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ACAT inhibition of alkamides identified in the fruits of *Piper nigrum*

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Abstract

In this study, via a bioactivity-guided fractionation of MeOH extracts of the fruits of *Piper nigrum*, alkamide (**5**) and five previously-identified alkamides were isolated. Their structures were elucidated via spectroscopic analysis (^{1}H, ^{13}C NMR and ESI-MS), as follows: retrofractamide A (**1**), pipercide (**2**), piperchabamide D (**3**), pellitorin (**4**), dehydroretrofractamide C (**5**) and dehydropipernonaline (**6**). The IC_{50} values determined for the compounds were 24.5 (**1**), 3.7 (**2**), 13.5 (**3**), 40.5 (**4**), 60 (**5**) and 90 μM (**6**), according to the results of an ACAT enzyme assay system using rat liver microsomes. These compounds all inhibited cholesterol esterification in HepG2 cells.

Keywords: *Piper nigum* L.; Piperaceae; Alkamides; Acyl CoA: cholesterol acyltransferase (ACAT); Atherosclerosis; HepG2 cells; Cellular cholesterol esterification

1. Introduction

Atherosclerosis is a complex disease which progresses via the accumulation of cholesterol, in particular, cholesterol ester-enriched foam cells, within the intima of the arteries. These foam cells manifest a high degree of activity of acyl CoA: cholesterol acyltransferase (ACAT), which catalyzes the esterification of free cholesterol (Suckling and Stange, 1985). ACAT is also involved in intestinal cholesterol absorption, steroid hormone production, and lipoprotein production in the liver, all of which are secreted into the blood and contribute to the accumulation of cholesterol ester within the macrophages (Steinberg, 2002). In addition, hepatic ACAT activity is a predominant factor in the maintenance of cholesterol homeostasis, which is one of the most important determinants of serum cholesterol levels (Heinonen, 2002). Therefore, ACAT has been studied extensively via biochemical and molecular biological methods.

ACAT has been identified as a potential target for the development of drugs for the prevention and treatment of atherosclerotic disease, as ACAT inhibitors have been shown to exhibit cholesterol lowering and anti-atherosclerotic activities, via the blockage of dietary cholesterol absorption, the inhibition of very low density lipoprotein (VLDL) secretion, and the prevention of foam cell formation (Chang et al., 1997; Sliskovic and White, 1991). Two ACAT isoforms, ACAT1 and ACAT2, have been identified in humans: there is evidence that these isoforms have different expression patterns and unique physiological functions. ACAT1 performs a pivotal function in foam cell formation in macrophages, whereas ACAT2 contributes to the cholesterol absorption process in intestinal enterocytes and lipoprotein generation in hepatocytes. ACAT1 has been suggested to be relevant to the homeostasis of intracellular cholesterol, rather than to atherosclerosis (Chang et al., 2001). A complete deficiency of ACAT1 in LDL-deficient mice was shown to result in an increase in atherosclerotic lesions. ACAT2 is localized in tissues that express

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apoB, and has been suggested to perform a function in the production of cholesterol esters. Therefore, ACAT2 has been identified as a target for improvement of plasma lipoprotein profiles in cases of hyperlipoproteinemia (Giovannoni et al., 2003).

Piper nigrum L. (Piperaceae) is one of the most popular spices in the world, and is also employed in the folk medical protocols of many countries. The pepper, the fruits of *Piper nigrum*, is important as spice and flavoring agents, and has also been used in the treatment of cholera and dyspepsia, as well as a variety of gastric ailments and arthritic disorders (Jung and Shin, 1998). Terpenes, steroids, lignans, flavones, and alkaloids/alkamides have been identified as the primary constituents of the peppers (Navickene et al., 2000; Parmar et al., 1997). Alkamides are particularly interesting, due to their various biological activities, including insecticidal (Kiuchi et al., 1988; Park et al., 2002), anti-bacterial (Reddy et al., 2004) and anti-inflammatory properties (Mujumdar et al., 1990).

In this paper, we report the isolation and structural elucidation of six alkamides, and describe their inhibitory effects against cholesterol esterification in rat liver microsomal ACAT enzyme and HepG2 cells.

