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Article

Human Acyl-CoA: Cholesterol Acyltransferase Inhibitory Effect of Flavonoids from Roots of *Glycine max* (L.) Merr.

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Isoflavones 1-3 and pterocarpans 4-8 were isolated from methanol extract of roots of *Glycine max*. In inhibitory effect against human acyl-CoA: cholesterol acytransferase (ACAT)-1 and ACAT-2, glyceollin I 5 showed potent hACAT-1 (IC_{50} = 29.0 μM) and hACAT-2 (IC_{50} = 82.7 μM) inhibitory activities.

Key wards: *Glycine max (L.) Merr., acyl-CoA, cholesterol acytransferase (ACAT), atherosclerosis, pterocarpan, glyceollin I*

During the last decades numerous papers have been published on soybeans, which are widely cultivated globally and their potential health effects including anti-cancer,[1,2] lowing of serum cholesterol level,[3,4] prevention of coronary heart disease,[5-7] and antiatherosclerotic activity.[8,9] In particular, flavonoids and saponins are the major components responsible for the biological activities of soybean.[10-14] However, even though there are many valuable secondary metabolites in soybean, many works have mainly focused on three isoflavones, genistein, daidzein, and glycitein.[15-17] Thus, evaluation of the biological function of other flavonoids in soybean and its roots are very important to level up the value of soybean as dietary supplement as well as the material of functional food material. Recently, we reported that pterocarpans and isoflavones from roots of *Glycine max* (L.) Merr. have a potent LDL oxidation inhibitory activities.[18] In particular, pterocarpan isolated from roots of *G. max* showed potent hACAT-1 and hACAT-2 inhibitory activities.

Acyl-CoA: cholesterol acyltransferase (ACAT, E.C. 2.3.1.26) is an allosteric enzyme that catalyzes the acylation of cholesterol into cholesteryl esters with long chain fatty acids,[19] which are very attractive target for the prevention and treatment of cardiovascular diseases and hypercholesterolemia.[20] Studies have found that ACAT is present as two isoforms, ACAT-1 and ACAT-2, in mammalian species,[21,22] particularly, in humans; ACAT-1 plays a considerable role in foam cell formation in macrophage, whereas ACAT-2 is in charge of the cholesterol absorption process in intestinal enterocytes.[23]

In this study, potential inhibitory effects of compounds **1-8** against hACAT-1 and hACAT-2 were for the first time evaluated from *G. max*. In particular, glyceollin I **5** belonging to pterocarpan showed significant hACAT-1 and hACAT-2 inhibitory activities.

Materials and Methods

Plant Material. The roots of *G. max* (Taekwangkong) were collected ten days after R8 at Moonsan, Jinju, Korea at the end of September 2003 and dried.

Instruments. All purifications were monitored by TLC (E. Merck Co., Darmstadt, Germany), using commercially available glass-backed plates sprayed with *p*-anisaldehyde solution. Column chromatography was carried out using 230-400 mesh silica gel (kieselgel 60, Merck, Germany). Melting points were measured on a Thomas Scientific capillary melting point apparatus (Electrothermal 9300, UK) and are uncorrected. IR spectra were recorded on a Bruker IFS66 (Bruker, karlsruhe, Germany) infrared Fourier transform spectrophotometer (KBr) and ^{1}H- and ^{13}C-NMR along with 2D-NMR data were obtained on a Bruker AM 500 (^{1}H-NMR at 500 MHz, ^{13}C-NMR at 125 MHz) spectrometer (Bruker, karlsruhe, Germany) in $CDCl_3$, acetone-d_6, DMSO-d_6, and CD_3OD. EIMS was obtained on a JEOL JMS-700 mass spectrometer (JEOL, Tokyo, Japan). All reagent-grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Extraction and isolation. Extraction and isolation methods of compounds **1-8** were previously reported.[18] The structures of compounds **1-8** were confirmed by spectroscopic data and herein, we report ^{1}H NMR of compounds **1-3**, which were

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Abbreviations: hACAT, Human ACAT; DEPT, distortionless enhancement by polarization transfer; HMBC, heteronuclear multiple-bond connectivity

