Identification of Antiadipogenic Constituents of the Rhizomes of *Anemarrhena asphodeloides*

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Three new phenolic compounds, (*E*)-4′-demethyl-6-methyleucomin (**1**), anemarcoumarin A (**2**), and anemarchalconyn (**3**), were isolated from an ethyl acetate extract of the rhizomes of *Anemarrhena asphodeloides*, together with seven known compounds (**4**−**10**). The structures of the new compounds (**1**−**3**) were determined on the basis of spectroscopic data interpretation. Compound **3** exhibited a potent inhibitory effect against the differentiation of preadipocyte 3T3-L1 cells with an IC_{50} value of 5.3 μM.

The rhizomes of *Anemarrhena asphodeloides* Bunge (Liliaceae) have been used as a traditional medicine for their anodyne, antidiabetic, antiphlogistic, antipyretic, diuretic, and sedative properties in Korea, mainland China, and Japan.[1] There have been several phytochemical reports on this species concerning its xanthones,[2] norlignans,[3,4] and steroidal saponins,[5−7] associated with various biological activities such as antidiabetic,[5] anticancer,[6] antioxidant,[2] antifungal,[3,4] and antidepressant effects.[7]

During a study to find novel adipocyte differentiation inhibitors of plant origin, the EtOAc extract of the rhizomes of *A. asphodeloides* exhibited an inhibitory effect against differentiation on preadipocyte 3T3-L1 cells at a concentration level of 100 μg/mL. The inhibitory activity of *A. asphodeloides* on adipocyte differentiation has not been reported previously. Mouse preadipocyte 3T3-L1 cells differentiate into mature adipocytes in the presence of specific factors such as insulin, dexamethasone, and PAPRγ activators, and they afford a well-known in vitro model system that reflects adipose tissue formation in vivo.[8,9] Therefore, the EtOAc fraction of *A. asphodeloides* was subjected to detailed phytochemical investigation, resulting in the isolation of three new phenolic compounds (**1**−**3**), along with seven known compounds (**4**−**10**). In the present study, the isolation and structure elucidation of **1**−**3** are reported as well as the evaluation of **1**−**10** for their inhibitory effects against differentiation of preadipocyte 3T3-L1 cells.

Compound **1** was obtained as a yellow powder. Its molecular formula was established as $C_{17}H_{14}O_5$ on the basis of the molecular ion peak at *m*/*z* 299.0922 $[M + H]^+$ (calcd for $C_{17}H_{15}O_5$, 299.0919) in the positive high-resolution FABMS. The UV spectrum showed an absorption maximum at 288 nm, indicating the presence of one or more separate aromatic group(s). In the IR spectrum of **1**, absorption bands for one or more hydroxy group(s) and a carbonyl functionality were observed at 3368 and 1730 cm^{-1}, respectively. The ^1H NMR spectrum of **1** (Table 1) showed two symmetrical doublets at δ 6.87 (2H, J = 8.4 Hz, H-3′ and H-5′) and 7.23 (2H, J = 8.4 Hz, H-2′ and H-6′), indicating the presence of a *para*-substituted benzyl group. The ^1H and ^{13}C NMR signals for one aromatic methine at δ_H 5.88/δ_C 95.1 (C-8), an oxygenated methylene at δ_H 5.27 (2H, d, J = 1.6 Hz)/δ_C 68.6 (C-2), and a carbonyl carbon at δ_C 186.6 (C-4) were indicative of the presence of an isoflavonoid skeleton. Besides these characteristics for the isoflavonoid skeleton, there was a benzylic methine signal at δ_H 7.72 (H-7′), which correlated with C-2, C-4, and C-2′ and C-6′ in the HMBC experiment of **1**. Therefore, compound **1** could be assigned with a benzylic methine group between C-3 and C-1′, thus displaying a typical homoisoflavanoid skeleton. These data were comparable with the known homoisoflavanone (*E*)-5,7-dihydroxy-3-(4′-hydroxybenzylidene)chroman-4-one (**9**),[10] except for the presence of a methyl group in **1**. The configuration of the vinylic proton was determined as *trans* (*E*) due to its typical chemical shift value at δ 7.72 (1H, s, H-7′), which appeared relatively more downfield than the *cis Z*-isomer (δ 7.02, s).[11] On the other hand, additional NMR data of **1** obtained in DMSO-d_6 were used to solve the position of the methyl group. Thus, a hydrogen-bonded hydroxy proton appeared at δ_H 13.18 (OH-5), which was correlated with C-5, C-6, and C-4a in the HMBC experiment. The methyl group resonated at δ_H 1.86 in DMSO-d_6 and exhibited two- and three-bond correlations with C-5, C-6, and C-7, indicating the position of the methyl group to be C-6. As a result, **1** [(*E*)-4′-demethyl-6-methyleucomin] was elucidated as the new compound (*E*)-5,7-dihydroxy-3-(4′-hydroxybenzylidene)-6-methylchroman-4-one.

