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Inhibition of Diacylglycerol Acyltransferase by Alkamides Isolated from the Fruits of *Piper longum* and *Piper nigrum*

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Pharmacological inhibition of acyl CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) has emerged as a potential therapy for the treatment of obesity and type 2 diabetes. Bioassay-guided isolation of $CHCl_3$ extracts of the fruits of *Piper longum* and *Piper nigum* (Piperaceae), using an in vitro DGAT inhibitory assay, lead to isolation of a new alkamide named (2*E*,4*Z*,8*E*)-*N*-[9-(3,4-methylenedioxyphenyl)-2,4,8-nonatrienoyl]piperidine (**2**), together with four known alkamides: retrofractamide C (**1**), pipernonaline (**3**), piperrolein B (**4**), and dehydropipernonaline (**5**). Compounds **2**−**5** inhibited DGAT with IC_{50} values of 29.8 (**2**), 37.2 (**3**), 20.1 (**4**), and 21.2 (**5**) μM, respectively, but the IC_{50} value for **1** was more than 900 μM. This finding indicates that compounds possessing piperidine groups (**2**−**5**) can be potential DGAT inhibitors.

KEYWORDS: ***Piper longum*; *Piper nigrum*; diacylglycerol acyltransferase (DGAT); alkamides**

INTRODUCTION

Triglycerides are the main storage form of energy. Excess accumulation of triglycerides in certain tissues leads to serious diseases such as obesity, type 2 diabetes, and hypertriglyceridemia (*1*). Therefore, the control of triglyceride synthesis is expected to have an impact on the treatment and prevention of these diseases. Triglyceride synthesis has been assumed to occur primarily through acyl CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), 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 triglyceride metabolism such as intestinal fat absorption, lipoprotein assembly, the regulation of plasma triglyceride concentrations, and fat storage in adipocytes (*3*, *4*). In particular, a study using DGAT deficient mice has provided a better understanding of the relationship between triglyceride synthesis and diseases, including obesity and diabetes. These mice have reduced body fat and are resistant to diet-induced obesity through the mechanism that increased energy expenditure (*5*). In addition, these mice tended to have low levels of plasma glucose and insulin (*6*). Therefore, DGAT inhibition may be a worthwhile strategy for treating obesity and type 2 diabetes.

While we screened DGAT inhibitors from natural products, *Piper longum* and *Piper nigrum* were additionally identified as active species. *P. longum* and *P. nigrum* belong to the family of Piperaceae and are commonly known as long pepper and black pepper, respectively. They are very important oriental medicinal plants, and their fruits have been used for the treatment of cholera, dyspepsia, various gastric ailments, and arthritic disorders (*7*). Terpenes, steroids, lignans, flavones, and alkaloids/alkamides were reported as their major constituents (*8*).

In this paper, we report the isolation and structure determination of five alkamides from the fruits of *Piper* species and structure−activity relationships on in vitro DGAT activity using rat liver microsome.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained on a Varian Unity 300 spectrometer using $CDCl_3$ as the solvent. UV spectra were obtained on a Hewlett-Packard model 8453 spectrophotometer, and IR spectra were recorded on a JASCO FT/IR-4100 spectrophotometer. Electrospray ionization (ESI)-MS was measured on a Finnigan navigator spectrometer. HRFAB-MS was measured on a JEOL HX 110A/HX 100A spectrometer. The high-performance liquid chromatography (HPLC) system consisted of a Shimaduz model LC-6AD pump, SPD-10A detector, and YMC J'sphere ODS H-80 column (4 μm, ∅ 20 mm × 250 mm). Reversed-phase column chromatography was accomplished with RP-C_{18} silica gel (YMC*GEL ODS-A, 12 nm S-150 μm, YMC Co. Ltd.), and silica gel column chromatography was carried out using Kieselgel 60 (70−230 and 200−400 mesh, Merck). 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 was purchased from Amersham Pharmacia Biotech Inc. (Little Chalfont, Buckinghamshire, England).

