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Minor Phenolic Constituents of the Anemarrhenae Rhizoma

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Abstract – A homoisoflavanone, 7,4'-dihydroxyhomoisoflavanone (**1**) and a flavanone, (2*S*)-7,4'-dihydroxy-5-methoxyflavanone (**2**), were isolated from the rhizomes of *Anemarrhena asphodeloides*, together with 4,4'-dihydroxychalcon (**3**), 2'-*O*-methylphlorethin (**4**), 1,3-bis-di-*p*-hydroxyphenyl-4-penten-1-one (**5**), and 2,4'-dihydroxy-4-methoxybenzophenone (**6**) on the basis of spectroscopic and physicochemical analyses including 1D- and 2D- NMR techniques as well as by comparison of their data with the published values. Compounds **1** - **4** were isolated for the first time from this plant source. Among isolates, compound **2** exhibited moderate inhibitory effect on the differentiation of pre-adipocyte 3T3-L1 cells.

Keywords – *Anemarrhena asphodeloides*, Liliaceae, Homoisoflavanone, Antiadipogenic activity

Introduction

The rhizomes of *Anemarrhena asphodeloides* Bunge (Anemarrhenae Rhizoma; Liliaceae) has been used as a traditional medicine for its antidiabetic, antiphlogistic, antipyretic, sedative, diuretic and anodyne properties in Korea, China, and Japan (Duke *et al*., 2002). There have been phytochemical reports on this species including xanthones (Pardo-Andreu *et al*., 2006), norlignans (Iida *et al*., 2000; Park *et al*., 2003), and steroidal saponins (Nakashima *et al*., 1993; Sy *et al*., 2008; Ren *et al*., 2006), associated with various biological activities such as antidiabetic (Nakashima *et al*., 1993), anticancer (Sy *et al*., 2008), antioxidant (Pardo-Andreu *et al*., 2006), antifungal (Iida *et al*., 2000; Park *et al*., 2003), and antidepressant (Ren *et al*., 2006).

During a study to find novel adipocyte differentiation inhibitors from natural sources, six known phenolic compounds (**1** - **6**) were isolated from the rhizomes of *A. asphodeloides*. This paper deals with the isolation and structure elucidation of **1** - **6**, as well as the evaluation for their inhibitory effects against differentiation of pre-adipocyte 3T3-L1 cells.

Experimental

General – Melting points were measured by using an Electrothermal apparatus. UV and IR spectra were recorded on a U-3000 spectrophotometer (Hitachi, Japan) and a FTS 135 FT-IR spectrometer (Bio-Rad, CA), respectively. 1D and 2D NMR experiments were performed on a UNITY INOVA 400 MHz FT-NMR instrument (Varian, CA) with tetramethylsilane (TMS) as internal standard. Thin layer chromatography (TLC) was performed on precoated silica gel 60 F254 (0.25 mm, Merck). Silica gel (230 - 400 mesh, Merck, Germany) and RP-18 (YMC-pack ODS-A, 12 nm, S150 μm) were used for column chromatography.

Plant material – The rhizomes of *A. asphodeloides* were purchased from OmniHerb.com Oriental Herb Store in Seoul, South Korea in September 2008, and were identified by Professor Je-hyun Lee (College of Oriental Medicine, Dongguk University). A voucher specimen (no. EA270) was deposited at the Natural Product Chemistry Laboratory, College of Pharmacy, Ewha Womans University.

Extraction and isolation – The rhizomes of *A. asphodeloides* (20 kg) were extracted with MeOH three times under reflux for 4 h. The MeOH solutions were concentrated in vacuo to yield a dried MeOH-soluble extract (4 kg). This extract was suspended in distilled water and fractionated with *n*-hexane, EtOAc, and *n*-BuOH, successively. The EtOAc extract (75 g) was chromatographed over a silica gel (1875 g) column, eluting with a gradient solvent system of *n*-hexaneEtOAc (100 : 1 to 1 : 1), to afford 25 fractions (E1 - E25).

