Natural Product Sciences
28(1) : 1-5 (2022)
https://doi.org/10.20307/nps.2022.28.1.1

Anti-inflammatory and Neurotrophic 2*H*-1-Benzopyran Derivatives of *Chaenomeles sinensis*

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Abstract – Two 2*H*-1-benzopyran derivatives, methyl 8-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-5-carboxylate (**1**) and methyl 8-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-6-carboxylate (**2**), including a new compound (**1**) were isolated from the twigs of *Chaenomeles sinensis*. Their chemical structures were characterized based on analysis of NMR data including ^{1}H and ^{13}C, COSY, HSQC, and HMBC and HRMS data. The isolated compounds (**1** and **2**) were assessed for their anti-neuroinflammatory activity by measuring inhibition levels of nitric oxide (NO) production in lipopolysaccharide (LPS)-activated BV-2 cells and for their neurotrophic activity by the secretion of nerve growth factor (NGF) in C6 cells. Compounds **1** and **2** exhibited powerful anti-neuroinflammatory effects with IC_{50} values of 17.14 and 19.30 μM, respectively, without cell toxicity, and also showed moderate effects on the stimulation of NGF secretion levels with 113.15 ± 3.54 and 130.20 ± 8.03%, respectively. The biosynthetic pathway of **1** and **2** was proposed that they would be derived from a protocatechuic acid and an isoprenyl unit.
Keywords – *Chaenomeles sinensis*, 2*H*-1-benzopyran derivatives, anti-neuroinflammation, neurotrophic activity

Introduction

Chaenomeles sinensis Koehne, commonly known as Chinese Quince, belongs to the Rosaceae and is a semi-evergreen tree which is widely distributed in Korea, Japan, and mainland China. The fruit of this plant has been used as Korean traditional medicine to treat vomiting, myalgia, and diarrhea[1] and also consumed as a tea.[2] Previous phytochemical research on *C. sinensis* reported triterpenoids,[3-5] flavonoids,[4,6,7] and phenolic compounds[8,9] with various biological activities.[1,5,10,11]

As a part of the ongoing studies to identify bioactive phytochemical constituents from the Korean medicinal plants, our previous phytochemical studies on the MeOH extract of the twigs of *C. sinensis* have resulted in the isolation and structure elucidation of triterpenoids,[10] biphenyls,[11] lignans,[1,12] oxylipins,[13] and norsesquiterpenoid glycoside[14] showing cytotoxic, anti-inflammatory, or potential neuroprotective activities. In a continuing search for minor constituents with biological activity from the twigs of *C. sinensis*, the MeOH extract was further investigated, which resulted in the isolation and characterization of a new 2*H*-1-benzopyran derivative (**1**) and a previously reported analog (**2**) (Fig. 1). The isolated compounds (**1** and **2**) were evaluated for their anti-neuroinflammatory effects on the inhibition levels of NO generation in LPS-stimulated BV-2 cell lines and for their neurotrophic activity through the secretion of NGF into C6 cells.

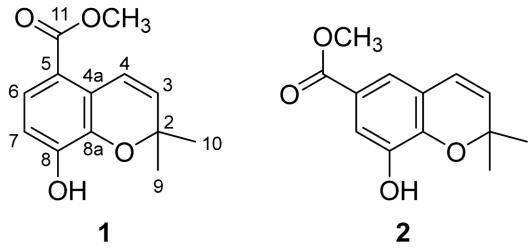

Fig. 1. Chemical structures of **1** and **2**.

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Experimental

General experimental procedures – Specific rotation values were recorded using a JASCO P-1020 polarimeter equipped with the sodium D line (590 nm) (JASCO, Easton, MD, USA). The NMR analysis was conducted by Bruker AVANCE III 700 NMR spectrometer and the NMR spectra (^{1}H, ^{13}C, COSY, HSQC, and HMBC) at 700 MHz (^{1}H) and 175 MHz (^{13}C) with chemical shifts given in ppm (δ) (Bruker, Karlsruhe, Germany). Waters SYNAPT G2 (Milford, MA, USA) was used to obtain HRFABMS. The semipreparative high-performance liquid chromatography (HPLC) equipped with a Gilson 306 pump (Middleton, WI, USA), a Shodex refractive index detector (New York, NY, USA), and an Apollo Silica 5 μ column (250 mm length × 10 mm i.d.) at a flow rate of 2 mL/min was used for isolation and purification of compounds. Both silica gel 60 (70−230 and 230−400 mesh; Merck, Darmstadt, Germany) and RP-C_{18} silica gel (Merck, 230−400 mesh) were used for column chromatography. Thin-layer chromatography (TLC) analysis was carried out using Merck precoated silica gel F_{254} plates and RP-C_{18} F_{254s} plates (Merck, Darmstadt, Germany), and spots were detected on TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid.