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Table 1. NMR Data of Compounds **2** and **5** (75 MHz for ^{13}C, 300 MHz for ^{1}H, $CDCl_3$)

	2		**5**	
no.	^{13}C	^{1}H	^{13}C	^{1}H
1	165.8 (s)		165.6 (s)	
2	121.3 (d)	6.36 (1H, d, J = 14.4 Hz)	119.1 (d)	6.31 (1H, d, J = 15.9 Hz)
3	137.3 (d)	7.59 (1H, dd, J = 15.0, 14.4 Hz)	141.1 (d)	7.23 (1H, dd, J = 10.5, 15.0 Hz)
4	120.6 (d)	6.17 (1H, t, J = 11.4 Hz)	127.8 (d)	6.21 (1H, dd, J = 10.8, 15.0 Hz)
5	138.4 (d)	5.79 (1H, dt, J = 10.2, 7.8 Hz)	142.5 (d)	6.07 (1H, dt, J = 6.0, 15.3 Hz)
6	28.1 (t)	2.48 (2H, m)	32.2 (t)	2.31 (2H, m)
7	32.9 (t)	2.29 (2H, m)	32.8 (t)	2.31 (2H, m)
8	121.3 (d)	6.03 (1H, dt, J = 15.6, 7.2 Hz)	129.4 (d)	6.02 (1H, m)
9	130.4 (d)	6.32 (1H, d, J = 15.6 Hz)	130.1 (d)	6.27 (1H, d, J = 14.7 Hz)
1′	132.4 (s)		132.1 (s)	
2′	105.7 (d)	6.88 (1H, s)	105.4 (d)	6.88 (1H, s)
3′	146.9 (s)		146.7 (s)	
4′	148.1 (s)		147.9 (s)	
5′	108.4 (d)	6.75 (2H, m)	108.2 (d)	6.73 (1H, br s)
6′	120.6 (d)		120.4 (d)	6.74 (1H, br s)
7′	101.1 (t)	5.94 (2H, s)	100.9 (t)	5.93 (2H, s)
1″	43.3 (t)	3.49 (2H, br s)	43.2 (t)	3.49 (2H, br s)
2″	26.9 (t)	1.58 (2H, m)	26.8 (t)	
3″	24.9 (t)	1.67 (2H, m)	24.7 (t)	1.52–1.68 (6H, m)
4″	25.8 (t)	1.58 (2H, m)	25.6 (t)	
5″	47.1 (t)	3.63 (2H, br s)	46.7 (t)	3.61 (2H, br s)

Plant Material. The fruits of *P. longum* and *P. nigrum* were purchased at an herbal market in Daejeon, Korea. The authenticity of the plant was confirmed by Prof. K. H. Bae, College of Pharmacy, Chungnam National University. Voucher specimens (PBC-413A and PBC-441A) were deposited in the Korea Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology.

Extraction and Isolation. The dried fruits of *P. longum* (100 g) were extracted with MeOH (1 L) for 7 days at room temperature. The MeOH extracts (32 g) were evaporated and suspended in distilled water and divided into two fractions with chloroform as the nonaqueous phase. The chloroform layer (7.5 g) was concentrated in vacuo, and its crude extracts were chromatographed on a silica gel (230−400 mesh, 1 kg, Merck) using a step gradient $CHCl_3$/MeOH solvent system (100:0, 80:1, 60:1, 40:1, 20:1, 10:1, 5:1, 1:1, each 3 L, v/v) to give eight fractions by TLC profile. Each fraction was concentrated in vacuo and monitored by the in vitro DGAT enzyme assay. The active compounds (3.86 g) eluted by the initial solvent ($CHCl_3$/MeOH = 100:0 fractions) were further fractionated using silica gel column chromatography (230−400 mesh, 400 g, Merck) eluting with hexane/EtOAc (50:1, 20:1, 10:1, 7:1, 5:1, 3:1, 1:1, each 2 L, v/v) to give eight subfractions. For further purification, the active fractions (F1−3, hexane/EtOAc = 5:1 fractions, 400 mg) were successively separated by semipreparative HPLC on a 20 mm × 250 mm i.d., 4 μm YMC J'sphere ODS H-80 column, at a flow rate of 4 mL/min, with UV detection at 210 nm, at elution with 80% aqueous MeOH. The compounds **1** (29.9 mg), **2** (8 mg), and **3** (11 mg) were eluted with retention times of 39, 41, and 54 min, respectively.

