Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 12 (2004) 3599–3605
Bioorganic & Medicinal Chemistry

# Evaluation of hesperetin 7-*O*-lauryl ether as lipid-lowering agent in high-cholesterol-fed rats

Gab-Sun Choi,[a] Sangku Lee,[b] Tae-Sook Jeong,[b] Mi-Kyung Lee,[c] Jeong-Sun Lee,[a] Un Ju Jung,[a] Hye-Jin Kim,[a] Yong Bok Park,[d] Song-Hae Bok[b] and Myung-Sook Choi[a,*]

[a]*Department of Food Science and Nutrition, Kyungpook National University, 1370 Sankyuk Dong Puk-Ku, 702-701 Daegu, South Korea*
[b]*Korea Research Institute of Bioscience and Biotechnology, Yusong, 305-333 Daejon, South Korea*
[c]*Food and Bio-Industry Research Institute, Kyungpook National University, 1370 Sankyuk Dong Puk-Ku, 702-701 Daegu, South Korea*
[d]*Department of Genetic Engineering, Kyungpook National University, 1370 Sankyuk Dong Puk-Ku, 702-701 Daegu, South Korea*

Received 19 February 2004; revised 16 April 2004; accepted 16 April 2004
Available online 18 May 2004

**Abstract**—The lipid-lowering efficacy of hesperetin was revealed in preliminary studies on experimental animals. As such, the current study compared the effect of hesperetin 7-*O*-lauryl ether, with that of hesperetin and lovastatin on the lipid profile and cholesterol-regulating mechanism in high-cholesterol-fed rats. Male rats were fed a high-cholesterol diet (1%, wt/wt) or high-cholesterol diet supplemented with lovastatin (**1**, 0.02%, wt/wt), hesperetin (**2**, 0.02%, wt/wt), or hesperetin 7-*O*-lauryl ether (**3**, 0.031%, wt/wt) for six weeks. The supplemental amount of **3** was 0.066 mmol/100 g diet as an equivalent to the supplemental amount of **2**. The plasma total cholesterol and triglyceride levels were significantly lowered by the **2** and **3** supplements compared with the control or **1**-supplemented group. The hepatic HMG-CoA reductase activities were also significantly lower in all the supplemented groups compared with the control group, and the hepatic ACAT activity was significantly lower in the **2**- and **3**-supplemented groups. The supplementation of **3** resulted in a higher excretion of total neutral sterol and total fecal sterol compared with the control or **1**-supplemented group. Accordingly, overall, compound **3**, exhibited a more potent plasma lipid-lowering effect than compound **1** based on inhibiting cholesterol biosynthesis and esterification, while also increasing the fecal sterol excretion.


## 1. Introduction

Cardiovascular disease (CVD) is the primary chronic disease afflicting industrialized societies today, and hypercholesterolemia is considered a major risk factor in the progression of coronary atherosclerosis and associated with an increase in the incidence of myocardial ischemia and cardiac events.[1]

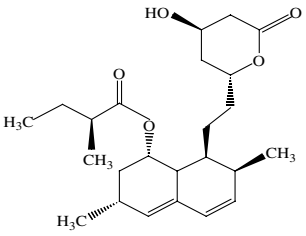
The inverse association of a high fruit and vegetable intake with the risk of CVD mortality in numerous epidemiologic studies[2,3] has led to many hypotheses regarding the physiologic role of flavonoids and an extensive review of their natural occurrence and biosynthesis, the methods of isolation and structural elucidation, and their application in food and pharmaceutical industries.[4]

Hesperidin (hesperetin-7-rutinoside), one of the most abundant flavonoids from citrus fruits, such as lemons, oranges, and grapefruit,[5,6] has already been reported to exert pharmacological effects, such as capillary permeability[7] and antioxidant activity.[8] In a previous study by the current authors, hesperidin and its aglycone, hesperetin, were found to reduce the levels of plasma cholesterol in rats fed a cholesterol-enriched diet.[9,10]

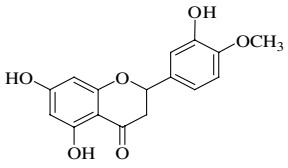
Numerous in vitro studies have revealed a close relationship between the chemical structure and biologic activity of flavonoids,[11,12] whereby their basic structure can be modified to increase or decrease their biologic activity.[13] As such, recent interest has focused on synthesizing a functional derivative with more potent

*Keywords*: Hesperetin derivative; Lipid lowering; HMG-CoA reductase; ACAT.
* Corresponding author. Tel.: +82-53-950-6232; fax: +82-53-950-6229; e-mail: mschoi@knu.ac.kr

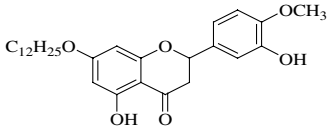
0968-0896/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2004.04.020



Lovastatin (**1**)



Hesperetin (**2**)



Hesperetin 7-*O*-lauryl ether (**3**)

