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Saucerneol B derivatives as human acyl-CoA: cholesterol acyltransferase inhibitors

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Abstract—A series of **2a**–**i** were prepared from a lead compound, saucerneol B (**1**) for evaluating their acyl-CoA: cholesterol acyltransferase inhibitory activities. Compounds **2a**–**g** exhibited the high specificity of hACAT-1 than hACAT-2, whereas **2h** and **2i** showed very weak inhibitory activities in both hACAT-1 and hACAT-2. Saucerneol B (**1**) exhibited strong cholesterol-lowering effect in high cholesterol-fed mice.

Inhibition of acyl-CoA: cholesterol acyltransferase (ACAT, E.C.2.3.1.26), the enzyme, which catalyzes the acylation of cholesterol to cholesteryl esters with long-chain fatty acids, is a very attractive target for the treatment of hypercholesterolemia and atherosclerosis.[1] It was found to be present as two isoforms in mammals,[2] ACAT-1 and ACAT-2, with different tissue distribution and membrane topology.[3] However, most ACAT inhibitors, which were screened by rat liver microsomal ACAT, have problems associated with low oral bioavailability and adrenal and/or hepatic toxicity in clinical trials.[4] ACAT-1 plays a critical role in foam cell formation in macrophages, whereas ACAT-2 is in charge of the cholesterol absorption process in intestinal mucosal cells.[5] These findings were consistent with the following results that atherosclerosis lesions were reduced at ACAT-1$^{-/-}$ mice, whereas ACAT-2$^{-/-}$ mice have limited cholesteryl absorption in the intestine, and decreased cholesterol ester content in the liver and plasma lipoproteins.[6] Because ACAT inhibitors, most without selectivity for ACAT-1 versus ACAT-2, have not yet been identified that are effective in plasma cholesterol lowering in humans,[7] the selective inhibitor of ACAT-1 or ACAT-2 may be effective for the development of a useful hypercholesterolemic or anti-atherogenic agent.

So, we reported recently mass production of hACAT-1 and hACAT-2 individually from Hi5 cells to screen isoform-specific inhibitors.[8] Also, we reported recently that the sesquineolignan, saucerneol B (**1**), were isolated from the methanolic extracts of *Saururus chinensis* root and exhibited the specificity of hACAT-1 than hACAT-2 with IC_{50} values of 43.0 and 124.0 μM.[9]

In general, many ACAT inhibitors having an alkyl chain, such as CI-976 and FR129169, have been reported.[10] It is considered that a long-chain aliphatic hydrocarbon moiety has structure similar to acyl residue of an acyl-CoA that is one of the substrates for ACAT. Therefore, alkyl chain of 1–9 carbons was introduced at position 7″-OH of saucerneol B (**1**) to develop more potent ACAT inhibitor. In this study, we describe in vitro human microsomal ACAT inhibitory activities for saucerneol B (**1**) and its *n*-alkoxy derivatives **2a**–**i**, and also cholesterol-lowering effect of saucerneol B (**1**) in high cholesterol-fed mice.

n-Alkoxy derivatives **2a**–**i** of saucerneol B (**1**) were prepared according to the method shown in Scheme 1. Treatment of saucerneol B (**1**) with various alkyl halides containing alkyl chain of 1–9 carbons gave *O*-alkylated compounds **2a**–**i** at position 7″-OH of saucerneol B (**1**) in good yields. The structure of all compounds **2a**–**i**

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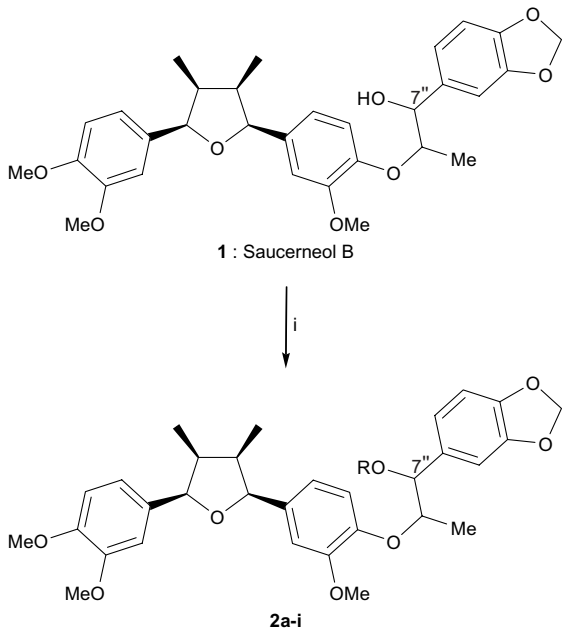

