Anti-adipogenic activity of a new cyclic diarylheptanoid isolated from *Alnus japonica* on 3T3-L1 cells via modulation of PPARγ, C/EBPα and SREBP1c signaling

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ABSTRACT

Total methanolic extract of *Alnus japonica* fruits exhibited significant anti-adipogenic activities in 3T3-L1 cells. A new cyclic diarylheptanoid (**1**) along with ten known compounds (**2–11**) were isolated by activity-guided fractionation. Compound **1**, determined to be 4-hydroxy-alnus-3,5-dione, showed the most potent anti-adipogenic effect. Compound **1** significantly down-regulated expression of peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα), and sterol regulatory element binding protein 1 (SREBP1c) in 3T3-L1 cells, as determined by quantitative real-time PCR and Western blot analysis. Furthermore, compound **1** suppressed mRNA expression of C/EBPβ and C/EBPδ during the early stage of adipogenesis as well as stearoyl coenzyme A desaturase 1 (SCD-1) and fatty acid synthase (FAS), target genes of SREBP1c. Upon investigating the mechanism of natural products, we propose that cyclic diarylheptanoid (**1**), the most potent constituent of *A. japonica*, can be a potent therapeutic agent against obesity through anti-adipogenesis via down-regulation of PPARγ, C/EBPα, and SREBP1c signaling.

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Obesity is now so common among the worldwide population that it has begun to replace malnutrition and infectious diseases as the most significant contributor to ill health. Despite rapid increases in obesity over the past several decades, treatments have remained disappointing due to serious limitations such as adverse effects or inadequate efficacy. Therefore, novel therapeutic approaches are needed instead of previously developed drugs.

Adipose tissue, which is composed of adipocytes as well as a small percentage of other cells, is an important metabolic organ that is crucial for insulin sensitivity, lipid metabolism, and energy homeostasis, and it serves as an integrator of various physiological pathways.[1] Disorders affecting adipose tissue, such as excess differentiation of adipocytes, increase the risk of metabolic diseases.[2] Therefore, modulation of adipocyte differentiation may provide an improved therapeutic approach to prevent initiation and progression of obesity.[3] Adipocyte differentiation (adipogenesis) is controlled by key adipogenic factors, such as peroxisome proliferator activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα), and sterol regulatory element binding protein 1 (SREBP1c). These transcription regulators, known as adipokines,

play important roles in balancing lipid metabolism by regulating the expression of target genes associated with lipid accumulation, fatty acid oxidation, and lipolysis.[2]

Alnus japonica Steud. (Betulaceae), an indigenous *Alnus* species found in Korea, is a deciduous broad-leaved tree growing in the damp areas of mountain valleys. *A. japonica* has been used to treat hemorrhages, burn injuries, antipyretic fever, diarrhea, and alcoholism in traditional Korean medicine.[4,5] These days, *A. japonica* is easily purchased from Oriental herb markets as a health tea for healing hangovers and alcoholism in Korea. Pharmacological studies on *A. japonica* have demonstrated anti-inflammatory, anti-tumor, anti-obesity, and anti-oxidative effects.[6] *A. japonica* contains various types of plant secondary metabolites such as diarylheptanoids, flavonoids, tannin, phenols, steroids, and triterpenoids.[6] We previously reported the inhibitory effect of adipocyte differentiation on diarylheptanoids isolated from leaves of *Alnus hirsuta* f. *sibirica*.[7] The present study describes the elucidated structures of cyclic diarylheptanoid (**1**) newly isolated from *A. japonica* and its anti-adipogenic and lipogenic activity mediating by PPARγ, C/EBPα, and SREBP1c signaling. It is newly reported that cyclic diarylheptanoid showed the suppression on adipogenesis in 3T3-L1 cells and can be a potent therapeutic agent against obesity.

A. japonica fruits were collected in Nambu forest in Seoul, Beagwoon Mountain, Gwangyang-si, Jeollanam-do, Korea and have

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been deposited as voucher specimen (SCNUP003-F1) in the laboratory of Pharmacognosy, College of Pharmacy, Sunchon National University, Suncheon-si, Jeollanam-do, Korea.