Compound **2** was obtained as a yellow powder. Its molecular formula was established as $C_{16}H_{12}O_4$ from the molecular ion peak at *m*/*z* 268.0738 $[M]^+$ (calcd for $C_{16}H_{12}O_4$, 268.0735) in the HREIMS. The IR spectrum showed the presence of a hydroxy group at 3298 cm^{-1} and a carbonyl group at 1690 cm^{-1}. The ^1H NMR spectrum of **2** showed the presence of a *para*-substituted benzene group at δ 6.73 (2H, d, J = 8.8 Hz) and 7.10 (2H, d, J = 8.8 Hz) and an ABX-type aromatic system at δ 6.68 (1H, d, J = 2.0 Hz), 6.75 (1H, dd, J = 2.0, 8.4 Hz), and 7.32 (1H, d, J = 8.4 Hz). The NMR signals at δ_H 7.45/δ_C 141.7 (C-4) and 164.3 (C-2) were characteristic for a coumarin structure. A methylene functionality resonated at δ_H 3.69/δ_C 36.5, which was correlated with C-2, C-3, C-4, and C-2′ and C-6′ in the HMBC experiment of **2**. These data were comparable to the known synthetic compound 3-benzyl-7-methoxychromen-2-one,[12] except for the presence of a hydroxy group at C-7 in compound **2**. Therefore, **2** (anemarcoumarin A) was determined as the new compound 7-hydroxy-3-(4-hydroxybenzyl)coumarin.

Compound **3** was obtained as a yellow powder, and its molecular formula of $C_{15}H_{10}O_3$ was established from the molecular ion peak at *m*/*z* 238.0630 $[M]^+$ (calcd for $C_{15}H_{10}O_3$, 238.0630) in the HREIMS. The IR absorption bands at 1620 and 2193 cm^{-1} suggested the presence of a carbonyl group and a C≡C triple bond, respectively.[13] The ^{13}C NMR signal at δ_C 178.5 (C-1) supported the presence of the carbonyl group, and the two quaternary carbons