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Fraction E11 (3.0 g) was chromatographed on a silica gel (75 g) column eluting with $CHCl_3$MeOH (50 : 1 to 5 : 1) to afford twenty subfractions (E11.1 to E11.20). Subfraction E11.14 (0.2 g) was subjected to semi-preparative HPLC (MeOHH_2O, 65 : 35] to yield compound **6** [14 mg (0.00035% w/w), t_R 160 min]. Fraction E11.16 (0.25 g) was subjected to semi-preparative HPLC (MeOHH_2O, 75 : 25) to yield compounds **4** [5 mg (0.00012% w/w), t_R 125 min) and **5** [10 mg (0.00025% w/w), t_R 115 min]. Fraction E22 (4.0 g) was chromatographed on a silica gel (100 g) column, eluted with $CHCl_3$-MeOH (50 : 1 to 5 : 1), to afford nine subfractions (E22.1 to E22.9). Subfraction E22.5 (0.1 g) was further purified by semi-preparative HPLC (MeOH-H_2O, 40 : 60) to yield compounds **1** [2 mg (0.00005% w/w), t_R 150 min] and **2** [5 mg (0.00012% w/w), t_R 115 min]. Subfraction E22.6 (0.05 g) was subjected to semi-preparative HPLC (MeOH-H_2O, 40 : 60) to yield compound **3** [5 mg (0.00012% w/w), t_R 180 min].

7,4'-Dihydroxyhomoisoflavanone (1) – Yellow powder, $[\alpha]_D^{20}$ = –55.20° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 288 (4.0), 315 (3.7) nm; IR ν_{max} (KBr) 3350, 1670, 1610, 1520, 1022, 930 cm^{-1}; EI-MS *m/z* 270 $[M]^+$; ^{1}H-NMR (400 MHz, CD_3OD) δ 2.60 (1H, dd, *J* = 8.0, 12.0 Hz, H-7a'), 3.01 (1H, dd, *J* = 4.0, 12.0 Hz, H-7b'), 2.70-2.78 (1H, m, H-3), 4.12 (1H, dd, *J* = 8.0, 12.0 Hz, H-2a), 4.28 (1H, dd, *J* = 4.0, 12.0 Hz, H-2b), 6.29 (1H, d, *J* = 2.4 Hz, H-8), 6.47 (1H, dd, *J* = 2.4, 8.4 Hz, H-6), 6.72 (2H, d, *J* = 8.4 Hz, H-3'/5'), 7.04 (2H, d, *J* = 8.4 Hz, H-2'/6'), 7.70 (1H, d, *J* = 8.4 Hz, H-5); ^{13}C-NMR (100 MHz, CD_3OD) δ 70.0 (C-2), 49.0 (C-3), 195.2 (C-4), 130.4 (C-5), 111.2 (C-6), 166.5 (C-7), 103.3 (C-8), 114.6 (C-4a), 165.0 (C-8a), 130.5 (C-1'), 131.4 (C-2'/C-6'), 116.2 (C-3'/C-5'), 157.9 (C-4'), 33.1 (C-7').

(2*S*)-7,4'-Dihydroxy-5-methoxyflavanone (2) – Yellow powder, $[\alpha]_D^{20}$ = –25.20° (*c* = 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 284 (4.0), 319 (3.8) nm, (addition $AlCl_3$) 284, 319 nm; IR ν_{max} (KBr) 3300, 1670, 1620, 1510 cm^{-1}; EI-MS *m/z* 286 $[M]^+$; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 2.53 (1H, dd, *J* = 2.8, 18.0 Hz, H-3a), 3.10 (1H, dd, *J* = 12.8, 18.0 Hz, H-3b), 5.31 (1H, dd, *J* = 2.8, 12.8 Hz, H-2), 5.94 (1H, d, *J* = 2.4 Hz, H-8), 6.04 (1H, d, *J* = 2.4 Hz, H-6), 6.76 (2H, d, *J* = 8.4 Hz, H-3'/5'), 7.27 (2H, d, *J* = 8.4 Hz, H-2'/H-6'), 3.73 (3H, s, OMe-5); ^{13}C-NMR (100 MHz, DMSO-d_6) δ 78.0 (C-2), 44.7 (C-3), 187.7 (C-4), 162.1 (C-5), 93.1 (C-6), 164.2 (C-7), 95.5 (C-8), 104.0 (C-4a), 164.2 (C-8a), 129.3 (C-1'), 128.0 (C-2'/C-6'), 115.0 (C-3'/C-5'), 157.5 (C-4'), 55.7 (OMe).

