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Pheophorbide A-methyl Ester, Acyl-CoA: Cholesterol Acyltransferase Inhibitor from *Diospyros kaki*

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In the course of our search for Acyl-CoA: cholesterol acyltransferase (ACAT) inhibitors from natural sources, a new type of ACAT inhibitor was isolated from a methanol extract of *Diospyros kaki*. On the basis of spectral and structural evidence, the compound was identified as pheophorbide A-methyl ester. Pheophorbide A-methyl ester inhibited ACAT activity in a dose dependent manner with an IC_{50} value of 1.85 μg/mL.

Key words: Acyl-CoA, cholesterol acyltransferase, Pheophorbide A-methyl ester, Diospyros kaki

INTRODUCTION

Atherosclerosis is a well known progressive disease characterized by the accumulation of lipids and fibrous elements in the artery. The relationship between blood cholesterol and atherosclerosis is no longer in doubt, and the presence of cholesterol esters as a principal constituent of the atheromatous gruel in atherosclerotic plaques has often been observed. Many of the cholesterol esters that accumulate in the artery are derived locally from lipoprotein cholesterol esters, which enter the intima from the plasma and which are hydrolysed and resynthesized in the cells of the developing plaque (Steinberg, 2002). Acyl-CoA: cholesterol acyltransferase (ACAT) is an integral membrane protein located in the endoplasmic reticulum (ER) of cells. ACAT catalyzes cholesterol esterification and plays important roles in the intestinal absorption of cholesterol, the hepatic production of lipoproteins and the accumulation of cholesterol ester within macrophages and the smooth muscle cells of the atheroma (Rudel *et al.*, 2001). Therefore, ACAT inhibitors have been proposed as an attractive target for the treatment of hypercholesterolemia and atherosclerosis (Buhman *et al.*, 2000).

The leaves of *Diospyros kaki* are well known in Chinese herbal medicine and used for the treatment of hypertension, cancer, diabetes and atherosclerosis. Tannins, phenols and many kinds of flavonoids are known to be amongst the active constituents of this plant (Tang *et al.*, 1992). In the course of our screening for ACAT enzyme inhibitors from natural sources, a methanol extract of *Diospyros kaki* exhibited potent inhibitory activity on ACAT enzyme prepared from rat liver. Bioactivity directed fractionation of the ethyl acetate extract of *Diospyros kaki* led to the isolation of pheophorbide A-methyl ester which exhibited strong ACAT inhibition activity.

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MATERIALS AND METHODS

Materials

Leaves of *Diospyros kaki* were collected in the suburbs of Taejon and identified by Prof. K.H. Bae, College of Pharmacy, Chungnam National University. A voucher specimen, PB-017-007, has been deposited in the Korea Plant Extract Bank, Korea Research Institute of Bioscience and Biotechnology. Bovine serum albumin and cholesterol were obtained from Sigma Chemical Co (ST. Louis, MO, USA). [1-^{14}C]oleoyl-CoA was purchased from Amersham.

Extraction and isolation

The fresh leaves of *Diospyros kaki* (9.5 kg) were extracted with CH_3OH (90 L). The CH_3OH extracts were evaporated and the remaining aqueous residue was extracted with ethyl acetate, dried with Na_2SO_4 and concentrated *in*

vacuo. The crude samples (33.2 g) were chromatographed on a silica gel (800 g, 230-400 mesh, Merck) eluting with $CHCl_3$-CH_3OH (100:0, 100:1, 50:1, 10:1, 1:1). The $CHCl_3$-CH_3OH (100:1) fraction (3.16 g) was purified by Sephadex LH-20 column chromatography (120×5 cm; solvent, $CHCl_3$:CH_3OH, 1:1). For further purification, semi-preparative HPLC (J'sphere ODS-H80; flow rate, 8 mL/min; UV, 254 nm) gave pure pheophorbide A-methyl ester (19 mg). Pheophorbide A-methyl ester was eluted with a retention time of 13.4 min.

