Natural Product Sciences
15(2) : 110-113 (2009)

ACAT Inhibition of Polyactylenes from *Gymnaster koraiensis*

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Abstract – Acyl-coenzyme A: cholesterol acyltransferase (ACAT) catalyzes cholesterol esterification and plays important roles in intestinal absorption of cholesterol, hepatic production of lipoproteins and accumulation of cholesteryl ester within macrophages and smooth muscle cells. In our study, eight polyacetylenes (**1** - **8**), were isolated from the roots of *Gymnaster koraiensis*, and their chemical structures were identified on the basis of spectroscopic analysis and mass. Compound **2** with the (10*S*)-15,16-epoxy group in skeleton strongly inhibited ACAT enzyme with IC_{50} value of 35.8 μg/mL, meanwhile the other compounds displayed significant inhibition of ACAT enzyme with the IC_{50} values from 45.5 to 55.1 μg/mL.
Keywords – *Gymnaster koraiensis,* Compositae, Polyacetylene, Acyl-coenzyme A: Cholesterol acyltransferase

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 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 and Chang, 1997; Sliskovic and White, 1991). Numerous ACAT inhibitors have been developed during the past 20 years, several of which have been reported to have undesirable toxic effects such as adrenal toxicity and induction of diarrhea. Recently, a number of ACAT inhibitors including R-755 (Matsui *et al*., 2001), F-1394 (Aragane *et al*., 2001), F-12511 (Rival *et al*., 2002) and avasimibe (Bocan *et al.,* 2000) have been reported and their pharmacological activities have been evaluated in animals and humans.

As a part of our continuing studies to discover cholesterol-lowering agents from natural sources, we have found that the CH_2Cl_2-soluble fraction of an 80% EtOH extract of the roots of *Gymnaster koraiensis* (Nakai) Kitamura (Compositae) had an appreciable ACAT inhibitory activity. *G. koraiensis* is an endemic species in Korea. In our previous studies, polyacetylenes had been identified as main constituents of the root of this plant, also their anticancer activities had been reported (Jung *et al*., 2002, Park *et al*., 2002). Recently, benzofuran derivatives (Dat *et al*., 2004), triterpenes as friedelinol, friedelin, and squalene were isolated from the aerial part of *G. koraiensis*, and the polyacetylenic compounds with epoxy groups showed strong inhibitory activity in NFAT transcription factor (Dat *et al*., 2005). However, no study

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has been specifically investigated the ability of these polyacetylenes on ACAT activity. In the present study, we report the inhibitory activity of some polyacetylenes in rat liver microsomes ACAT enzyme.

Experimental

Plant material – Roots of *G. koraiensis* were collected in May 1997 at Gurae, Chunnam Province, Korea. A voucher specimen (CNU96003) is deposited in the herbarium of College of Pharmacy, Chungnam National University, Taejon, Korea.

Extraction and purification – The air-dried roots (4.8 kg) of *G. koraiensis* were extracted with 80% aqueous EtOH (700 g). The EtOH extract was suspended in H_2O and extracted with CH_2Cl_2 and BuOH, successively, to give the CH_2Cl_2 (140 g) and BuOH-soluble fractions (123 g), respectively. The CH_2Cl_2-soluble fraction was chromatographed on a silica gel column. The column was eluted using a stepwise gradient of hexane and EtOAc to give nine fractions. Repeated column chromatography of each fraction on silica gel (hexane-acetone) and preparative TLC (hexane-acetone), followed by HPLC on RP-C_{18} (50 - 70% aqueous MeOH) afforded **1** (30 mg), **2** (30 mg), **3** (40 mg), **4** (50 mg), **5** (50 mg), **6** (40 mg), **7** (35 mg) and **8** (40 mg) (Fig. 1).

(3*S*)-8-Decen-4,6-diyn-1,3-diol (1): yellow oil; $[\alpha]_D^{20}$ –14° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 240 (0.68), 252 (1.18), 266 (1.59), 282 (1.25) nm; IR (CCl_4) ν_{max} 3324, 2928, 2856, 2232, 2640, 1640, 1010, 870 cm^{-1}; EIMS *m/z* 164 $[M]^+$ (5), 146 (25), 119 (100), 89 (42); ^1H and ^{13}C NMR data were in accordance with our previous paper (Jung *et al.*, 2002).

