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Sesquiterpenoids Isolated from the Flower Buds of *Tussilago farfara* L. Inhibit Diacylglycerol Acyltransferase

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Inhibition of acyl CoA:diacylglycerol acyltransferase (DGAT), which is a key enzyme in triglyceride synthesis in eukaryotic organisms, has been proposed as one of the drug targets for treating obesity, type II diabetes mellitus, and metabolic syndrome. Bioassay-guided fractionation of EtOH extract of the flower buds of *Tussilago farfara*, using an in vitro DGAT enzyme assay, resulted in the isolation of four known sesquiterpenoids, tussilagonone (**1**), tussilagone (**2**), 7β-(3-ethyl-*cis*-crotonoyloxy)-1α-(2-methylbutyryloxy)-3,14-dehydro-*Z*-notonipetranone (**3**), and 8-angeloylxy-3,4-epoxy-bisabola-7(14),10-dien-2-one (**4**). DGAT1 inhibitory activity was studied by in vitro DGAT assay using rat liver microsomes and HepG2 cell microsomes. They showed DGAT1 inhibition with IC_{50} values of 99.2 (**1**), 18.8 (**2**), 47.0 (**3**), and 211.1 (**4**) μM (for rat liver microsomes) and >1 mM (**1**), 49.1 (**2**), 160.7 (**3**), and 294.4 (**4**) μM (for HepG2 cell microsomes), respectively. Compound **2** showed the most potent inhibition against microsomal DGAT1 derived from rat liver and human hepatocellular carcinoma HepG2 cells and also significantly inhibited triglyceride synthesis by suppressing incorporation of [^{14}C]acetate or [^{14}C]glycerol into triglycerides in HepG2 cells. These findings suggest that tussilagone is a potential lead compound in the treatment of obesity and type 2 diabetes.

KEYWORDS: *Tussilago farfara*; sesquiterpenoid; diacylglycerol acyltansferase (DGAT); triglyceride synthesis; obesity; type II diabetes

INTRODUCTION

Triglycerides (TG) are the major form of energy storage in eukaryotic organisms. The excess supply of TG in a tissue could lead to serious diseases such as obesity, type II diabetes mellitus, and metabolic syndrome (*1*). TG synthesis has been assumed to occur primarily through acyl CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), a key enzyme in TG biosynthesis, a microsomal enzyme that catalyzes the final step in the glycerol phosphate pathway (*2*). DGAT plays a fundamental role in the metabolism of cellular diacylglycerol and is important for TG metabolism such as dietary fat absorption, lipoprotein assembly, the regulation of plasma TG concentrations, and fat storage in fat tissue (*3, 4*). Two enzymes that display DGAT activity have been characterized: DGAT1 and DGAT2. Although both enzymes utilize the same substrate, there is no homology between DGAT1 and DGAT2. In particular, the study of DGAT1-deficient mice showed the effects of increased energy expenditure, obesity resistance, and insulin sensitivity, decreased tissue levels of TG, and improvement of glucose metabolism (*5, 6*). Thus, DGAT1 inhibition may be a worthwhile strategy for treating obesity and type II diabetes. In this study, DGAT assay was carried out in the presence of 100 mM $MgCl_2$ because there was no detectable increase in DGAT2 activity when the assay contained 100 mM $MgCl_2$ (*25*).

During the screening of DGAT inhibitors from natural products, *Tussilago farfara* L. was additionally selected as an active natural resource. *T. farfara* L. (Compositae) is a perennial herb that is widely spread in China, North Africa, Siberia, and Europe. The flower buds of *T. farfara* are known as an important folk medicine used in the treatment of cough and wheezing (*12*). Terpenes, steroids, and flavonoids such as tussilagone derivatives, faradiol, phytosterol, rutin, and tussilagone were reported as its major constituents (*13*−*15*). Recently, it has been reported that the flower buds of *T. farfara* exhibit antioxidant effect, antimicrobial activity,

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and inhibitory effect on nitric oxide synthesis in lipopolysaccharide (LPS)-activated macrophages (*16*−*18*).