Plant material – The twigs of *C. sinensis* were collected from Seoul, Republic of Korea in January 2012. A voucher specimen (SKKU-NPL 1206) was authenticated by Prof. Dr. Kang Ro Lee (Sungkyunkwan University) and deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Republic of Korea.

Extraction and isolation – The dried twigs of *C. sinensis* (7.0 kg) were extracted with 80% aqueous MeOH under reflux condition and filtered. The filtrate was concentrated under reduced pressure to obtain MeOH extract (280 g). The extract was suspended in distilled H_2O and then was partitioned with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH, yielding 3, 15, 6, and 30 g of each organic residue. The *n*-hexane-soluble fraction (3 g) was chromatographed on silica open column (*n*-hexane-EtOAc, 3:1 → 1:1) to give seven fractions (H1–H7). Fraction H3 (400 mg) was separated over RP-C_{18} silica gel column and eluted with 90% aqueous MeOH to obtain twelve subfractions (H3-1–H3-12). Compounds **1** (2 mg) and **2** (3 mg) were purified from the subfraction H3-1 (20 mg) by semipreparative normal-phase HPLC (2 mL/min, hexanes-EtOAc, 5:1) under isocratic conditions.

Methyl 8-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-5-carboxylate (1) – Colorless gum; ^{1}H (700 MHz) and ^{13}C NMR (175 MHz) data in $CDCl_3$, see Table 1; HRFABMS (positive-ion mode) *m/z* 235.0962 $[M + H]^+$ (calcd. for $C_{13}H_{15}O_4^+$, 235.0965).

Methyl 8-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-6-carboxylate (2) – Colorless gum; ^{1}H (700 MHz) and ^{13}C NMR (175 MHz) data in $CDCl_3$, see Table 1; HRFABMS (positive-ion mode) *m/z* 235.0970 $[M + H]^+$ (calcd. for $C_{13}H_{15}O_4^+$, 235.0965).

Assessment of NO production from BV-2 cells – Analogous as described in the previous paper.[15] The BV-2 cells, developed by Dr. V. Bocchini at the University of Perugia (Perugia, Italy), were used for this study.[16,17] The cells were seeded in a 96-well plate (4×10^4 cells/well) and incubated in the presence or absence of various doses

Table 1. ^{1}H and ^{13}C NMR data for compounds **1** and **2** in $CDCl_3$

Position	1		2	
	δ_C	δ_H [mult. (*J* in Hz)]	δ_C	δ_H [mult. (*J* in Hz)]
2	76.8	-	78.6	-
3	132.4	5.77, d (10.2)	131.0	5.66, d (9.9)
4	120.7	7.39, d (10.2)	121.9	6.35, d (9.9)
4a	122.8	-	120.6	-
5	117.9	-	120.0	7.32, d (2.0)
6	124.8	7.52, d (8.6)	122.9	-
7	113.8	6.81, d (8.6)	116.4	7.48, d (2.0)
8	148.6	-	144.3	-
8a	139.6	-	143.6	-
9/10	27.7	1.47, s	28.4	1.48, s
11	167.3	-	166.9	-
OCH_3	51.9	3.86, s	52.1	3.87, s
OH	-	5.86, brs	-	5.44, brs

of tested compounds (**1** and **2**). LPS (100 ng/mL) was added to all the pre-treated wells except the control one and grown for 1 day. The produced levels of nitrite (NO_2), a soluble oxidized product of NO, was evaluated with 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid. After 10 min, the absorbance at λ 540 nm was measured using a microplate reader.

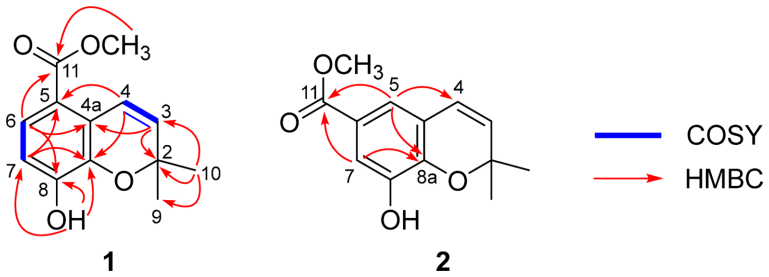
Assays for NGF release from C6 cells – Analogous as described in the previous paper.[10] C6 glioma cell lines were used to measure the NGF of the culture medium, which was fixed with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) in an incubator filled with 5% CO_2. The cells were seeded 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** and **2**), 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 using an ELISA development kit (R&D System, Minneapolis, MN, USA). Besides, the cell viability was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay by comparison with 6-shogaol as a positive control and the results are expressed as percentage of the control group.