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Chart 1

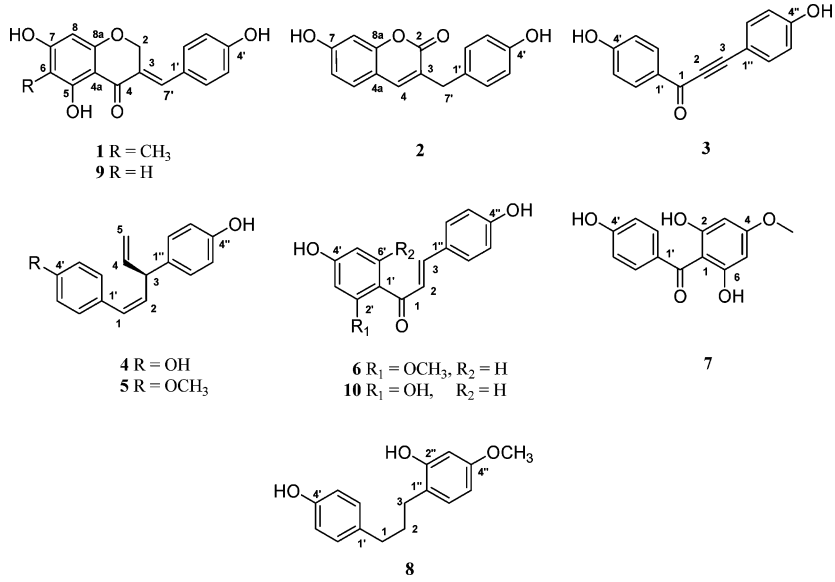


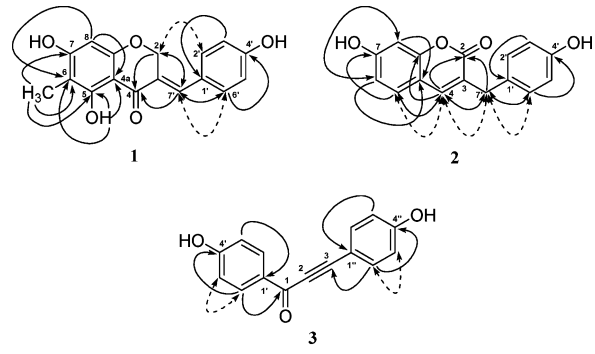
Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for (*E*)-4′-Demethyl-6-methyleucomin (**1**), Anemarcoumarin A (**2**), and Anemarchalconyn (**3**)

	1[a]		**2**[a]		**3**[a]	
position	δ_C, mult.	δ_H, (*J* in Hz)	δ_C, mult.	δ_H, (*J* in Hz)	δ_C, mult.	δ_H, (*J* in Hz)
1					178.5, qC	
2	68.6, CH_2	5.27, d (1.6)	164.3, qC		87.2, qC	
3	128.9, qC		126.2, qC		95.7, qC	
4	186.6, qC		141.7, CH	7.45, s		
4a	103.4, qC		113.8, qC			
5	163.5, qC		130.1, CH	7.32, d (8.4)		
6	105.7, qC		114.5, CH	6.75, dd (8.4, 2.0)		
7	166.3, qC		162.3, qC			
8	95.1, CH	5.88, s	103.1, CH	6.68, d (2.0)		
8a	161.6, qC		156.2, qC			
1′	127.2, qC		130.6, qC		130.3, qC	
2′	133.6, CH	7.23, d (8.4)	131.3, CH	7.10, d (8.8)	133.2, CH	8.07, d (8.8)
3′	116.9, CH	6.87, d (8.4)	116.5, CH	6.73, d (8.8)	116.6, CH	6.90, d (8.8)
4′	160.7, qC		157.3, qC		165.2, qC	
5′	116.9, CH	6.87, d (8.4)	116.5, CH	6.73, d (8.8)	116.6, CH	6.90, d (8.8)
6′	133.6, CH	7.23, d (8.4)	131.3, CH	7.10, d (8.8)	133.2, CH	8.07, d (8.8)
7′	137.9, CH	7.72, s	36.5, CH_2	3.69, s		
1″					111.5, qC	
2″					136.3, CH	7.55, d (8.8)
3″					117.1, CH	6.85, d (8.8)
4″					161.8, qC	
5″					117.1, CH	6.85, d (8.8)
6″					136.3, CH	7.55, d (8.8)
CH_3-6	7.1, CH_3	1.95, s				

[a] Spectrum recorded at 400 MHz (^{1}H NMR) and 100 MHz (^{13}C NMR) in CD_3OD.

at δ_C 87.2 and 95.7 indicated the occurrence of a triple bond. Two sets of *para*-substituted aromatic groups resonated at δ_H 6.90 (2H, d, J = 8.8 Hz, H-3′ and H-5′), 8.07 (2H, d, J = 8.8 Hz, H-2′ and H-6′), 6.85 (2H, d, J = 8.8 Hz, H-3″ and H-5″), and 7.55 (2H, d, J = 8.8 Hz, H-2″ and H-6″). The position of the carbonyl group was assigned at C-1 by the three-bond connectivity between the carbonyl group and H-2′ and H-6′ of the HMBC spectrum. In turn, the triple bond was positioned between C-2 and C-3 from the three-bond connectivity between C-3 and H-2′ and H-6′ in the HMBC spectrum of **3**. These data for **3** were comparable to those of the known synthetic compound 1,3-diphenylpropynone,[14] except for the presence of two separate *para*-hydroxy groups at the two phenyl groups in **3**. Therefore, **3** (anemarchalconyn) was elucidated as the new compound 1,3-bis(4-hydroxyphenyl)prop-2-yn-1-one.

