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Six New Chalcones from *Angelica keiskei* Inducing Adiponectin Production in 3T3-L1 Adipocytes

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***Angelica keiskei* (Ashitaba in Japanese), a traditional herb in Japan, contains abundant prenylated chalcones. It has been reported that the chalcones from *A. keiskei* showed such bioactivities as anti-bacterial, anti-cancer and anti-diabetic effects. Xanthoangelol, 4-hydroxyderricin and six new chalcones were isolated in this study from an ethanol extract of *A. keiskei* by octadecyl silyl (ODS) and silica gel chromatography, and identified by 1D- and 2D-nuclear magnetic resonance (NMR) and high-resolution mass spectrometric analyses. The chalcones from *A. keiskei* markedly increased the expression of the adiponectin gene and the production of adiponectin in 3T3-L1 adipocytes. These results suggest that the chalcones from *A. keiskei* might be useful for preventing the metabolic syndrome.**

Key words: *Angelica keiskei*; chalcone; adiponectin; adipocyte

Chalcones are naturally occurring flavonoids with the 1,3-diphenyl prop-2-en-1-one structure. Chalcones are synthesized from malonyl CoA and cinnamic acid by chalcone synthase in higher plants, and are the starting material for such flavonoids as flavanone, flavonol, isoflavone, catechin and anthocyanidin. Recent studies have revealed chalcones to have such bioactivities as anti-bacterial, anti-fungal, anti-viral, anti-cancer, anti-oxidative and anti-inflammatory effects.[1–4] Chalcones have very limited distribution in such edible plants as *Angelica keiskei* Koidzumi (Ashitaba in Japanese),[5] hops,[6] licorice[7] and safflower.[8]

The *A. keiekei* herb is grown along the Pacific coast of Japan, and its leaves are used as a folk medicine and for health-promoting foods. *A. keiskei* abundantly contains such nutrients as vitamin A, vitamin K and dietary fiber, as well as various chalcones, flavanones and coumarins in its leaves, stems and roots. It has been reported that the major chalcones, xanthoangelol (XA) and 4-hydroxyderricin (4HD), from *A. keiskei* exhibited anti-bacterial,[9] anti-ulcer,[10] anti-cancer[11,12] and anti-inflammatory[13] actions. XA and 4HD have improved the cholesterol metabolism of spontaneously hypertensive rats.[14,15] We have previously reported that XA and 4HD induced adipogenesis in 3T3-L1 preadipocytes, and enhanced the glucose uptake into mature adipocytes.[16] Mature adipocytes have recently been reported to produce and secrete various hormones called "adipocytokines."[17] Adiponectin, one of the adipocytokines, has enhanced glucose and lipid metabolism by binding its receptors in liver and skeletal muscle.[18,19] Since adiponectin improves insulin resistance and dyslipidemia, the enhancing effect on adiponectin production can be expected to prevent the metabolic syndrome. Recent studies have shown that several phytochemicals enhanced adiponectin production in adipocytes.[20–24] The objective of this study was to isolate new chalcones from *A. keiskei* and to evaluate their potential for producing adiponectin in 3T3-L1 adipocytes.

Materials and Methods

Instruments and reagents. The ^{1}H (600 MHz), ^{13}C (150 MHz), DEPT-135, DQF-COSY, TOCSY, HMQC and HMBC NMR spectra were measured with an Avance 600 NMR spectrometer (Bruker, Billerica, MA, USA). High-resolution MALDI-TOFMS data were obtained with an Ultraflex II mass spectrometer (Bruker), using 2,5-dihydroxybenzoic acid as a matrix. Dulbecco's modified Eagle's medium (DMEM)-low glucose, ascorbic acid and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Calf serum was purchased from Dainippon Sumitomo Pharma (Osaka, Japan), and fetal bovine serum was purchased from Thermo Scientific (Waltham, MA, USA). Triphenylphosphine was purchased from Tokyo Chemical Industry (Tokyo, Japan), all other reagents being of analytical grade.

Isolation of the chalcones from A. keiskei. *A. keiskei* was cultivated in Kagoshima Prefecture in Japan. The dried root powder of *A. keisikei* (20 kg) was extracted with 60 L of ethanol at room temperature for 30 min. After filtration, the residue was extracted twice with the same volume of ethanol. The ethanol extracts were

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Abbreviations: DEPT, distortionless enhancement by polarization transfer; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; DQF-COSY, double quantum filtered correlation spectroscopy; 4HD, 4-hydroxyderricin; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum correlation; MALDI-TOFMS, matrix assisted laser desorption ionization-time of flight mass spectrometry; NMR, nuclear magnetic resonance; ODS, octadecyl silyl; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcription polymerase chain reaction; TOCSY, total correlated spectroscopy; XA, xanthoangelol

concentrated with a rotary evaporator. The resulting concentrate was dissolved in 25% ethanol and subjected to ODS chromatography (Cosmosil C-18 OPN, Nacalai Tesque, Kyoto, Japan), successively eluting with 30, 40, 75 and 100% ethanol. The elute with 40% ethanol from ODS chromatography was subjected to silica gel chromatography (BW-300SP, Shin-Etsu Chemical, Tokyo, Japan) and eluted with a gradient of chloroform/methanol (50:1–5:1, v/v) to give **1**, 21 mg; **2**, 114 mg; **3**, 27 mg; and **4**, 70 mg. The elute with 75% ethanol from ODS chromatography was subjected to silica gel chromatography, eluting with chloroform/hexane (2:1 and 5:2) and ethyl acetate to give 4HD, 8.7 g; **5**, 22 mg; XA, 14.4 g; and **6**, 245 mg.