(10*S*)-15,16-Epoxy-1,8-heptadecadien-11,13-diyn-10-ol (2): yellow oil; $[\alpha]_D^{20}$ +267° (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 237 (1.15), 250 (1.17), 264 (0.82) nm; IR (KBr) ν_{max} 3603, 2930, 2857, 2154, 1641, 1549, 990 cm^{-1}; CIMS *m/z* 259 $[M + H]^+$ (14), 241 (60), 197 (100), 161 (15), 155 (50), 135 (56), 95 (68), 55 (53); ^1H and ^{13}C NMR data were in accordance with our previous paper (Jung *et al.*, 2002).

(3*S*,8*S*)-1,9,16-Heptadecatrien-4,6-diyn-3,8-diol-3-acetate (3): yellow oil; $[\alpha]_D^{20}$ +263° (*c* 0.5, MeOH); UV λ_{max} (log ε) 233 (1.34), 246 (1.19), 260 (0.71) nm; IR (KBr) ν_{max} 3466, 2929, 2857, 2159, 1750, 1640, 1222, 990, 910, 868 cm^{-1}; CIMS *m/z* 301 $[M + H]^+$ (14), 283 (84), 241 (100), 171 (76), 131 (51), 93 (36); ^1H and ^{13}C NMR data were in accordance with our previous paper (Jung *et al.*, 2002).

(8*S*)-2,9,16-Heptadecatrien-4,6-diyn-1,8-diol-1-acetate (4): yellow oil; $[\alpha]_D^{20}$ +281° (*c* 0.5, MeOH); UV λ_{max} (log ε) 242 (0.81), 255 (1.39), 269 (1.82), 285 (1.50) nm; IR (KBr) ν_{max} 3465, 2929, 2857, 2360, 1748, 1640, 1382, 1225, 1000, 910 cm^{-1}; CIMS *m/z* 301 $[M + H]^+$ (16), 283 (43), 241 (100), 171 (76), 131 (51), 99 (10), 93 (36); ^1H and ^{13}C NMR data were in accordance with our previous paper (Jung *et al.*, 2002).

***erythro*-(10*S*)-1,8-Heptadecadien-11,13-diyn-10,15,16-triol (5):** yellow oil; $[\alpha]_D^{20}$ +260° (*c* 0.5, MeOH); UV λ_{max} (log ε) 236 (0.66), 250 (0.69), 263 (0.37) nm; IR (KBr) ν_{max} 3436, 2927, 2856, 2347, 1639, 1550, 1383, 1000 cm^{-1}; CIMS *m/z* 277 $[M + H]^+$ (70), 259 (39), 243 (50), 223 (12), 197 (91), 135 (100), 97 (47), 95 (82); ^1H and ^{13}C NMR data were in accordance with our previous paper (Jung *et al.*, 2002).

(8*S*)-2,9,16-Heptadecatrien-4,6-diyn-1,8-diol (6): yellow oil; $[\alpha]_D^{20}$ +296° (*c* 0.5, MeOH); UV λ_{max} (log ε) 241 (0.73), 254 (1.26), 268 (1.70), 284 (1.37) nm; IR (KBr) ν_{max} 3324, 2928, 2856, 2232, 1721, 1640, 1549, 1371, 1010, 990, 910, 860 cm^{-1}; CIMS *m/z* 259 $[M + 1]^+$ (14), 241 (100), 223 (54), 171 (75), 131 (71), 91 (85); ^1H and ^{13}C NMR data were in accordance with our previous paper (Jung *et al.*, 2002).