The dried fruits of *P. nigrum* (5 kg) were extracted with MeOH (10 L) at room temperature. The MeOH extracts were combined and evaporated under vacuum to give a residue. The residues were dispersed in H_2O and then extracted with $CHCl_3$, and the solvents were evaporated in vacuo. The $CHCl_3$ extract (157.7 g) was chromatographed by silica gel (600 g) with a step gradient of hexane and EtOAc (50:1, 30:1, 10:1, 1:1, 1:6, 0:100, each 3 L, v/v) to give 14 fractions by TLC profile. Each fraction was tested for DGAT inhibitory activity. The active fraction (F10, 8.2 g) was subjected to reverse-phase column chromatography (83 g) eluting with methanol and H_2O (50:50, 60:40, 70:30, 80:20, 90:10, 100:0, each 1 L, v/v) to afford eight subfractions. F10-5 (4.3 g) was further separated by low-pressure liquid chromatography on a 310 mm × 25 mm, 40−63 μm Lichroprep RP-18 Lobar column, at a flow rate of 8 mL/min, with UV detection at 210 nm, eluted with MeOH/H_2O (80:20) to give four subfractions. F10-5-2 (637.5 mg) was subjected to semi-prep HPLC (20 mm × 250 mm i.d., 4 μm YMC J'sphere ODS H-80 column, at a flow rate of 4 mL/min, with UV detection at 210 nm, at elution with 80% aqueous MeOH) to furnish compound **4** (42.4 mg, t_R = 45 min). Fraction F11 (1.2 g) was subjected to reverse-phase column chromatography (24 g) eluting with methanol and H_2O (50:50, 60:40, 70:30, 80:20, 90:10, 100:0, each 300 mL, v/v) to afford nine subfractions. Fraction F11-4 (1.07 g) was further separated by low-pressure liquid chromatography (CH_3CN/H_2O, 70:30) to give five subfractions. F11-4-3 was subjected to semi-prep HPLC (CH_3CN/H_2O, 70:30) to furnish **5** (123 mg, t_R = 43 min).

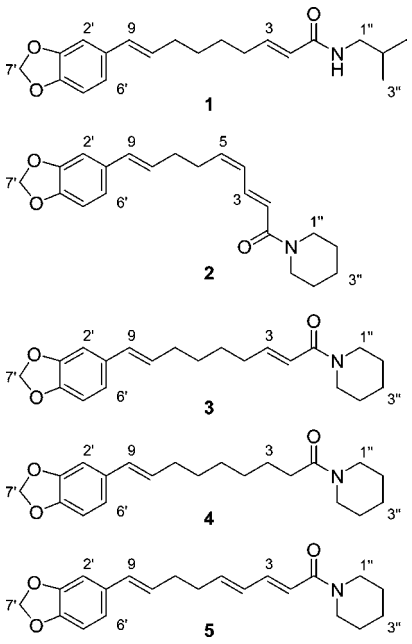

Figure 1. Structures of alkamide compounds isolated from *P. longum* and *P. nigrum.*

Retrofractamide C (1). $C_{20}H_{27}NO_3$; crystalline solid. IR (KBr): ν_{max} 1667, 3303 cm^{-1}. UV λ_{max} nm 212, 261, 295−305 (MeOH). ESI-MS: m/z = 352 $[M + Na]^+$. ^1H NMR (300 MHz, $CDCl_3$): δ 0.91 (3H, s, H-3″), 0.93 (3H, s, H-4″), 1.49 (4H, m, H-5 and 6), 1.79 (1H, m, H-2″), 2.19 (4H, m, H-4 and 7), 3.14 (2H, t, J = 6.5 Hz, H-1″), 5.51 (NH, br s), 5.76 (1H, d, J = 15.3 Hz, H-2), 5.93 (2H, s, H-7′), 6.02 (1H, dt, J = 15.6, 6.6 Hz, H-8), 6.28 (1H, d, J = 15.6 Hz, H-8), 6.74 (4H, m, H-5′ and 6′), 6.83 (1H, dt, J = 15.3, 7.2 Hz, H-3) and 6.88 (1H, s, H-2′).

(2*E*,4*Z*,8*E*)-*N*-[9-(3,4-Methylenedioxyphenyl)-2,4,8-nonatrienoyl]-piperidine (2). $C_{21}H_{25}NO_3$; colorless plates. IR (KBr): ν_{max} 1654 cm^{-1}. UV λ_{max} nm 214, 266 (MeOH). HRFAB-MS m/z 340.1909 $[M + H]^+$,

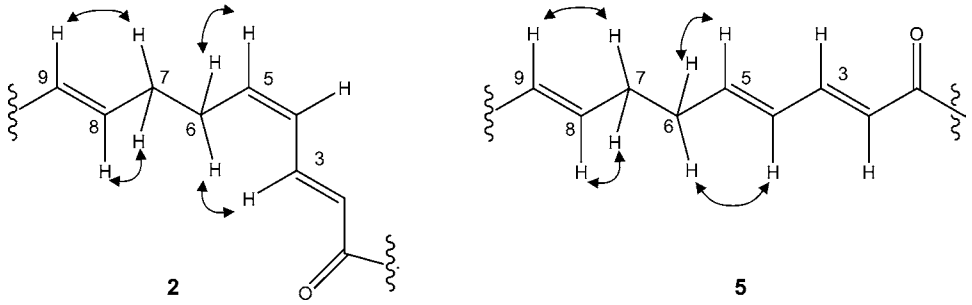

Figure 2. NOESY correlations (H and H) of compounds **2** and **5**.

