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Ergosterol Peroxide from Flowers of *Erigeron annuus* L. as an Anti-Atherosclerosis Agent

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Flowers of *Erigeron annuus* L. were extracted with 80% aqueous MeOH, and the concentrated extract was partitioned with EtOAc, *n*-BuOH, and H_2O. Repeated silica gel and ODS column chromatography of the EtOAc fraction led to the isolation of a sterol, through activity-guided fractionation, using ACAT inhibitory activity measurements. From the physico-chemical data, including NMR, MS, and IR, the chemical structure of the compound was determined to be an ergosterol peroxide (**1**), which has been isolated for the first time from this plant. This compound exhibited hACAT-1 and Lp-PLA_2 inhibitory effects, with inhibitory values of 51.6 ± 0.9 and 51.7 ± 1.2%, at a treatment concentration of 0.23 mM.

Key words: *Erigeron annuus* L., Compositae, hACAT1 Inhibitory effect, Lp-PLA_2 Inhibitory effect, Ergosterol peroxide

INTRODUCTION

The enzyme, acyl-CoA: cholesterol acyltransferase (ACAT, E.C.2.3.1.26), which catalyzes the acylation of cholesterol to cholesteryl esters with long chain fatty acids, is an inhibition target for the treatment of hypercholesterolemia and atherosclerosis (Brown *et al.*, 1975). In mammals, this enzyme exists in two isoforms, (Anderson *et al.*, 1998; Coses *et al.*, 1998), ACAT-1 and ACAT-2, with different tissue distribution and membrane topology (Joyce *et al.*, 2000). However, most ACAT inhibitors screened by rat liver microsomal ACAT, have had problems associated with low oral bioavailability and adrenal and/or hepatic toxicity in clinical trials (Dominick *et al.*, 1993; Matsuo *et al.*, 1996). ACAT-1 plays a critical role in macrophage foam cell formation, whereas ACAT-2 controls the cholesterol absorption process in intestinal mucosal cells (Rudel *et al.*, 2001). These findings were consistent with the results that atherosclerosis lesions were reduced in ACAT-1 knockout mice, whereas ACAT-2 knockout mice had limited cholesteryl absorption in the intestine and decreased cholesterol ester contents in the liver and plasma lipoproteins (Accad *et al.*, 2000; Yagyu *et al.*, 2000; Buhman *et al.*, 2000). Because ACAT inhibitors, most of which are not selective to either ACAT-1 or ACAT-2, effective in lowering plasma cholesterol in humans have not been identified (Sliskovic *et al.*, 2002), a selective inhibitor of ACAT-1 or ACAT-2 may be effective in the development of useful hypercholesterolemic or anti-atherogenic agents.

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2), a platelet-activating factor acetylhydrolase (PAF-AH, EC 3.1.1.47), is a calcium-independent member of the phospholipase A_2 superfamily, which is produced and secreted mainly by cells of the monocyte-macrophage series, T-lymphocytes, and mast cells (Stafforini *et al.*, 1990). During the early stage of its action, the lipoprotein-associated phospholipase A_2 (Lp-PLA_2) enzyme hydrolyzes the *sn*-2 fatty acid of oxidatively modified LDL to generate lysophosphatidylcholine (lyso-PC) and oxidized free fatty acids (oxFFA) (Macphee *et al.*, 1999). Lyso-PC has been shown to be a chemoattractant for monocytes, which promotes the chronic inflammation associated with macrophage accumulation. Lp-PLA_2 inhibitors have been shown

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to reduce atherosclerotic plaque development in a Watanabe heritable hyperlipidemic (WHHL) rabbit study (Leach *et al.*, 2001). Therefore, the Lp-PLA_2 enzyme is a very attractive target for the treatment of atherosclerosis.

Erigeron annuus L. (Bignoniaceae) is a biennial herbaceous plant of the Compositae family, and a naturalized plant imported from the Northern American Continent, commonly found all over Korea. The flower of this plant has been used for medical treatment in Korea, and the chemical constituents of *Erigeron annuus* L. have been reported by some researchers. For example, 4-hydroxycinnamic acid and 3,4-dihydroxycinnamic acid methyl ester, which showed a germination inhibitory effect on lettuce seeds, and 3-hydroxy-pyran-4-one and 5-butyl-3-oxo-2,3-dihydrofuran-2-yl acetic acid have been isolated from the stem, leaf and flower of *Erigeron annuus* L., respectively (Oh *et al*, 2002). Also, 3,5-di-*O*-caffeoylquinic acid and methyl 3,5-di-*O*-caffeoylquinate, from *E. annuus,* have been reported as angiotensin converting enzyme inhibitors (Oh *et al.*, 2002). However, an extensive study for the chemical components of the plant has not been yet reported.