**Figure 1.** Structures of compounds **1**, **2**, and **3**.

hypocholesterolemic activity than compound **2**, and fellow researchers found that hesperetin 7-*O*-lauryl ether (**3**), which possesses a long alkyl chain linked to the 7-hydroxyl position of hesperetin (**2**) (Fig. 1), lowered the plasma total cholesterol level in high-cholesterol-fed C59BC/6J mice in a preliminary screening test.[14] Lovastatin (**1**), a secondary metabolite of *Aspergillus terreus*, is an effective hypocholesterolemic agent that belongs to the new class of cholesterol-lowering agents, which inhibit HMG-CoA reductase, the rate-limiting conversion of HMG-CoA to mevalonate in cholesterol biosynthetic pathway.[15] Accordingly, the current study examined the effect of compound **3** in high-cholesterol-fed rats, its cholesterol-regulating enzyme activity, plasma and tissue lipid profile, and excretion of sterol in comparison to **1** and **2**.

## 2. Results

### 2.1. Food intake, weight gain, and organ weights

There were no differences in the weight gain and food intake among the groups (Table 1), indicating no effect from the dietary supplements. The liver and kidney weights were also not different among the groups, yet the heart weight was significantly lower in the **2**- and **3**-supplemented groups than in the **1**-supplemented group.

### 2.2. Plasma and hepatic lipids

The supplementation of **2** and **3** significantly lowered the plasma total cholesterol ($2.85 + 0.18$ and $2.70 \pm 0.12$ mmol/L vs $4.09 \pm 0.16$ mmol/L, $p < 0.05$) and triglyceride ($0.89 \pm 0.06$ and $0.86 \pm 0.05$ mmol/L vs $1.21 \pm 0.07$ mmol/L, $p < 0.05$) concentrations compared to the control group (Table 2). The HDL-cholesterol concentration was not different among the groups, yet the supplementation of **2** and its derivative, compound **3**, significantly raised the HDL-C/total-C ratios compared to the control group. Accordingly, **2** and **3** significantly lowered the atherogenic index compared to the control group. The hepatic cholesterol content was significantly lower in the **2**-supplemented group than in the control group, and slightly lower in the **3**-supplemented group. The hepatic triglyceride content was not significantly different among the groups (Table 3).

### 2.3. Hepatic HMG-CoA reductase and ACAT activities

The hepatic HMG-CoA reductase activity was significantly lower in all the experimental groups (**1**, **2**, and **3**) compared to the control group ($150 \pm 9$, $149 \pm 5$, and

**Table 1.** Effect of supplementation with compound **3** on food intake, weight gain, and organ weights in high-cholesterol-fed rats*

| Group | Weight gain (g/day) | Food intake (g/day) | Organ weights | | |
|---|---|---|---|---|---|
| | | | Liver (g) | Heart (g) | Kidney (g) |
| Control | $8.48 \pm 0.10^{NS}$ | $29.03 \pm 0.17^{NS}$ | $4.57 \pm 0.11^{NS}$ | $0.32 \pm 0.01^{a,b}$ | $0.76 \pm 0.01^{NS}$ |
| **1** | $8.12 \pm 0.18$ | $28.95 \pm 0.19$ | $4.57 \pm 0.23$ | $0.34 \pm 0.01^{a}$ | $0.74 \pm 0.02$ |
| **2** | $8.32 \pm 0.16$ | $29.27 \pm 0.21$ | $4.47 \pm 0.15$ | $0.30 \pm 0.01^{b}$ | $0.71 \pm 0.01$ |
| **3** | $8.26 \pm 0.19$ | $29.43 \pm 0.19$ | $4.35 \pm 0.13$ | $0.31 \pm 0.01^{b}$ | $0.71 \pm 0.01$ |

$^{NS}$Not significantly different ($p < 0.05$) between groups.

**1**: lovastatin, **2**: hesperetin, **3**: hesperetin 7-*O*-lauryl ether.

$^{a,b}$Means in the same column not sharing a common superscript are significantly different ($p < 0.05$) between groups.

* Mean ± SE.

**Table 2.** Effect of supplementation with compound **3** on plasma lipids in high-cholesterol-fed rats*

| Group | TC (mmol/L) | TG (mmol/L) | HDL-C (mmol/L) | HDL-C/TC (%) | Atherogenic index |
|---|---|---|---|---|---|
| Control | $4.09 \pm 0.16^{a}$ | $1.21 \pm 0.07^{a}$ | $0.82 \pm 0.05^{NS}$ | $20.05 \pm 1.15^{a}$ | $4.00 \pm 0.35^{a}$ |
| **1** | $3.85 \pm 0.28^{a}$ | $1.35 \pm 0.12^{a}$ | $0.86 \pm 0.04$ | $22.25 \pm 2.43^{a}$ | $3.50 \pm 0.56^{a,b}$ |
| **2** | $2.85 \pm 0.18^{b}$ | $0.89 \pm 0.06^{b}$ | $0.82 \pm 0.03$ | $28.59 \pm 1.93^{b}$ | $2.50 \pm 0.24^{b}$ |
| **3** | $2.70 \pm 0.12^{b}$ | $0.86 \pm 0.05^{b}$ | $0.81 \pm 0.05$ | $30.02 \pm 2.60^{b}$ | $2.38 \pm 0.33^{b}$ |

$^{NS}$Not significantly different ($p < 0.05$) between groups.

TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; atherogenic index: (total cholesterol – HDL-cholesterol)/HDL-cholesterol; **1**: lovastatin, **2**: hesperetin, **3**: hesperetin 7-*O*-lauryl ether.

$^{a,b}$Means in the same column not sharing a common superscript are significantly different ($p < 0.05$) between groups.

* Mean ± SE.

**Table 3.** Effect of supplementation with compound **3** on hepatic lipids in high-cholesterol-fed rats*

| Group | Total cholesterol (mmol/g) | Triglyceride (mmol/g) |
|---|---|---|
| Control | $0.53 \pm 0.02^{a}$ | $0.20 \pm 0.01^{NS}$ |
| **1** | $0.41 \pm 0.01^{b}$ | $0.20 \pm 0.02$ |
| **2** | $0.43 \pm 0.03^{b}$ | $0.19 \pm 0.02$ |
| **3** | $0.48 \pm 0.04^{a,b}$ | $0.20 \pm 0.02$ |

[NS]Not significantly different ($p < 0.05$) between groups.
**1**: lovastatin, **2**: hesperetin, **3**: hesperetin 7-*O*-lauryl ether.
[a,b]Means in the same column not sharing a common superscript are significantly different ($p < 0.05$) between groups.
* Mean ± SE.

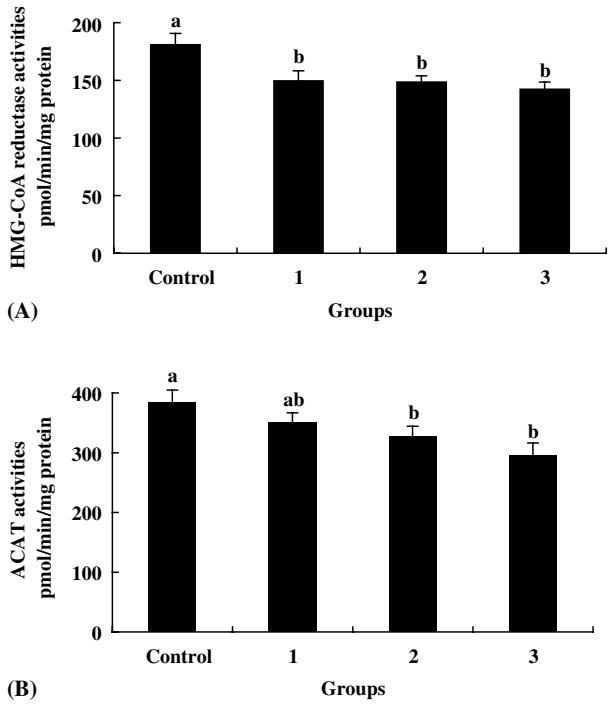



**Figure 2.** Effect of supplementation with compound **3** on hepatic HMG-CoA reductase (A) and ACAT (B) activities in high-cholesterol-fed rats. Mean ± SE. The means not sharing a common letter are significantly different between groups ($p < 0.05$). Compounds **1**: lovastatin, **2**: hesperetin, **3**: hesperetin 7-*O*-lauryl ether.

$142 \pm 6$ pmol/min/mg protein vs $181 \pm 10$ pmol/min/mg protein, $p < 0.05$), while the ACAT activity was significantly lower in the **2**- and **3**-supplemented groups compared to the control group (Fig. 2). Thus, compound **3** was found to inhibit both hepatic cholesterol biosynthesis and esterification.

## 2.4. Fecal sterols

Acidic sterol excretion was higher in the **2**- and **3**-supplemented groups than in the control and **1**-supplemented group, whereas the total neutral sterol excretion was significantly higher in the **3**-supplemented group. Thus, the combined fecal excretion level of neutral sterol (cholesterol, coprostanol, and coprostanone) and acidic sterol was significantly higher in the **2**- and **3**-supplemented groups by 116% and 139%, respectively, when compared to the control group (Fig. 3).

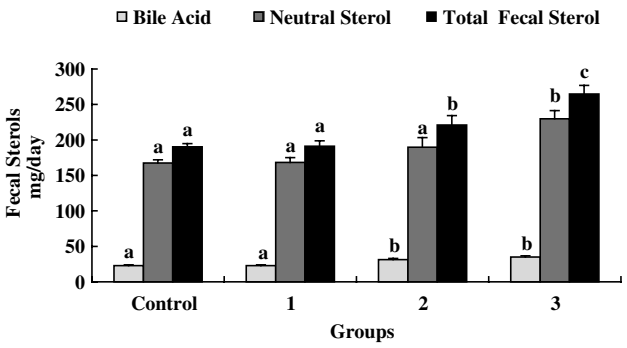



**Figure 3.** Effect of supplementation with compound **3** on fecal sterol content in high-cholesterol-fed rats. The daily neutral sterol excretion was calculated based on the sum of the cholesterol, coprostanol, and coprostanone from the gas chromatographic peaks, while the bile acids were measured using an enzymatic method to represent the total bile acids. The total fecal sterol refers to the sum of the neutral sterols and bile acids. Mean ± SE. The means not sharing a common letter are significantly different between groups ($p < 0.05$). Compounds **1**: lovastatin, **2**: hesperetin, **3**: hesperetin 7-*O*-lauryl ether.