The present study was to isolate a series of sesquiterpenes from the flower buds of *T. farfara* and to examine their inhibitory activity on DGAT1 and triglyceride synthesis in rat liver and HepG2 cells.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained on a Varian Unity Inova 400 spectrometer using $CDCl_3$ as the solvent. HRFAB-MS was measured on a JEOL HX 110A/HX 100A spectrometer. Optical rotations were determined on a JASCO P-1020 polarimeter using a 100 mm glass microcell. HPLC was carried out using a Shimadzu System LC-6AD pump equipped with a model SPD-10Avp UV detector and YMC-Pack SIL column (5 μm, ∅ 20 × 250 mm). Column chromatography was conducted on silica gel (70−230 mesh, Merck) and Sephadex LH-20 (25−100 μm, Sigma). Thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} plates (Merck).

Materials. Bovine serum albumin and *sn*-1,2-dioleoylglycerol were obtained from Sigma Chemical Co (St. Louis, MO). [1-^{14}C]Oleoyl-CoA (250 μCi) was purchased from Amersham Pharmacia Biotech. Inc. (Little Chalf-ont, Buckinghamshire, U.K.). [1-^{14}C]Acetic acid (250 μCi) and [U-^{14}C]glycerol (50 μCi) were purchased from Amersham Biosciences (Buckinghamshire, UK). For the cell culture, HepG2 cells, human hepatocellular carcinoma, were obtained from the American Type Culture Collection (ATCC; Manassas, VA), and Dulbecco's modified Eagle's medium (DMEM), L-glutamine, kanamycin sulfate, and fetal bovine serum (FBS) were purchased from GIBCO-BRL (Gaithersburg, MD).

Plant Material. The plant material was purchased from a herbal store in Daejeon, Republic of Korea, and was identified by a botanist, Prof. K. H. Bae (College of Pharmacy, Chungnam National University, Daejeon, Republic of Korea). A voucher specimen (CA02-016) was deposited at the Korea Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

Extraction and Isolation. The dried flower buds of *T. farfara* (7 kg) were extracted with ethanol (60 L) at room temperature for 7 days. The extract solutions were filtered and then evaporated at 40 °C under reduced pressure. The ethanol extract (649 g) was suspended in 10 L of water and then extracted with an equal volume of $CHCl_3$. A part of the $CHCl_3$-soluble layer (80 g) was subjected to chromatography on a silica gel column (Merck, 70 − 230 mesh, 1.2 kg, ∅ 14.0 × 40 cm) using a stepwise gradient of hexane/ethyl acetate (from 50:1 to 1:1) to give 13 fractions (fraction A, 5.6 g; fraction B, 4.1 g; fraction C, 3.2 g; fraction D, 19.0 g; fraction E, 15.4 g; fraction F, 4.5 g; fraction G, 5.1 g; fraction H, 10.3 g; fraction I, 3.7 g; fraction J, 3.1 g; fraction K, 2.9 g; fraction L, 1.8 g; fraction M, 0.8 g). Fraction E (15 g), which exhibited the most potent DGAT inhibition, was further purified by a Sephadex LH-20 column using $CHCl_3$/MeOH (1:1) to yield 11 fractions (fraction E1, 1.4 g; fraction E2, 1.5 g; fraction E3, 2.1 g; fraction E4, 1.8 g; fraction E5, 1.7 g; fraction E6, 0.4 g; fraction E7, 0.8 g; fraction E8, 1.1 g; fraction E9, 0.9 g; fraction E10, 1.3 g; fraction E11, 1.8 g). Fraction E5 (1.7 g) was finally purified through a semipreparative HPLC (YMC-Pack SIL column, 250 × 20 mm i.d., 5 μm) at a flow rate 6 mL/min with UV detection at 210 nm, eluting with an isocratic solvent system of hexane/acetone (20:1) over 60 min to afford compounds **1** (20 mg, t_R = 20.9 min) and **2** (69 mg, t_R = 38.9 min). Fraction D (19 g) was rechromatographed on a silica gel column (Merck, 70−230 mesh, 700 g, ∅ 7.0 × 35 cm) with a gradient of hexane/ethyl actate (from 20:1 to 1:1) to afford 13 subfractions, which finally led to the isolation of 702 mg of **3** and 10 mg of **4**.