Scheme 1. Reagents and conditions: (i) NaH (2.0 equiv), R–X (2.0 equiv, X = I, Br, Cl), DMF, 0 °C, reflux.

was determined by their spectroscopic data analysis.[11] Also, the stereochemistry of saucerneol B (**1**) was elucidated by NOE experiments, namely, a NOE of 8.22% for the H-8 (or H-8′) was observed upon irradiation of H-7 (H-7′), inversely, a NOE of 8.19% for the H-7 (or H-7′) was showed. Therefore, a NOE correlation signals between H-7/H-8 (H-7′/H-8′) and H-8/H-7 (H-8′/H-7′) indicated the 7,8-*cis*-8,8′-*cis*-7′,8′-*cis* configuration (Fig. 1).

The potential of compounds **2a**–**i** was evaluated as an inhibitor of hACAT-1 or -2 that was expressed and characterized from Hi5 cells by recombinant baculoviruses.[8] Then, the rate of incorporation of [1-^{14}C]-oleoyl-CoA into cholesteryl ester was determined using the expressed hACAT-1 or -2. The ACAT inhibitory activities of the compounds **2a**–**i** were confirmed by the positive control with oleic acid anilide, which inhibited hACAT-1 and hACAT-2 with IC_{50} values of 0.14 and 0.17 μM, respectively.[12] The biological data for saucerneol B (**1**) and its *n*-alkoxy derivatives **2a**–**i** substituted on the 7″-OH has been shown in Table 1. Compounds **2a** and **2b** introduced with methyl and ethyl residues at 7″-OH of **1**, showed the increased inhibitory activities of hACAT-1 with IC_{50} value of 35 μM for **2a** and 31 μM for **2b**, respectively. Compounds **2c** and **2d**, which had *n*-propyl and *n*-butyl residues at 7″-OH, exhibited more potent and optimized hACAT-1 inhibitory activities with IC_{50} value of 14 μM for **2c** and 17 μM for **2d** compared to 43 μM for saucerneol B (**1**). However, the hACAT-2 inhibitory activities of **2a**–**d** were decreased somewhat than saucerneol B (**1**). Compounds **2e**–**g** containing alkyl chain of 5–7 carbons showed less potent inhibitory activities than **2c** and **2d** in hACAT-1 and the specificity of hACAT-1 over hACAT-2, whereas compounds **2h** and **2i** showed very weak inhibitory activities in both hACAT-1 and hACAT-2. In contrast to these results, manassantin A showed the specificity hACAT-2 (IC_{50} = 8.0 μM) over hACAT-1 (IC_{50} = 39.0 μM).[9] Also, pyripyropene A inhibited only hACAT-2 with IC_{50} values of 0.64 μM, whereas manassantin B dominantly inhibited hACAT-1 with IC_{50} values of 82.0 μM.[8,9] Therefore, human microsomal ACAT-1 and -2 enzymes have proven to be very sensitive to the property of the compounds. According to Rudel's results,[5] topology orientation of African green monkey ACAT-1 and ACAT-2 in the membrane of the endoplasmic reticulum (ER) shows that serine residue is located on opposite sides of the membrane for either enzyme. This result means that functional differences between the enzymes may occur, even though the role of this serine in enzyme function is not yet known. Also, it may be not only influenced at the substrate binding site but also at lipophilicity of inhibitors that are through ER membrane. Therefore, these results may be rationalized that more specificity of compounds **2a**–**h** against hACAT-1 may be due to the substrate binding effect between various alkyl chains on 7″-OH and enzyme.

Table 1. ACAT inhibitory activities of saucerneol B (**1**) and **2a**–**i**

Compounds	R	Yield (%)[a]	hACAT-1, IC_{50} (μM)[b]	hACAT-2, inhibition % at 125 μM[b]
1	H	—	43	57
2a	Me	76	35	45
2b	Et	65	31	29
2c	*n*-Pr	56	14	33
2d	*n*-Bu	61	17	28
2e	*n*-C_5H_{11}	60	20	33
2f	*n*-C_6H_{13}	53	34	33
2g	*n*-C_7H_{15}	63	50	26
2h	*n*-C_8H_{17}	73	44% at 125 μM	19
2i	*n*-C_9H_{19}	75	25% at 125 μM	10

[a] Isolated yield.
[b] In vitro ACAT inhibitory activity was measured using the expressed hACAT-1 or hACAT-2. Data are shown as mean values of two independent experiments performed in duplicate.