## 3. Discussion

The relation between elevated plasma cholesterol levels and the risk of coronary heart disease (CHD) has already been established in numerous large-scale epidemiologic trials.[16] The regulation of plasma cholesterol levels involves factors that influence both the extracellular and intracellular cholesterol metabolism. A number of cholesterol-lowering drugs are already available for use in humans,[17,18] plus, the last decade has seen the development of a new class of agents that specifically inhibits 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase.[19] Current clinical data on HMG-CoA reductase inhibitors also demonstrate their efficacy and safety in treating hypercholesterolemia and improving long-term morbidity and mortality related to CHD.[20]

The role of naringenin and the structurally related citrus flavanone, hesperetin, in the prevention and treatment of disease has also recently received considerable attention,[21] with particular interest in the use of these flavanones as anticancer[22] and antiatherogenic[23] compounds. A previous report by the current authors already speculated on the cholesterol-lowering action of a 0.02% (wt/wt) compound **2** diet via the inhibition of HMG-CoA reductase and acyl-CoA: cholesterol acyltransferase (ACAT) activities in high-cholesterol-fed rats.[10]

In a recent report by Jeong et al.,[14] ester and ether derivatives of **2**, with a different lipophilic chain length connected to the 7-hydroxyl position of **2**, were synthesized to study the relationship between the structure and the hypocholesterolemic activity. The ether derivatives of **2** showed more potent activities than the ester derivatives, and hesperetin 7-*O*-ether analogues with

lauryl and oleyl moieties exhibited strong cholesterol-lowering activities.

Meanwhile, in the present study, supplementation with **2** and **3** was found to lower the plasma cholesterol and triglyceride levels in rats fed a high-cholesterol diet, plus this cholesterol-lowering action was also accompanied by the inhibition of hepatic HMG-CoA reductase and ACAT activities and an elevation of fecal sterol excretion. In the plasma lipids, the HDL-C/total-C ratio was higher in the **2**- and **3**-supplemented groups than in the control group due to a reduction of the total cholesterol concentration. Consequently, the atherogenic index was significantly lowered by the **2** and **3** supplements. As such, compound **3** appeared to be as potent as compound **2** in lowering the atherosclerotic risk based on the HDL-C/total-C ratio and atherogenic index.

The two key enzymes involved in regulating cholesterol metabolism are HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthetic pathway, and ACAT, the cholesterol-esterifying enzyme in tissue, including the liver. The inhibition of HMG-CoA reductase reduces cholesterol synthesis and its inhibitors are very effective in lowering serum cholesterol in most animal species, including humans.[24–26] The blockade of cholesterol synthesis by an inhibitor of HMG-CoA reductase results in a lower intracellular supply of cholesterol, thereby triggering an over-expression of hepatic LDL receptors and enhancing the clearance of circulating LDL particles.[27] ACAT is primarily responsible for the esterification of cholesterol in all mammalian cells and has been implicated as a key enzyme involved in cholesterol absorption, VLDL secretion, and the formation of lipid-filled macrophages and smooth muscle cells.[28] Inhibition of ACAT has been shown to limit atherosclerotic lesion development, cholesteryl ester (CE) enrichment, and monocyte-macrophage foam cell involvement.[29] Thus, ACAT inhibitors are also expected to be cholesterol-lowering and antiatherosclerotic agents. In this study, the hepatic HMG-CoA reductase activity was significantly lower in all the experimental groups (**1**, **2**, and **3**) compared to the control group, while the ACAT activity was significantly lower in the **2**- and **3**-supplemented groups compared to the control group. Interestingly, compound **3** was found to inhibit both hepatic HMG-CoA reductase and ACAT activities.

Hepatic ACAT utilizes cholesterol as its substrate, which is either synthesized endogenously or acquired via the LDL and chylomicron remnant receptors. Wilcox et al.[30] recently reported that compound **2** decreases the availability of lipids for the assembly of apoB-containing lipoproteins in a cell culture of HepG2 by reducing the activities of ACAT1 and ACAT2, and selectively decreases ACAT expression along with an enhanced expression of LDL-receptors and an inhibition of microsomal triglyceride transfer protein (MTP) activity. The treatment of different species with tissue-selective inhibitors of ACAT has led to variable decreases in the plasma cholesterol content.[31–33] Accordingly, the present study indicated that the supplementation of **2** or **3** significantly lowered hepatic cholesterol biosynthesis and esterification, leading to hypocholesterolemic effects. Borradaile and co-workers[34] provided evidence that **2** not only decrease cholesterol synthesis but also inhibit ACAT activity in vitro. Other researchers have already reported that high concentrations of HMG-CoA reductase inhibitors can inhibit ACAT,[35] presumably by limiting the free cholesterol pool available for esterification.

