Human ACAT Inhibitory Effects of Flavonoids from *Sophora flavescens*

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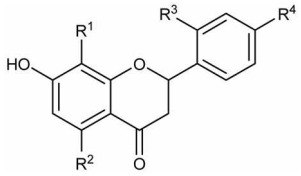
Acyl-coenzyme A:cholesterol acyltransferase (ACAT, EC 2.3.1.26) is the primary cellular enzyme that catalyzes the formation of cholesteryl esters from cholesterol and fatty acyl-coenzyme A.[1] It has been identified that two isoenzymes, ACAT-1 and ACAT-2, have different cell locations, membrane orientations, and metabolic functions.[2] Atherosclerotic lesions are associated with the accumulation of cholesteryl ester lipid droplets *via* ACAT-1 in macrophages-derived foam cells.[1,3] In contrast, ACAT-2 plays a key role in the intestinal absorption of cholesterol and the assembly of very low density lipoprotein (VLDL) in the liver.[4] Therefore, ACAT inhibitors may act as antihypercholesterolemic and antiathero-sclerotic agents. For the past 20 years, numerous ACAT inhibitors have been developed,[5,6] and many of them showed promise in animal studies by inhibiting intestinal or hepatic ACAT and lowering plasma cholesterol levels.[7-9] But several clinical studies showed that ACAT inhibitors lacked efficacy for lowering cholesterol,[10,11] suggesting that ACAT inhibitors with different preferences on ACAT-1 and ACAT-2 may exert more favorable effects on atherosclerosis.

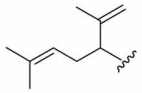
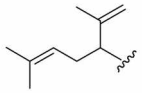
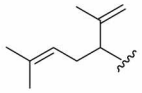
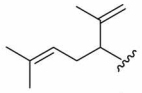
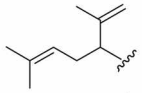
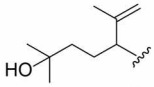
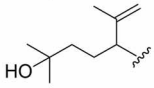
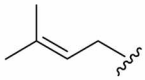
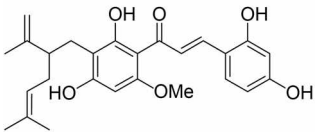
Recently, we reported that sesquineolignan, saucerneol B, and dineolignans, manassantin A and B, were isolated by bioassay-guided fractionation of the methanolic extracts of the root of *Saururus chinensis* and showed specificity of inhibitory activity against hACAT-1 and -2.[12] More recently, we found that shikonin derivatives isolated from *Lithospermum erythrorhizon* roots inhibited hACAT-1 and -2 activities.[13] During search for new ACAT inhibitors from natural sources, we found the ethyl acetate extracts of the *S. flavescens* exhibited significant hACAT inhibitory activity (69% inhibition at 100 μg/mL for hACAT-1, 62% inhibition at 100 μg/mL for hACAT-2, respectively). *S. flavescens*, which is polyphenol-rich plant from the legume family known to possess numerous medicinal properties such as antipyretic, analgesic, anthelmintic, stomachic, and glycosidase inhibitory activities,[14,19,20] but there is no report as to inhibit ACAT activity. In this study, we isolated nine flavonoids [sophoraflavanone G (**1**), (−)-kurarinone (**2**), leachianone A (**3**), kushenol A (**4**), (2*S*)-2-methoxykurarinone (**5**), kushenol T (**6**), kurarinol (**7**), isoxanthohumol (**8**), and kuraridin (**9**)][14-18] from the roots of *S. flavescens* and describe hACAT-1 and -2 inhibitory activities of compounds **1-9** in vitro assay system and compounds **3**, **4**, and **9** in cell-based assay system.