2,9,16-Heptadecatrien-4,6-diyn-8-ol (7): bright yellow oil; $[\alpha]_D^{20}$ +280° (*c* 0.5, MeOH); UV λ_{max} (log ε) 241 (0.83), 254 (1.35), 267 (1.76), 284 (1.41) nm; IR (KBr) ν_{max} 3366 (OH), 2928, 2855 (C-H) cm^{-1}; CIMS *m/z* 242 $[M]^+$ (3.1), 115 (100); ^1H and ^{13}C NMR data were in accordance with our previous paper (Jung *et al.*, 2002).

1,9,16-Heptadecatriene-4,6-diyn-3,8-diol (8): bright yellow oil; $[\alpha]_D^{20}$ +310° (*c* 0.5, MeOH); UV λ_{max} (log ε) 233 (1.32), 246 (1.18), 260 (0.73) nm; IR (KBr) ν_{max} 3398 (broad, OH), 2927, 2856 (C-H), 2151 (C ≡ C) cm^{-1}; CIMS *m/z* 258 $[M]^+$ (10.4), 203 (49.8), 107 (100.0); ^1H and ^{13}C NMR data were in accordance with our previous paper (Jung *et al.*, 2002).

ACAT enzyme assay using rat liver microsomes – ACAT activity was assayed as previously reported (Rho *et al.*, 2005). 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

Table 1. Inhibitory effect of isolated compounds against ACAT *in vitro*.

Extract/Compounds	IC_{50} (μg/mL)
80% EtOH extract[a)]	50.3%
$CHCl_3$ fraction[a)]	80.7%
BuOH fraction[a)]	< 20%
Aqueous fraction[a)]	< 20%
1	> 100
2	35.8
3	50.2
4	45.7
5	45. 5
6	45.7
7	55.1
8	50.8
Panaxydol[b)]	11.7

[a)]% inhibition at concentration of 20 μg/mL
[b)]used as positive control

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.

Results and Discussion

In search for ACAT inhibitors from natural sources, we found that 80% ethanol extract of the roots of *G. koraiensis* inhibited rat liver ACAT enzyme activity (50% inhibition at a final concentration of 20 μg/mL). The 80% EtOH extract was partitioned into CH_2Cl_2-, BuOH- and aqueous fractions. In the preliminary experiment, we tested the inhibitory activity of these fractions at the concentration of 20 μg/mL against ACAT enzyme, which was used rat liver as the sources. The result showed that CH_2Cl_2-soluble fraction (80.7% inhibition) was approximately 4.1-fold more potent than BuOH- and aqueous soluble fractions (< 20% inhibition) at the same test concentration (Table 1). Considering that the CH_2Cl_2-soluble fraction was the most potent, it was selected for the isolation of the active constituents. In the next study, the CH_2Cl_2-soluble fraction was purified by silica gel column chromatography using a stepwise gradient (hexane and EtOAc), preparative TLC on silica gel, and preparative HPLC (Metachem C_{18}, MeOH and H_2O) to afford eight polyacetylenes (**1** - **8**). These compounds were

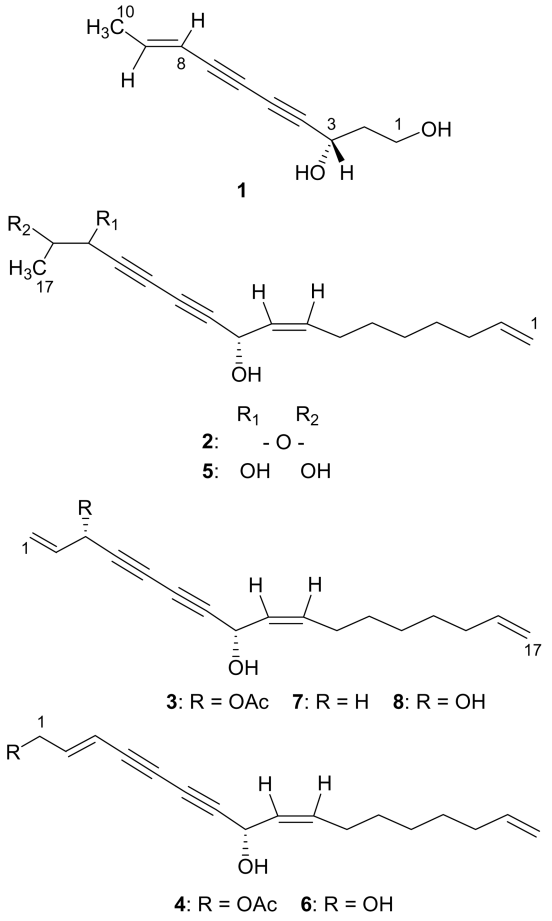

Fig. 1. Chemical structures of isolated compounds (**1** - **8**).