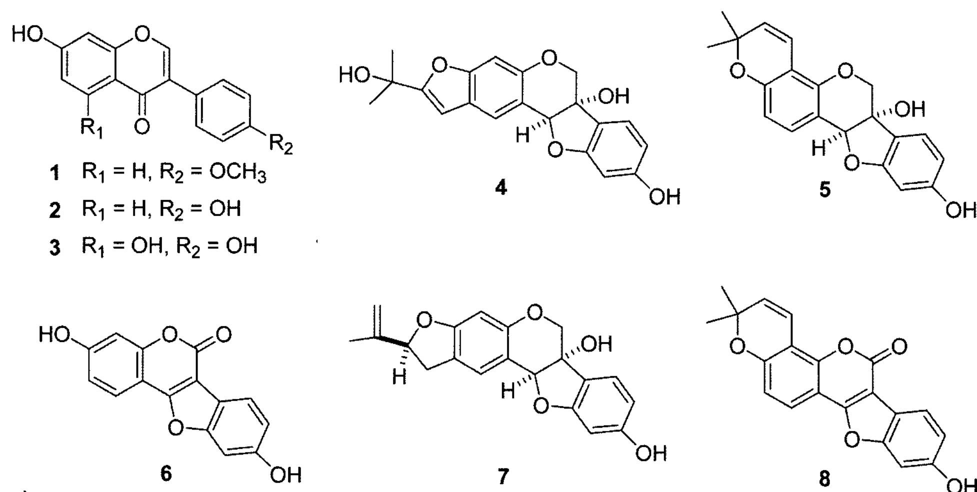

Fig. 1. Structures of isolated compounds 1-8 isolated from roots of *Glycine max* (L.) Merr..

compared with those previously reported.[24-26] In addition, we report ^{1}H NMR, ^{13}C NMR, and HMBC spectroscopic data of the significant hACAT inhibitor **5**.

Compound **1**: amorphous yellow powder; mp 264-267°C; IR (KBr) ν_{max} 3405, 1634, 1480 cm^{-1}; UV λ_{max} nm 302, 248, 205 (MeOH); ^{1}H NMR (500 MHz, acetone d_6) δ 3.79 (3H, s, OCH_3), 6.88 (1H, d, *J* = 2.3 Hz, H-8), 6.96 (1H, d, *J* = 8.8 Hz, H-6), 6.99 (2H, d, *J* = 8.9 Hz, H-3 and H-5), 7.52 (2H, d, *J* = 8.9 Hz, H-2 and H-6), 7.99 (1H, d, *J* = 8.8 Hz, H-5), and 8.34 (1H, s, H-2).

Compound **2**: yellow neddles; mp 317-320°C; IR (KBr) ν_{max} 3412, 1645 cm^{-1}; UV λ_{max} nm 303, 259, 249, 238 (MeOH); ^{1}H NMR (500 MHz, DMSO d_6) δ 6.85 (1H, d, *J* = 2.0 Hz, H-8), 6.93 (1H, dd, *J* = 8.8, 2.1Hz, H-6), 6.99 (2H, d, *J* = 8.6 Hz, H-3 and H-5), 7.38 (2H, d, *J* = 8.6 Hz, H-2 and H-6), 7.96 (1H, d, *J* = 8.8 Hz, H-5), and 8.28 (1H, s, H-2).

Compound **3**: amorphous yellow powder; mp 297-299°C; IR (KBr) ν_{max} 3414, 1653, 1570 cm^{-1}; UV λ_{max} nm 305, 262 (MeOH); ^{1}H NMR (500 MHz, DMSO d_6) δ 6.12 (1H, d, *J* = 2.1 Hz, H-6), 6.28 (1H, d, *J* = 2.1 Hz, H-8), 6.99 (2H, dd, *J* = 6.7, 1.8 Hz, H-3 and H-5), 7.28 (2H, dd, *J* = 6.7, 1.8 Hz, H-2 and H-6), 8.20 (1H, s, H-2), and 12.8 (1H, s, 5-OH).

Compound **5**: amorphous yellow powder; mp 102-104°C; EIMS *m/z* (relative intensity) 338 (M^{+}, 22%), 323 (100%), 321 (38%), 280 (6%); IR (KBr) ν_{max} 3460, 1860, 1650 cm^{-1}; UV λ_{max} nm, 350, 298, 280, 262, 230 (EtOH); The ^{1}H NMR and ^{13}C NMR spectroscopic data are shown in Table 1.

