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The Inhibition of Diacylglycerol Acyltransferase by Terpenoids from *Youngia koidzumiana*

Nguyen Tien Dat, Xing Fu Cai, Mun-Chual Rho[1], Hyun Sun Lee[1], KiHwan Bae, and Young Ho Kim

College of Pharmacy, Chungnam National University, Deajeon 305-764, Korea and [1]Cardiovascular Research Laboratory, Korea Research Institute of Bioscience and Biotechnology, P.O.Box 115, Yusong, Daejeon 305-600, Korea

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The EtOAc extract of *Youngia koidzumiana* significantly inhibited the diacylglycerol acyltransferase (DGAT) from rat liver microsomes. Bioactivity-guided fractionation led to the isolation of nine compounds, the structures of which were established using physicochemical and spectral data. Of the isolated compounds, oleanolic acid (**2**), methyl ursolate (**7**) and corosolic aicd (**8**) inhibited DGAT, with IC_{50} values of 31.7, 26.4, and 44.3 μM, respectively. However, sesquiterpenoids showed only weak inhibitory effects toward DGAT.

Key words: *Youngia koidzumiana*, Germanicol acetate, Brachynereolide, Ixerin Y, Methyl ursolate, Corosolic acid, Crepidiaside C, Diacylglycerol acyltransferase

INTRODUCTION

Triglycerides represent the major form of stored energy in eukaryotes, and play important roles in the metabolic processes of the liver, intestine, mammary gland and adipose tissue. However, a high triglyceride level is known as a major risk factor of coronary heart disease, obesity and hypertriglyceridemia (Lehner and Kuksis, 1996). Triglyceride synthesis is presumed to occur primarily through acyl CoA: diacylglycerol acyltransferase (DGAT), a microsomal enzyme that catalyses the final step in the glycerol phosphate pathway, and has been cloned (Cases *et al.*, 1998). By the catalysis of DGAT, acyl residue transfers from the acyl-CoA to diacylglycerol and then to the triacylglycerol forms in the final step of the glycerol-phosphate pathway of triglyceride synthesis. The enzyme is also believed to catalyze the final step of the monoacylglycerol pathway, which is important for intestinal fat absorption, and is found predominantly in the enterocytes of the small intestine (Lehner and Kuksis, 1996). Therefore, DGAT inhibition may be a worthwhile strategy for the treatment of triglyceride metabolic disorders, such as obesity and hypertriglyceridemia (Smith *et al.*, 2000; Gray and Tartaglia, 2000). Although no synthetic inhibitors of DGAT are known, several naturally occurring compounds have been reported to inhibit DGAT activity. Amidepsin AD (*Humicola* sp. FO-2942, IC_{50}: 10.2-51.6 μM), xanthohumol and xanthohumol B (*Humulus lupulus*, IC_{50}: 50.3 μM and 194.0 μM), and an extract from roselipins (*Gliocladium roseum* KF-1040, IC_{50}: 15.0-22.0 μM) have been reported as DGAT inhibitors (Tomoda *et al.*, 1995; Tabata *et al.*, 1997; Tomoda *et al.*, 1999). Quinolone alkaloids (*Evodia rutaecarpa*, IC_{50}: 13.5-69.5 mM), tanshinones (*Salvia miltiorrhiza*, IC_{50}: 10.5-11.1 mg/mL), polyacetylenes (*Panax ginseng*, IC_{50}: 9.0-32.0 mg/mL) and prenylflavonoids (*Sophora flavescence*, IC_{50}: 8.6-10.9 mg/mL) have also recently been reported to inhibit DGAT (Ko *et al.*, 2002; Lee *et al.*, 2004).

Youngia koidzumiana (Asteraceae) is plant endemic to the Mt. Chiri region of Korea, but its chemical constituents and biological activities remain to be fully elucidated. Previously, four compounds, including germanicol acetate (**1**), oleanolic acid (**2**), brachynereolide (**3**), and ixerin Y (**4**) were isolated from this plant in our laboratory (Dat *et al.*, 2002). In our screening for DGAT inhibitors from medicinal plants, the MeOH extract of *Y. koidzumiana* was found to inhibit the DGAT enzyme from rat liver. In order to identify the natural compounds involved, five other compounds were isolated from the EtOAc fraction of *Y. koidzumiana*. In total, seven compounds (**1-4**, **7-9**) isolated from *Y. koidzumiana* were examined for their inhibitory effects toward DGAT.