Dried and pulverized *A. japonica* fruits were extracted with 80% methanol (4 L, 3 h × 5) by using an ultrasonic apparatus at room temperature. Methanolic extract of *A. japonica* fruits was concentrated in vacuo to give a crude extract (419.1 g). This methanolic extract was suspended in H_2O and partitioned successively with *n*-hexane (4 L), $CHCl_3$ (4 L), *n*-BuOH (4 L), and H_2O, providing solid residues of 29.5 g, 47.6 g, 251.6 g, and 62.7 g, respectively. Each fraction was evaluated for its anti-adipogenic activity in 3T3-L1 cells. Total methanolic extract of *A. japonica*, $CHCl_3$, and *n*-butanol soluble fractions showed significant inhibitory effects on adipocyte differentiation at a concentration of 50 µg/mL. Of these fractions, $CHCl_3$ and *n*-BuOH fractions with significantly inhibitory effects on adipocyte differentiation (67.7 ± 5.9% and 31.4 ± 6.8% at concentration of 50 µg/mL, respectively) were subjected to follow-up isolation work. $CHCl_3$ fraction was subjected to silica gel column chromatography and eluted by a $CHCl_3$–MeOH–water gradient to yield 15 fractions (C1–C7). C4 was subjected to MPLC (RediSep silica gel; $CHCl_3$–MeOH, 15:1 → MeOH, 30 mL/min) to yield six fractions (C4-1–C4-6). Compound **1** (8.1 mg, t_R 302.60 min) and compound **3** (3.1 mg, t_R 292.46 min) were obtained from C4-5-4 through preparative HPLC (polymeric gel filtration, 500 × 20 mm, Hexane/EtOAc 3:1 → 1:1, 4 mL/min). C4-5-6 was subjected to preparative HPLC (polymeric gel filtration, 500 × 20 mm, *n*-hexane/EtOAc 3:1 → 1:1, 4 mL/min) to yield 30 fractions (C4-5-6-1–C4-5-6-30). Compound **2** (3.2 mg) was obtained from C4-5-6-27 by column chromatography on Sephadex LH-20 ($CHCl_3$ MeOH, 1:1). *n*-BuOH fraction (251.6 g) was eluted from Diaion HP-20 resin using a H_2O–MeOH gradient as the mobile phase to yield six fractions (B1–B6). B3 was subjected to silica gel CC and eluted by a $CHCl_3$–MeOH–water gradient to yield 11 fractions (B3-1–B3-11). Compound **4** (6.7 mg, t_R 68.14 min) and compound **9** (1.5 mg, t_R 346.12 min) were obtained from B3-5 through preparative HPLC (C_{18}, 500 × 20 mm, MeOH–H_2O, 30:70, 4 mL/min). Compound **10** (2.4 mg, t_R 80.98 min) was obtained from B3-6 through preparative HPLC (C_{18}, 500 × 20 mm, MeOH–H_2O, 30:70, 4 mL/min). B4 yielded compound **5** (1.9 mg, t_R 182.12 min) and **8** (10.5 mg, t_R 90.48 min) by preparative HPLC (C_{18}, 500 × 20 mm, CH_3CN–H_2O, 10:90, 4 mL/min). B5 was subjected to RP C_{18} column chromatography and eluted by a H_2O–MeOH gradient to yield 65 subfractions (B5-1–B5-65). Compounds **6** (2.6 mg) and **7** (5.1 mg) were refined from B5-40 and B5-32, respectively, by column chromatography on Sephadex LH-20 (MeOH).

Compound **1** was obtained as a brownish syrup and $[\alpha]_D^{25}$ −0.5 (c 1.0 MeOH). The IR spectrum revealed the presence of carbonyl (1700 cm^{-1}) and aromatic ring (1588 and 1430 cm^{-1}) functional groups. The signals in the ^1H and ^{13}C NMR and HSQC experiments indicated that compound **1** possesses a carbonyl carbon at δ_C 193.7 (C-3), hydroxy-bearing methine carbon at δ_C 107.0 (C-4), and methylene carbons at δ_C 28.3 (C-1) and δ_C 38.1 (C-2). Furthermore, the ^1H NMR spectroscopic data revealed signals for 1,3,4-trisubstituted aromatic rings [δ_H 7.13 (1H, d, J = 2.0 Hz, H-2′), 6.98 (1H, dd, J = 8.1, 2.0 Hz, H-6′), and 6.83 (1H, d, J = 8.1 Hz, H-5′)], signals for two neighboring methylene groups at δ_H 3.36 (1H, t, J = 13.2 Hz, H-1a), 2.77 (1H, m, H-1b), 2.69 (1H, m, H-2a), and 2.46 (1H, t, J = 13.2 Hz, H-2b), and a signal for a hydroxy-bearing methane at δ_H 5.88 (1H, s, H-4). HMBC analysis demonstrated correlations of H-2′ to C-1′ and C-4′, H-5′ and H-6′ to C-4′, and H-2 and H-4 to C-3 (Fig. 1). Taking into account all of the above described spectroscopic data, compound **1** was designated as arylbutanoid. However, the molecular formula of compound **1** was established as $C_{19}H_{22}O_6$ by the observed pseudo-molecular ion peak at *m/z* 326.1152 $[M]^+$ (Calcd for $C_{19}H_{18}O_5$ 326.1154) in the positive HRFABMS. In the ^1H NMR spectrum, the area ratio of four peaks
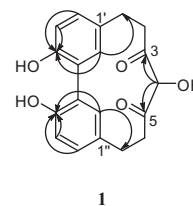

Figure 1. The key correlations of HMBC spectral data of compounds **1**.

at δ_H 7.13 (H-2′), 6.98 (H-5′), 6.83 (H-6′), and 5.88 (H-4) was 2:2:2:1. Based on the above information, the structure of compound **1** was considered to be symmetrical and was determined as 4-hydroxy-alnus-3,5-dione (Fig. 1).