Table 2. DGAT Inhibitory Activities of Compounds **1**–**5**

	compound					
	1	**2**	**3**	**4**	**5**	kuraridine[b]
IC_{50}[a] (μM)	>900	29.8 ± 2.4	37.2 ± 3.8	20.1 ± 1.5	21.2 ± 2.1	9.8 ± 0.3

[a] The in vitro DGAT inhibitory activity was measured using the rat liver microsomal DGAT. Data are shown as mean values of three independent experiments performed in duplicate. [b] Kuraridine was used as a positive control.

calcd 340.1913. ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}CNMR (75 MHz, $CDCl_3$): See **Table 1**.

Pipernonaline (3). $C_{21}H_{27}NO_3$; pale yellow oil. IR (neat): ν_{max} 1656 cm^{-1}. UV λ_{max} nm 210, 268 (MeOH). ESI-MS: *m*/*z* 364 [M + Na]$^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.49 (4H, m, H-5 and 6), 1.56 (4H, m, H-2″ and H-4″), 1.63 (2H, m, H-3″), 2.21 (4H, m, H-4 and 7), 3.47 (2H, br s, H-1″), 3.59 (2H, br s, H-5″), 5.93 (2H, s, H-7′), 6.02 (1H, dt, J = 15.6, 6.6 Hz, H-2), 6.23 (1H, d, J = 14.7 Hz, H-8), 6.28 (1H, d, J = 15.6 Hz, H-9), 6.73 (4H, br s, H-5′ and 6′), 6.83 (1H, dt, J = 15.3, 6.6 Hz, H-3) and 6.88 (1H, br s, H-2′).

Piperrolein B (4). $C_{21}H_{29}NO_3$; colorless oil. IR (neat): ν_{max} 1654 cm^{-1}. UV λ_{max} nm 260.5, 305 (MeOH). ESI-MS: *m*/*z* 366 [M + Na]$^+$. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.35 (4H, m, H-3 and 5), 1.45 (2H, m, H-6), 1.54 (4H, m, H-4 and 3″), 1.61 (4H, m, H-2″ and 4″), 2.16 (2H, q, J = 6.6 Hz, H-7), 2.30 (2H, t, J = 7.5 Hz, H-2), 3.38 (2H, t, J = 5.5 Hz, H-5″), 3.54 (2H, t, J = 5.4 Hz, H-1″), 5.75 (1H, d, J = 15.3 Hz, H-2), 5.92 (2H, s, H-7′), 6.03 (1H, dt, J = 15.9, 6.9 Hz, H-8), 6.27 (1H, d, J = 15.3 Hz, H-9), 6.73 (1H, br s, H-6′), 6.74 (1H, m, H-5′) and 6.88 (1H, br s, H-2′).

Dehydropipernonaline (5). $C_{21}H_{25}NO_3$; colorless plate. IR (KBr): ν_{max} 1660 cm^{-1}. UV λ_{max} nm 211, 268 (MeOH). ESI-MS: *m*/*z* 362 [M + Na]$^+$. ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}CNMR (75 MHz, $CDCl_3$): See **Table 1**.

Preparation of Microsomes from Rat Liver and Measurement of in Vitro DGAT Activity. The microsomal fractions from rat liver were prepared as described earlier (*9*). In brief, rat liver (Male Sprague−Dawley rat, 250−300 g) were rinsed and then homogenized in 9 volumes of STE buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 10 mM EDTA) with a Teflon-glass homogenizer by 10 up-and-down strokes at medium speed. 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 a 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.

DGAT activity was measured as reported previously (*9*). 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, 30 μM [1-^{14}C]oleoyl-CoA (0.02 μCi), and a test sample (5 μL) dissolved in DMSO in a total volume of 200 μL, was initiated by the addition of rat liver microsomal fraction (100 μg), followed by gentle and brief vortexing. After incubation for 10 min at 25 °C, the reaction was stopped by addition of 1.5 mL of 2-propanol/heptane/water (80:20:2, v/v) and 1.0 mL of heptane and 0.5 mL of water to extract lipid. After it was vortexed, 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)]. 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, and kuraridine was used as a positive control (*10*). All inhibitors were added as solutions in DMSO.