Results and Discussion

Compound **1** was isolated as colorless gum and its molecular formula was determined as $C_{13}H_{14}O_4$ from the protonated molecular ion at *m/z* 235.0962 $[M+H]^+$ in the HRFABMS data (calcd. for $C_{13}H_{15}O_4^+$, 235.0965). The ^1H and ^{13}C NMR data of **1** show the presence of a 1,2,3,4-tetrasubstitued benzene [δ_H 7.52 (1H, d, $J = 8.6$ Hz, H-6) and 6.81 (1H, d, $J = 8.6$ Hz, H-7); δ_C 148.6 (C-8), 139.6 (C-8a), 124.8 (C-6), 122.8 (C-4a), 117.9 (C-5), and 113.8 (C-7)], an olefinic group [δ_H 7.39 (1H, d, $J = 10.2$ Hz, H-4) and 5.77 (1H, d, $J = 10.2$ Hz, H-3); δ_C 132.4 (C-3) and 120.7 (C-4)], a hydroxy group [δ_H 5.86 (1H, brs, OH-8)], a methoxy group [δ_H 3.86 (3H, s, OCH_3-11); δ_C 51.9 (OCH_3-11)], two methyl groups [δ_H 1.47 (6H, s, CH_3-9/10); δ_C 27.7 (CH_3-9/10)], an ester carbon [δ_C 167.3 (C-11)], and an oxygenated carbon [δ_C 76.8 (C-2)]. These ^1H and ^{13}C NMR data are very similar to those of methyl 8-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-6-carboxylate (**2**) (Table 1),[18] but the major difference is that the coupling constant between two aromatic protons of **1** (H-6 and H-7) is 8.6 Hz whereas that of **2** (H-5 and H-7) is 2.0 Hz. This observation suggests that the two aromatic protons of **1** are in *ortho* position rather than *meta* position as in **2** and this initial assignment is further supported by presence of HMBC correlation of H-6 (δ_H 7.52) with C-11 (δ_C 167.3) and absence of HMBC correlation of H-7 (δ_H 6.81) with C-11 (δ_C 167.3) (Fig. 2). Additional 2D NMR data analysis of **1** including COSY, HSQC, and HMBC spectra confirmed full structural characterization. The position of methoxy group can be assigned at C-11 based on the HMBC correlation of OCH_3-11 (δ_H 3.86) with C-11 (δ_C 167.3) and the methyl ester unit is located at C-5 based on the HMBC correlations of H-4 (δ_H 7.39) and H-7 (δ_H 6.81) with C-5 (δ_C 117.9). The location of a hydroxy group at C-8 was confirmed by the HMBC correlation of OH-8 (δ_H 5.86) with C-7 (δ_C 113.8), C-8 (δ_C 148.6) and C-8a (δ_C 139.6) (Fig. 2). Thus, the structure of **1** was defined as 8-hydroxy-2,2-dimethyl- 2*H*-1-benzopyran-5-carboxylic acid methyl ester.

Compound **2** exhibited the same molecular formula $C_{13}H_{14}O_4$ as that of **1** deduced from the protonated molecular ion at *m/z* 235.0970 $[M+H]^+$ in the HRFABMS data (calcd. for $C_{13}H_{15}O_4^+$, 235.0965). The structure of **2** was assumed to be methyl 8-hydroxy-2,2-dimethyl-2*H*-1-benzopyran-6-carboxylate by observing the almost identical ^1H and ^{13}C NMR data to those of previously reported by Orjala et al.[18] and this initial structural proposal was confirmed through detailed inspection of 2D NMR data of **2** (Fig. 2).

With these data in hand, we then were able to propose the biosynthetic pathway of **1** and **2** as follow. Since the demethylated analog of **2** has been proposed to be formed



1 **2**

Fig. 2. Key COSY (blue bold) and HMBC (red arrow) correlations of compounds **1** and **2**.