Results and Discussion

The biological activities of flavanones **1-8** and chalcone **9** were assessed against expressed hACAT-1 or -2 from Hi5 cells[21] and confirmed by the positive control with oleic acid anilide which inhibited hACAT-1 and -2 with IC_{50} values of 0.14 and 0.17 μM, respectively. The biological data for **1-9** has been shown in Table 1. Sophoraflavanone G (**1**) inhibited hACAT-1 and -2 in 49.9% and 32.9%, respectively, at 25 μM. Methylated hydroxyl group at C-5 position in (−)-kurarinone (**2**) is almost same as that of compound **1**. Interestingly, leachanone A (**3**) which was substituted with methoxy group at C-2' position exhibited higher activity against both hACAT-1 and -2, with IC_{50} values of 23.8 and 37.8 μM, respectively, whereas absence hydroxyl group at C-4' position in kushenol A (**4**) showed slightly an attenuated potency, with IC_{50} values of 35.2 and 56.5 μM, respectively. However, (2*S*)-2'-methoxykurarinone (**5**) having methoxy group at C-5 position was less potent in both of enzymes. Kushenol T (**6**) involving hydroxyl group at C-8" position exhibited significantly decreased activity against both hACAT-1 and -2 compared with the corresponding analogue kushenol (**4**). Also, kurarinol (**7**) substituted with hydroxyl group at C-8" position showed an attenuated potency. Prenyl flavanone, isoxanthohumol (**8**), resulted in a significantly loss in potency. Kuraridin (**9**) having chalcone moiety showed inhibitory activity with IC_{50} values of 38.7 and 22.1 μM, respectively. To investigate that these compounds function as ACAT inhibitor in physiological condition, we established stable cell line expressing hACAT-1 or -2 from AC-29 cells, ACAT-deleted CHO cells, using Flp-In™ system.[13] The cell-based ACAT assay was performed using NBD cholesterol which property to be strongly fluorescent in a neutral cholesteryl ester form and weakly fluorescent as a free cholesterol form. Among compounds **1-9**, the most potent ACAT inhibitors, compounds **3**, **4**, and **9** were tested in cell-base fluorescent ACAT assay system. As shown in Table 1, compound

Table 1. ACAT inhibitory activities of flavonoids (**1-9**) in *in vitro*[a] and in cell-base[b] assay system



Compound	R^1	R^2	R^3	R^4	Inhibition (%)[c]	
					hACAT-1	hACAT-2
1		OH	OH	OH	49.9	32.9
2		OMe	OH	OH	28.1	32.7
3		OH	OMe	OH	23.8 μM[a] 16.4 μM[b]	37.8 μM[a] 13.6 μM[b]
4		OH	OH	H	35.2 μM[a] 19.7 μM[b]	56.5 μM[a] 20.4 μM[b]
5		OMe	OMe	OH	30.1	19.7
6		OH	OH	H	35.8	32.8
7		OMe	OH	OH	30.2	29.0
8		OMe	H	OH	12	16
9					38.7 μM[a] 8.9 μM[b]	22.1 μM[a] 6.4 μM[b]
10	Oleic acid amide				0.14 μM[a]	0.17 μM

[a]*In vitro* and [b]In cell-base ACAT inhibitory activity was measured using expressed human ACAT-1 and -2. Data are shown as mean values of two independent experiments performed in duplicate. [c]Percentage at 25 μM.

3 inhibited significantly the enzymatic activities of both hACAT-1 and -2 in cell-based assay system with even lower IC_{50} values (IC_{50} = 16.4 μM for hACAT-1 and IC_{50} = 13.6 μM for hACAT-2) than that of in vitro assay system. Also, compound **4** exhibited a slight decrease in potency in both hACAT-1 and -2 with IC_{50} values of 19.7 and 20.4 μM, respectively. In addition, the lavandulyl chalcone, kuraridin (**9**), showed a significantly increase in potency in both hACAT-1 and -2 with IC_{50} values of 8.9 and 6.4 μM, respectively. However, these compounds **3**, **4**, and **9** exhibited no selectivity against hACAT isoforms in both assay systems.

According to these results, it was found that the inhibitory potency was strongly dependent on the lipophilicity of functional groups, such as methoxy, hydroxyl, and lavandulyl groups. Especially, different inhibitory potency of flavanone derivatives **4** and **6** against hACAT-1 and -2 may be resulted from the degree of lipophilicity. Lavanduly chain at C-8 position on **4** could increase a chance to interact with catalytic site of hACAT-1 and -2 located in the hydrophobic membrane, whereas C-8"-hydroxyl lavanduly at C-8 on **6** resulted in a significant lose in potency.

Conclusion

In summary, we have discovered a new class of hACAT inhibitors. Nine flavonids **1-8** and chalcone **9** were isolated from the MeOH extracts of roots of *S. flavescens* and tested for their inhibitory activities against hACAT-1 or hACAT-2. Among them, compounds **3**, **4**, and **9** exhibited hACAT-1 and -2 inhibitory activities. Further structural-activity relationship (SAR) studies are being pursued to find more potent and selective inhibitors.