2. Results and discussion

In our ongoing search for ACAT inhibitors obtained from natural sources, we found that the MeOH extracts of *Piper nigrum* inhibited ACAT enzyme inhibitory activity. The extracts were fractionated via open-column chromatography on silica gel and subjected to semi-preparative HPLC, to yield a new compound, dehydroretrofractamide C (**5**), as well as five known alkamide compounds: retrofractamide A (**1**), pipercide (**2**), piperchabamide D (**3**), pellitorin (**4**) and dehydropipernonaline (**6**).

Compound **5** was isolated as a white powder, which exhibited a UV spectrum with λ_{max} at 216, 261, and 306 (sh) nm. The IR spectrum revealed the presence of a NH (3332 cm^{-1}) and a carbonyl group (1645 cm^{-1}). Its molecular formula, $C_{20}H_{29}NO_3$, was determined via HRFAB-MS (*m*/*z* 332.2223 $[M+H]^+$, calc. 332.2226). In the ^1H NMR spectrum, a broad singlet NH peak (δ 5.46, *brs*) was observed. The presence of an isobutyl moiety was confirmed by a methylene proton signal at δ 3.08 (2H, *t*, $J = 6.6$ Hz, H-1″), a methane proton at δ 1.77 (1H, *m*, H-2″), and two methyl protons at δ 0.92 (3H, *s*, H-3″ or H-4″) and 0.91 (3H, *s*, H-3″ or H-4″). The low field-shifted methylene proton signal at δ 5.94 (2H, *s*) is suggestive of the presence of a methylenedioxy group, and six methylene protons were observed at δ 1.35 (4H, *m*), 1.45 (2H, *m*), 1.64 (2H, *m*) and 2.17 (4H, *m*). Two coupled olefinic protons at δ 6.04 (1H, *dt*, $J = 15.6$, 7.2 Hz, H-8) and 6.28 (1H, *d*, $J = 15.6$ Hz, H-9) were also detected. The ^{13}C NMR spectrum (Table 1) revealed the presence of 20 carbons, and the connectivity of proton and carbon atoms was elucidated via DEPT and HMQC spectroscopic analyses. In the HMBC experiment, long-range couplings were observed from H-8 (δ 6.04) to C-1′ (δ 132.4), C-7 (δ 32.8) and C-6 (δ 29.2), and from H-9 (δ 6.28) to C-2′ (δ 105.4), C-6′ (δ 120.2), C-1′ (δ 132.4) and C-7 (δ 32.8). These results indicated that the olefinic C-9 was connected to the C-1′ of the methylenedioxyphenyl group. These signals were found to be similar to those of retrofractamide C, but the molecular formula of compound **5** differed by two hydrogen atoms from that of retrofractamide C (Banerji et al., 1985). This suggests that compound **5** may represent a dehydro derivative of retrofractamide C. Based on the evidence given above, the structure of compound **5** was determined as 1-[(*E*), 9-(3,4-methylenedioxyphenyl)-8-nonenoyl] isobutyl amide, and named dehydroretrofractamide C (see Fig. 1).

Table 1
^1H (300 MHz) and ^{13}C (75 MHz) NMR chemical shift assignments for dehydroretrofractamide C (5)

No	^{13}C ($CDCl_3$)	^1H ($CDCl_3$)	HMBC correlations (H→C)
1	172.9	–	–
2	36.9	2.17 (2H, *m*)	C-3, C-6, C-1
3	25.8	1.64 (2H, *m*)	–
4	29.2	1.35 (2H, *m*)	–
5	28.9	1.35 (2H, *m*)	–
6	29.2	1.45 (2H, *m*)	–
7	32.8	2.17 (2H, *m*)	C-6, C-8, C-9
8	129.2	6.04 (1H, *dt*, $J = 15.6$, 7.2 Hz)	C-6, C-7, C-1′
9	129.3	6.28 (1H, *d*, $J = 15.6$ Hz)	C-7, C-2′, C-6′, C-1′
1′	132.4	–	–
2′	105.4	6.89 (1H, *s*)	–
3′	147.9	–	–
4′	146.5	–	–
5′	108.2	6.74 (1H, *m*)	–
6′	120.2	6.75 (1H, *m*)	–
7′	100.9	5.94 (2H, *s*)	–
1″	46.8	3.08 (2H, *t*, $J = 6.6$ Hz)	C-3″, C-4″, C-2″, C-1
2″	28.5	1.77 (1H, *m*)	–
3″, 4″	20.1	0.92, 0.91 (each 3H, *s*)	–
NH	–	5.46 (*brs*)	–