4,4'-Dihydroxychalcon (3) – Yellow powder, UV (MeOH) λ_{max} (log ε) 234 (3.70), 350 (3.98) nm; IR ν_{max} (KBr) 3500, 1750, 1655 cm^{-1}; EI-MS *m/z* 240 $[M]^+$; ^{1}H-NMR (400 MHz, CD_3OD) δ 6.83 (2H, d, *J* = 8.4 Hz, H-3''/H-5''), 6.88 (2H, d, *J* = 8.4 Hz, H-3'/H-5'), 7.55 (2H, d, *J* = 8.4 Hz, H-2''/H-6''), 7.61 (1H, d, *J* = 16.0 Hz, H-2), 7.68 (1H, d, *J* = 16.0 Hz, H-3), 7.99 (2H, d, *J* = 8.4 Hz, H-2'/H-6'); ^{13}C-NMR (100 MHz, CD_3OD) δ 190.5 (C-1), 125.3 (C-2), 145.0 (C-3), 134.2 (C-1'), 130.1 (C-2'/6'), 120.0 (C-3'/5'), 168.4 (C-4'), 132.4 (C-1''), 129.8 (C-2''/6''), 121.9 (C-3''/5''), 156.6 (C-4'').

2'-*O*-Methylphloretin (4) – Colourless needle, UV (MeOH) λ_{max} (log ε) 288 (3.98) nm; IR ν_{max} (KBr) 3400, 1670, 1620, 1510, 930 cm^{-1}; EI-MS *m/z* 288 $[M]^+$; ^{1}H-NMR (400 MHz, CD_3OD) δ 2.81-2.85 (2H, m, H-3), 3.18-3.22 (2H, m, H-2), 5.88 (1H, d, *J* = 2.0 Hz, H-3'), 5.95 (1H, d, *J* = 2.0 Hz, H-5'), 6.68 (2H, d, *J* = 8.8 Hz, H-3''/5''), 7.02 (2H, d, *J* = 8.8 Hz, H-2''/H-6''), 3.84 (3H, s, OMe-6'); ^{13}C-NMR (100 MHz, CD_3OD) δ 206.1 (C-1), 47.5 (C-2), 31.5 (C-3), 106.1 (C-1'), 166.6 (C-2'), 97.1 (C-3'), 168.4 (C-4'), 92.2 (C-5'), 165.0 (C-6'), 133.9 (C-1''), 130.4 (C-2''/6''), 116.3 (C-3''/5''), 156.6 (C-4''), 56.2 (OMe-6').

1,3-bis-di-*p*-Hydroxyphenyl-4-penten-1-one (5) – Yellow powder, $[\alpha]_D^{20}$ = +5.0° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 265 nm (4.09); IR ν_{max} (KBr) 3370, 1653, 1511, 1460 cm^{-1}; EI-MS *m/z* 268 $[M]^+$; ^{1}H-NMR (400 MHz, CD_3OD) δ 3.32 (2H, dd, *J* = 3.2, 7.2 Hz, H-2), 4.00 (1H, q, *J* = 7.2 Hz, H-3), 4.95 (2H, m, H-5), 6.03 (1H, m, H-4), 6.74 (2H, d, *J* = 8.8 Hz, H-3'/H-5'), 6.89 (2H, d, *J* = 8.4 Hz, H-3''/H-5''), 7.11 (2H, d, *J* = 8.8 Hz, H-2'/H-6'), 7.85 (2H, d, *J* = 8.4 Hz, H-2''/H-6''); ^{13}C-NMR (100 MHz, CD_3OD) δ 196.0 (C-1), 44.2 (C-2), 45.1 (C-3), 143.1 (C-4), 113.9 (C-5), 135.2 (C-1'), 139.7 (C-2'/6'), 116.0 (C-3'/5'), 156.7 (C-4'), 130.6 (C-1''), 131.4 (C-2''/6''), 116.0 (C-3''/5''), 162.6 (C-4'').