Tussilagonone (1): colorless gum; $[\alpha]_D^{24}$ −50.3 ° (*c* 0.3, $CHCl_3$); HR-FABMS, *m/z* 330.2202 (M^+, calcd for $C_{21}H_{30}O_3$ 330.2195); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.81 (3H, d, *J* = 7.0 Hz, H-13), 0.96 (3H, d, *J* = 7.0 Hz, H-12), 1.07 (3H, t, *J* = 7.3 Hz, H-5′), 1.48 (1H, m, H-6b), 2.05 (1H, m, H-6a), 2.09 (1H, m, H-5), 2.10 (1H, m, H-4), 2.12 (3H, d, *J* = 7.2 Hz, H-15), 2.15 (2H, m, H-4′a,b), 2.16 (3H, d, *J* = 1.2 Hz, H-6′), 2.22 (1H, dd, *J* = 17.0, 3.3 Hz, H-1b), 2.34 (1H, m, H-11), 2.38 (1H, dd, *J* = 17.0, 6.2 Hz, H-1a), 2.61 (1H, m, H-9), 4.79 (1H, br s, H-10b), 5.14 (1H, br s, H-10a), 5.54 (1H, dd, *J* = 3.0, 3.0 Hz, H-7), 5.65 (1H, q, *J* = 1.2 Hz, H-2′), 6.23 (1H, q, *J* = 7.3 Hz, H-14); ^{13}C ($CDCl_3$, 100 MHz) δ 12.1 (C-5′), 14.7 (C-15), 15.5 (C-13), 19.0 (C-6′), 21.5 (C-12), 27.2 (C-11), 30.8 (C-6), 34.0 (C-4′), 40.1 (C-5), 41.2 (C-1), 41.9 (C-9), 51.0 (C-4), 73.3 (C-7), 110.2 (C-10), 114.8 (C-2′), 132.6 (C-14), 141.2 (C-3), 146.3 (C-8), 162.1 (C-3′), 166.2 (C-1′), 206.6 (C-2).

Tussilagone (2): white powder; $[\alpha]_D^{24}$ −40.2 ° (*c* 1.0, $CHCl_3$); ESI-MS, *m/z* 390 $[M]^+$; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.78 (3H, d, *J* = 6.6 Hz, H-13), 0.98 (3H, d, *J* = 6.6 Hz, H-12), 1.07 (3H, t, *J* = 7.2 Hz, H-5′), 1.22 (3H, d, *J* = 6.6 Hz, H-15), 1.46 (1H, m, H-4), 2.10 (3H, s, OAC), 2.14 (1H, H-1a), 2.15 (1H, d, *J* = 1.2 Hz, H-6′), 2.18 (1H, H-4′), 2.31 (1H, m, H-11), 2.37 (1H, dd, *J* = 4.8, 16.5 Hz, H-1b), 2.50 (1H, dd, *J* = 3.3, 10.6 Hz, H-3), 2.59 (1H, m, H-9), 4.79 (1H, s, H-10a), 5.14 (1H, s, H-10b), 5.58 (1H, br t, *J* = 3.0 Hz, H-7), 5.63 (1H, d, *J* = 1.1 Hz, H-2′); ^{13}C ($CDCl_3$, 100 MHz) δ 42.6 (C-1), 214.8 (C-2), 57.2 (C-3), 49.1 (C-4), 43.9 (C-5), 31.2 (C-6), 73.3 (C-7), 146.1 (C-8), 42.3 (C-9), 110.1 (C-10), 27.6 (C-11), 21.6 (C-12), 15.4 (C-13), 69.6 (C-14), 15.2 (C-15), 21.4 ($\underline{C}H_3CO$), 170.9 ($CH_3\underline{C}O$), 166.0 (C-1′), 114.6 (C-2′), 162.0 (C-3′), 33.8 (C-4′), 11.9 (C-5′), 18.9 (C-6′).