Data Analysis. Each experiment was performed at least in triplicate. Results are expressed as the mean value ± standard error (SE).

RESULTS AND DISCUSSION

In our search for DGAT inhibitors from natural sources, we found that MeOH extracts of *Piper* species plants (*P. longum* and *P. nigrum*) inhibited DGAT enzyme activity. The extracts were fractionated by open column chromatography on silica gel and semipreparative HPLC to afford a new compound (**2**) and four known active constituents (**1**, **3**−**5**). Four known compounds were identified as retrofractamide A (**1**), pipernonaline (**3**), piperrolein B (**4**), and dehydropipernonaline (**5**) (**Figure 1**) by spectroscopic analyses, including MS and NMR, and in comparison with published data (*11*−*13*).

Compound **2** was newly isolated as a colorless plate. The UV spectrum exhibited λ_{max} at 214 and 266 nm, and the IR spectrum showed the presence of a carbonyl group (1654 cm^{-1}). It displayed a peak at *m*/*z* 340.1909 in the HRFAB-MS, corresponding to [M + H]$^+$, indicating a molecular formula of $C_{21}H_{26}NO_3$. In the ^{1}H NMR spectrum of **2** (**Table 1**), the signals of six olefinic protons were shown at δ 6.36 (1H, d, J = 14.4 Hz, H-2), 7.59 (1H, dd, J = 15.0, 14.4 Hz, H-3), 6.17 (1H, t, J = 11.4 Hz, H-4), 5.79 (1H, dt, J = 10.2, 7.8 Hz, H-5), 6.03 (1H, dt, J = 15.6, 7.2 Hz, H-8) and 6.32 (1H, d, J = 15.6 Hz, H-9). The signal of low field-shifted methylene proton at δ 5.94 (2H, *s*) suggests the presence of methylenedioxy group. Allylic methylene protons were shown at δ 2.48 (2H, m, H-6) and 2.29 (2H, m, H-7). Also, two broad singlet peaks of piperidine ring were shown at δ 3.49 (2H, br s) and 3.63 (2H, br s), and three methylene protons of piperidine ring were observed at δ 1.58 (4H, m) and 1.67 (2H, m). As shown in **Table 1**, the NMR spectrum of **2** was similar to that of dehydropipernonaline (**5**) except for some proton signals. The proton signal of H-3 shifted to downfield (δ 7.59), and the H-5 shifted to up field (δ 5.79) in comparison with **5**. The peaks of allylic methylene protons (H-6 and H-7) of **2** were observed at δ 2.48 (H-6) and 2.29