Five known compounds (see Fig. 1) were also isolated from the MeOH extracts of the *Piper nigrum* fruits, and were identified as retrofractamide A (**1**), pipercide (**2**), piperchabamide D (**3**), pellitorin (**4**), and dehydropipernonaline (**6**), via comparisons with previously published data (Park et al., 2002; Miyakado et al., 1979; Morikawa et al., 2004; Reddy et al., 2004; Shoji et al., 1986).

The alkamides can be divided into two groups: those with isobutyl (**1**–**5**) and piperidine moieties (**6**), all of which possess identical methylenedioxyphenyl and carbonyl groups. But compound **4** possesses an aliphatic acyl group rather than a methylenedioxyphenyl group. Whereas compounds **1**, **2**, **4**, and **6** possess conjugated dienamide groups, compounds **3** and **5** possess conjugated mono- and non-conjugated amide systems, respectively.

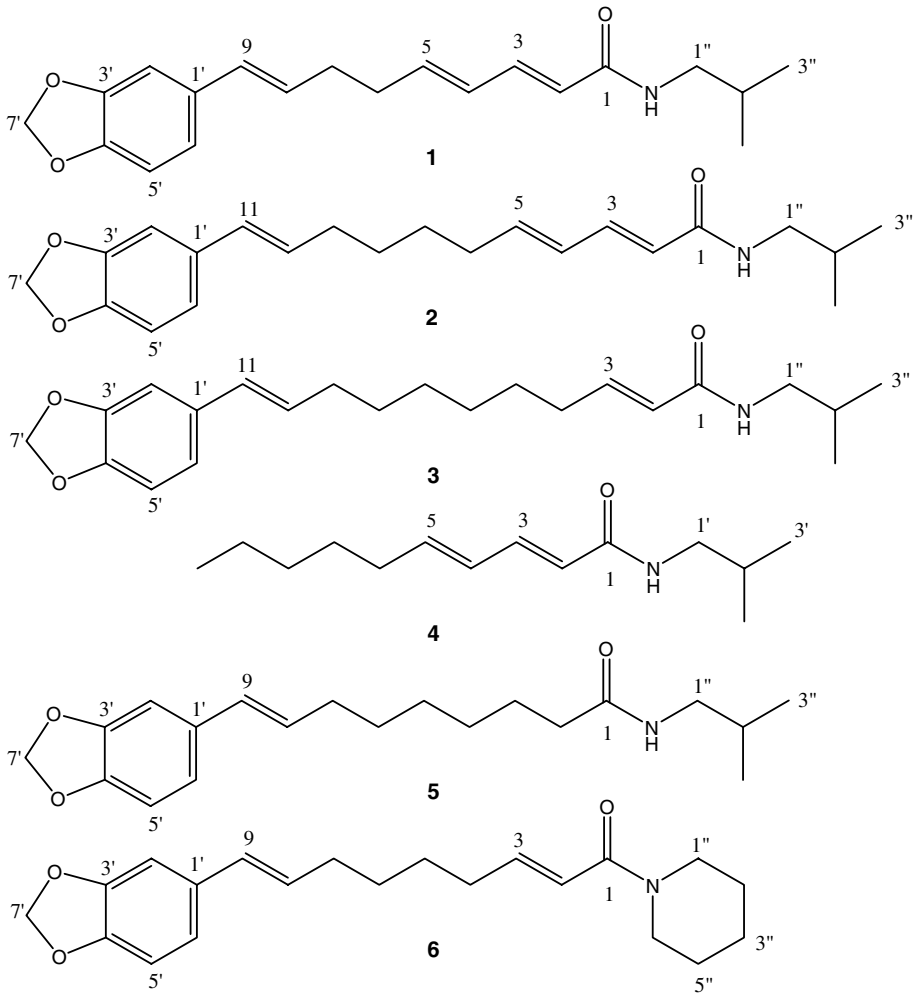

Fig. 1. Structure of alkamide compounds isolated from *Piper nigrum*.