Correspondence to: Young Ho Kim, College of Pharmacy, Chungnam National University, Deajeon 305-764, Korea
Tel: 82-42-821-5933, Fax: 82-42-823-6566
E-mail: yhk@cnu.ac.kr

MATERIALS AND METHODS

General experimental procedure

Melting points were measured using a Yanagiomoto micro hot-stage melting point apparatus, and are uncorrected. Optical rotations were determined on a JASCO DIP-370. Both ^{1}H- (300 MHz) and ^{13}C-NMR (75 MHz) spectra were obtained using a Bruker DRX-300 NMR spectrometer. FAB-MS spectra were obtained from a JEOL JMS-HX/HX-100A tandem mass spectrometer, and IR spectra from a Jasco IR Report-100 spectrophotometer. Silica gel (230-400 mesh, Merck) was used for column chromatography and silica gel 60 F_{254} (Merck) for TLC. Bovine serum albumin and *sn*-1, 2-dioleoylglycerol were obtained from the Sigma Chemical Co (St. Louis, MO), and the [1-^{14}C]-palmitoyl CoA was purchased from Amersham (London, UK).

Plant material

The whole plants of *Y. koidzumiana* were collected in the Mt. Chiri region during November 2000 and identified by Prof. KiHwan Bae, of the College of Pharmacy, Chungnam National University. Voucher specimens (CNU 20046) were deposited at the herbarium in the College of Pharmacy, Chungnam National University.

Extraction and isolation

The whole plants (2 kg) were dried, ground and extracted 3 times with MeOH, yielding 120 g of dried extract upon removal of the solvent under vacuum. The resulting extract was suspended in water and partitioned with hexane, ethyl acetate and butanol to afford 41.8, 48.8 and 18.1 g of residues, respectively. The ethyl acetate fraction was subjected to Si-gel column chromatography, with solvent gradient elution using hexane-ethyl acetate (6:1 v/v to 100% ethyl acetate), to afford 7 fractions (Fr. A~G). Fr. B was subjected to further chromatography on a Si-gel column, using hexane-chloroform (15:1-5:1) as eluent, to give compounds **5** (136 mg) and **6** (120 mg). Compound **7** (16.5 mg) was obtained from Fr. E by Si-gel column chromatography, with hexane-ethyl acetate (4:1→1:1) as the eluent. Fr. F was subjected to chromatography on a Si-gel column, yielding 4 subfractions (subfr. A~D); subfr. C was subjected to C_{18} reverse phase column chromatography, using methanol-water (8:1) to give compound **8** (7.6 mg). Compound **9** (14.1 mg) was eluted from Fr. D using C_{18} reverse phase column chromatography, with methanol-water (2:1) as the eluent.

Nonadecan-1-ol (5)

White powder, mp 124-126 °C; FAB-MS m/z 307 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$): δ 3.66 (2H, t, J = 6.6 Hz, H-1), 1.54 (2H, m, H-2), 1.27 (32H, br s, H-3~18), 0.90 (3H, t, J = 6.6 Hz, H-19); ^{13}C-NMR ($CDCl_3$): δ 63.5 (C-1), 33.2 (C-2), 32.3 (C-17), 29.8~30.0 (C-4~15), 26.1 (C-3), 23.1 (C-18), 14.5 (C-19).

β-Sitosterol (6)

White powder, mp 132-135°C; FAB-MS m/z 437 $[M+Na]^+$; ^{1}H-NMR (300 MHz, $CDCl_3$) δ : 5.37 (1H, d, J = 4.8 Hz, H-6), 3.54 (1H, m, H-3), 0.70 (3H, s, H-18).