Pheophorbid A-methyl ester, a dark-green amorphous solid, exhibited a molecular ion peak at *m/z* 607.2 (M+H) in the FAB-MS and the molecular formula was established as $C_{36}H_{38}N_4O_5$ by high resolution FAB-MS. The UV spectrum of pheophorbide A-methyl ester exhibited at 408 and 666 nm and similar to those of porphyrin type compounds (Fig. 1). ^{1}H-NMR ($CDCl_3$): δ1.62 (3H, t, *J*=7.8 Hz, 31-H), 1.76 (3H, d, *J*=7.2 Hz, 25-H), 2.20 (2H, m, 2-H), 2.51 (2H, m, 1-H), 3.14 (3H, s, 29-H), 3.33 (3H, s, 26-H), 3.51 (3H, s, 36-H), 3.58 (2H, q, *J*=7.6 Hz, 30-H), 3.62 (3H, s, 32-H), 3.81 (3H, s, 34-H), 4.15 (1H, m, 3-H), 4.40 (1H, dq, *J*=7.2, 1.8 Hz, 4-H), 6.11 (1H, dd, *J*=11.4, 1.2 Hz, 28-Ha), 6.11 (1H, dd, *J*=17.9, 1.2 Hz, 28-Hb), 6.19 (1H, s, 21-H), 7.91 (1H, dd, *J*=17.9, 11.4 Hz, 27-H), 8.52 (1H, s, 6-H), 9.30 (1H, s, 11-H), 9.45 (1H, s, 16-H); ^{13}C-NMR ($CDCl_3$): δ11.23 (q, C-29), 12.08 (q, C-26), 12.11 (q, C-32), 17.37 (q, C-31), 19.56 (t, C-30), 23.07 (q, C-25), 29.85 (t, C-2), 31.04 (t, C-1), 50.11 (d, C-4), 51.12 (d, C-3), 51.65 (q, C-36), 52.84 (q, C-34), 64.72 (d, C-21), 93.22 (d, C-6), 97.55 (d, C-11), 104.43 (d, C-16), 105.26 (s, C-22), 122.84 (t, C-28), 129.00 (s, C-19), 129.06 (d, C-27), 129.18 (s, C-18), 131.89 (s, C-8), 136.24 (s, C-9), 136.24 (s, C-10), 136.54 (s, C-13), 137.95 (s, C-17), 142.08 (s, C-7), 145.21 (s, C-14), 149.70 (s, C-24), 149.70 (s, C-15), 155.41 (s, C-12), 161.30 (s, C-23), 169.54 (s, C-33), 172.21 (s, C-5), 173.31 (s, C-35), 189.56 (s, C-20).

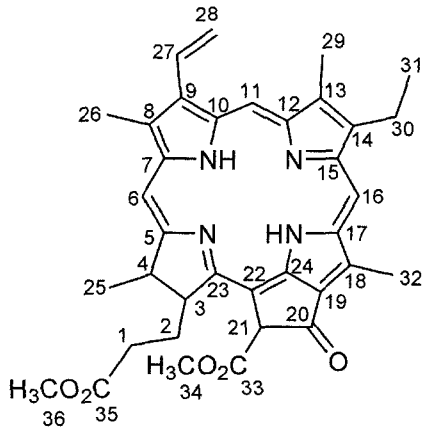

Fig. 1 Structure of pheophorbide A-methyl ester isolated from the leaves of *Diospyors kaki*

Acyl-CoA: cholesterol acyltransferase assay

Microsomes prepared from rat liver were used as a source of the enzyme. ACAT activity was measured as reported previously (Kim *et al.*, 1996). In brief, the reaction mixture, containing 10 μL of rat liver microsomes (10 mg/mL protein), 20 μL of 1 M potassium phosphate buffer (pH 7.4, 10 mM dithiothreitol), 10 μL of bovine serum albumin (fatty acid free, 180 mg/mL), 2.0 μL of cholesterol in acetone (20 mg/mL, added last), 130 μL of water and 10 μL of test sample in a total volume of 190 μL was preincubated for 30 min at 37°C. The reaction was initiated by the addition of 10 μL of [1-^{14}C]oleoyl-CoA solution (0.05 μCi : final concentration 10 μM). After 30 min of incubation at 37°C, the reaction was stopped by the addition of 1.0 mL of isopropanol-heptane (4:1, v/v) solution. A mixture of 0.6 mL of heptane and 0.2 mL of 0.1 M assay buffer was then added to the terminated reaction mixture. This was mixed for 2 min and allowed to separate into phases. Cholesterol oleate was recovered in the upper phase. The radioactivity in 100 μL of the upper phase was measured in a 7 mL liquid scintillation vial with 4 mL of scintillation cocktail (Lipoluma, Lumac Co.) using a liquid scintillation counter (Packard Delta-2000).