The hepatic cholesterol content was lower in the **1**- and **2**-supplemented groups than in the control group, and slightly lower in the **3**-supplemented group. In the current study, although the hepatic triglyceride content did not differ significantly among the groups, the plasma triglyceride concentration was lower in the **2**- and **3**-supplemented groups. As such, the reduced ACAT activity in these groups may have led to less CE being available for VLDL packing, thereby resulting in a reduction of VLDL secretion from the liver, as suggested by Carr et al.[36] Thus, the inhibition of hepatic ACAT may be one of the mechanisms through which compounds **2** and **3** exert their hypocholesterolemic effects.

The fecal neutral sterol and acidic sterol excretion were both highest in the **3**-supplemented group among the groups, as shown in the total fecal sterol. Thus, it is speculated that the elevated fecal bile acid excretion lowered the reabsorption of bile acid by the enterohepatic circulation, thereby lowering the bile acid pool and activating the bile acid-synthesizing enzyme in the liver. As an increase of bile acid excretion with a simultaneous inhibition of HMG-CoA reductase may stimulate the removal of blood cholesterol in vivo, the increased utilization of hepatic cholesterol seemed to result in a lower plasma cholesterol concentration. In addition, it would appear that the decrease in the plasma cholesterol concentration resulting from the compounds **2** and **3** supplements may have been due to the increased fecal sterol, which in turn led to a decreased absorption of dietary cholesterol. Although the intestinal ACAT activity was not measured in the current study, intestinal ACAT inhibitors are expected to reduce the plasma cholesterol level by inhibiting the intestinal esterification of cholesterol, thereby lowering cholesterol absorption in the small intestine.[37] Some ACAT inhibitors exhibit tissue specificity for both the intestine and the liver.[38]

## 4. Conclusion

The major implication of the current study is that supplementation with compound **3** markedly lowered the plasma total cholesterol and improved the fecal sterol excretion in high-cholesterol-fed rats. The plasma lipid-lowering action of **2** and **3** seemed to be due to the inhibition of hepatic HMG-CoA reductase and ACAT activities and increased fecal sterol excretion. Accordingly, supplementation with compound **2** and its synthetic derivative, **3** (0.066 mmol/100 g diet) may provide potential benefits to animals fed a high-cholesterol diet through improving the lipid metabolism. Compound **3**

also appeared to be an inhibitor of HMG-CoA reductase and ACAT activities and modulator of fecal sterol excretion in high-cholesterol-fed rats. However, more extensive investigation of compound **3** is still required to further elucidate the usefulness of this synthetic hesperetin derivative in animal models and humans.

## 5. Experimental

### 5.1. Synthesis of hesperetin 7-*O*-lauryl ether (3)

Compound **3**[14] was derived from compound **2**. A mixture of compound **2** (2.0 g, 6.6 mmol), 1-bromododecane (1.9 g, 7.6 mmol), and $Na_2CO_3$ (1.0 g, 9.4 mmol) was maintained at 80 °C for 14 h. The reaction mixture was then cooled to room temperature, diluted with water, extracted with ethyl acetate, washed with water, dried over $MgSO_4$, and concentrated in vacuo. Thereafter, the resultant solid was recrystallized from petroleum ether to yield 1.8 g of product, while the mother liquid was concentrated and chromatographed on a silica gel (6:1 hexane/EtOAc) to afford 0.5 g of additional product.

### 5.2. Spectral data of hesperetin 7-*O*-lauryl ether (3)

The structure of compound **3** was confirmed by NMR spectra. The $^1H$ and $^{13}C$ NMR spectra were recorded on a Varian Mercury 400 NMR spectrometer using the solvent peak as a reference ($^1H$ 7.25 ppm and $^{13}C$ 77.0 ppm of $CDCl_3$, respectively), while the HRFABMS was recorded on a high-resolution mass (JMS-700 Mstation, Jeol 1D) spectrometer. The structure was confirmed as shown in Figure 1. Compound **3** (hesperetin 7-*O*-lauryl ether): yellow solid; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$ 12.01 (s, 1H), 7.04 (d, $J = 2.0$ Hz, 1H), 6.93 (dd, $J = 8.4$, 2.0 Hz, 1H), 6.88 (d, $J = 8.0$ Hz, 1H), 6.05 (d, $J = 2.0$ Hz, 1H), 6.03 (d, $J = 2.8$ Hz, 1H), 5.32 (dd, $J = 12.8$, 2.8 Hz, 1H), 3.95 (t, $J = 6.4$ Hz, 2H), 3.91 (s, 3H), 3.07 (dd, $J = 17.2$, 2.8 Hz, 1H), 2.77 (dd, $J = 17.2$, 2.8 Hz, 1H), 1.75 (m, 2H), 1.26 (m, 18H), 0.88 (t, $J = 6.4$ Hz, 3H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 196.1, 167.8, 164.3, 163.0, 147.2, 146.1, 131.8, 118.4, 112.9, 110.8, 103.2, 95.8, 94.8, 79.1, 68.8, 56.3, 43.4, 32.1, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.1, 26.1, 22.9, 14.4 ppm; HRFABMS $m/z$ found for 471.2751 (calcd for $C_{28}H_{39}O_6$ 471.2747).