Methyl ursolate (7)

White powder, mp 230-232°C; $[\alpha]_D^{20}$ +34.5° (*c* 0.5, $CHCl_3$); FAB-MS m/z 493 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$): δ 5.20 (1H, m, H-12), 3.29 (3H, s, OCH_3), 3.15 (1H, m, H-3), 2.19 (1H, d, J = 13.0 Hz, H-18), 1.07 (3H, s, H-27), 0.94 (3H, s, H-23), 0.92 (3H, s, H-25), 0.91 (3H, s, H-30), 0.87 (3H, s, H-29), 0.80 (3H, s, H-24), 0.74 (3H, s, H-26); ^{13}C-NMR ($CDCl_3$): δ 181.3 (C-28), 139.7 (C-13), 126.4 (C-12), 79.4 (C-3), 56.0 (C-5), 53.8 (C-18), 50.0 ($COOCH_3$), 48.6 (C-9), 47.2 (C-17), 42.9 (C-14), 42.6 (C-19), 40.4 (C-8), 40.1 (C-4), 39.9 (C-20), 39.6 (C-1), 39.5 (C-10), 37.8 (C-22), 34.0 (C-7), 31.5 (C-21), 28.9 (C-15), 28.6 (C-23), 27.5 (C-2), 25.0 (C-16), 24.1 (C-27), 24.1 (C-11), 21.6 (C-30), 19.5 (C-6), 17.6 (C-26), 17.5 (C-29), 16.1 (C-25), 16.0 (C-24).

Corosolic acid (8)

White powder, mp 240-244°C; $[\alpha]_D^{20}$ +49.9° (*c* 0.3, $CHCl_3$); FAB-MS m/z 495 $[M+Na]^+$; ^{1}H-NMR ($CDCl_3$): δ 5.27 (1H, m, H-12), 3.63 (1H, m, H-2), 2.92 (1H, d, J = 9.5 Hz, H-3), 2.23 (1H, d, J = 11.0 Hz, H-18), 1.18 (3H, s, H-27), 1.14 (3H, s, H-23), 1.03 (3H, s, H-26), 0.96 (3H, s, H-24), 0.92 (3H, s, H-30), 0.86 (3H, s, H-25), 0.82 (3H, s, H-29); ^{13}C-NMR ($CDCl_3$): δ 180.5 (C-28), 138.7 (C-13), 125.7 (C-12), 83.5 C-3), 68.5 (C-2), 55.6 (C-5), 53.3 (C-18), 48.2 (C-17), 47.9 (C-9), 47.2 (C-1), 42.3 (C-14), 41.7 (C-19), 39.8 (C-8), 39.6 (C-4), 39.4 (C-20), 38.2 (C-10), 37.0 (C-22), 32.9 (C-7), 30.7 (C-21), 28.3 (C-23), 28.1 (C-15), 24.3 (C-16), 23.4 (C-11), 23.0 (C-27), 20.5 (C-30), 18.2 (C-6), 16.8 (C-29), 16.7 (C-26), 16.6 (C-25), 16.5 (C-24).

Crepidiaside C (9)

White powder, mp 128-130°C; $[\alpha]_D^{20}$ +40.5° (*c* 0.5, CH_3OH); FAB-MS m/z 449 $[M+Na]^+$; ^{1}H-NMR (CD_3OD): δ 5.98 (1H, br s, H-3), 1.75 (3H, br s, H-14), 1.35 (3H, d, J = 7.0 Hz, H-13); ^{13}C-NMR (CD_3OD): δ 181.2 (C-12), 142.4 (C-4), 137.7 (C-1), 130.9 (C-3), 127.9 (C-10), 102.9 (C-1′), 84.1 (C-6), 78.3 (C-3′), 78.0 (C-5′), 75.2 (C-2′), 71.9 (C-4′), 71.4 (C-8), 69.0 (C-15), 63.1 (C-7), 62.9 (C-6′), 52.9 (C-5), 46.6 (C-9), 42.9 (C-11), 38.3 (C-2), 23.2 (C-14), 16.1 (C-13).

Preparation of microsomes from rat liver

Rat livers (Male Sprague-Dawley rat, 250-300 g) were minced and then homogenized in 9 volumes of STE

buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4 and 1.0 mM EDTA) in a Teflon-glass homogenizer. The homogenate was then centrifuged at 14,000 g and 4°C for 20 min and the supernatant further centrifuged at 100,000 g and 4°C for 1 h to obtain the microsomal pellet, which was then suspended in STE buffer, without EDTA, and centrifuged at 100,000 g and 4 °C for 1h. The final pellet was re-suspended in STE buffer without EDTA. The microsomal fractions of rat livers were prepared, and aliquots stored at -70 °C until used (Coleman, 1992).