RESULTS AND DISCUSSION

The methanol extract of the leaves of *Diospyros kaki* showed potent inhibitory activity of ACAT prepared from the liver membrane of rats. The extract was fractionated by a series of normal and reverse column chromatographic techniques. One active compound was isolated by bioactivity guided fractionation and was identified as pheophorbide A-methyl ester. The structure of pheophorbide A-methyl ester was determined by the direct comparison of its physicochemical and spectroscopic properties with previously reported findings (Gerlach *et al.*, 1998; Wongsinkongman *et al.*, 2002).

Pheophorbide A-methyl ester inhibited ACAT activity in a dose dependent fashion with an IC_{50} value of 1.85 μg/mL in an enzyme assay using rat liver microsomes (Fig. 2). The enzyme assay was carried out with positive control phenylpyropene A (Kwon *et al.*, 2002) which inhibits ACAT activity with an IC_{50} value of 0.86 μM in this assay system. Since pheophorbide A-methyl ester is known to be a derivative product of chlorophyll A (Gerlach *et al.*, 1998), we investigated the inhibitory effects of chlorophyll A on rat liver microsomal ACAT inhibition. It was shown that Chlorophyll A at a concentration of 20 μM, did not affect ACAT activity (data not shown). We reported that pheophorbide A isolated from *Persicaria vulgaris* inhibited ACAT activity with an IC_{50} value of 1.35 μg/mL (Song *et al.*, 2002) which means that pheophorbide A and pheophorbide A-methyl ester inhibit ACAT activity to a similar degree.

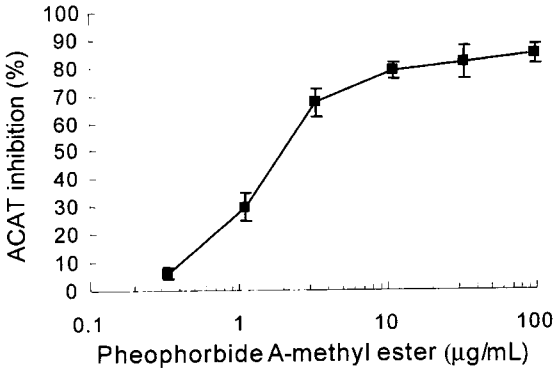

Fig. 2. Inhibitory effects of pheophorbide A-methyl ester on rat liver microsomal ACAT. The enzyme reaction was carried out at 37°C for 30 mins. Results are expressed as the mean ± S. E. of three experiments.

Pheophorbide A was not detected in HPLC tests on the fresh leaves of *Diospyros kaki* suggesting that pheophorbide A-methyl ester is not present as a direct result of chemical reactions during the isolation procedure. It is widely known that pheophorbide A-related compounds, including pheophorbide A-methyl ester, are biologically useful as antioxidants (Sakata *et al.*, 1990), anticancer agents (Wongsinkongman *et al.*, 2002) and endothelin receptor antagonists (Ohshima *et al.*, 1994). Our studies demonstrated a new biological activity of pheophorbide A-related compounds.

Considerable knowledge concerning cholesterol ester metabolism has accumulated during the past century. However, rapid advances have occurred in the past 7 years since the cloning of an ACAT gene, and the discovery that two ACATs function in mammalian systems (Buhman *et al.*, 2000). Due to ACATs role in the absorption, storage and production of lipids, it has been explored as a potential target for pharmacological intervention. Therefore our results will be very useful for the design of new and potent ACAT inhibitors leading to future treatments for hyperlipidaemia and atherosclerosis.

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