### 5.3. Animals and diets

Forty male Sprague-Dawley rats weighing between 50 and 55 g were purchased from Bio Genomics Inc. (Seoul, Korea), which shares research technology on the commercial production of experimental animals with the Charles Rivers Laboratory (Wilmington, USA). The animals were individually housed in stainless steel cages in a room with controlled temperature (24 °C) and lighting (alternating 12 h periods of light and dark). Upon arrival, all the rats were fed a pelletized commercial chow diet for 10 days and then randomly divided into four groups ($n = 10$). The rats were fed a 1% (wt/wt) high-cholesterol diet (control diet) or a diet supplemented with **1** (0.02% wt/wt), **2** (0.02% wt/wt), or **3** (0.03% wt/wt) for six weeks. The amount of **2** included in the diet was based on the results of a previous study,[11] which were also used to determine an equivalent amount of **3** at 0.066 mmol/100 g diet. The composition of the experimental diet was based on the AIN-76 semisynthetic diet.[39,40]

The animals were given food and distilled water ad libitum throughout the experimental period. The food consumption and weight gain were measured daily and weekly, respectively. The feces were collected during the last three days using metabolic cages and used to determine the fecal sterol. At the end of the experimental period, the rats were anesthetized with Ketamine following a 12 h fast. Blood samples were collected from the inferior vena cava to determine the plasma lipid profile, plus the livers were removed and rinsed with physiological saline. All samples were stored at −70 °C until analyzed.

### 5.4. Plasma and hepatic lipids

The plasma cholesterol and HDL-cholesterol concentrations were determined using a commercial kit (Sigma) based on a modification of the cholesterol oxidase method of Allain et al.[41] The HDL-fractions were separated using a Sigma kit based on the heparin-manganese precipitation procedure.[42] The plasma triglyceride concentrations were measured enzymatically using a kit from Sigma Chemical Co., a modification of the lipase-glycerol phosphate oxidase method.[43] The hepatic lipids were extracted using the procedure developed by Folch et al.[44] The dried lipid residues were dissolved in 1 mL of ethanol for the cholesterol and triglyceride assays. Triton X-100 and a sodium cholate solution (in distilled $H_2O$) were added to 200 μL of the dissolved lipid solution to produce final concentrations of 5 g/L and 3 mmol/L, respectively. The hepatic cholesterol and triglycerides were analyzed with the same enzymatic kit as used in the plasma analysis.

### 5.5. HMG-CoA reductase and ACAT activities

The microsomes were prepared according to the method developed by Hulcher and Oleson[45] with a slight modification. Two grams of liver tissue were homogenized in 8 mL of an ice-cold buffer (pH 7.0) containing 0.1 M of triethanolamine, 0.02 M of EDTA, and 2 mM of dithiothreitol, pH 7.0. The homogenates were centrifuged for 10 min at 10,000$g$ and then at 12,000$g$ at 4 °C. Next, the supernatants were ultracentrifuged twice at 100,000$g$ for 60 min at 4 °C. The resulting microsomal pellets were then redissolved in 1 mL of a homogenation buffer for protein determination[46] and finally analyzed for HMG-CoA reductase and ACAT activities.

Using freshly prepared hepatic microsomes, the HMG-CoA reductase activities were determined by slightly modifying the method of Shapiro et al.[47] An incubation

mixture (120 μL) containing microsomes (100–150 μg) and 500 nmol of NADPH (dissolved in a reaction buffer containing 0.1 M of triethanolamine and 10 mM of EDTA) was preincubated at 37 °C for 5 min. Next, 50 nmol of [$^{14}$C]HMG-CoA (specific activity; 2.1420 GBq/mmol; NEM™ Life Science Products, Inc., Boston, MA) was added and the incubation continued for 15 min at 37 °C. The reaction was terminated by the addition of 30 μL of 6 M HCl, then the resultant reaction mixture was incubated at 37 °C for a further 15 min to convert the mevalonate into mevalonolactone. Thereafter, the incubation mixture was centrifuged at 10,000*g* for 5 min, and the supernatant spotted on a Silica Gel 60 $F_{254}$ TLC plate with a mevalonolactone standard. The plate was then developed in benzene/acetone (1:1, v/v) and air-dried. Finally, the $R_f$ 0.3–0.6 region was removed by scraping using a clean razor blade, and the $^{14}$C radioactivity determined using a liquid scintillation counter (Packard Tricarb 1600TR; Packard Instrument Company, Meriden, CT). The results were expressed as pmol mevalonate synthesized per min per mg protein.