NMR and MS analyses of the chalcones. Compound **1**. Yellow amorphous powder; ^{1}H-NMR (DMSO-d_6) δ: 1.18 (3H, s, 3″-Me), 1.28 (3H, s, 3″-Me), 3.07 (2H, m, H-1″), 3.87 (3H, s, 4′-OMe), 4.72 (1H, s, 3″-OH), 4.78 (1H, t, J = 8.7 Hz, H-2″), 6.65 (1H, d, J = 9.0 Hz, H-5′), 6.82 (2H, d, J = 8.4 Hz, H-3, H-5), 7.57 (2H, d, J = 8.4 Hz, H-2, H-6), 7.59 (1H, d, J = 15.6 Hz, H-β), 7.69 (1H, d, J = 9.0 Hz, H-6′), 7.81 (1H, d, J = 15.6 Hz, H-α), 10.02 (1H, s, 4-OH). ^{13}C-NMR (DMSO-d_6) δ: 26.2 (3″-Me), 26.8 (3″-Me), 27.6 (C-1″), 56.5 (4′-OMe), 70.9 (C-3″), 91.5 (C-2″), 105.2 (C-5′), 115.7 (C-3′), 116.0 (C-1′), 116.7 (2C, C-3, C-5), 123.8 (C-α), 127.0 (C-1), 131.0 (2C, C-2, C-6), 131.3 (C-6′), 142.7 (C-β), 160.5 (C-4′), 160.6 (C-4), 161.8 (C-2′), 186.5 (C=O). HR MALDI-TOFMS m/z (M)$^+$: calcd. for $C_{21}H_{22}O_5$, 354.1467; found, 354.1471.

Compound **2**. Yellow amorphous powder; ^{1}H-NMR (DMSO-d_6) δ: 1.20 (3H, s, 3″-Me), 1.36 (3H, s, 7″-Me), 1.57 (3H, s, 7″-Me), 1.68 (2H, m, H-4″), 2.10 (2H, m, H-5″), 2.41 (1H, dd, J = 9.0, 16.8 Hz, H-1″), 2.85 (1H, dd, J = 6.0, 16.8 Hz, H-1″), 3.76 (1H, m, H-2″), 5.01 (1H, m, H-6″), 5.23 (1H, d, J = 4.8 Hz, 2″-OH), 6.47 (1H, d, J = 8.4 Hz, H-5′), 6.80 (2H, d, J = 8.4 Hz, H-3, H-5), 7.38 (1H, d, J = 8.4 Hz, H-6′), 7.44 (1H, d, J = 15.6 Hz, H-β), 7.47 (1H, d, J = 15.6 Hz, H-α), 7.50 (2H, d, J = 8.4 Hz, H-2, H-6), 9.96 (1H, s, 4-OH), 10.19 (1H, s, 4′-OH). ^{13}C-NMR (DMSO-d_6) δ: 18.1 (3″-Me), 18.2 (7″-Me), 22.1 (C-5″), 26.3 (7″-Me), 27.2 (C-1″), 38.7 (C-4″), 66.7 (C-2″), 80.2 (C-3″), 107.8 (C-5′), 109.1 (C-3′), 116.7 (2C, C-3, C-5), 121.0 (C-1′), 125.1 (C-6″), 125.1 (C-α), 127.0 (C-1), 130.3 (C-6′), 130.8 (2C, C-2, C-6), 131.6 (C-7″), 141.5 (C-β), 154.6 (C-2′), 160.4 (C-4), 160.4 (C-4′), 189.9 (C=O). HR MALDI-TOFMS m/z (M)$^+$: calcd. for $C_{25}H_{28}O_5$, 408.1937; found, 408.1934.