Methanol extracts of this plant have especially shown significant inhibitory activity toward ACAT. Therefore, in this paper the authors report on the ACAT-1, ACAT-2 and Lp-PLA_2 inhibitory effects of a sterol isolated from flowers of *Erigeron annuus* L.

MATERIALS AND METHODS

Plant materials

The flowers of *Erigeron annuus* L. were collected by the authors from the fields and mountains of Yongin, Kyunggi-Do, in August, 2002. A voucher specimen (KHU-02034) was reserved at the Laboratory of Natural Products Chemistry, KyungHee University, Suwon, Korea.

Instrumentation

The melting point was determined on a Fisher-John apparatus (Pittsburgh, U.S.A.), and is reported uncorrected. The specific rotation, $[\alpha]_D$, was measured on a JASCO P-1010 digital polarimeter (Tokyo, Japan). The EI-MS was recorded on a JEOL JMSAX 505-WA. The IR spectrum (Tokyo, Japan) was run on a Perkin Elmer Spectrum One FT-IR spectrometer (Boston, U.S.A.). The ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained on a Varian Unity Inova AS 400 FT-NMR spectrometer (Washington, D.C., U.S.A.). A scintillation counter was used with 1450 Microbeta Trilux, Qallac Oy (Turku, Finland) for Lp-PLA_2 and ACAT acyivity assay.

Chemicals

The [1-^{14}C]oleoyl-CoA (56.0 mCi/mmol, 50 μCi/mL, CFA634) was purchased from Amersham Biosciences (Stockholm, Sweden), the [^{3}H]PAF (100 Ci/mL, 21.5 Ci/mmol, NET 910) from Perkin Elmer Life Sciences, Inc, and the PAF(L-α-phosphatidylcholine, β-acetyl γ-O-hexadecyl) from Sigma-Aldrich (St. Louis, U.S.A.). The KH_2PO_4, dithiothreitol, bovine serum albumin (fatty acid free) and all the reagent grade chemicals were purchased from Sigma-Aldrich.

Isolation of a sterol from the flower of *Erigeron annuus* L.

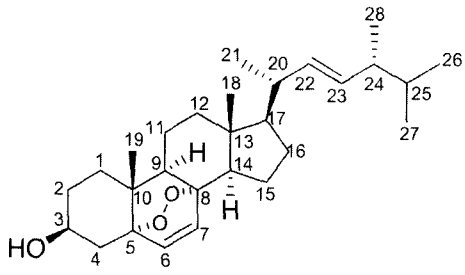
The dried and powdered flowers of *E. annuus* (1.2 kg) were extracted three times with 80% aqueous methanol (MeOH, 15 L×3) at room temperature. The extracts were successively partitioned with water (2 L), ethyl acetate (EtOAc, 2L×3) and *n*-butanol (*n*-BuOH, 2L×3). The EtOAc extract (50 g) was applied to the silica gel column (9×23 cm), and subjected to chromatography (c.c.), with *n*-hexane:EtOAc (7:1) as the eluent. The eluted fractions were monitored by thin layer chromatography (TLC), and twenty three fractions were produced (EAE1 to EAE23). EAE9 (2.4 g) was subjected to the silica gel c.c. (5×12 cm), and eluted with *n*-hexane:EtOAc (4:1) to afford six fractions (EAE9-1 to EAE9-6). EAE9-6 (363 mg) was purified by ODS c.c. (4×10 cm), using MeOH:H_2O (10:1→15:1) as eluent, to yield compound **1** (27 mg, Rf value: 0.27 on ODS TLC developed in MeOH:H_2O = 15:1).

5α,8α-Epidioxy-24(*R*)-methylcholesta-6,22-dien-3α-ol [ergosterol peroxide, (1)]

White powder ($CHCl_3$); m.p. 180-181; $[\alpha]_D$ = -26.0° (*c*=0.2, $CHCl_3$); EI/MS *m/z*: 428, 410, 369, 253; IR_ν ($CHCl_3$, cm^{-1}) 3360, 1460, ^{1}H-NMR (400 MHz, $CDCl_3$), δ 6.48 (1H, d, *J*=8.4 Hz, H-7), 6.22 (1H, d, *J*=8.4 Hz, H-6), 5.18 (1H, d, *J*=15.1 Hz, H-22), 5.09 (1H, d, *J*=15.1 Hz, H-23), 3.92 (1H, m, H-3), 0.95 (3H, d, *J*=6.4 Hz, H-21), 0.86 (3H, d, *J*=7.2 Hz, H-28), 0.84 (3H, s, H-19), 0.79 (3H, d, *J*=6.6 Hz, H-26), 0.77 (3H, s, H-18), 0.77 (3H, d, *J*=6.6 Hz, H-27), ^{13}C-NMR (100 MHz, $CDCl_3$, δ) 135.3 (C-7), 135.1 (C-23), 132.2 (C-22), 130.6 (C-6), 82.1 (C-8), 79.4 (C-5), 66.4 (C-3), 56.2 (C-17), 51.7 (C-14), 51.1 (C-9), 44.6 (C-13), 42.8 (C-24), 39.8 (C-20), 39.3 (C-1), 39.3 (C-12), 37.0 (C-10), 34.7 (C-4), 33.1 (C-25), 30.1 (C-2), 28.7 (C-15), 23.4 (C-16), 20.9 (C-27), 20.7 (C-11), 20.0 (C-26), 19.7 (C-21), 18.2 (C-19), 17.6 (C-28), 12.9 (C-18).