4-hydroxy-alnus-3,5-dione (**1**) brownish syrup; $[\alpha]_D^{25}$: −0.5 (c 1.0 MeOH); UV λ_{max} (MeOH) (log ε) (nm): 251 (3.55), 289 (3.43); IR (KBr) v_{max} (cm^{-1}): 3024, 2916, 1700, 1588, 1508, 1430, 1410, 1238, 1011, 951, 816; ^1H NMR (400 MHz, $CDCl_3$): δ 7.13 (2H, d, J = 2.0 Hz, H-2′,2″), 6.98 (2H, dd, J = 8.1, 2.0 Hz, H-6′,6″), 6.83 (2H, d, J = 8.1 Hz, H-5′,5″), 5.88 (1H, s, H-4), 3.36 (2H, t, J = 13.2 Hz, H-1a,7a), 2.77 (2H, m, H-1b, 7b), 2.69 (2H, m, H-2a, 6a), and 2.46 (2H, t, J = 13.2 Hz, H-2b, 6b); ^{13}C NMR (100 MHz, $CDCl_3$): δ 193.7 (C-3,5), 151.2 (C-4′,4″), 133.7 (2′,2″), 132.3 (1′,1″), 128.6 (6′,6″), 126.2 (3′,3″), 116.3 (5′,5″), 107 (C-4), 38.1 (2,6), 28.3 (1,7); HRFABMS (positive mode) *m/z* 326.1152 $[M]^+$ (Calcd for $C_{19}H_{18}O_5$, 326.1154).

The ten known compounds were identified as dihydroalnusone (**2**),[8] alnusol (**3**),[8] alnusonone (**4**),[8] betulatetraol (**5**),[9] 2″″-cinnamoyloregonin (**6**),[10] oregonyl A (**7**),[11] oregonyl B (**8**),[11] (5S)-O-methylhirsutanonol (**9**),[12] hitsutanonol-5-O-β-D-glucopyranoside (**10**),[13] and aceroside VII (**11**)[14] by comparing the measured spectroscopic data with published values.

The effect of these compounds on the adipogenic activity were evaluated using an established in-house assay system. Confluent 3T3-L1 preadipocytes were treated with various concentrations of diarylheptanoids from *A. japonica* during differentiation (days 0–8).[15] Relative lipid contents were measured by Oil Red O staining, on day 8.[16] The protein and gene expression of PPARγ and C/EBPα were detected by Western blot analysis and real time PCR according to established protocol, respectively.[17,18]

As shown in Figure 2, anti-adipogenic activity of compounds **1–11**, together with epigallocatechin-3-gallate (EGCG) as a positive control, were evaluated by assessing fat accumulation of 3T3-L1 cells. Inhibitory activity of (−)-EGCG, a positive control, was 54.5% at 100 µM. These eleven compounds consisted of cyclic diarylheptanoids and non-cyclic diarylheptanoids. Among cyclic diarylheptanoids, compounds **1**, **2**, and **5** showed significant inhibitory effects on adipocyte differentiation of 3T3-L1 cells (11.6%, 54.4%, and 58.5% relative lipid contents at 100 µM, respectively). For non-cyclic diarylheptanoids, Lee et al. elucidated that ketone functionality in heptanol and/or heptane plays an important role in anti-adipogenesis.[7] However, compounds **6–8** showed weak inhibitory effects on adipocyte differentiation (85.6%, 95.7%, and 71.3% relative lipid contents at 100 µM, respectively), compared to other non-cyclic diarylheptanoids with ketone functionality at C-3. These results suggest that substitution of phenylpropanoid at the xylosyl moiety of oregonin reduced lipid accumulation. Of the diarylheptanoids tested in the present assay system, compound **1** showed the most potent inhibitory effect on adipocyte differentiation at a concentration of 100 µM. Hence, further investigation using compound **1** was carried out to reveal its anti-adipogenic mechanism in 3T3-L1 cells.

Adipocyte differentiation results in a series of programmed alterations of specific genes. Adipogenesis is regulated by adipokines such as PPARγ, C/EBPα, and SREBP1c, which are known to be critical activators of adipogenesis.[19] Expression levels of adipokines