The inhibitory effects of these compounds on ACAT enzyme activity was studied using microsomes prepared from the livers of rats. The compounds all inhibited ACAT activity in a dose-dependent manner, with IC_{50} values of 24.5 (**1**), 3.7 (**2**), 13.5 (**3**), 40.5 (**4**), 60 (**5**) and 90 μM (**6**) (see Fig. 2). This activity was verified using phenylpyropene A as a positive control, which inhibited ACAT activity with an IC_{50} value of 0.8 μM in the assay system (Kwon et al., 2002). As shown in Fig. 2, ACAT inhibitory activity was influenced positively by the presence of the isobutyl group (**1**–**5**), but not by the piperidine group (**6**). Increased carbon numbers between the carbonyl and methylenedioxyphenyl groups of these compounds might exert some influence on ACAT inhibitory activity, as compound **2** had the most potent activity. In addition, compound **2** has more profound levels of activity than did compound **3**, which suggested that ACAT inhibitory activity is also affected by the conjugated dienamide system. The methylenedioxyphenyl moiety also appears to contribute to ACAT inhibitory activity, because compound **4** had very weak activity as compared to other dienamide-harboring alkamides.

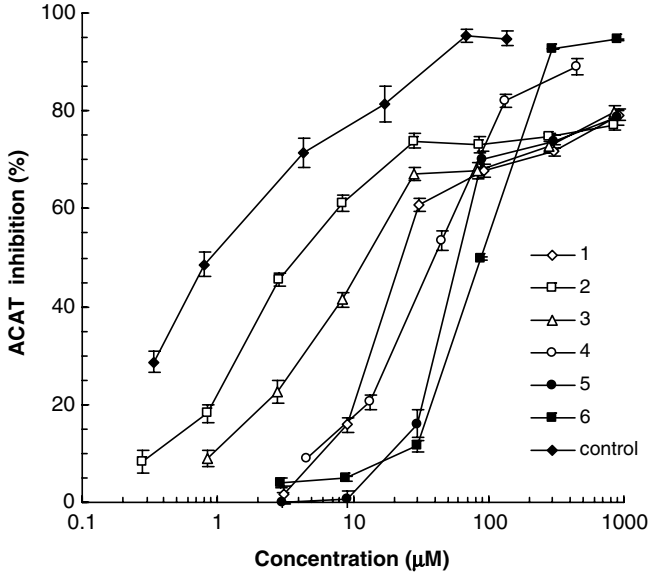

Fig. 2. Inhibition of rat liver microsomal ACAT by alkamide compounds. The enzyme reactions were carried at 37 °C for 30 min. The phenylpyropene A was used as a positive control. Data represented the means ± SE of three independent experiments.

Table 2
Effects of compounds **1**–**6** on cholesterol ester formation in HepG2 cells

Samples (μg/ml)	Inhibition (%)					
	1	2	3	4	5	6
10	92.3	94.0	94.4	99.2	99.6	99.7
3	87.1	80.5	70.3	81.3	66.0	79.9
1	47.1	36.8	31.5	20.0	36.5	45.0
0.3	43.2	34.7	26.6	13.4	26.6	31.2
0.1	26.9	22.5	16.5	7.2	19.7	15.3

*Cells were incubated with [1-^{14}C] oleic acid (0.5 μCi, 17.9 μM) in the presence and absence of the compounds **1**–**6** at varying concentrations (10–0.1 μg/ml) for 6 h at 37 °C. Total lipids were extracted and separated on TLC. The amounts of radioactivity of cholestrol ester were analyzed with a bio-imaging analyzer. Results are expressed as means ± SE ($n = 3$). (+): HepG2 cells + [1-^{14}C] oleic acid, protein concentration: 1.5 ± 0.8 mg/ml.
*Control amount of [1-^{14}C] cholesterol ester: 275 nM.