ACAT activity assay

Microsomal fractions of Hi5 cells, containing baculovirally expressed hACAT-1 or hACAT-2, and rat liver microsomes were used as sources of the enzyme. The activities of hACAT-1 and hACAT-2 were measured using the method of Brecher and Chan (Brecher *et al.*, 1980), with slight modification (Jeong *et al.*, 1995; Lee *et al.*, 2001).



(1)
Ergosterol peroxide

Fig. 1. The chemical structure of the ergosterol peroxide isolated from flowers of *Erigeron annuus* L.

The reaction mixture, containing 4 μL of microsomes (8 mg/mL protein) and 20 μL 0.5 M potassium phosphate buffer (pH 7.4), with 10 mM dithiothreitol, 15 μL bovine serum albumin (fatty acid free, 40 mg/mL), 2 μL cholesterol in acetone (20 μg/mL, added last), 41 μL water and 10 μL of test sample, in a total volume of 92 μL, was preincubated for 20 min at 37°C, with brief vortexing and sonication. The reaction was initiated by the addition of 8 μL of [1-^{14}C] oleoyl-CoA solution (0.05 μCi, final conc. 10 μM). After 25 min of incubation at 37°C, the reaction was stopped by the addition of 0.1 mL isopropanol-heptane (4:1; v/v). A mixture of 0.6 mL heptane and 0.4 mL 0.1 M potassium phosphate buffer (pH 7.4), with 2 mM dithiothreitol, was subsequently added. This solution was mixed and allowed to phase separate under gravity for 2 min. Cholesterol oleate was recovered from the upper heptane phase (total volume 0.9-1.0 mL). The radioactivity in 100 μL of the upper phase was measured in a liquid scintillation vial, containing 3 mL of scintillation cocktail (Lipoluma, Lumac Co.), using a liquid scintillation counter (1450 Microbeta Trilux Wallac Oy, Turku, Finland). Background values were obtained by preparing heat inactivated or normal insect cell lysate microsomes; the background value was usually 200-250 cpm, at 8000 cpm for the ACAT reaction. The ACAT activity was expressed as defined units: cholesteryl oleate pmol/min/mg protein.

Lp-PLA$_2$ activity assay

LDL isolated from the plasma of normolipidemic volunteers (Jeong *et al.*, 2004) was used as the enzyme source, as the plasma isoform of Lp-PLA$_2$ is 80% bound to LDL (Macphee *et al.*, 1999). The activity of Lp-PLA$_2$, also known as platelet-activating factor acetylhydrolase (PAF-AH), was measured using [^{3}H]PAF as a substrate (Tew *et al.*, 1996). Briefly, a micelle substrate was prepared with unlabelled PAF and [^{3}H]PAF (100 μCi/mL, 21.5 μCi/mmol, NET 910) in 10 mM phosphate-buffered saline (PBS), pH 7.4, containing 2.7 mM EDTA (PBS-EDTA). The reaction mixture, containing 20 μL of diluted human LDL (4-5 μg protein), 120 μL PBS-EDTA and 20 μL of test sample, was preincubated at 37°C for 15 min. The reaction was initiated by the addition of 40 μL micelle substrate (0.1 μCi, final conc. 80 μM PAF) to measure the initial PAF-AH activity rate. The reaction was stopped by vortexing with 600 μL $CHCl_3$/MeOH (2:1, v/v). The $CHCl_3$ and aqueous layers were separated by centrifugation. The aqueous layer was removed (250 μL) and vortexed with 250 μL $CHCl_3$. The aqueous layer was again removed, and the [^{3}H]acetate determined by scintillation counting (1450 Microbeta Trilux, Qallac Oy, Turku, Finland). The raw counts were background corrected using a non enzyme-containing blank, and expressed as nanomoles of PAF degraded/hour/milligram of protein.

RESULTS AND DISCUSSION

Flowers of *Erigeron annuus* L. were extracted with MeOH. The extracts were sequentially partitioned with EtOAc, *n*-BuOH, and water. Activity-guided fractionation for the inhibitory activity on ACAT-1, using repeated silica gel and ODS column chromatography of the EtOAc fraction, led to the isolation of compound **1**, an ergosterol peroxide.