7*β*-(3-Ethyl-*cis*-crotonoyloxy)-1α-(2-methylbutyryloxy)-3(14)-dehydro-*Z*-notonipetranone (3): colorless gum; $[\alpha]_D^{24}$ −56.2 ° (*c* 0.3, $CHCl_3$); HR-FABMS, *m/z* 430.2736 (M^+, calcd for $C_{26}H_{38}O_5$ 430.2719); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (3H, t, *J* = 7.2 Hz, H-4″), 0.90 (3H, t, *J* = 7.2 Hz, H-13), 0.98 (3H, d, *J* = 6.4 Hz, H-12), 1.07 (3H, t, *J* = 7.6 Hz, H-5′), 1.12 (3H, d, *J* = 7.2 Hz, H-5″), 2.02 (1H, m, H-11), 2.15 (3H, s, H-6′), 2.18 (3H, d, *J* = 7.2 Hz, H-15), 2.40 (1H, m, H-2″), 4.80 (1H, s, H-10a), 5.17 (1H, s, H-10b), 5.51 (1H, d, *J* = 3.2 Hz, H-7), 5.53 (1H, d, *J* = 4.2 Hz, H-1), 5.64 (1H, d, *J* = 1.6 Hz, H-2′), 6.39 (1H, q, *J* = 6.8 Hz, H-14); ^{13}C ($CDCl_3$, 100 MHz) δ 11.6 (C-4″), 12.0 (C-5′), 15.2 (C-15), 15.7 (C-13), 16.7 (C-5″), 19.0 (C-6′), 21.4 (C-12), 26.9 (C-3″), 27.6 (C-11), 29.9 (C-6), 33.9 (C-4′), 40.6 (C-5), 41.1 (C-2″), 44.8 (C-4), 45.9 (C-9), 72.5 (C-1), 73.4 (C-7), 112.7 (C-10), 114.7 (C-2′), 136.9 (C-14), 139.3 (C-3), 141.0 (C-8), 162.2 (C-3′), 166.0 (C-1′), 175.6 (C-1″), 200.3 (C-2).

8-Angeloylxy-3,4-epoxy-bisabola-7(14),10-dien-2-one (4): colorless oil; $[\alpha]_D^{24}$ −5.6° (*c* 0.6, DMSO); HR-FABMS, *m/z* 332.4346 (M^+, calcd for $C_{20}H_{28}O_4$ 332.4339); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.41 (3H, s, H-15), 1.61 (3H, br s, H-13), 1.68 (3H, br s, H-12), 1.89 (3H, dq, *J* = 1.6, 1.6 Hz H-5′), 1.98 (3H, dq, *J* = 7.2, 1.6 Hz, H-4′), 2.04 (1H, dd, *J* = 15.2, 11.6 Hz, H-5a), 2.16 (1H, m, H-1b), 2.36 (2H, m, H-9a,9b), 2.40 (1H, m, H-5b), 2.60 (1H, m, H-6), 2.87 (1H, dd, *J* = 14.0, 12.8 Hz, H-1a), 3.45 (1H, d, *J* = 4.8 Hz, H-4), 5.00 (1H, s, H-14b), 5.04 (1H, tq, *J* = 7.2, 1.2 Hz, H-10), 5.16 (1H, dd, *J* = 7.0, 3.2 Hz, H-8), 5.17 (1H, s, H-14a), 6.08 (1H, qq, *J* = 7.2, 1.6 Hz, H-3′); ^{13}C ($CDCl_3$, 100 MHz) δ 15.0 (C-15), 16.0 (C-4′), 18.2 (C-13), 20.8 (C-5′), 26.0 (C-12), 31.2 (C-5), 33.2 (C-9), 40.6 (C-6), 41.5 (C-1), 59.4 (C-3), 65.0 (C-4), 75.1 (C-8), 111.6 (C-14), 119.1 (C-10), 128.0 (C-2′), 134.9 (C-11), 138.4 (C-3′), 150.5 (C-7), 167.3 (C-1′), 208.1 (C-2).