Using freshly prepared hepatic microsomes, the ACAT activities were determined according to the method developed by Erickson et al.[48] and modified by Gillies et al.[49] To prepare the cholesterol substrate, 6 mg of cholesterol and 600 mg of Tyloxapol (Triton WR-1339, Sigma) were each dissolved in 6 mL of acetone, mixed well, and completely dried in $N_2$ gas. The dried substrate was then redissolved in 20 mL of distilled water to a final concentration of 300 μg of cholesterol/mL. Next, reaction mixtures containing 20 μL of a cholesterol solution (6 μg of cholesterol), 20 μL of a 1 M potassium-phosphate buffer (pH 7.4), 5 μL of 0.6 mM bovine serum albumin, 50–100 μg of the microsomal fraction, and distilled water (up to 180 μL) were preincubated at 37 °C for 30 min. The reaction was then initiated by adding 5 nmol of [$^{14}$C]oleoyl-CoA (specific activity; 2.0202 GBq/mmol; NEM™ Life Science Products, Inc.) to a final volume of 200 μL; the reaction time was 30 min at 37 °C. The reaction was stopped by the addition of 500 μL of isopropanol/heptane (4:1, v/v), 300 μL of heptane, and 200 μL of 0.1 M potassium phosphate (pH 7.4), then the reaction mixture was allowed to stand at room temperature for 2 min. Finally, an aliquot (200 μL) of the supernatant was subjected to scintillation counting. The ACAT activity was expressed as pmol cholesteryl oleate synthesized per min per mg protein.

### 5.6. Fecal sterols

The fecal neutral sterols were determined using a simplified micro-method developed by Czubayk et al.[50] The gas–liquid chromatography was carried out using a Hewlett-Packard gas chromatograph (Model 5890; Palo Alto, CA) equipped with a hydrogen flame-ionization detector and Sac™-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film; Supelco Inc., Bellefonate, PA, USA). Helium was used as the carrier gas, the temperatures were set at 230 °C for the column and 280 °C for the injector/detector temperature, and 5-α-cholestane (Supelco Inc.) was used as the internal standard. The daily neutral sterol excretion was calculated based on the sum of the cholesterol, coprostanol, and coprostanone found in each sample. The fecal bile acid was extracted with *tert*-butanol and quantified enzymatically with 3-α-hydroxysteroid dehydrogenase.[51]

### 5.7. Statistical analysis

All data is presented as the mean ± SE. The data was evaluated by one-way ANOVA using an SPSS program, and the differences between the means assessed using Duncan's multiple-range test. Statistical significance was considered at $p < 0.05$.

## Acknowledgements

This work was supported by a grant from the Korean Ministry of Science and Technology (M1-0015-00-0012, 01-J-BP-01-B-66).

## References and notes

1. Smith, G. D.; Shipley, M. J.; Marmot, M. G.; Rose, G. *JAMA* **1992**, *267*, 70.
2. Rimm, E. B.; Katan, M. B.; Ascherio, A.; Stampfer, M. J.; Willett, W. C. *Ann. Int. Med.* **1996**, *125*, 384.
3. Yochum, L.; Kushi, L. H.; Meyer, K.; Folsom, A. R. *Am. J. Epidemiol.* **1999**, *149*, 943.
4. Benavente-Garcia, O.; Castillo, J.; Marin, F. R.; Ortuno, A.; Del Rio, J. A. *J. Agric. Food Chem.* **1997**, *45*, 4505.
5. Mouly, P. P. M.; Arzouyan, C. G.; Gaydou, E. M.; Estienne, J. M. *J. Agric. Food Chem.* **1994**, *42*, 70.
6. Rouseff, R. L.; Martin, S. F.; Youtsey, C. O. *J. Agric. Food Chem.* **1987**, *35*, 1027.
7. Middleton, E., Jr.; Kandaswami, C. *Biochem. Pharmacol.* **1992**, *43*, 1167.
8. Kimura, Y.; Kubo, M.; Tani, T.; Arichi, S.; Okuda, H. *Chem. Pharm. Bull.* **1981**, *29*, 2610.
9. Lee, S. H.; Jeong, T. S.; Park, Y. B.; Kwon, Y. K.; Choi, M. S.; Bok, S. H. *Nutr. Res.* **1999**, *19*, 1245.
10. Kim, H. K.; Jeong, T. S.; Lee, M. K.; Park, Y. B.; Choi, M. S. *Clin. Chim. Acta* **2003**, *327*(1–2), 129.
11. Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. *Lancet* **1993**, *342*, 1007.
12. Krol, W.; Czuba, Z.; Scheller, S.; Paradowski, Z.; Shani, J. *J. Ethnopharmacol.* **1994**, *41*, 121.
13. Ferriola, P. C.; Cody, V.; Middleton, E. *Biochem. Pharmacol.* **1989**, *38*, 1617.
14. Jeong, T. S.; Kim, E. E.; Lee, C. H.; Oh, J. H.; Moon, S. S.; Lee, W. S.; Oh, G. T.; Lee, S. K.; Bok, S. H. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2663.
15. Cortner, J. A.; Bennett, M. J.; Le, N.-A.; Coates, P. M. *J. Inherit. Metab. Dis.* **1993**, *16*, 127.
16. Gotto, A. M., Jr. *Hosp. Pract.* **1997**, *32*, 145.
17. National Cholesterol Education Program *Circulation* **1994**, *89*, 1329.
18. Havel, R. J.; Rapaport, E. *New Engl. J. Med.* **1995**, *332*, 1491.
19. Endo, A. *J. Lipid Res.* **1992**, *33*, 1569.
20. Nash, D. T. *Am. J. Cardiol.* **1996**, *78*(Suppl 6A), 26.