Compound **3**. Yellow amorphous powder; ^{1}H-NMR (DMSO-d_6) δ: 0.81 (3H, s, 7″-Me), 1.03 (3H, s, 7″-Me), 1.24 (3H, s, 3″-Me), 1.54 (1H, m, H-5″), 1.61 (1H, dd, J = 4.8, 13.2 Hz, H-2″), 1.71 (1H, m, H-5″), 1.75 (1H, m, H-4″), 1.87 (1H, m, H-4″), 2.34 (1H, dd, J = 13.2, 16.8 Hz, H-1″), 2.67 (1H, dd, J = 4.8, 16.8 Hz, H-1″), 3.27 (1H, m, H-6″), 4.65 (1H, d, J = 4.8 Hz, 6″-OH), 6.47 (1H, d, J = 8.4 Hz, H-5′), 6.83 (2H, d, J = 8.4 Hz, H-3, H-5), 7.39 (1H, d, J = 8.4 Hz, H-6′), 7.42 (1H, d, J = 15.6 Hz, H-β), 7.48 (1H, d, J = 15.6 Hz, H-α), 7.51 (2H, d, J = 8.4 Hz, H-2, H-6), 9.97 (1H, brs, 4-OH), 10.22 (1H, brs, 4′-OH). ^{13}C-NMR (DMSO-d_6) δ: 15.3 (7″-Me), 18.8 (C-1″), 20.7 (3″-Me), 28.1 (7″-Me), 28.9 (C-5″), 38.3 (C-4″), 38.9 (C-7″), 46.4 (C-2″), 76.8 (C-6″), 77.9 (C-3″), 107.7 (C-5′), 110.4 (C-3′), 116.8 (2C, C-3, C-5), 120.8 (C-1′), 125.2 (C-α), 127.1 (C-1), 130.2 (C-6′), 130.8 (2C, C-2, C-6), 141.2 (C-β), 154.9 (C-2′), 160.3 (C-4), 160.6 (C-4′), 189.8 (C=O). HR MALDI-TOFMS m/z (M)$^+$: calcd. for $C_{25}H_{28}O_5$, 408.1937; found, 408.1932.

Compound **4**. Yellow amorphous powder; ^{1}H-NMR (DMSO-d_6) δ: 0.96 (3H, s, 7″-Me), 1.02 (3H, s, 7″-Me), 1.16 (1H, m, H-5″), 1.61 (1H, m, H-5″), 1.73 (3H, s, 3″-Me), 1.85 (1H, m, H-4″), 2.15 (1H, m, H-4″), 3.01 (1H, m, H-6″), 3.24 (1H, m, H-1″), 3.31 (1H, m, H-1″), 4.00 (1H, s, 7″-OH), 4.23 (1H, d, J = 6.0 Hz, 6″-OH), 5.19 (1H, t, J = 7.2 Hz, H-2″), 6.47 (1H, d, J = 8.4 Hz, H-5′), 6.84 (2H, d, J = 8.4 Hz, H-3, H-5), 7.75 (1H, d, J = 5.4 Hz, H-α), 7.75 (1H, d, J = 5.4 Hz, H-β), 7.75 (2H, d, J = 8.4 Hz, H-2, H-6), 8.03 (1H, d, J = 8.4 Hz, H-6′), 10.11 (1H, s, 4-OH), 10.55 (1H, s, 4′-OH), 14.00 (1H, s, 2′-OH). ^{13}C-NMR (DMSO-d_6) δ: 17.0 (3″-Me), 22.1 (C-1″), 25.4 (7″-Me), 27.2 (7″-Me), 30.3 (C-5″), 37.5 (C-4″), 72.4 (C-7″), 78.0 (C-6″), 108.2 (C-5′), 113.6 (C-1′), 115.4 (C-3′), 116.7 (2C, C-3, C-5), 118.3 (C-α), 122.4 (C-2″), 126.7 (C-1), 130.7 (C-6′), 132.0 (2C, C-2, C-6), 135.7 (C-3″), 145.0 (C-β), 161.1 (C-4), 163.2 (C-4′), 164.4 (C-2′), 192.6 (C=O). HR MALDI-TOFMS m/z (M)$^+$: calcd. for $C_{25}H_{30}O_6$, 426.2042; found, 426.2036.

Compound **5**. Yellow amorphous powder; ^{1}H-NMR (DMSO-d_6) δ: 1.40 (1H, m, H-5″), 1.56 (1H, m, H-5″), 1.62 (3H, s, 7″-Me), 1.72 (3H, s, 3″-Me) , 1.89 (2H, m, H-4″), 3.27 (1H, m, H-1″), 3.31 (1H, m, H-1″), 3.91 (3H, s, 4′-OMe), 4.07 (1H, t, J = 6.9 Hz, H-6″), 4.79 (1H, s, H-8″), 4.84 (1H, s, H-8″), 5.14 (1H, t, J = 6.6 Hz, H-2″), 6.68 (1H, d, J = 9.0 Hz, H-5′), 6.85 (2H, d, J = 8.4 Hz, H-3, H-5), 7.78 (2H, d, J = 8.4 Hz, H-2, H-6), 7.78 (1H, d, J = 15.0 Hz, H-β), 7.83 (1H, d, J = 15.0 Hz, H-α), 8.24 (1H, d, J = 9.0 Hz, H-6′), 10.15 (1H, s, 4-OH), 11.25 (1H, s, 6″-OOH), 13.81 (1H, s, 2′-OH). ^{13}C-NMR (DMSO-d_6) δ: 16.7 (3″-Me), 17.7 (7″-Me), 22.0 (C-1″), 29.5 (C-5″), 36.0 (C-4″), 56.9 (4′-OMe), 88.2 (C-6″), 103.6 (C-5′), 114.0 (C-8″), 114.9 (C-1′), 116.7 (C-3′), 116.7 (2C, C-3, C-5), 118.1 (C-α), 122.9 (C-2″), 126.5 (C-1), 131.3 (C-6′), 132.3 (2C, C-2, C-6), 134.9 (C-3″), 145.3 (C-7″), 145.7 (C-β), 161.3 (C-4), 163.0 (C-2′), 163.8 (C-4′), 193.3 (C=O). HR MALDI-TOFMS m/z (M)$^+$: calcd. for $C_{26}H_{30}O_6$: 438.2042; found: 438.2045.