Preparation of Microsomes from Rat Liver and Measurement of in Vitro DGAT Activity. The microsomal fractions from rat livers (male Sprague−Dawley rats, 250−300 g) were prepared as described earlier (*22*). In brief, rat livers were rinsed and then homogenized in 9 volumes of STE buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA) with a Teflon−glass homogenizer. The homogenate was centrifuged at 14000*g* for 20 min at 4 °C. The supernatant was centrifuged at 100000*g* for 1 h at 4 °C to obtain microsomal pellet. The pellet was suspended in STE buffer without EDTA and centrifuged at 100000*g* for 1 h at 4 °C. The pellet containing microsomes was resuspended in STE buffer without EDTA and stored at −70 °C.

DGAT1 activity was measured according to the method of Coleman et al. (*22*). The reaction mixture, containing 175 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$, 0.2 mM *sn*-1,2-diacylglycerol, 0.25 mg of fatty acid free bovine serum albumin, and 10 μM [1-^{14}C]oleoyl-CoA (2.75 μCi) in a total volume of 200 μL, was initiated by the addition of rat liver microsomal fraction, followed by gentle and brief vortexing. After incubation for 30 min at 25 °C, the reaction was stopped by the addition of 1.5 mL of 2-propanol/heptane/water (80:20:2, v/v/v), 1.0 mL of heptane, and 0.5 mL of water to extract lipid. After vortexing, 1.2 mL

of the organic phase was transferred to a glass tube and washed once with 2.0 mL of an alkaline ethanol solution [ethanol/0.5 N NaOH/water (50:10:40, v/v/v)]. The amount of radioactivity was determined in a liquid scintillation counter (1450 micro beta TRIUX). The samples were tested for DGAT inhibitory activity in three independent experiments. All inhibitors were added as solutions in DMSO. The presence of DMSO in assay medium at 2.5% concentration had no effect on the enzyme activity.

Preparation of Microsomes from HepG2 Cells and in Vitro DGAT Assay. The microsomal fractions from HepG2 cells were prepared as described previously (*22*). Briefly, 70−80% confluent HepG2 cells were washed twice with cold PBS and were collected in 10 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA (STE buffer) by scraping under cold conditions (4 °C) and were homogenized with a Branson Sonifier 450. The homogenate was centrifuged at 25000*g* for 15 min at 4 °C. The supernatant was then centrifuged at 100000*g* for 1 h at 4 °C to obtain the microsomal pellet. The microsomes were resuspended in STE buffer and stored at −70 °C. The protein concentration of the microsomes was determined according to the method of Bradford using a protein assay kit (Bio-Rad Laboratory, Hercules, CA).

The DGAT1 activity in the microsomal fractions was measured by previously described procedures using [1-^{14}C]oleoyl-CoA and *sn*-1,2-dioleoylglycerol as substrates (*22*). The reaction mixture contains 175 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$, 200 μM *sn*-1,2-diacylglycerol in acetone, 200 μg of fatty acid free bovine serum albumin, 20 μM [1-^{14}C]oleoly-CoA (5.5 μCi), and 15 μg of HepG2 microsomal fraction in a total volume of 200 μL.