2,4'-Dihydroxy-4-methoxybenzophenone (6) – Yellow needle crystals, UV (MeOH) λ_{max} (log ε) 290 (3.85), 328 (3.80) nm; IR ν_{max} (KBr) 3682, 2900, 2367, 2345, 1710, 1543, 773 cm^{-1}; LREIMS *m/z* (rel. int., %) 244 $[M]^+$; ^{1}H-NMR (400 MHz, CD_3OD) δ 6.47 (1H, d, *J* = 2.4 Hz, H-3), 6.51 (1H, d, *J* = 6.4 Hz, H-5), 6.89 (2H, d, *J* = 8.4 Hz, H-3'/H-5'), 7.58 (2H, d, *J* = 8.4 Hz, H-2'/H-6'), 7.58 (1H, d, *J* = 8.4 Hz, H-6), 3.85 (3H, s, OCH_3); ^{13}C-NMR (100 MHz, CD_3OD) δ 114.7 (C-1), 166.8 (C-2), 107.9 (C-3), 167.5 (C-4), 102.3 (C-5), 136.3 (C-6), 130.7 (C-1'), 132.9 (C-2'/6'), 116.3 (C-3'/5'), 163.1 (C-4'), 56.2 (OCH_3), 200.4 (C = O).

Differentiation of 3T3-L1 pre-adipocytes – 3T3-L1 pre-adipocytes were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum. For adipocyte differentiation, cells were grown to

confluence for 48 h and the medium was changed to DMEM containing insulin (5 μg/mL), 10 μM rosiglitazone, 1 μM dexamethasone, and 10% fetal bovine serum (FBS), to differentiate into adipocytes (day 0). Cells were then replaced with 10% FBS/DMEM supplemented with 5 μg/mL insulin after 48 h and refreshed with 10% FBS/DMEM every other day during differentiation. In order to observe the effect of compounds on adipocyte differentiation, cells were treated with the indicated amounts of compounds on differentiation day 0, refreshed every 2 days, and stained with Oil-red O at day 7.

Oil-red O staining. At differentiation day 7, cells were washed with phosphate buffered saline (PBS) and fixed in 10% formalin for 10 min. Cells were subsequently rinsed twice with PBS and stained with Oil-red O staining solution for 1 h at room temperature. Stained cells were washed with distilled water and dissolved in 100% isopropyl alcohol for measuring the absorbance at 500 nm.

Results and Discussion

Repeated chromatography of the EtOAc-soluble fraction of the MeOH extract from the rhizomes of *A. asphodeloides* on silica gel, YMC-pack RP-C_{18} columns led to the isolation of six compounds (**1 - 6**). Compounds **5** and **6** were identified by comparing the ^{1}H-, ^{13}C-NMR, and MS spectral data with the literature values to be 1,3-bis-di-*p*-hydroxyphenyl-4-penten-1-one (**5**) (Jeong *et al*., 1999) and 2,4'-dihydroxy-4-methoxybenzophenone (**6**) (Triana *et al*., 2009). Compounds **1-4** were isolated for the first time from this plant source.

Compound **1** was obtained as yellow powder and it showed a molecular ion peak at *m/z* 270 $[M]^+$ in the EIMS. The IR spectrum showed the presence of hydroxyl group at 3350 cm^{-1} and aromatic ring at 1610 cm^{-1}. The ^{1}H-NMR spectrum of **1** showed the presence of a *para*-substituted benzene group at δ 6.72 (2H, d, J = 8.4 Hz) and 7.04 (2H, d, J = 8.4 Hz), and an ABX-type aromatic system at δ 6.29 (1H, d, J = 2.4 Hz), 6.47 (1H, dd, J = 2.4, 8.4 Hz), and 7.70 (1H, d, J = 8.4 Hz). The ^{1}H- and ^{13}C-NMR signals for a methine signal at δ_H 2.70-2.78/δ_C 49.0 (C-3), an oxygenated methylene at δ_H 4.12, 4.28/δ_C 70.0 (C-2), and a carbonyl carbon at δ_C 195.2 (C-4), were indicative of the presence of a homoisoflavonoid skeleton. A methylene functionality resonated at δ_H 2.60, 3.01/δ_C 33.1, which was correlated with C-2, C-3, C-4, and C-2' and C-6' in the HMBC experiment of **1** (Fig. 2). Therefore, compound **1** was identified as 7,4'-dihydroxyhomoisoflavanone, which had previously been isolated from *Dracaena draco* (Camarda *et al*., 1983).