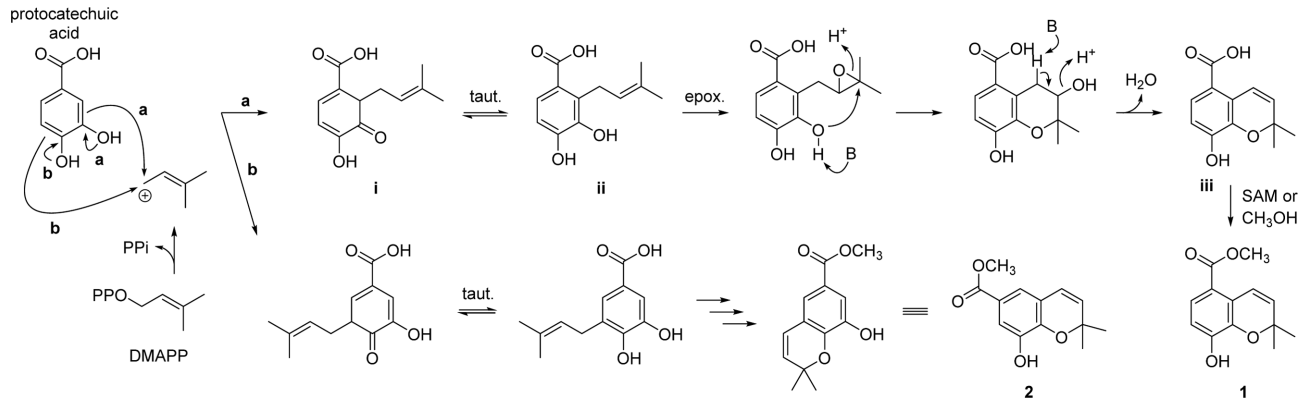


Fig. 3. Proposed biosynthetic pathway of compounds **1** and **2**. taut., tautomerization. epox., epoxidation.

from 4-hydroxybenzoic acid and isoprenoid in fungi[19] we assumed that the similar biosynthetic precursors, protocatechuic acid and dimethylallyl pyrophosphate (DMAPP), would be used for biosynthesis of **1** and **2**. Base-catalyzed C-C bond formation between protocatechuic acid and carbocation (route **a** in Fig. 3), released from DMAPP, would produce **i** and then **ii** by tautomerization of **i**. Epoxidation at the double bond in the side chain in **ii** followed by cyclization and dehydration would occur to form 2*H*-1-benzopyran-containing molecule **iii**. A methyl group in *S*-adenosyl methionine (SAM) molecule or in methanol used for extraction and isolation would be transferred to the carboxylic acid in **iii** to produce **1**. Compound **2** is thought to be synthesized via the similar pathway of **1** with different C-C bond formation between protocatechuic acid and DMAPP-derived carbocation (route **b** in Fig. 3).

In continuing search for secondary metabolites with anti-neuroinflammatory and neurotrophic activity from *C. sinensis* twigs,[1,10-14] we first tested the isolated compounds **1** and **2** for their inhibitory effect on NO release from LPS-stimulated murine microglia BV-2 cell lines. As shown in Table 2, both compounds showed more potent activity with IC_{50} values of 17.14 (**1**) and 19.30 μM (**2**) without cell toxicity, than a well-known inhibitor of NO synthase (NOS), N^G-monomethyl-L-arginine (NMMA, IC_{50} 21.35 μM). Also, these two compounds exhibited mild induction of NGF from C6 glioma cell lines with stimulation levels of 113.15 ± 3.54% (**1**) and 130.20 ± 8.03% (**2**) at 20 μM whereas 6-shogaol, a positive control substance, showed NGF secretion level of 149.53 ± 5.36 % (Table 3).

In sum, two 2*H*-1-benzopyran analogs (**1** and **2**) including a new one (**1**) were isolated and structurally characterized from the twigs of *C. sinensis*, which has been used for traditional Korean medicine. The biosynthetic pathway of these two compounds were proposed based on the previously reported literature and compounds **1** and **2** showed strong anti-inflammatory and weak neurotrophic activities. This study suggests that these two characterized secondary metabolites **1** and **2** could be a starting point for development of anti-

Table 2. Effects of compounds **1** and **2** on NO generation in LPS-stimulated BV-2 cells

Comp.	IC_{50}[a] (μM)	Cell viability[b] (%)
1	17.14	101.50 ± 5.05
2	19.30	109.48 ± 4.16
L-NMMA[c]	21.35	104.56 ± 4.20

[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 after treatment with 20 μM of each compound was determined by MTT assay and is expressed in percentage (%). The results are averages of three independent experiments, and the data are expressed as mean ± SD.
[c]L-NMMA as positive control.

Table 3. Effects of compounds **1** and **2** on NGF Secretion in C6 cells

Comp.	NGF secretion[a] (%)	Cell viability[b] (%)
1	113.15 ± 3.54	99.00 ± 1.15
2	130.20 ± 8.03	94.35 ± 4.72
6-shogaol[c]	149.53 ± 5.36	97.01 ± 0.17

[a]C6 cells were treated with 20 μM of each compound. After 24 h, the content of NGF secreted into 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%).
[b]Cell viability after treatment with 20 μM of each compound was determined by an MTT assay and is expressed as a percentage (%). Results are the means of three independent experiments and the data are expressed as mean ± SD.
[c]Positive control substance.

neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.

Acknowledgments

This work was supported by the National Research Foundation (NRF) of Korea grant funded by the government of Korea (MSIT) (No. 2012M3A9C4048775) and by the BK21 FOUR Project. We are thankful to the Korea Basic Science Institute (KBSI) for the measurements on mass spectra.

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Received August 14, 2021
Revised October 1, 2021
Accepted October 18, 2021