Cellular Triglyceride Formation Assay Using HepG2 Cells. The cell-based assay for DGAT was conducted with human hepatocellular carcinoma HepG2 cells as described earlier (*23*). Briefly, the HepG2 cells were grown in DMEM supplemented with 2 mM L-glutamine, 60 mg/L kanamycin, 2 g/L sodium bicarbonate, and 10% (v/v) FBS. Cells were maintained in a standard culture with humidified air containing 5% CO_2 at 37 °C. HepG2 cells (1×10^6 cells/mL) were incubated with vehicle (control) or compound **2** at the indicated doses (20, 30 μM) in the presence of [U-^{14}C]glycerol (0.6 μCi) for 18 h or [1-^{14}C]acetate (1.25 μCi) for 6 h. At the end of the incubation, intracellular lipids were extracted with a mixture of hexane/isopropanol (3:2, v/v). Intracellular lipids were separated by thin-layer chromatography (Kieselgel 60 F_{254} plates, Merck) using petroleum ether/diethyl ether/acetic acid (80:20:1, v/v) as a developing solvent, and the amount of radioactivity was analyzed with a bioimaging analyzer (BAS 1500, Fuji). Data were expressed as percentages of control values for TGs.

Cell Viability Assay. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (*29*). For the cell viability assay HepG2 cells were grown in 100 mm Petri dishes with 10 mL of Minimum Essential Medium (MEM) containing L-glutamine, 10% FBS, 1 mM sodium pyruvate, and antibiotics. Viable cells were seeded in the growth medium (200 μL) into 96-well plates (2×10^4 cells/well) and incubated at 37 °C in a 5% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 1 to 100 μM. Each sample was prepared in triplicate. After the samples had been allowed to stand for 24 h, 0.4 μL of the test sample was added to each well. The same volume of DMSO was added to the control wells. Forty-eight hours after the test sample was added, 25 μL of MTT was also added to the each well (final concentration = 62.5 μg/ml). Three hours later, the medium was removed, and the resulting formazan crystals were dissolved with 100 μL of DMSO. The optical density (OD) was measured at 540 nm using a tunable microplate reader. The effect of test sample on growth inhibition was assessed as percent cell viability, where vehicle-treated cells (i.e., control) were taken as 100% viable.

RESULTS AND DISCUSSION

In our search for DGAT1 inhibitors from natural sources, we found that the ethanolic extract of the flower buds of *T. farfara* inhibited DGAT1 enzyme activity of microsomal protein prepared from rat liver in a dose-responsive manner with IC_{50} values of 86.2 μg/mL. Thus, the extract was subjected to divide into two portions by serial solvent partition, that is, $CHCl_3$ and remaining water residue. The $CHCl_3$-soluble part exhibited a more potent DGAT1 inhibitory effect with an IC_{50} value of 45.8 μg/mL. The $CHCl_3$-soluble part was further purified by repeated column chromatography, which finally afforded three oplopane-type sesquiterpenoids, tussilagonone (**1**), tussilagone (**2**), and 7β-(3-ethyl-*cis*-crotonoyloxy)-1α-(2-methylbutyryloxy)-3,14-dehydro-*Z*-notonipetranone (**3**), and a bisabolane-type sesquiterpenoid, 8-angeloyloxy-3,4-epoxy-bisabola-7(14),10-dien-2-one (**4**) (**Figure 1**). These compounds were identified by direct comparison of their physical and spectral properties (^{1}H NMR and ^{13}C NMR) with those in the literature (*15, 19*−*21*).