Compound **6**. Yellow amorphous powder; ^{1}H-NMR ($CDCl_3$) δ: 1.34 (3H, s, 3″-Me), 1.57 (2H, m, H-4″), 1.65 (3H, s, 7″-Me), 1.71 (3H, s, 7″-Me), 1.79 (1H, s, 3″-OH), 2.11 (1H, m, H-5″), 2.19 (1H, m, H-5″), 3.19 (2H, d, J = 8.7 Hz, H-1″), 4.82 (1H, t, J = 8.7 Hz, H-2″), 5.15 (1H, t, J = 6.7 Hz, H-6″), 5.21 (1H, s, 4-OH), 6.44 (1H, d, J = 8.4 Hz, H-5′), 6.89 (2H, d, J = 7.2 Hz, H-3, H-5), 7.46 (1H, d, J = 15.0 Hz, H-α), 7.58 (2H, d, J = 7.2 Hz, H-2, H-6), 7.80 (1H, d, J = 8.4 Hz, H-6′), 7.84 (1H, d, J = 15.0 Hz, H-β), 13.51 (1H, s, 2′-OH). ^{13}C-NMR ($CDCl_3$) δ: 18.1 (7″-Me), 22.4 (C-5″), 23.2 (3″-Me), 26.1 (7″-Me), 27.3 (C-1″), 37.1 (C-4″), 74.2 (C-3″), 91.6 (C-2″), 102.1 (C-5′), 114.2 (C-3′), 115.4 (C-1′), 116.4 (2C, C-3, C-5), 118.6 (C-α), 124.4 (C-6″), 128.2 (C-1), 130.9 (2C, C-2, C-6), 132.1 (C-6′), 132.7 (C-7″), 144.3 (C-β), 158.3 (C-4), 161.9 (C-2′), 167.0 (C-4′), 192.5 (C=O). HR MALDI-TOFMS m/z (M)$^+$: calcd. for $C_{25}H_{28}O_5$, 408.1937; found, 408.1928.

Reduction of **5**. Compound **5** (1.8 mg) was treated by equivalent triphenylphosphine in methanol at room temperature for 1 h. The reaction product was subjected to TLC (Silica 60, Merck, Darmstadt, Germany) in chloroform/methanol (100:1) to give compound **5R** (1.0 mg).

Cell culture. 3T3-L1 preadipocytes (JCRB 9014) were obtained from Health Science Research Resources Bank (Tokyo, Japan) and were maintained in DMEM-low glucose supplemented 10% calf serum, 0.2 mM ascorbic acid, 50 U/mL of penicillin and 50 µg/mL of streptomycin at 37 °C in a humidified CO_2 atmosphere. Two d after confluence, the cells were differentiated for 5 d in DMEM-low glucose containing 10% fetal bovine serum, 0.2 mM ascorbic acid and 0.25 µM dexamethasone (0–2 d) in the presence of chalcones dissolved in DMSO or DMSO alone (0.1%). The medium was changed after two d.

Adiponectin mRNA expression. Total RNA was isolated from 3T3-L1 cells by RNA Iso Plus (Takara Bio, Shiga, Japan). cDNA was synthesized from total RNA by using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan), and then subjected to real-time RT-PCR using SYBR Premix Ex Taq II (Perfect Real Time; Takara Bio) according to the manufacturer's protocol with the Thermal Cycler Dice real time system (Takara Bio). The PCR conditions were as follows.

1 cycle of 95 °C for 10 s, 45 cycles of 95 °C for 5 s and 60 °C for 30 s, followed by 1 cycle of 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. β-Actin was used as an internal control gene. The primer sequences were adiponectin (forward, 5′-tgatggcagagatggcactc-3′; reverse, 5′-cctgtcattccaacatctcc-3′) and β-actin (forward, 5′-ttctttgcagc-tccttcgttg-3′; reverse, 5′-acatgccggagccgttg-3′).

Adiponectin protein production. Adiponectin in the medium was measured by using a commercial sandwich ELISA kit for mouse adiponectin (R&D Systems, Minneapolis, MN, USA).