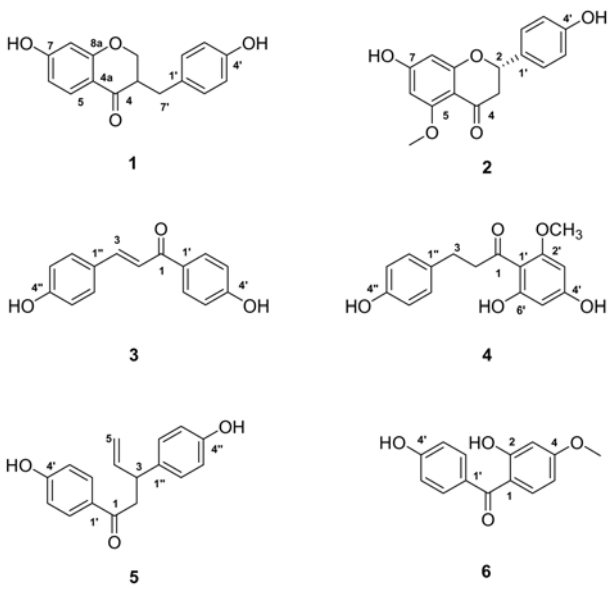

Fig. 1. Chemical structures of compounds **1 - 6** from rhizomes of *A. asphodeloides*.

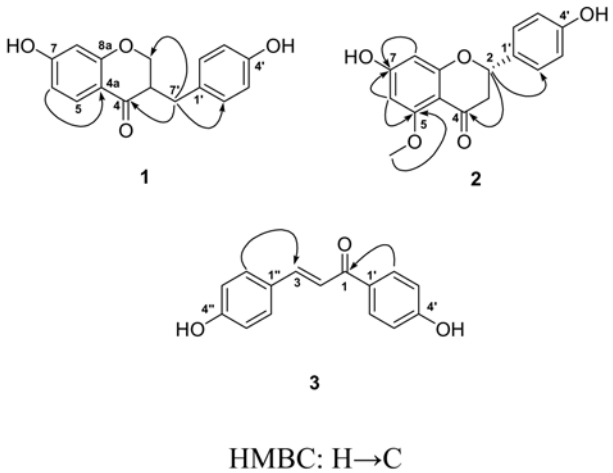

Fig. 2. Important HMBC correlations of **1 - 3**.

Compound **2** was obtained as yellow powder and it showed a molecular ion peak at *m/z* 286 $[M]^+$ in the EIMS. The IR spectrum showed the presence of hydroxyl group at 3300 cm^{-1} and aromatic ring at 1620 cm^{-1}. The ^{1}H-NMR spectrum of **2** showed the presence of *meta*-coupled aromatic protons at δ 5.94 (1H, d, J = 2.4 Hz) and 6.04 (1H, d, J = 2.4 Hz), a *para*-substituted benzene group at δ 6.76 (2H, d, J = 8.4 Hz) and 7.27 (2H, d, J = 8.4 Hz), and a methoxy proton signal at δ 3.73 (3H, s). In the ^{1}H- and ^{13}C-NMR spectra, an oxygenated methine proton signal at δ_H 5.31 (1H, dd, J = 2.8, 12.8 Hz)/δ_C 78.0 (C-2), a methylene at δ_H 2.53 (1H, dd, J = 2.8, 18.0 Hz), 3.10 (1H, dd, J = 12.8, 18.0 Hz)/δ_C 44.7