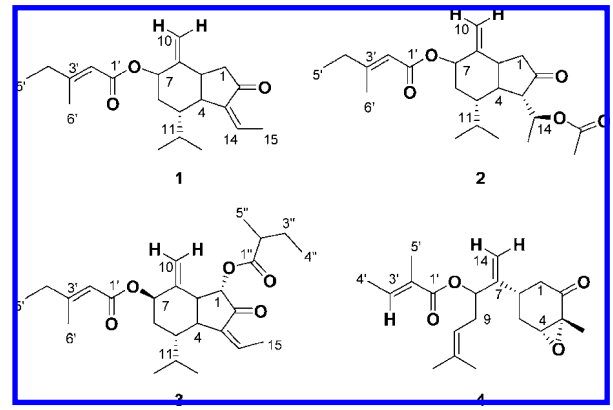

Figure 1. Chemical structures of compounds **1**−**4** isolated from flower buds of *T. farfara* L.

The DGAT inhibitory effect of these compounds was evaluated by measuring the DGAT activity of microsomal protein prepared from rat liver and human hepatocellular carcinoma, HepG2 cells. They showed DGAT1 inhibition with IC_{50} values of 99.2 (**1**), 18.8 (**2**), 47.0 (**3**), and 211 (**4**) μM (for rat liver microsome) and >500 (**1**), 49.1 (**2**), 160.7 (**3**), and 294.4 (**4**) μM (for HepG2 cell microsomes), respectively, in a dose-dependent manner (**Figure 2**). The inhibitory activities of these compounds were confirmed by using a positive control of kurarinone, which inhibited DGAT1 activity with IC_{50} values of 10.9 and 28.8 μM in the assay system (*8*). Compound **2** showed the most potent inhibition against microsomal DGAT1 prepared from rat liver and human hepatocellular carcinoma.

We investigated the effect of compound **2** on cellular TG synthesis by incubating HepG2 with [^{14}C]acetate or [^{14}C]glycerol in the presence of compound **2** (**Figure 3**). When the cells were incubated in the presence of compound **2** with radiolabeled glycerol or acetate, compound **2** showed 49 and 59% (for radiolabeled glycerol) and 34 and 61% (for radiolabeled acetate) inhibition against cellular triglyceride synthesis at concentrations of 20 and 30 μM, respectively. Compound **2** dose-dependently inhibited the incorporation of [^{14}C]acetate or [^{14}C]glycerol into TG, suggesting decreased de novo synthesis of TG by compound **2** using HepG2 cells as an in vitro model system. Because the cell viability was not decreased in HepG2 cells at the concentrations used in this study, the inhibitory effect of compound **2** was not due to the cytotoxicity (data not shown). 5-Aminoimidazole-4-carboxamide 1-β-ribofuranoside (AICAR), which is known to inhibit TG synthesis through AMP-activated protein kinase (AMPK) activation, was used as positive control.

Excessive deposition of TG in various tissues leads to obesity and, particularly, when it occurs in nonadipose tissues (e.g., skeletal muscle, liver, and β-cell) is associated with tissue dysfunction referred to as lipotoxicity (*24*). Compounds that can decrease the synthesis of triglycerides from diacylglycerol by inhibiting or lowering the activity of the enzyme would be