Statistical analysis. All data are expressed as the mean ± SEM. The statistical analysis was performed by using a one-way analysis of variance (ANOVA) followed by Dunnett's test. $p < 0.05$ was considered as significant.

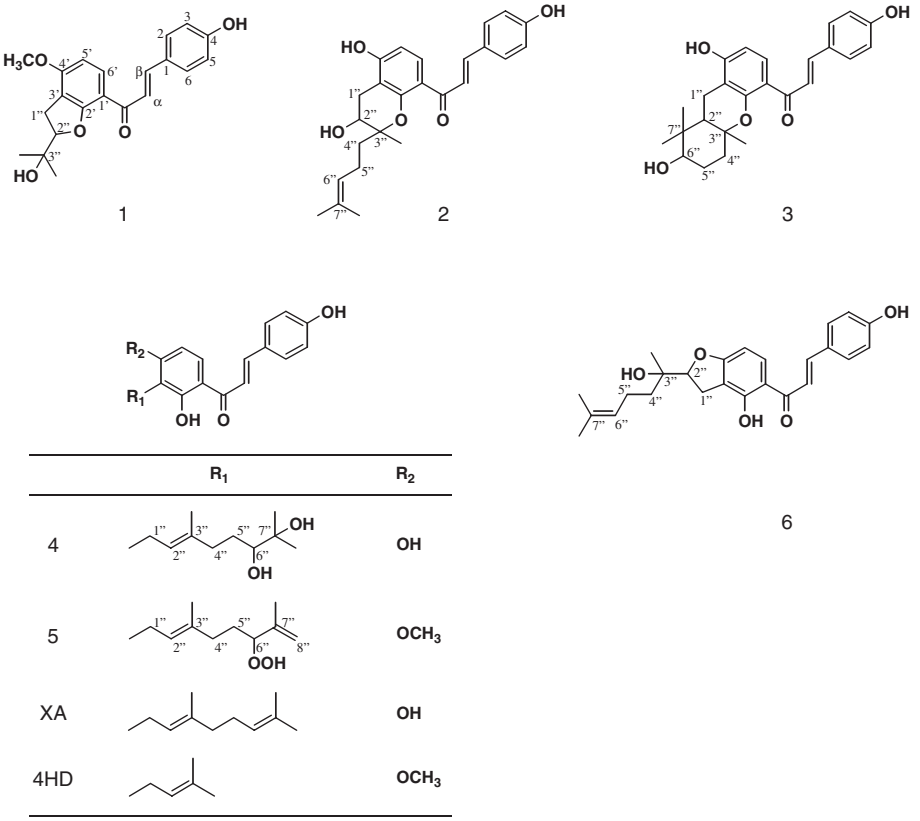

Fig. 1. Structures of the Chalcones Isolated from *A. keiskei*.

Results and Discussion

Isolation and structural elucidation of the new chalcones from A. keiskei

We isolated XA, 4HD and six new chalcones from the ethanol extract of *A. keiskei* by ODS and silica gel chromatography (Fig. 1). The structures of XA and 4HD were confirmed by comparing with the literature data.[25)]

Compound **1** had the molecular formula of $C_{21}H_{22}O_5$ determined by its HR MALDI-TOFMS value ($[M]^+$, m/z 354.1471). The ^{1}H-, ^{13}C-NMR and HMQC spectra indicated **1** to contain 21 carbons, including three methyls, an aliphatic methylene, an aliphatic methine, eight olefinic methines, an aliphatic quaternary, six olefinic quaternary carbons, and a carbonyl carbon. The DQF-COSY correlations (H-α/H-β; H-2/H-3; H-5/H-6; H-5′/H-6′) and HMBC correlations (H-α/C-β, C=O and C-1; H-β/C=O, C-1 and C-2, 6; H-2, 6/C-α, C-β, C-1, C-2, 6, C-3, 5, and C-4; H-3, 5/C-1, C-2, 6 and C-3, 5; H-5′/C-1′, C-3′ and C-4′; H-6′/C=O, C-2′ and C-4′) revealed **1** to have a 2′,3′,4,4′-tetra substituted chalcone skeleton. The presence of 4-OH and 4′-OMe were also determined from the HMBC correlations (4-OH/C-3, C-4 and C-5; 4′-OMe/C-4′). The functional group linked to C-2′ and C-3′ was determined as a 2″-(hydroxy isopropyl)-dihydrofuran ring by the HMBC long-range correlations (H-1″/C-2′, C-3′, C-4′, C-2″ and C-3″; H-2″/3″-Me; 3″-OH/C-2″, C-3″ and 3″-Me; 3″-Me/C-2″, C-3″ and 3″-Me). These results suggested the structure of **1** to be that shown in Fig. 1.