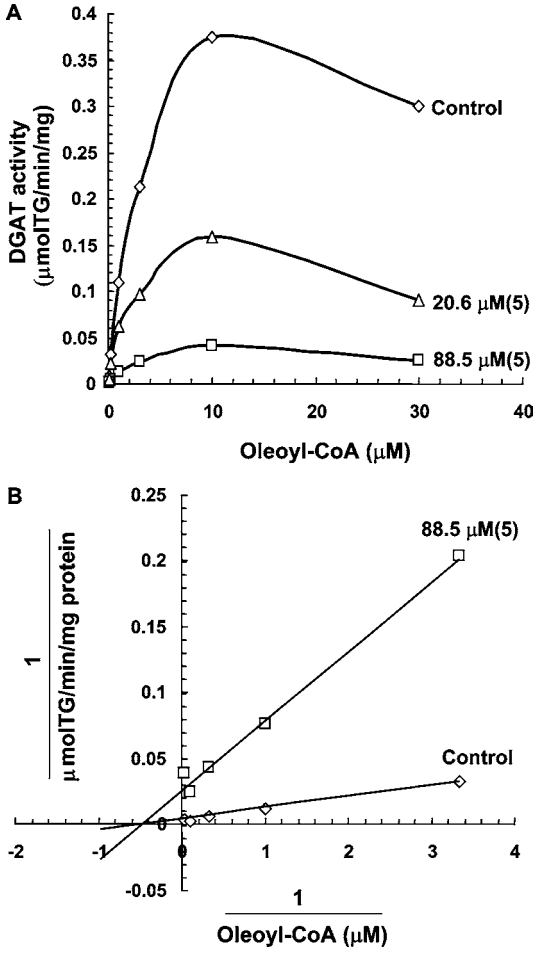

Figure 3. Characteristics of enzyme inhibition by compound **5**. (**A**) The initial velocity plot showing the dependence of DGAT activity on [1-^{14}C] oleoyl-CoA. The DGAT activity was measured at varying concentrations of [1-^{14}C] oleoyl-CoA (0.1–30 μM) in the absence (control) and presence of compound **5** (20.6 and 88.5 μM) as described in the Materials and Methods. (**B**) Lineweaver–Burk (LB) plot of inhibition kinetics of DGAT for compound **5**. The reciprocal of the rate of formation of TG was plotted against the reciprocal of [1-^{14}C] oleoyl-CoA concentration. The data are presented as the means of three independent experiments performed in duplicate.

(H-7), whereas the peak of **5** was shown at δ 2.31 (4H, m). Also, coupling constants of olefinic protons (H-4 and H-5) of **2** were 11.4 (H-4) and 10.2 (H-5) Hz, whereas the coupling constants of **5** were 15.0 (H-4) and 15.3 (H-5) Hz. In the ^{1}H−^{1}H NOESY spectrum of **2** (**Figure 2**), correlations from H-6 to H-3 and H-5 and from H-7 to H-8 and H-9 were observed. The configuration of C-4 and C-5 of **2** was confirmed cis form in comparison with the previously reported data of **5** (*11*). The ^{13}C NMR spectrum of **2** (**Table 1**) showed the presence of 21 carbons: one carbonyl carbon, one methylenedioxy carbon, four methylene carbons, five methylene carbons of piperidine ring, six olefinic carbons, and carbons of a benzene ring. The connectivity of proton and carbon atoms was assigned by DEPT, HMQC, and HMBC spectra. On the basis of the above evidence, the structure of **2** was determined as (2*E*,4*Z*,8*E*)-*N*-[9-(3,4-methylenedioxyphenyl)-2,4,8-nonatrienoyl]piperidine (**Figure 1**). Compound **2** was newly isolated from *P. longum* fruits.

The inhibitory effect of these compounds on the DGAT enzyme was investigated with microsome prepared from rat liver. They inhibited DGAT activity in a dose-dependent fashion with IC_{50} values of 29.8 (**2**), 37.2 (**3**), 20.1 (**4**), and 21.2 (**5**) μM except for **1** (IC_{50}: >900 μM) (**Table 2**). The inhibitory activities of these compounds were confirmed using a positive control of kuraridine, which inhibited the DGAT activity with an IC_{50} value of 9.8 μM in the assay system (*10*). As shown in **Table 2**, the DGAT inhibitory activity was influenced by the presence of piperidine group (**2**−**5**) rather than by isobutyl group (**1**). In addition, a kinetic analysis using a Lineweaver−Burk plot revealed that compound **5** was a noncompetitive type inhibitor of DGAT (**Figure 3**). The apparent Michaelis constant (K_m) value for [l-^{14}C]oleoyl-CoA and inhibition constant (K_i) value were calculated to be 4.6 and 5.4 μM, respectively.

Excess deposition of triglyceride leads to obesity and, particularly when it occurs in nonadipose tissues (e.g., skeletal muscle, liver, and B-cell), is associated with insulin resistance. Therefore, inhibition of triglyceride synthesis represents a potential therapeutic for obesity and type 2 diabetes. For that reason, we have searched for DGAT inhibitors from natural sources and reported DGAT inhibitors such as quinolone alkaloids from *Evodia rutaeacapa* (*14*), polyacetylenens from *Panax ginseng* (*15*), prenylflavonoids from *Sophora flavenscens* (*10*), and betulinic acid from *Alnus hirsuta* (*16*). In this study, we isolated five active constituents from $CHCl_3$ extracts of *Piper* species through bioassay-guided isolation. Among them, compound **2** was newly isolated from *P. longum*. They showed a significant inhibitory effect on rat liver microsomal DGAT. Recently, it was reported that alkamide compounds isolated from *Piper* species had various biological activities such as insecticidal (*12*, *17*), antibacterial (*18*), and antiinflammatory activities (*19*). However, their DGAT inhibitory activity was first reported by this study.

In conclusion, these compounds may be useful for the design of new DGAT inhibitors leading to antiobesity and antitype 2 diabetic agents. In addition, these plants may be used as a dietary supplement to improve and prevent obesity and type 2 diabetes.

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