DGAT assay using rat liver microsomes

The DGAT assay was performed as previously reported, but with some modification (Coleman, 1992). In brief, the reaction mixture, containing 175 mM Tris-HCl (pH 8.0), 8.0 mM $MgCl_2$, 0.2 mM *syn*-1,2-diacylglycerol, 0.25 mg of fatty acid free bovine serum albumin and 30 μM [1-^{14}C] palmitoyl-CoA (0.02 μCi), in a total volume of 200 μL, was initiated by the addition of a rat liver microsomal fraction, followed by gentle and brief vortexing. After incubating for 10 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 mL of heptane and 0.5 mL of water to extract the lipid. After vortexing, 1.2 mL of the organic phase was transferred to a glass tube and washed once with 2.0 mL of alkaline ethanol solution [ethanol-0.5 N NaOH-water (50:10:40 v/v/v)]. The amount of radioactivity was then determined using a liquid scintillation counter (1450 micro beta TRIUX). Triplicate samples were then tested for their DGAT inhibitory activities. All inhibitors were added as solutions in DMSO.

RESULTS AND DISCUSSION

The extracts of the medicinal herbs were screened using an *in vitro* assay, employing rat liver microsomes as the DGAT enzyme sources. The MeOH extract of whole plants of *Y. koidzumiana* was found to moderately inhibit the DGAT activity. The MeOH extract of *Y. koidzumiana* was sequentially partitioned with hexane, ethyl acetate and butanol. Each partitioned residue was tested for DGAT activity, with the eight compounds isolated from the ethyl acetate fraction, showing the strongest DGAT activity (Table I).

In the ^{1}H- and ^{13}C-NMR spectra, compound **5** showed the characteristic pattern of a long chain hydrocarbon compound. A hydroxy methylene peak, at δ 3.66, and a large broad peak, due to the integration of equivalent CH_2 groups, at δ 1.27 were observed in the ^{1}H-NMR spectrum. A terminal methyl group was present at δ 0.90 and at δ 14.5 in the ^{1}H-NMR and ^{13}C-NMR spectra, respectively. The FAB-MS spectrum revealed a $[M+Na]^+$ peak at m/z 307, corresponding to a molecular weight of 284. Thus,

Table I. DGAT inhibitory activity of the extracts and fractions from *Y. koidzumiana*

Sample	Conc. (μg/mL)	Inhibition (%)
MeOH extract	25	– [b]
	125	13.5
	250	62.4
Hexane extract	25	–
	125	15.4
	250	27.6
EtOAc extract	25	–
	125	23.0
	250	61.2
BuOH extract	25	–
	125	–
	250	–
H_2O residue	25	–
	125	23.0
	250	22.8
Cryptotanshinone [a]	25	68.4

[a] Cryptotanshinone was used as a positive control
[b] '–': less than 10% inhibition

compound **5** was tentatively identified as nonadecan-1-ol (Kang and Son, 2000). Compound **6** was identified as a common constituent, β-sitosterol, by comparison with an authentic sample (Kang and Son, 2000).

Compounds **7** and **8** were found to be ursane type

Table II. DGAT inhibitory activity of the compounds isolated from *Y. koidzumiana*

Sample	Conc. (μg/mL)	Inhibition (%)	IC_{50} (μM)
Germanicol acetate (**1**)	25	– [b]	>250
	125	–	
Oleanolic acid (**2**)	5	29.9	31.7
	25	64.2	
	125	82.9	
Brachynereolide (**3**)	25	–	>250
	125	–	
Ixerin Y (**4**)	25	–	>250
	125	–	
Methyl ursolate (**7**)	5	38.2	26.4
	25	59.5	
	125	78.9	
Corosolic acid (**8**)	5	27.9	44.3
	25	51.6	
	125	79.0	
Crepidiaside C (**9**)	25	–	>250
	125	–	
Cryptotanshinone [a]	5	41.8	27.3
	25	68.4	
	125	80.0	