The other seven isolates obtained were identified as the previsouly known compounds nyasol (**4**),[15] 4′-*O*-methylnyasol (**5**),[16] 2′-*O*-methylisoliquiritigenin (**6**),[17] 2,4′,6-trihydroxy-4-methoxyben-



HMBC: H—C and NOESY: H---H

Figure 1. Important HMBC and NOESY correlations of **1**−**3**.

Table 2. Inhibitory Activities of Compounds **1**−**10** on Differentiation of Preadipocyte 3T3-L1 Cells

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	resveratrol[c]
IC_{50}	>100[a]	nd[b]	5.3	>100[a]	45.9	41.8	nd[a]	74.5	>100[a]	96.4	31.4

[a] IC_{50} values of greater than 100 μM are considered to be inactive. [b] Not determined. [c] Positive control substance.

zophenone (**7**),[18] broussonin A (**8**),[19] (*E*)-5,7-dihydroxy-3-(4′-hydroxybenzylidene)chroman-4-one (**9**),[10] and 2′,4′,4-trihydroxychalcone (**10**),[20] by comparison of their physical and spectroscopic data with published values. To the best of our knowledge, compounds **6**, **9**, and **10** have been isolated from the genus *Anemarrhena* for the first time.

Compounds **1**−**10** were tested in vitro for their inhibitiory effects on the adipogenic differentiation of preadipocyte 3T3-L1 cells. Of these, the new compound **3** exhibited a potent inhibitory effect with an IC_{50} value of 5.3 μM. Compounds **5**, **6**, **8**, and **10** showed less potent inhibitory activities, with IC_{50} values of 45.9, 41.8, 74.5, and 96.4 μM, respectively. Compounds **1**, **4**, and **9** had no significant inhibition effects on adipogenic differentiation, while compounds **2** and **7** could not be evaluated in this manner, due to their insufficient amounts available for testing (Table 2).

Experimental Section

General Experimental Procedures. Melting points were measured using an Electrothermal apparatus. Optical rotations were measured with a P-1010 polarimeter (JASCO, Japan) at 20 °C. 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. Mass spectrometry was carried out with a JEOL JMS-700 Mstation mass spectrometer. 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 gel ODS-A, 12 nm, S-150 μm) were used for column chromatography. Preparative HPLC was run on an Acme 9000 HPLC (Young Lin, South Korea) using a YMC-pack ODS-A column, with a the flow rate of 1 mL/min.