Experimental Section

General Experimental Methods. All purifications were monitored by TLC using commercially available glass-backed plates. Column chromatography was carried out using 230-400 mesh silica gel. Melting points were measured on a Thomas Scientific Capillary Point Apparatus and are uncorrected. Infrared spectra were recorded on a Bruker IFS 66. Proton and carbon nuclear magnetic resonance spectra were measured downfield relative to tetramethylsilane in $CDCl_3$, MeOD and DMSO-d_6 unless otherwise noted (values in ppm). ^{1}H- and ^{13}C-NMR experiments were conducted on a Bruker DRX-500. Elemental analyses were performed on Leco CHNS-932. CD spectra were recorded on a JASCO J-715 spectropolarimeter. Absorption measurements were redored using an ADAP-2020 spectrophotometer. [1-^{14}C] Oleoyl-CoA was purchased from Amersham Biosciences Korea. Cell culture media and reagents were from Invitrogen. NBD-cholesterol was from molecular probe. HPLC grade organic solvents were from Merck. All the other reagent grade chemicals were purchased from the Sigma.

Plant Material. *Sophora flavescens* was collected in Hamyang (Republic of Korea) and identified by Prof. Myon Gi Chung. A voucher specimen (Park, K. H. 112) of this raw material is deposited at Herbarium of Gyeongsang National University (GNUC).

Extraction and Isolation. Dried roots of *S. flavescens* (2.4 kg) were air-dried, pulverized, and extracted with methanol for a week at room temperature. The combined methanol extract was concentrations in vacuo to yield a dark orange gum (53 g). Vacuum liquid chromatography (VLC) of methanol extract on celite was performed using $CHCl_3$ and MeOH. The $CHCl_3$ fraction (23 g) was chromatographed on silica gel (4 × 60 cm, 230-400 mesh, 400 g) using hexane/EtOAc [80:1 (0.1 L), 40:1 (0.1 L), 20:1 (0.1 L), 10:1 (0.2 L), 5:1 (0.4 L), 1:1 (1 L)] mixtures to furnish fractions A-G. Fraction C (2.9 g) was subjected to silica gel chromatography (2 × 50 cm) eluting with hexane/EtOAc (40:1 → 1:2) to give five fractions (fr. C.1-C.5); fr. C.2 was resubjected to silica gel chromatography with hexane/acetone (30:1 → 1:1) followed by column chromatography on Sephadex LH-20 [elunt: MeOH (2 × 90 cm)] to yield compound **1** (19 mg) and subsequently compound **4** (28 mg) was isolated from fr. C.4. Fr. D (2.7 g) was applied to a silica gel column (2 × 60 cm, 230-400 mesh, 120 g) [eluent:hexane/EtOAc (20:1 → 1:1)] to afford three subfractions fr. D.1-D.3. Fr. D.2 was resubjected to silica gel column {(1.5 × 30 cm, 230-400 mesh, 90 g) [eluent: hexane/Et_2O (40:1 → 1:2)]} to yields compound **3** (8 mg). Fr E (3.3 g) was subjected to silica gel column (2 × 60 cm, 230-400 mesh, 180 g) chromatography with hexane/EtOAc (10:1 → 1:1) and then purified by an second flash silica gel column (1 × 30 cm, 230-400 mesh, 80 g) using a gradient of hexane/EtOAc [40:1 (80 mL), 20:1 (80 mL), 10:1 (100 mL), 8:1 (100 mL), 6:1 (200 mL), 4:1 (200 mL), 2:1 (200 mL), 1:1 (400 mL)] to yield compound **2** (32 mg). Fraction F (4.7 g) was subjected to silica gel column chromatography [eluent: $CHCl_3$/acetone (40:1 → 1:1)] followed by Sephadex LH-20 [elunt: MeOH (2 × 90 cm)] yielding **5** (40 mg) and compound **8** (27 mg). MeOH phase (28 g) was chromatographed on silica gel {(6 × 60 cm, 230-400 mesh, 800 g, eluent: $CHCl_3$/acetone [40:1 (1 L), 30:1 (1 L), 20:1 (1 L), 10:1 (1 L), 5:1 (1 L), 1:1 (3 L)]; followed by $CHCl_3$/MeOH [20:1 (1 L), 10:1 (1 L), 5:1 (1 L), 1:1 (3 L)]} to give fraction A-I. Fr. G (3.1 g) was applied to a silica gel column [3 × 60 cm, 230-400 mesh, 250 g, eluent: $CHCl_3$/MeOH (90:1 → 4:1)] to afford seven subfractions fr. G.1-G.7. Fr. G.3-G.5 were subjected to silica gel column [3 × 70 cm, 230-400 mesh, 280 g, eluent: $CHCl_3$/MeOH (60:1 → 2:1)] to yields compound **9** (12 mg). Fr. H (2.2 g) was chromatographed on silica gel [eluent: $CHCl_3$/MeOH (20:1 → 1:1)] to yield compound **6** (34 mg). Fr. I was repeatedly chromatogaphed over silica gel using [eluent: $CHCl_3$/MeOH (20:1 → 1:1)] and then on Sephadex LH-20 [elunt: MeOH (2 × 90 cm)] to yield compound **7** (26 mg). All of isolated compounds (**1-9**) were agree with those previously published.[14,18]