[a] Cryptotanshinone was used as a positive control
[b] '–': less than 10% inhibition

triterpenoids, containing seven methyl groups, one carboxyl group and one double bond between C-12 and C-13. Compound **7** was identified as methyl ursolate by comparison of the MS, ^{1}H-NMR and ^{13}C-NMR data reported in the literature (Piozzi and Paternostro, 1986). By comparison of the ^{13}C-NMR data of **8** with that of **7**, an additional hydroxy-attached carbon signal was observed at δ 68.5. The large differences in the C-1, C-2 and C-3 chemical shifts versus those for **7** suggested that **8** possessed two OH groups, one each at the C-2 and C-3 positions. Thus, **8** was identified as corosolic acid (Kuang and Kasai, 1989, Kozima and Ogura, 1989).

Compound **9** was obtained as a white powder. Its IR spectrum showed hydroxy group (3400 cm^{-1}) and γ-lactone ring (1750 cm^{-1}) absorptions. The ^{1}H-NMR spectrum showed a characteristic doublet methyl signal at δ 1.35 (J = 6.9 Hz, H-13); the other signals were similar to compound **4** (Dat *et al.*, 2002). Based on the NMR spectral evidence, and by comparison with reported data (Adegawa and Miyase, 1985), compound **9** was identified as crepidiaside C.

The DGAT activities of compounds **1**-**4** and **7**-**8** (Table II) were studied using microsomes prepared from rat livers. The sesquiterpenoid constituents (**3**, **4** and **9**) showed no inhibitory activities toward DGAT at 125.0 μg/mL. However, the triterpenoids **2**, **7**, and **8** showed moderate inhibition in the 26.4-44.3 μM range of the IC_{50} values. The inhibitory activities of the compounds were compared with that of crytotanshinone (IC_{50} value of 27.3 μg/mL), which was used as a positive control (Ko *et al.*, 2002). In particularly, compound **1**, with no carboxy group at C-28, had a lower inhibitory value (IC_{50} > 250 μM). From these results, the DGAT inhibition is suggested to depend on the presence of a carboxylic group at the C-28 position and a hydroxy group in the triterpene structure. The structure-activity relationships remain for further studies.

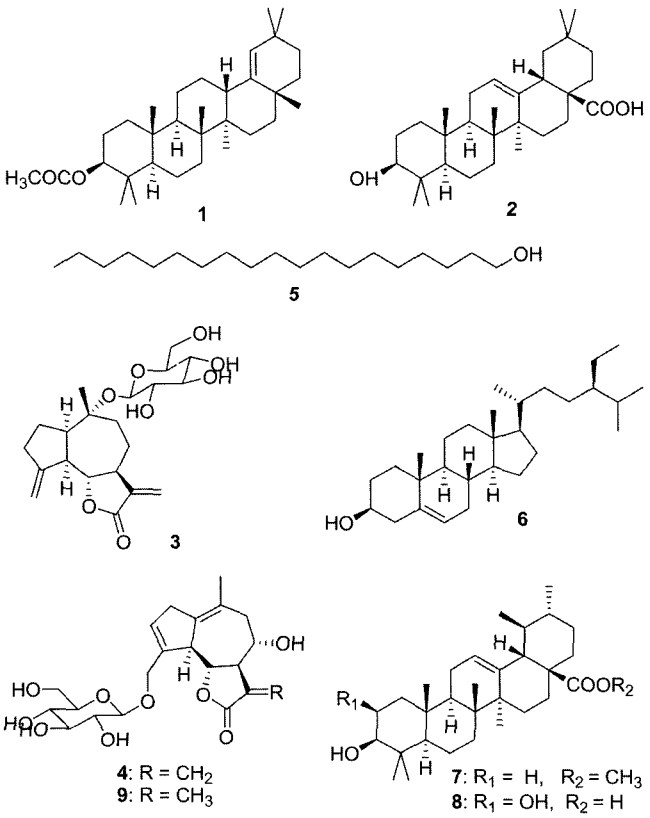

Fig. 1. Structures of the compounds isolated from *Y. koidzumiana*

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