Inhibitory activity against Acyl-CoA: cholesterol acyltransferase (ACAT) The microsomal fractions of Hi5 cells containing baculovirally expressed hACAT-1 and hACAT-2 were used as the enzyme sources.[27] The hACAT-1 and hACAT-2 activity assays were measured according to a previously described method with slight modifications.[28] The tubes were sequentially added 4 μ*l* microsomes (8 mg/m*l* protein), 20 μ*l* KH_2PO_4 buffer (0.5 M, pH 7.4) with 10 mM dithiothreitol, 15 μ*l* bovine serum albumin (fatty acid-free, 40 mg/m*l*), 41 μ*l* water, 10 μ*l* test compounds, and 2 μ*l* cholesterol in acetone (20 μg/m*l*), and mix (total volumn: 92 μ*l*). The tube containing the reaction mixture was incubated for 20 min in a shaking water bath set at 37°C. After adding 8 μ*l* [1-^{14}C] oleoyl-CoA solution (0.05 μCi, final con. 10 μM), the tube was vortexed and placed back into the water bath for 25 min at 37°C. To stop the reaction, 1.0 m*l* isopropanol-heptane (4 : 1; v/v) solution was added to the tube. A solution mixture of 0.6 m*l* heptane and 0.4 m*l* potassium phosphate buffer (0.1 M, pH 7.4) with 2 mM dithiothreitol was added to the terminated reaction mixture. After centrifugation for 2 min, cholesterol oleate was recovered in the upper phase (total volume 0.9-1.0 m*l*). The radioactivity in 100 μ*l* of the upper phase was measured in a 3 m*l* liquid scintillation vial with 3 m*l* scintillation cocktail (Lipoluma, Lumac Co.) using a liquid scintillation counter (1450 Micerobeta Trilux Wallac Oy, Turku, Finland). The ACAT activity was expressed as a defined unit, cholesteryl oleate pmol/min/mg protein.

Results and Discussions

Structural identifications of eight compounds were carried out by interpretation of spectral data.[18] In partically, significant hACAT inhibitor, compound **5**, was obtained as yellow amorphous powder, and a molecular ion peak at *m/z* 338. The structure of **5** was inferred from a detailed analysis of ^{1}H- and ^{13}C-NMR data, together with 2D-NMR experiments (Table 1). The ^{1}H-NMR spectrum of **5** showed the presence of 16 protons as 2 methyl protons [δ 1.36 (1H, s, H-15) and 1.39 (1H, s, H-16)], 7 aromatic protons [δ 5.52 (1H, d, *J* = 10.0 Hz, H-13), 6.23 (1H, s, H-10), 6.29 (1H, d, *J* = 8.1 Hz, H-8), 6.55 (1H, d, *J* = 10.0 Hz, H-12), 6.49 (1H, d, *J* = 8.4 Hz, H-2), 7.05 (1H, d, *J* = 8.1 Hz, H-7), and 7.15 (1H, d, *J* = 8.4 Hz, H-1)], AB quartet protons [δ 3.95 (1H, d, *J* = 6.9 Hz, H-6α) and 4.15 (1H, d, *J* = 6.9 Hz, H-6β)], and 1 proton [δ 5.18 (1H, s, H-11α)]. In addition, ^{13}C-NMR data with DEPT experiments showed the presence of 20 carbon atoms as 8 methins [δ (85.5, C-11a), (99.2, C-10), (109.2, C-8), (111.5, C-2), (116.8,

Table 1. NMR data of compound 5 (500 MHz, CD_3Cl)[a]

Position	^1H	$^{13}C^b$	HMBC
1	7.15 (1H, d, J = 8.4 Hz)	131.3 (d)	H-2, H-11a
2	6.49 (1H, d, J = 8.4 Hz)	111.5 (d)	
3		154.4 (s)	H-1, H-2, H-12
4		110.7 (s)	H-1, H-2, H-12, H-13
4a		150.7 (s)	
5			
6	6α, 3.95 (1H, d, J = 6.9 Hz) 6β, 4.15 (1H, d, J = 6.9 Hz)	70.1 (t)	
6a		77.1 (s)	H-7
6b		120.3 (s)	H-8, H-11a, H-6α/β, H-10
7	7.05 (1H, d, J = 8.1 Hz)	124.6 (d)	
8	6.29 (1H, d, J = 8.1 Hz)	109.2 (d)	H-10
9		161.1 (s)	H-7, H-8, H-10
10	6.23 (1H, s)	99.2 (d)	H-7, H-10
10a		158.8 (s)	H-7, H-10
11			
11a	5.18 (1H, s)	85.5 (d)	H-1, H-6α/β
11b		112.7 (s)	H-11a
12	6.55 (1H, d, J = 10.0 Hz)	116.8 (d)	H-13
13	5.52 (1H, d, J = 10.0 Hz)	129.8 (d)	
14		76.8 (s)	H-12, H-13, H-15, H-16
15	1.36 (3H, s)	28.2 (q)	H-12, H-13
16	1.39 (3H, s)	28.2 (q)	H-12, H-13

[a]Assignments were made by ^1H-^1H COSY, HMQC, and HMBC data. [b]Multiplicity was established from DEPT data.

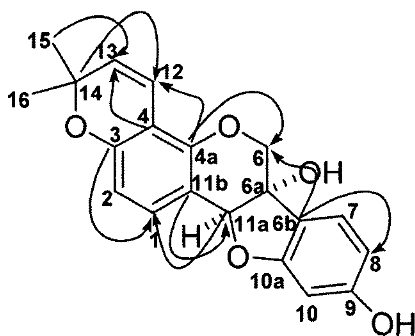

Fig. 2. Important HMBC correlations in compound 5.