ACAT Activity Assay. The rate of incorporation of oleoyl-CoA into cholesteryl ester was determined using the expressed hACAT-1 or hACAT-2 according to the method described. Microsomal fractions of Hi5 cells containing baculovirally expressed hACAT-1 or hACAT-2 were used as the sources of enzymes. Generation of cell lines expressing hACAT-1 or hACAT-2: We generated stable cell line expressing human ACAT-1 or -2 (Accession No. BC028940 and AF059203 for ACAT-1 and ACAT-2, respectively) from AC-29 cells, ACAT-deleted CHO cells, using Flp-In™ system (Invitrogen) according to the manufacturer's suggestion. AC-29 cells were kindly gifted from Dr. Rudel, L. L. Briefly, Flp-In AC-29 cells containing five copies of integrated FRT sequence, were generated by transfection of pFRT/lacZeo into AC-29 cells. FRT site-integrated clones were selected by 100 mg/mL zeocin (invitrogen) for 1 week. Several colonies were picked and maintained in F-12 medium supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL zeocin. The presence and number of FRT-site was verified by β-galactosidase assay and southern blot analysis, respectively. To construct stable cell lines expressing hACAT-1 or hACAT-2, Flp-In AC-29 cells were plated on 60-mm dishes and transfected with 1.8 μg of the Flp-In recombinase-encoding pOG44 vector and either 0.2 μg of pcDNA5/FRT-hACAT1 or pcDNA5/FRT-hACAT-2. After 2 days, we proceeded to select the single colony with 500 μg/mL hygromycin (Invitrogen) for 2 weeks. Colonies were picked up, expanded, and assayed for expression of hACAT-1 or -2. Established cell lines were maintained in F-12/10% FBS containing 100 μg/mL hygromycin.

Cell-based fluorescent ACAT assay: A total of 30,000 cells per well were plated on 96-well culture plates and allowed to recover overnight. Assays were done with cells at least 80% confluent. Cells were incubated in Ham's F-12 medium supplemented with 1% Eagle's vitamins and 10% heat-inactivated FBS containing 5 μg/mL NBD-cholesterol as methyl-β-cyclodextrin complex with or without ACAT inhibitor for 9 h. NBD-cholesterol was added from a 5 mg/

mL stock solution in methanol, and methanol concentrations in the medium did not exceed 0.1%. Flp-In AC-29 cells incubated with NBD-cholesterol were used to determine background fluorescence attributable to free NBD-cholesterol. After incubation, the medium was removed, and the cells were washed two times with cold balanced salt solution (BSS, Invitrogen). The fluorescent intensities of 96-well culture plates were read from the top using VICTOR3 fluorescent plate reader (PerkinElmer) equipped with 485-nm excitation and 535-nm emission filters. After measurement, cellular protein was digested through incubation with 25 μL of 0.4 N NaOH for 2 h. The fluorescent intensity was normalized by cellular protein content and calculated by subtracting the background fluorescent intensity from the total fluorescent intensity.

Methyl-β-cyclodextrin (MbCD)-NBD-cholesterol complex preparation: One hundred μM of MβCD was dissolved in 10 mL of F-12 medium, and 6 μM NBD-cholesterol and 10 μg/mL BSA was added to this solution with vigorous vortex. The dispersion was sonicated at room temperature for 5 min using a Fisher model 60 sonic dismembrator at setting 5. The solution was filtered with 0.45-μm membrane filter and kept in a glass tube under argon at 4 °C for up to a week.

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