identified as (3*S*)-8-decen-4,6-diyn-1,3-diol (**1**), (10*S*)-15, 16-epoxy-1,8-heptadecadien-11,13-diyn-10-ol (**2**), (3*S*,8*S*)-1,9,16-heptadecatrien-4,6-diyn-3,8-diol-3-acetate (**3**), (8*S*)-2, 9,16-heptadecatrien-4,6-diyn-1,8-diol-1-acetate (**4**), *erythro*-(10*S*)-1,8-heptadecadien-11,13-diyn-10,15,16-triol (**5**), (8*S*)-2, 9,16-heptadecatrien-4,6-diyn-1,8-diol (**6**), 2,9,16-heptadecatrien-4,6-diyn-8-ol (**7**) and 1,9,16-heptadecatrien-4,6-diyn-3,8-diol (**8**) (Fig. 1). The compounds **1** - **6** were named as gymnasterkoreayne A-F, which were described in detail in our previous paper (Jung *et al.*, 2002). Polyacetylenes have been found in many families of higher plants, such as Araliaceae, Campanulaceae, Compositae, Olacaceae, Pottosporaceae, Santalaceae, and Umbelliferae (Chritensen, 1992; Krause *et al.*, 1998). It has been reported that these compounds exhibit potent cytotoxic, antimicrobial, antiviral, RNA-cleaving, sedative, and enzyme-inhibitory activities, as well as brine-shrimp lethality (Kobaisy *et al.*, 1997, Jung *et al.*, 2002). In this paper, these isolated polyacetylenes were tested for their inhibitory activity against ACAT enzyme using rat liver microsomes, and the results was presented in Table 1. Of

the compounds tested, compound **1** showed weak inhibitory activity (IC_{50} > 100 μg/mL). Compound **2** with the (10*S*)-15,16-epoxy group in skeleton strongly inhibited ACAT enzyme with IC_{50} value of 35.8 μg/mL, meanwhile the other compounds also displayed significant inhibition of ACAT enzyme with the IC_{50} values from 45.5 to 55.1 μg/mL. In this experiment, panaxydol, a C_{17} polyacetylene compound isolated from *Panax ginseng* (Rho *et al*., 2005), was used as positive control, and exhibited IC_{50} value of 11.7 μg/mL.

Due to its roles in the absorption, storage, and production of cholesterol, ACAT has been explored as a potential target for pharmacological intervention of hyperlipidemia and atherosclerotic disease. There are many known synthetic and naturally occurring inhibitors of ACAT; however, some of them have problems associated with oral bioavailability, adrenal and/or hepatic toxicity, drug interactions, and accumulation of un-esterified cholesterol (Matsui *et al*., 2001; Aragane *et al*., 2001). Recently, a few candidate drugs have reached the stage of clinical trials as potential lipid-lowering and atherosclerotic agents (Chang *et al*., 2001, Giovannoni *et al*., 2003). Of our results, the ACAT inhibitory ability of compound **2** and others were comparable to those of C_{17} polyacetylenic compounds from ginseng, which was very famous to use in reducing serum total cholesterol and low density lipoprotein-cholesterol levels in clinical used. Recent studies reported that the polyacetylenes were used as acyl CoA: diacylglycerol acyltransferase (DGAT) enzyme inhibitors (Lee *et al*., 2004, Rho *et al*., 2005). Thus, *G. koraiensis* extract and its polyacetylenes with the ACAT inhibitory activity might be able the other source to provide potent lipid-lowering agents.

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Received June 16, 2009
Revised June 25, 2009
Accepted June 25, 2009