In order to determine the effects of isolated ACAT inhibitors (**1**–**6**) on cellular cholesterol esterification, the incorporation of [1-^{14}C] oleic acid into cellular lipids was determined in an intact cell assay using HepG2 cells. The compounds inhibited cholesterol ester synthesis in a dose-dependent manner, with IC_{50} values of 4.6 (**1**), 5.0 (**2**), 6.4 (**3**), 7.9 (**4**), 10.8 (**5**) and 5.3 μM (**6**) (Table 2). However, almost no inhibition of [1-^{14}C] triglyceride synthesis was observed under high concentrations (up to 30 μg/ml), and the compounds were not observed to be toxic at the concentrations employed in this study (data not shown). The isolated compounds evidenced dose-dependent inhibitory effects on cholesterol ester formation. This inhibitory effect was more profound with the HepG2 cells than with the rat liver microsomes. The result suggests that the accessibility of hydrophobic compounds to intact cell membranes probably make a difference to the inhibitory activity in the two assays (Namatame et al., 2004).

In conclusion, several active compounds (**1**–**6**) were isolated from the MeOH extracts of *Piper nigrum*. Among them, a new compound, designated dehydroretrofractamide C (**5**), was isolated from this plant. The isolated compounds inhibited ACAT activity in both rat liver microsomes and HepG2 cells. These compounds may prove useful for the design of new potent ACAT inhibitors, which could be utilized in the prevention and treatment of hyperlipidemia and atherosclerosis, operating via the inhibition of ACAT activity.

3. Experimental

3.1. General experimental procedures

^{1}H NMR (300 MHz), ^{13}C NMR (75 MHz), HMQC and HMBC spectra were acquired using a Varian Unity 300 spectrometer, with $CDCl_3$ as a solvent. ESI-MS was determined using a Finnigan™ navigator spectrometer, whereas HRFAB-MS was obtained using a JEOL HX 110A/HX 100A spectrometer. The HPLC system consisted of a Shimaduz Model LC-6AD pump, SPD-10A detector, and YMC J′sphere ODS H-80 column (4 μm, ϕ 20 × 150 mm, YMC Co. Ltd.). Reversed-phase CC was conducted using RP-C_{18} silica gel (YMC*GEL ODS-A, 12 nm S-150 μm, YMC Co. Ltd.), and silica gel CC was conducted using Kieselgel 60 (70–230 and 200–400 mesh, Merck). TLC was conducted using Kieselgel 60 F_{254} plates (Merck).

3.2. Plant material

The *Piper nigrum* fruits were purchased at an herbal market in Daejon, Korea. The authenticity of the plants was confirmed by Prof. K.H. Bae, at the College of Pharmacy of Chungnam National University. A voucher specimen (PBC-441A) was deposited in the Korea Plant Extract Bank, at the Korea Research Institute of Bioscience and Biotechnology.

3.3. Extraction and isolation

The dried fruits of *Piper nigrum* (5 kg) were extracted with MeOH (10 L) at room temperature. The MeOH extract was evaporated *in vacuo*, yielding a residue (500 g). The residue was suspended in H_2O (1.5 L) and extracted with $CHCl_3$ (5 L). The $CHCl_3$ solubles were then evaporated *in vacuo*, with the resulting extract (157.7 g) subjected to silica gel (600 g) CC using a gradient of hexane–EtOAc (50:1, 30:1, 10:1, 1:1, 1:6, 0:100; each 3 L, v/v) as eluant to yield 14 fractions (F1–F14) by TLC profile. Each of the fractions was evaluated with regard to ACAT inhibitory activity. F10 (8.2 g) was subjected to reversed-phase CC (83 g) eluted with MeOH–H_2O (50:50, 60:40, 70:30, 80:20, 90:10, 100:0; each 1 L, v/v), to yield eight subfractions (F10-1–F10-8). F10-4 (1.6 g) and F10-5 (4.3 g) were further separated via low-pressure liquid chromatography [Lichroprep RP-18 Lobar column, 40–63 mm; flow rate, 6 and 8 ml/min; UV, 210 nm] with elution of MeOH–H_2O (75:25 and 80:20, v/v), to yield three and four subfractions (F10-4-1–F10-4-3 and F10-5-1–F10-5-4), respectively. F10-5-2 (85.3 mg) and F10-5-4 (637.5 mg) were subjected to semi-prep HPLC (MeOH–H_2O, 80:20, v/v), yielding compounds **1** (36 mg, t_R 31 min), **2** (27 mg, t_R 43 min) and **3** (5 mg, t_R 53 min). F10-4-3 (837 mg) was successively separated via semi-prep HPLC (YMC *J′* sphere ODS H-80, 4 μm, ϕ 20 × 150 mm; UV, 254 nm; flow rate, 4 ml/min) with MeOH–H_2O elution (75:25, v/v) to yield compound **4** (11 mg, t_R 25 min). F11 (1.2 g) was subjected to reversed-phase CC (24 g) with MeOH–H_2O elution (50:50, 60:40, 70:30, 80:20, 90:10, 100:0; each 300 ml, v/v), yielding nine subfractions (F11-1–F11-9). Fractions F11-4,5 (1.07 g) were further separated via low-pressure liquid chromatography (CH_3CN–H_2O = 80:20, v/v) to yield five subfractions (F11-4,5-1–F11-4,5-5). F11-4,5-2 and F11-4,5-3 were subjected to semi-prep HPLC (CH_3CN–H_2O = 70:30, v/v) to yield compounds **5** (60 mg, t_R 31 min) and **6** (123 mg, t_R 43 min), respectively.