Compound **2** had the molecular formula of $C_{25}H_{28}O_5$ determined by its HR MALDI-TOFMS value ($[M]^+$, m/z 408.1934). The ^{1}H-, ^{13}C-NMR, HMQC and DEPT spectra indicated **2** to contain 25 carbons, including three methyls, three methylenes, an aliphatic methine, nine olefinic methines, an aliphatic quaternary, seven olefinic quaternary carbons, and a carbonyl carbon. The presence of a 2′,3′,4,4′-tetrasubstituted chalcone skeleton was confirmed by the DQF-COSY and HMBC correlations as in the case of **1**. The ^{1}H- and ^{13}C-NMR spectra of **2** were very similar to those of xanthoangelol I, 2′, 3′-[2-methyl-2-(4-methyl-3-pentenyl)-dihydropyrano]-4, 4′-dihydroxychalcone, except for the presence a hydroxyl group (δ_H 5.23) at C-2″ in **2**.[26)] The functional group linked to C-2′ and C-3′ was determined to be a 2″,3″,3″-trisubsutituted dihydropyran ring as shown Fig. 1 by DQF-COSY correlations (H-1″/H-2″; H-2″/2″-OH; H-4″/H-5″; H-5″/H-6″) and HMBC long-range correlations (H-1″/C-2′, C-3′, C-4′, C-2″ and C-3″; 2″-OH/C-1″, C-2″ and C-3″; 3″-Me/C-2″, C-3″ and C-4″; H-4″/C-5″; H-6″/7″-Me; 7″-Me/C-6″, C-7″ and 7″-Me). The structure of **2** was therefore established to be that shown in Fig. 1.

Compound **3** had the molecular formula of $C_{25}H_{28}O_5$ determined by its HR MALDI-TOFMS value ($[M]^+$, m/z 408.1932). The ^{1}H-, ^{13}C-NMR, HMQC and DEPT spectra indicated **3** to contain 25 carbons, including three methyls, three methylenes, two aliphatic methines, eight olefinic methines, two aliphatic quaternary, six olefinic quaternary carbons, and a carbonyl carbon. The presence of a trans-*p*-coumaroyl moiety in **3** was confirmed by the DQF-COSY correlations (H-α/H-β; H-2/H-3; H-5/H-6) and HMBC correlations (H-α/C-β, C=O and C-1; H-β/C-α, C=O, C-1 and C-2, 6; H-2, 6/C-β, C-2, 6, C-3, 5 and C-4; H-3, 5/C-1, C-4 and C-3, 5). The presence of a hexahydroxanthene moiety in **3** was also indicated by the HMBC correlations (H-5′/C-1′ and C-3′; H-6′/C-2′ and C-4′; H-1″/C-2″ and C-3″;

H-2″/C-1″, C-3″ and C-7″), DQF-COSY correlations (H-5′/H-6′; H-1″/H-1″ and H-2″; H-4″/H-5″; H-5″/H-6″) and TOCSY correlation (H-4″/H-6″). ^{1}H-, ^{13}C long-range couplings in the HMBC spectrum (3″-Me/C-2″, C-3″ and C-4″; H-2″/3″-Me and 7″-Me; H-6″/7″-Me; 7″-Me/C-2″, C-6″, C-7″ and 7″-Me) showed **3** to possess a methyl group at C-3″ and *gem*-dimethyl group at C-7″ in the hexahydroxanthene moiety. Moreover, the HMBC correlations (6″-OH/C-5″, C-6″ and C-7″), DQF-COSY correlations (6″-OH/H-6″) and TOCSY correlations (6″-OH/H-4″ and H-5″) indicated the presence of a hydroxyl group at C-6″. The structure of **3** was therefore established to be that shown in Fig. 1.

Compound **4** had the molecular formula of $C_{25}H_{30}O_6$ determined by its HR MALDI-TOFMS value ($[M]^+$, m/z 426.2036). The ^{1}H-, ^{13}C-NMR and HMQC spectra indicated **4** to contain 25 carbons, including three methyls, three aliphatic methylenes, an aliphatic methine, nine olefinic methines, an aliphatic quaternary, seven olefinic quaternary carbons, and a carbonyl carbon. The ^{1}H- and ^{13}C-NMR spectra of **4** were similar to those of xanthoangelol, except for the signals arising from the geranyl moiety. The HRMS data predicted that **4** corresponded to the dihydroxylated xanthoangelol. The ^{1}H-, ^{13}C long-range couplings in the HMBC spectrum (6″-OH/C-5″, C-6″ and C-7″; 7″-OH/C-6″, C-7″ and 7″-Me) showed **4** to possess two hydroxyl groups at C-6″ and C-7″. The HMBC correlations (H-1″/C-2′, C-3′, C-4′, C-2″ and C-3″; H-2″/C-1″, 3″-Me and C-4″; 3″-Me/C-2″, C-3″ and C-4″; H-4″/C-2″, C-3″ and C-5″; H-6″/C-4″; 7″-Me/C-6″, C-7″ and 7″-Me) and DQF-COSY correlations (H-1″/H-2″; H-4″/H-4″ and H-5″; H-5″/H-5″ and H-6″) supported the structure of the side chain shown Fig. 1. These results elucidated **4** to be 2′,4,4′-trihydroxy-3′-(6,7-dihydroxy-3,7-dimethyl-2-octaenyl) chalcone.