Compound **1**, a white powder, showed absorbance bands due to hydroxyl (3360 cm^{-1}) and olefine (1460 cm^{-1}) groups in the IR spectrum ($CHCl_3$) and a molecular ion peak (M^+) at *m/z* 428 in the EI/MS. In the ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), two olefinic methine signals (δ_H 6.48, 6.22), with a coupling constant of 8.4 Hz, two other olefinic methine signals (δ_H 5.18, 5.09), with a coupling constant of 15.1 Hz, and one oxygenated methine signal (δ_H 3.92) were observed. Also, in the high magnet field region, four doublet (δ_H 0.95, 0.86, 0.79, and 0.77) and two singlet (δ_H 0.84 and 0.77) methyl signals were observed. In the ^{13}C-NMR (100 MHz, $CDCl_3$), twenty eight signals, consisting of four olefinic (δ_C 135.3, 135.1, 132.2, and 130.6), two oxygenated quaternary (δ_C 82.1 and 79.4) and one oxygenated methine (δ_C 66.4), as well as six further methine, seven methylene, two quaternary and six methyl (δ_C 20.9, 20.0, 19.7, 18.2, 17.6, and 12.9) signals were observed, leading to the conclusion that compound **1** was a sterol, with two double bonds, three oxygenated carbons and four doublet and two singlet methyls. Compound **1** was finally identified as 5α,8α-epidioxy-24(*R*)-methylcholesta-6,22-dien-3β-ol (ergosterol peroxide) by comparison of several physical and spectral data with those in the literature (Bok *et al.*, 1999; Nam *et al.*, 2001). It is worth noting ergosterol peroxide has been isolated for the first time from *E. annuus*, a biennial herb, even though previously this compound has mainly been isolated from fungi, such as mushrooms.

For the development of a useful hypercholesterolemic or anti-atherogenic agent, compound **1** was examined for

Table I. hACAT-1 and hACAT-2 inhibitory activity of the ergosterol peroxide (**1**) from flowers of *Erigeron annuus* L.

Sample	hACAT-1	hACAT-2
Positive control (oleic acid anilide at 0.3 M)	45.2 ± 1.4 %	50.8 ± 1.7 %
Compound **1** (0.23 mM)*	51.6 ± 0.9 %	16.0 ± 0.6 %

The data are presented as the mean ± standard deviation of three replications.
*The value denotes the concentration of each compound.

Table II. Lp-PLA_2 inhibitory activity of the ergosterol peroxide (**1**) from flowers of *Erigeron annuus* L.

Sample	Lp-PLA_2
Positive control (SB 402564 at 6.5 nM)	58.2 ± 1.8 %
Compound **1** (0.23 mM)*	51.7 ± 1.2 %

The data are presented as the mean ± standard deviation of three replications.
*The value denotes the concentration of each compound.

ACAT inhibitory activity, with oleic acid anilide used as a positive control (Roth *et al.*, 1992; Kim *et al.*, 1994). As shown in Table I, compound **1** exhibited relatively high hACAT-1 inhibitory activity, with an inhibitory value of 51.6 ± 0.9% at 0.23 mM, although inhibition of the hACAT-2 activity was barely detected. The inhibitory activities of compound **1** were confirmed by the oleic acid anilide positive control, which inhibited both hACAT-1 and hACAT-2, with inhibitory values of 45.2 ± 1.4 and 50.8 ± 1.7% at a concentration of 0.3 μM. Therefore, the authors have discovered a sterol showing significant specific enzyme inhibitory activity toward hACAT-1.

The Lp-PLA_2 inhibitory activity of compound **1** was confirmed using a positive control, with SB381320 (provided by GlaxoSmithKline), which inhibited Lp-PLA_2, with an IC_{50} value of 8.8 nM in LDL (IC_{50} value of 8.0 nM in recombinant Lp-PLA_2) and 67% inhibition in whole human plasma at 100 nM (Bloomer *et al.*, 2001). Compound **1** exhibited moderate Lp-PLA_2 inhibitory activity, with an inhibitory value of 51.7 ± 1.2% at 0.23 mM. Although various synthetic compounds have been developed as potent Lp-PLA_2 inhibitors (Blackie *et al.*, 2003), thus far; however, naturally occurring Lp-PLA_2 inhibitors have not been described, with the exceptions of SB-253514 and analogues isolated from *Pseudomonas fluorescens* DMS 11579 (Thirkettle *et al.*, 2000). Compound **1** was isolated from flowers of *Erigeron annuus* L., and its Lp-PLA_2 inhibitory activity reported for the first time.

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