21. Montanari, A.; Chen, J.; Widmer, W. Citrus Flavonoids: A Review of Past Biological Activity Against Disease. In *Flavonoids in the Living System*; Manthey, J. A., Buslig, B. S., Eds.; Plenum: New York, 1998; pp 103–113.
22. Guthrie, N.; Carroll, K. K. Inhibition of Mammary Cancer by Citrus Flavonoids. In *Flavonoids in the Living System*; Manthey, J. A., Buslig, B. S., Eds.; Plenium: New York, 1998; pp 227–236.
23. Samman, S.; Wall, P. M. L.; Cook, N. C. Flavonoids and Coronary Heart Disease: Dietary Perspectives. In *Flavonoids in the Living System*; Manthey, J. A., Buslig, B. S., Eds.; Plenum: New York, 1999; pp 469–481.
24. Alberts, A. W.; Chen, J.; Kuron, G. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3957.
25. Amin, D.; Gustafson, S. K.; Weinacht, J. M. *Pharmacology* **1993**, *47*, 13.
26. Alberts, A. W. *Am. J. Cardiol.* **1988**, *62*, 10J.
27. Billheimer, D. W.; Grundy, S. M.; Brown, M. S.; Goldstein, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4124.
28. Krause, B. R.; Bocan, T. M. A. ACAT inhibitors: Physiologic mechanisms for hypolipidemic and anti-atherosclerotic activities in experimental animals. In *Inflammation, Mediators, and Pathways*; Ruffolo, R. R., Hollinger, M. A., Eds.; CRC: Boca Raton, FL, 1995; p 173.
29. Bocan, T. M. A.; Bak-Mueller, S.; Uhlendorf, P. D.; Newton, R. S.; Krause, B. R. *Arterioscler. Thromb.* **1991**, *11*, 1830.
30. Wilcox, L. J.; Borradaile, N. M.; Dreu de, L. F.; Huff, M. W. *J. Lipid Res.* **2001**, *42*, 725.
31. Largis, E. F.; Wnag, C. H.; DeVries, V. G.; Schaffer, S. A. *J. Lipid Res.* **1989**, *30*, 681.
32. Williams, R. J.; McCarthy, A. D.; Sutherland, C. D. *Biochim. Biophys. Acta* **1989**, *1003*, 213.
33. Marezetta, C. A.; Savoy, Y. E.; Ferrman, A. M.; Long, C. A.; Pettini, J. L.; Hagar, R. E.; Inskeep, P. B.; Davis, K.; Stucchi, A. F.; Nicolosi, R. J.; Hamanaka, E. S. *J. Lipid Res.* **1994**, *35*, 1829.
34. Borradaile, N. M.; Carroll, K. K.; Kurowska, E. M. *Lipids* **1999**, *34*, 591.
35. Ishida, F.; Sata, A.; Iizuka, Y.; Kamei, T. *Chem. Pharm. Bull.* **1989**, *37*, 1635.
36. Carr, T. P.; Park, J. S.; Rudel, L. L. *Arterioscler. Thromb.* **1992**, *12*, 1274.
37. Suckling, K. E.; Stange, E. F. *J. Lipid Res.* **1985**, *26*, 637.
38. Krause, B. R.; Anderson, M.; Bisgaier, C. L.; Bocan, T.; Bousley, R.; Dehart, P.; Essenberg, A.; Hamelehle, K.; Homan, R.; Kieft, K.; Mcnally, W.; Stanfield, R.; Newton, R. S. *J. Lipid Res.* **1993**, *34*, 279.
39. American Institute of Nutrition *J. Nutr.* **1977**, *107*, 1340.
40. American Institute of Nutrition *J. Nutr.* **1980**, *110*, 1717.
41. Allain, C. C.; Poon, L. S.; Chan, C. S. G. *Clin. Chem.* **1974**, *20*, 470.
42. Waenic, R. G.; Albers, J. J. *J. Lipid Res.* **1978**, *19*, 65.
43. McGowan, M. W.; Artiss, J. D.; Strandbergh, D. R.; Zak, B. *Clin. Chem.* **1983**, *29*, 538.
44. Folch, J.; Lees, M.; Sloan-Stanley, G. H. *J. Biol. Chem.* **1957**, *226*, 497.
45. Hulcher, F. H.; Oleson, W. H. *J. Lipid Res.* **1973**, *14*, 625.
46. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
47. Shapiro, D. J.; Nordstrom, J. L.; Mitschelen, J. J.; Rodwell, V. W.; Schimke, R. T. *Biochim. Biophys. Acta* **1974**, *370*, 369.
48. Erickson, S. K.; Schrewsbery, M. A.; Brooks, C.; Meyer, D. J. *J. Lipid Res.* **1980**, *21*, 930.
49. Gillies, P. J.; Rathgeb, K. A.; Robinson, C. S. *Exp. Mol. Path.* **1986**, *44*, 320.
50. Czubayk, F.; Beumers, B.; Lutjohann, D.; von Bergmann, K. *J. Lipid Res.* **1992**, *32*, 1861.
51. Michael, J. C.; Ian, A. M. *Clin. Chem.* **1980**, *26*, 1298.