Compound **5** had the molecular formula of $C_{26}H_{30}O_6$ determined by its HR MALDI-TOFMS value ($[M]^+$, m/z 438.2045). The ^{1}H-, ^{13}C-NMR and HMQC spectra indicated **4** to contain 26 carbons, including three methyls, three aliphatic methylenes, an olefinic methylene, an aliphatic methine, nine olefinic methines, eight olefinic quaternary carbons, and a carbonyl carbon. The ^{1}H- and ^{13}C-NMR spectra of **5** were similar to those for the xanthoangelol F, 3′-geranyl-2′,4-dihydroxy-4′-methoxychalcone structure, except for the signals arising from the geranyl moiety.[27] The structure of the side chain was predicted to be the 6-hydroperoxy-3,7-dimethyl-octa-2,7-di-ene moiety by HMBC correlations (H-1″/C-2′, C-3′, C-4′, C-2″ and C-3″; H-2″/3″-Me and C-4″; 3″-Me/C-2″, C-3″ and C-4″; H-4″/C-2″, C-3″ and C-5″; H-5″/C-4″; H-6″/C-5″, C-7″ and C-8″; 7″-Me/C-6″ and C-7″; H-8″/C-6″ and 7″-Me) and DQF-COSY correlations (H-1″/H-2″; H-4″/H-5″; H-5″/H-5″ and H-6″; H-8″/7″-Me). Baba *et al.* have also reported the presence of chalcone with the hydroperoxylated side chain, xanthoangelol E, in *A. keiskei*.[25] To confirm the presence of a hydroperoxy group at C-6″, **5** was reduced by triphenylphosphine, which converted the hydroperoxy group to a hydroxyl group, according to their procedure. The reduced product (**5R**) was identified as xanthoangelol G, 2′,4-dihydroxy-3′-(6-hydroxy-3,7-dimethyl-2,7-octadienyl)-4′-methoxychalcone by the ^{1}H-, ^{13}C- and HMBC NMR spectra and MS spectrum (data not shown).[27] The ^{1}H- and ^{13}C-NMR spectra of **5R** were very similar to those of **5**, except for the presence of 6″-OH (δ_H 4.62) in **5R** instead of 6″-OOH in **5** (δ_H 11.25). These results elucidated **5** as 2′,4-dihydroxy-3′-(6-hydroperoxy-3,7-dimethyl-2,7-octadienyl)-4′-methoxychalcone.

Compound **6** had the molecular formula of $C_{25}H_{28}O_5$ determined by its HR MALDI-TOFMS value ($[M]^+$, m/z 408.1928). The ^{1}H-, ^{13}C-NMR and HMQC spectra indicated **6** to contain 25 carbons, including three methyls, three aliphatic methylenes, an aliphatic methine, nine olefinic methines, an aliphatic quaternary, seven olefinic quaternary carbons, and a carbonyl carbon. The presence of a 2′,3′,4,4′-tetrasubstituted chalcone skeleton was confirmed by the DQF-COSY and HMBC correlations, as in the case of **1**. In addition, 2′-OH and 4-OH were determined by their HMBC correlations (2′-OH/C-1′, C-2′ and C-3′; 4-OH/C-3, C-4 and C-5). The functional group linked to C-3′ and C-4′ was determined to be a 2″-substituted dihydrofuran ring as shown Fig. 1 by the DQF-COSY correlations (H-1″/H-2″; H-4″/H-5″; H-5″/H-6″) and HMBC long-range correlations (H-1″/C-2′, C-3′, C-4′, C-2″ and C-3″; 3″-Me/C-2″, C-3″ and C-4″; H-4″/C-3″, C-5″ and C-6″; H-5″/C-4″, C-6″ and C-7″; H-6″/C-5″ and 7″-Me; 7″-Me/C-6″, C-7″ and 7″-Me). The structure of **6** was thus established as that shown in Fig. 1.

This is the first report on the isolation of compounds **1**–**6** from a natural source, although the total chemical synthesis of **3** has previously been reported.[28]

Enhanced mRNA expression and protein secretion of adiponectin by the chalcones from A. keiskei

The effects of XA, 4HD and the six new chalcones isolated from *A. keiskei* were evaluated on adiponectin production in 3T3-L1 adipocytes. Table 1 shows the effects of the chalcones on adiponectin gene expression. All the chalcones, except for compound **3**, enhanced the expression of adiponectin mRNA. In particular, compound **4** (8.27-fold) and **6** (7.80-fold) markedly increased the expression of adiponectin mRNA.

