **1** which had glycoside moiety in its structure showed moderate activity compared to the other compounds at the concentration tested. However, compound **1a**, the hydrolyzed product of compound **1** showed more potent inhibitory activity, which is comparable to compound **2**. The inhibitory activity of 8-methoxy-N-methylflindersine (**3**) was most potent, which showed almost complete inhibition on LPS-induced NO production at the concentration of 12.25 μM. In 4-methoxyfuroquinoline alkaloids (compounds **4**–**7**), the oxygenation at C-7 and C-8 resulted in a moderate increase in inhibitory activity on NO production, while weak activity was found in compounds **4** and **5,** which had no substitution at C-7. Especially, the methoxy group at C-7 led to a significant increase in inhibitory potency: skimmianine (**7**), halopine (**6**), γ-fagarine (**5**) > dictamine (**4**). On the other hand, in 4-oxo-N-methylfuroquinoline alkaloids (compounds **8**–**10**), the substitutions at C-6 or C-8 decreased the inhibitory activity at the concentration over 50 μM. To verify whether the reduced cell numbers caused by the cytotoxicity of these compounds resulted in decrease of NO production, cell viability was measured employing MTT assay. Cell viability was not significantly altered by the treatment of compounds at the concentrations used (data not shown).

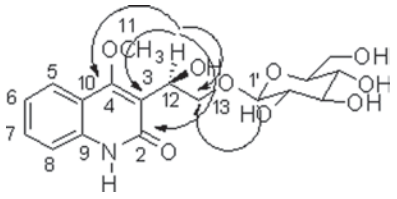

Figure 3. Key HMBC (→) Correlations of **1.**

In the previous study, we reported that limonoids isolated from *D. dasycarpus* root barks significantly protected primary cultures of rat cortical cells against glutamate-induced neurotoxicity[8]. In addition, it was revealed that neuroprotection by these limonoids was exhibited through inhibiting calcium influx, overproduction of cellular NO and reactive oxygen species, and to preserve mitochondrial membrane potential and activities of antioxidant enzymes[9]. Limonoids and alkaloids isolated herein are thought to contribute to

Table 1. Inhibitory effects of compounds **1**–**10** isolated from *D. dasycarpus* on LPS-induced NO production in BV2 cells.

	Relative NO production (%)					
Compound	1μM	12.25 μM	25 μM	50 μM	100 μM	IC_{50} (μM)
1	92.9±3.0	89.9±2.3	83.6±3.9	66.1±3.4*	65.0±4.4*	> 100
1a	90.7±2.1	79.8±1.9*	44.5±1.9**	22.2±2.8***	12.4±3.1***	17.2
2	87.9±2.5	77.3±3.3*	52.6±2.2**	30.4±2.3**	8.7±2.2***	18.5
3	42.3±0.8**	5.50±2.1***	1.0±0.7***	0.0±1.0***	0.0±1.1***	0.4
4	98.0±1.9	94.2±2.2**	87.9±1.3**	82.0±2.5**	49.7±1.1**	99.8
5	98.9±2.0	73.2±4.0*	86.7±5.3	60.2±3.4*	43.1±2.5**	85.3
6	90.6±1.7	74.7±1.3*	68.2±3.0*	36.8±2.1**	21.0±1.8**	27.4
7	75.8±2.1*	58.9±2.0**	18.9±1.8**	0.0±1.8***	0.0±1.5***	7.0
8	99.9±1.7	99.0±3.8	48.7±2.2**	12.6±2.8***	12.0±2.3***	25.0
9	97.6±1.4	88.2±1.4	47.2±2.5**	28.4±1.3**	28.7±2.7**	24.7
10	99.8±0.2	98.0±2.9	67.4±3.1*	38.9±3.5**	30.8±3.1**	38.1
L-NNA	99.9±0.3	90.3±3.0	75.6±2.4*	67.7±4.1*	49.4±2.2**	53.5

BV2 cells were pre-treated with compound for 1 h before exposure to LPS for 24 h. The concentration of nitrite in culture medium was measured as described in the Methods. The values shown are mean ± SD of data from three independent experiments. NO production (NP) of control and LPS-treated cultures were 7.7±0.2 and 46.7±0.3 mM, respectively. LPS-stimulated value differs significantly from control at a level of $p<0.001$. Results differ significantly from the LPS-treated, *$p<0.05$, **$p<0.01$ and ***$p<0.001$, respectively. Compound **1a** is the hydrolyzed product of compound **1**. L-NNA: N^G-Nitro-L-arginine, a well-known inhibitor of NOS.

neuroprotective effects of *D. dasycarpus*, and these compounds are expected to be potential drug candidates for treatment of various neurodegenerative diseases.

Declaration of interest

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