C-12), (124.6, C-7), (129.8, C-13), and (131.3, C-1)], one methylene (70.1, C-6), and nine quaternary carbons [δ (76.8, C-17), (77.1, C-6α), (110.7, C-4), (112.7, C-11β), (120.3, C-6β), (154.4, C-3), (150.7, C-4α), (161.1, C-9), and (158.8, C-10α)] and 2 methyl carbons (28.2, C-15 and 16). The spectrum showed a set of proton signals AB quartet [δ 3.95 (1H, d, J = 6.9 Hz, H-6α), 4.15 (1H, d, J = 6.9 Hz, H-6β)], and 5.18 (1H, s, H-11α) a characteristic of pterocarpan, as well as the presence of an ABX-type aromatic proton system appearing at H-1 and H-2 due to the A-ring protons, and AB-type aromatic proton signals resonating at H-7, H-8, and H-10 due to the B-ring protons. The remaining signals at δ 6.55 (1H, d, J = 10.0 Hz), 5.52 (1H, d, J = 10.0 Hz), and δ (3H, 1.36 and 1.39) were due to olefinic protons H-12 H-13, and two methyl group of

Table 2. Human ACAT inhibitory activities of compounds 1-8

Compound	Inhibition[a]	
	hACAT-1	hACAT-2
1	35%[b]	NI
2	20%	NI
3	NI	NI
4	25%	NI
5	29.0[c]	82.7
6	37%	22%
7	NI	NI
8	NI	NI
Oleic acid anilide[d]	0.14[c]	0.17

[a]*In vitro* ACAT inhibitory activity was measured using expressed hACAT-1 and hACAT-2. Data are shown as mean values of two independent experiments performed in duplicate. [b]Percentage at 25 μg/m*l*. [c]IC_{50} (μM). [d]Oleic acid anilide was used as a positive control. NI = no inhibition.

the chromene ring. The correction of H-12 to C-3, C-4, and C-4α suggested that the chomene ring was connected to C-3 and C-4 (Fig. 2). Important HMBC correlations of compound **5** were observed in HMBC spectrum and the complete HMBC data (Table 1) and comparison with the data described in the literature confirmed the structure of glyceollin I (**5**).[29]

In search of new biological functions, we found that pterocarpan showed potent hACAT-1 and hACAT-2 inhibitory

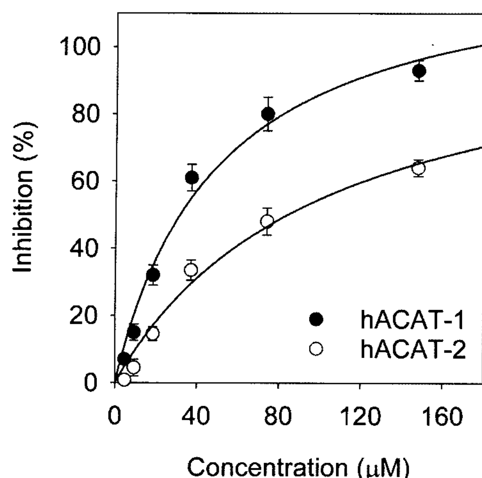

Fig. 3 Effects of compound 5 on the activities of hACAT-1 and hACAT-2 inhibitory activities. Data are shown as means ± S.D. of duplicate experiments.

activities. The catalytic activity of cholesteryl esters formed from cholesterol and long-chain fatty acyl-coenzyme A was determined using expressed hACAT-1 and hACAT-2 from Hi5 cells. Oleic acid anilide was used as the reference ACAT inhibitor.[27] All isolated compounds were examined for hACAT-1 and hACAT-2 inhibitory activities. Compounds **1**, **2**, and **4** showed weak hACAT-1 inhibitory activities of 35, 20, and 25%, respectively and potent LDL-antioxidant coumestrol **6** showed weak hACAT-1 and hACAT-2 inhibitory activities of 37 an 22% at 25 μg/m*l*. On the other hand, compound **5** exhibited more potent hACAT-1 and hACAT-2 inhibitory activities with IC_{50} values of 29.0 and 82.7 μM, respectively (Table 2). In particular, a significant hACAT inhibitor, **5** showed dose-dependent hACAT-1 and hACAT-2 inhibitory activities (Fig. 3).

In conclusion, glyceollin I (**5**) including pterocarpan derivative showed potent inhibitory activities, with IC_{50} values of 29.0 (hACAT-1) and 82.7 μM (hACAT-2). Furthermore, compounds **1**, **2**, **4**, and **6** showed weak hACAT-1 and hACAT-2 inhibitory activities at 25 μg/m*l*. These results show the hACAT inhibitory activities of pterocarpan could enhance the value of soybean and its roots as functional materials and human diet.

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