3.3.1. Dehydroretrofractamide C (5)

White powder; $C_{20}H_{30}NO_3$; IR (KBr) ν_{max} 3332, 1645 cm^{-1}; UV λ_{max} (MeOH) nm: 216, 261, 306; for ^{13}C NMR (75 MHz, $CDCl_3$) and ^1H NMR (300 MHz, $CDCl_3$) spectra, see Table 1; HRFAB-MS: m/z 332.2223 $[M + H]^+$ (calc. 332.2260 for $C_{20}H_{30}NO_3$).

3.4. ACAT enzyme assay using rat liver microsomes

ACAT activity was assayed as previously reported (Kwon et al., 2002). In brief, the reaction mixture, which contained 10 μL of rat liver microsomes (10 mg/ml protein), 20 μL of 0.5 M potassium phosphate buffer (pH 7.4, 10 mM dithiothreitol), 10 μL of bovine serum albumin (180 mg/ml), 2.0 μL of cholesterol in acetone (20 mg/ml), 130 μl of water, and 10 μl of the test sample in a total volume of 190 μl, was pre-incubated for 30 min at 37 °C. The reaction was initiated via the addition of 10 μl of [1-^{14}C] oleoyl-CoA (0.05 μCi: final concentration 10 μM). After 30 minutes of incubation at 37 °C, the reaction was halted via the addition of 1.0 ml of *i*-PrOH-heptane (4:1, v/v) solution. A mixture of 0.6 ml of heptane and 0.4 ml of 0.1 M potassium phosphate buffer was then added to the reaction mixture. This was mixed for 2 min and allowed to separate into phases. Cholesterol oleate was recovered in the upper (heptane) phase. The radioactivity in the 100 μl of the upper phase was measured in a 4 ml liquid scintillation vial with 3 ml of scintillation cocktail (Lipoluma, Lumac Co.), using a Wallac microbeta liquid scintillation counter (Boston, MA, USA). All inhibitors were added as solutions in DMSO.

3.5. Cholesteryl oleate formation in HepG2 cells

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum. The HepG2 cells were incubated in the presence of 2.5 μl of sample or 0.1% DMSO as a vehicle and [1-^{14}C] oleic acid (complexed with BSA, 0.5 μCi, 17.9 μM) for 6 h in a 24-well plate. The medium was then removed, and the cells were washed three times in PBS. The cells were lysed via the addition of 0.5 ml of PBS containing 0.1% (wt/v) SDS, and the intracellular lipids were extracted with hexane-*i*-PrOH (3:2 v/v), and the organic phase was evaporated under nitrogen. The total lipids were separated on silica gel TLC plates (silica gel F254, 0.5 mm, Merck) in petroleum ether–Et_2O–AcOH (90:10:1, v/v/v), and the amount of radioactivity was assessed using a bio-imaging analyzer (BAS 1500, Fuji).

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