Figure 2 shows the protein concentration of adiponectin protein in the supernatant of 3T3-L1 adipocytes treated by the chalcones. Compounds **2**, **3** and **6** showed growth inhibition of 3T3-L1 adipocytes at 20 μM. All the chalcones, except for compound **3**, appreciably enhanced the production of adiponectin. In particular, compounds **4** and **6** exhibited marked enhancement of adiponectin production, compound **1** showing weak

Table 1. Effect of the Chalcones from *A. keiskei* on the Genetic Expression of Adiponectin in 3T3-L1 Adipocytes

Compounds	Relative genetic expression
Control (DMSO)	1.00 ± 0.08
1	$3.43 \pm 0.45^{**}$
2	$5.15 \pm 0.31^{***}$
3	0.85 ± 0.09
4	$8.27 \pm 1.47^{***}$
5	$3.92 \pm 0.49^{*}$
6	$7.80 \pm 0.79^{***}$
XA	$5.27 \pm 0.20^{***}$
4HD	$4.74 \pm 1.13^{***}$

Data are shown as the mean ± SEM (n = 4–6).
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (*vs.* the Control)

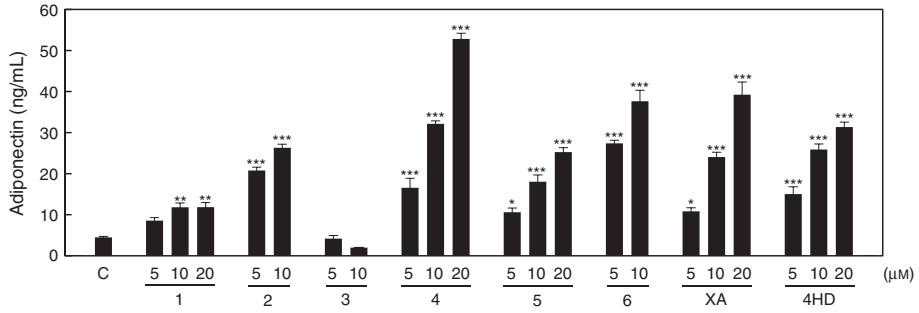

Fig. 2. Effect of the Chalcones from *A. keiskei* on Adiponectin Production in 3T3-L1 Adipocytes.

3T3-preadipocytes were incubated for 5 d in DMEM containing 10% fetal bovine serum, 0.2 mM ascorbic acid and 0.25 μM dexamethasone in the presence or absence of chalcones. Adiponectin in the medium was measured by ELISA. Data are shown as the mean ± SEM (n = 4–6), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (*vs.* the Control).

activity and **3** having no activity. These results indicate that the side chains of chalcones played an important role in enhancing adiponectin production. We have also previously indicated the structural importance of the side chains to the activity of glucose uptake in adipocytes by using various natural or synthetic chalcones.[29)]

The adipocyte-specific genetic expression including adiponectin was regulated *via* the peroxisome proliferator-activated receptor (PPAR)-γ signaling pathway. Such thiazolidinediones as pioglitazone are powerful PPAR-γ agonists and enhance adipogenesis and adiponectin production in 3T3-L1 adipocytes.[30)] Yamazaki *et al.* have reported that synthetic phenolamides enhanced the expression of adiponectin mRNA in 3T3-L1 preadipocytes by activating the PPAR-γ/retinoid X receptor-α heterodimeric complex.[31)] However, we have previously shown that XA and 4HD did not exhibit PPAR-γ activation in the GAL4-PPAR-γ ligand domain chimeric system.[16)] Moreover, our preliminary study indicated that XA and 4HD enhanced the expression of PPAR-γ mRNA in 3T3-L1 preadipocytes, and that 4HD and pioglitazone synergistically enhanced adiponectin production (Ohnogi, H. and Tahara, K., unpublished data). These results show that the chalcones from *A. keiskei* may enhance adiponectin production by a different mechanism from that of thiazolidinediones.

We have previously reported that orally administered XA and 4HD suppressed the progression of diabetes in KK-A^{y} type II diabetic mice.[16)] Moreover, an *A. keiskei* extract containing chalcones improved insulin resistance and tended to increase the serum adiponectin level in fructose-drinking rats (Ohnogi, H. and Hayami, S., unpublished data). The results of our human study have shown that 12 weeks ingestion of the green juice of *A. keiskei* appreciably increased the serum adiponectin level of mild diabetes when compared with placebo juice of *A. keiskei* with the chalcones removed.[32)] Taken together, it is possible that the chalcones from *A. keiskei* prevented the metabolic syndrome as an adiponectin inducer.

In conclusion, we isolated new chalcones from *A. keiskei* and showed that they induced the production of adiponectin in 3T3-L1 adipocytes. These chalcones also displayed other functions of aldose reductase inhibition and HMG-CoA reductase inhibition.[33–35)] Further studies are required to clarify the beneficial roles of the chalcones from *A. keiskei* in preventing the metabolic syndrome and related diseases.

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