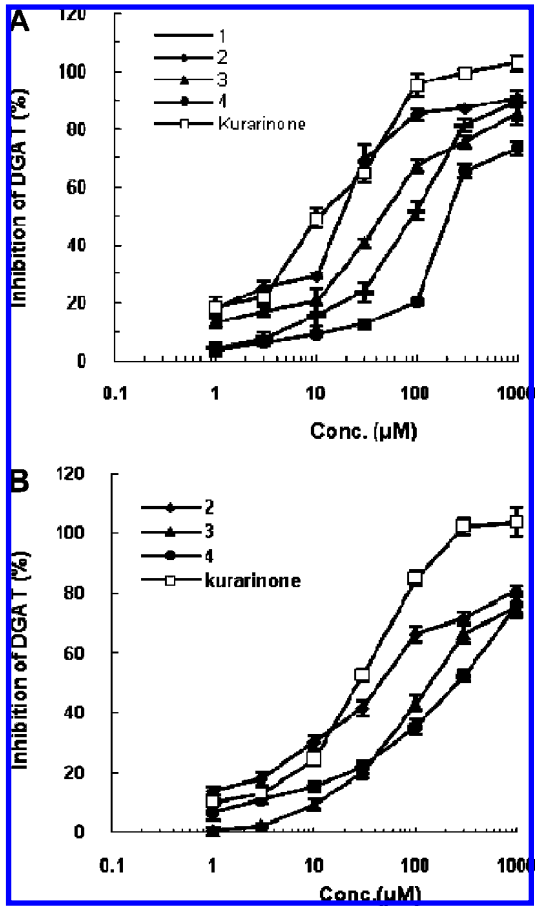

Figure 2. Inhibitory effects of compound **1** (—), **2** (◆), **3** (▲), and **4** (●) on DGAT1 activity of rat liver microsomes (**A**) and HepG2 cell microsomes (**B**). Enzyme reactions were carried out at 25 °C for 30 min in the presence of 100 mM $MgCl_2$. Kurarinone (□) was used as a positive control. Data represent the mean ± SE ($n = 3$).

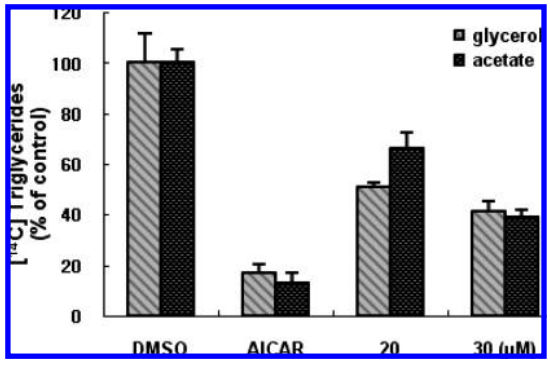

Figure 3. Inhibition of triglyceride synthesis by tussilagone (**2**) in HepG2 cells. HepG2 cells (1×10^6 cells/1 mL) were incubated in the absence (control) or presence of compound **2** (20, 30 μM) or 2 mM AICAR with [^{14}C]glycerol for 18 h or [^{14}C]acetate for 6 h at 37 °C. Intracellular [^{14}C] lipids were extracted and analyzed as indicated under Materials and Methods. AICAR was used as a positive control. Values are expressed as percentages of control and are means ± SD ($n = 3$).

of value as therapeutic agents for the treatment diseases associated with abnormal metabolism of TG. For that reason, we have already studied and reported several DGAT inhibitors such as tanshinones from *Salvia miltiorrhiza* (*7*), prenylflavonoids from the *Sophora flavescens* (*8*), substituted quinolone alkaloids from *Evodia rutaecarpa* (*9*), polyacetylenens from *Panax ginseng* (*10*), and alkamides from *Piper* species (*11*) from natural sources.

In this study, we isolated four sesquiterpenes from *T. farfara* extracts through bioassay-guided isolation. Among them, compound **2** showed strong inhibitory effects on both rat liver and HepG2 cell microsomal DGAT1. Tussilagone (**2**) has been reported to possess several pharmacological activities, namely, inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression (*26*), antagonistic activity on platelet-activating factor receptor (*27*), and cardiovascular effects (*28*). However, in vitro DGAT inhibition and cellular TG synthesis inhibition of tussilagone (**2**) was first reported by this study.

In conclusion, the chloroform extract of flower buds of *T. farfara* markedly inhibited rat liver microsomal DGAT1 enzyme activity. Especially, tussilagone (**2**) is confirmed to be the main active compound and may be useful for the design of DGAT1 inhibitors for the treatment of obesity and type II diabetes. In addition, this plant may be used as a dietary supplement to improve and prevent obesity and type II diabetes. Because we have the experimental evidence for the inhibitory activities of compound **2** in in vitro DGAT assay and cellular TG synthesis, further studies should be required to determine the enzyme specificity and biological efficacy of tussilagone (**2**) and *T. farfara* extract in an in vivo model.

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