(C-3), and a carbonyl carbon at δ_C 187.7 (C-4), were indicative of the presence of a flavanone skeleton. These data were comparable with the known flavanone, 5,4'-dihydroxy-7-methoxyflavanone (Mizuno *et al*., 1987). No bathochromic shift was observed after addition of $AlCl_3$ in the UV, indicating that the methoxy group was attached at 5-position in the molecule. This observation was further supported by HMBC spectroscopic assignment, correlations of *meta*-coupled two aromatic protons at δ_H 5.94 (H-8)/6.04 (H-6) with δ_C 164.2 (C-6) and δ_H 6.04 (H-6)/3.73 (OCH_3) with δ_C 162.1 (C-5) (Fig. 2). The stereochemistry at C-2 was determined to be *S* on the basis of optical rotation ($[\alpha]_D^{20}$ –25.2°), in comparison with published data ($[\alpha]_D^{20}$ –14.0°) (Hammami *et al*., 2004). Thus, compound **2** was identified as (2*S*)-7,4'-dihydroxy-5-methoxyflavanone, which had previously been isolated from *Coptis japonica* var. *dissecta* (Mizuno *et al*., 1987).

Compound **3** was obtained as yellow powder and it showed a molecular ion peak at *m/z* 240 $[M]^+$ in the EIMS. In the IR spectrum of **3**, absorption bands for one or more hydroxy group(s) and a carbonyl functionality were observed at 3500 cm^{-1} and 1750 cm^{-1}, respectively. The ^{1}H-NMR spectrum of **3** showed the presence of two sets of *para*-substituted benzene groups at δ 6.83 (2H, d, J = 8.4 Hz, H-3"/H-5"), 7.55 (2H, d, J = 8.4 Hz, H-2"/H-6") and at δ 6.88 (2H, d, J = 8.4 Hz, H-3'/H-5'), 7.99 (2H, d, J = 8.4 Hz, H-2'/H-6'). The ^{1}H- and ^{13}C-NMR spectra showed a α,β-unsaturated carbonyl group at δ_H 7.61 (1H, d, J = 16.0 Hz)/δ_C 125.3 (C-2), 7.68 (1H, d, J = 16.0 Hz)/δ_C 145.0 (C-3), and a carbonyl carbon at δ_C 190.5 (C-1). The connectivity of two symmetrical aromatic groups was further supported by the HMBC spectrum (Fig. 2), which showed the correlation between δ_H 7.55 (H-2"/H-6") with δ_C 145.0 (C-3) and δ_H 7.99 (H-2'/H-6') with δ_C 190.5 (C-1). Therefore, compound **3** was identified as 4,4'-dihydroxychalcon, which had previously been isolated from the heartwood of *Chamaecyparis obtusa* (Ohashi *et al*., 1988).

Compound **4** was obtained as colourless needle and it showed a molecular ion peak at *m/z* 288 $[M]^+$ in the EIMS. The IR spectrum showed the presence of absorption bands for one or more hydroxy group(s) at 3400 cm^{-1} and aromatic ring at 1620 cm^{-1}. The ^{1}H-NMR spectrum of **4** showed the presence of a *para*-substituted benzene group at δ 6.68 (2H, d, J = 8.8 Hz, H-3"/5") and 7.02 (2H, d, J = 8.8 Hz, H-2"/H-6"), and an *ortho* coupled benzene moiety at δ 5.88 (1H, d, J = 2.0 Hz, H-5') and 5.95 (1H, d, J = 2.0 Hz, H-3'). The ^{1}H- and ^{13}C-NMR spectra showed two methylene signals at δ_H 2.81-2.85 (2H, m, H-3)/δ_C 31.5 (C-3), 3.18-3.22 (2H, m, H-2)/47.5 (C-2), a carbonyl carbon at δ_C 206.1 (C-1), and a methoxy group at δ_H 3.84/56.2 (C-2'), were indicative of the presence of dihydrochalcone skeleton. On the basis of the above evidence, **4** was identified as 2',4",4"-trihydroxy-6'-methoxydihydrochalcone, which had previously been isolated from the aerial parts of *Goniothalamus gardneri* and *Goniothalamus thwaitesii*. (Seidel *et al*., 2000).

Compounds **1 - 6** were tested *in vitro* for their inhibitiory effects on the adipogenic differentiation of pre-adipocyte 3T3-L1 cells. Of these, compound **2** exhibited moderate inhibitory effect with an IC_{50} value of 150 μM. Compounds **1** and **3 - 6** showed no inhibitory effect on this cell line.

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