Plant Material. The rhizomes of *A. asphodeloides* were purchased from Oriental Herb Store (OmniHerb.com) in Seoul, South Korea, in September 2008, and were identified by one of the authors (J.-H.L.). 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 active EtOAc extract (75 g) was chromatographed over a silica gel (1875 g) column, eluting with a gradient solvent system of *n*-hexane−EtOAc (100:1 to 1:1), to afford 25 fractions (E1−E25). Fraction E8 (10.0 g) was chromatographed on a silica gel (250 g) column eluting with $CHCl_3$−MeOH (100:1 to 10:1) to afford five subfractions (E8.1 to E8.5). Subfraction E8.3 (5.1 g) was chromatographed on a silica gel (125 g) column using $CHCl_3$−MeOH (50:1 to 10:1) to give five subfractions (E8.3.1 to E8.3.5). Subfraction E8.3.2 (0.2 g) was subjected to semipreparative HPLC (MeOH−H_2O, 75:25 to 90:10] to yield compound **5** [15 mg (0.000375% w/w), t_R 120 min]. Fraction E8.3.3 (3.5 g) was chromatographed over a silica gel (90 g) column using *n*-hexane−EtOAc (100:1 to 50:50) as gradient solvent system to afford compounds **4** (1500 mg, 0.375% w/w) and **8** (10 mg, 0.00025% w/w), which were eluted with 80:20 and 60:40 *n*-hexane−EtOAc, respectively. 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 20 subfractions (E11.1 to E11.20). Subfraction E11.16 (0.2 g) was subjected to semipreparative HPLC (MeOH−H_2O, 75:25) to yield compounds **1** [5 mg (0.000125% w/w), t_R 125 min], **9** [4 mg (0.0001% w/w), t_R 115 min], and **10** [8 mg (0.0002% w/w), t_R 90 min]. Fraction E14 (5.0 g) was chromatographed on a silica gel (125 g) column, using a gradient solvent system of $CHCl_3$−MeOH (50:1 to 5:1), to give compounds **6** [5 mg (0.000125% w/w)] and **7** [1500 mg (0.375% w/w)], which were eluted with 40:1 and 30:1 $CHCl_3$−MeOH, respectively. 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 semipreparative HPLC (MeOH−H_2O, 40:60) to yield compound **2** [2 mg (0.00005% w/w), t_R 150 min]. Subfraction E22.6 (0.05 g) was subjected to HPLC (MeOH−H_2O, 40:60) to yield compound **3** [1.5 mg (0.0000375% w/w), t_R 180 min].

(*E*)-4′-Demethyl-6-methyleucomin (1): yellow powder; UV (MeOH) λ_{max} (log ε) 349 (3.7), 288 (3.8) nm; IR ν_{max} (KBr) 3368, 2913, 1730, 1595, 1467 cm^{-1}; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data (in CD_3OD), see Table 1; ^1H NMR (400 MHz, DMSO-d_6) δ 5.27 (2H, s, H-2), 5.84 (1H, brs, H-8), 6.85 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.29 (2H, d, J = 8.8 Hz, H-2′, 6′), 7.62 (1H, brs, H-7′), 1.86 (3H, s, CH_3-6), 13.18 (1H, brs, OH-5); ^{13}C NMR (100 MHz, DMSO-d_6) δ 66.9 (CH_2, C-2), 126.7 (C, C-3), 183.4 (C, C-4), 101.0 (C, C-4a), 161.5 (C, C-5), 103.7 (C, C-6), 166.1 (C, C-7), 94.4 (CH, C-8), 159.3 (C, C-8a), 124.9 (C, C-1′), 132.6 (CH, C-2′, 6′), 115.7 (CH, C-3′, 5′), 159.3 (C, C-4′), 135.7 (CH, C-7′); HRFABMS *m*/*z* 299.0922 $[M + H]^+$ (calcd for $C_{17}H_{15}O_5$, 299.0919).

Anemarcoumarin A (2): yellow powder; UV (MeOH) λ_{max} (log ε) 320 (3.9), 250 (3.7) nm; IR ν_{max} (KBr) 3298, 2918, 1690, 1610, 1454 cm^{-1};^1H (400 MHz) and ^{13}C NMR (100 MHz) data, see Table 1; HREIMS *m*/*z* 268.0738 $[M]^+$ (calcd for $C_{16}H_{12}O_4$, 268.0735).

Anemarchalconyn (3): yellow powder; UV (MeOH) λ_{max} (log ε) 286 (4.2) nm; IR ν_{max} (KBr) 3350, 2193, 1620, 1159 cm^{-1}; ^1H (400 MHz) and ^{13}C NMR (100 MHz) data, see Table 1; HREIMS *m*/*z* 238.0630 $[M]^+$ (calcd for $C_{15}H_{10}O_3$, 238.0630).

Differentiation of 3T3-L1 Preadipocytes. 3T3-L1 preadipocytes 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 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.

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Supporting Information Available: Spectroscopic data including ^1H and ^{13}C NMR, 2D NMR, and HRMS of new compounds **1**−**3**. This information is available free of charge via the Internet at http://pubs.acs.org.

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