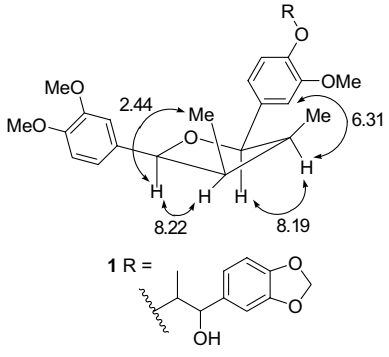

Figure 1. NOE experiments of saucerneol B (**1**).

For the development of a useful hypercholesterolemic or anti-atherogenic agent having ACAT inhibitory activity, we have examined firstly the preliminary cholesterol-lowering activity of saucerneol B (**1**) in high cholesterol-fed C59BL/6J mice. The plasma total cholesterol

Table 2. The effect of saucerneol B (**1**) on plasma total cholesterols in high cholesterol-fed mice

Group	*N*	Body weight (g)[a]		Total cholesterol (mg/dL)[b]	
		0 day	6 weeks	0 day	6 weeks
Control	10	22.8 ± 0.9	28.6 ± 2.3	97.8 ± 11.1	238.0 ± 27.7
Saucerneol B (3.5 mg/kg diet)	10	22.8 ± 1.1	29.5 ± 1.5	102.4 ± 7.0*	197.8 ± 20.9*

* Significantly different ($p < 0.05$) from control group.
[a] All values are expressed as mean ± SD.
[b] Mean ± SD.

levels were measured after feeding a high cholesterol diet supplemented with 3.5 mg/kg diet of saucerneol B (**1**) for six weeks.[13] Saucerneol B (**1**) exhibited strong cholesterol-lowering effect (−16.9%) in high cholesterol-fed mice (Table 2).

In conclusion, we have discovered a novel class of hACAT-1 specific enzyme inhibitors, its *n*-alkoxy derivatives **2a**–**h** of saucerneol B (**1**). Furthermore, process for mass production, cell-based fluorescence assay, and the efficacy test of cholesterol-lowering and anti-atherogenic activities of the derivatives **2a**–**f** will be the subject of future publications.

Acknowledgements

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11. Physical and spectroscopic data: **2c**: colorless oil, ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.68 (3H, d, J = 6.1 Hz), 0.69 (3H, d, J = 6.1 Hz), 0.87 (3H, t, J = 7.4 Hz), 1.08 (3H, d, J = 6.3 Hz), 2.24 (2H, m), 3.35 (2H, m), 3.85 (3H, s), 3.88 (3H, s), 3.89 (3H, s), 4.43 (1H, d, J = 6.1 Hz), 4.50 (1H, m), 5.40 (2H, d, J = 6.0 Hz), 5.95 (2H, s), 6.76– 6.98 (9H, m); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 10.7, 14.6, 14.7, 16.4, 23.1, 43.8, 55.9, 56.1, 71.0, 78.9, 83.7, 84.3, 100.9, 107.0, 107.8, 110.5, 110.6, 110.7, 116.2, 118.6, 119.4, 120.2, 131.9, 134.6, 135.6, 146.4, 147.1, 147.5, 148.5, 148.8, 150.1.
12. ACAT activity assay: microsomal fractions of Hi5 cells containing baculovirally expressed ACAT-1 or -2 were used as the sources of enzymes.[8] The activity of the hACAT-1 and hACAT-2 was measured according to the method of Brecher and Chan[14] with slight modification.[15] The reaction mixture, containing 4 μL of microsomes (5 μg/mL protein), 20 μL of 0.5 M potassium–phosphate buffer (pH 7.4, 10 mM dithiothreitol), 15 μL of bovine serum albumin (fatty acid free, 40 mg/mL), 2 μL of cholesterol in acetone (20 μg/mL, added last), 41 μL of water, and 10 μL of test sample in a total volume of 92 μL, was preincubated for 20 min at 37 °C. The reaction was initiated by the addition of 8 μL of [1-^{14}C]oleoyl-CoA solution (0.05 μCi, final concn 10 μM). After 25 min of incubation at 37 °C, the reaction was stopped by the addition of 1.0 mL of isopropanol–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 (pH 7.4, 2 mM dithiothreitol) was then added to the terminated reaction mixture. The above solution was mixed and allowed to phase separation under gravity for 2 min. Cholesterol oleate was recovered in the upper heptane phase (total volume 0.9 ~ 1.0 mL). The radioactivity in 100 μL of the upper phase was measured in scintillation vial with 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 microsomes. The ACAT activity was expressed as a defined unit, cholesteryl oleate pmol/min/mg protein.
13. The hypocholesterolemic effect of saucerneol B (**1**) was investigated in male C57BL/6J mice maintained at Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The mice were housed in a room with controlled temperature (22 ± 2 °C), relative humidity

(55 ± 5%), and lighting (alternating 12 h cycle of light and dark). At seven weeks of age, 20 male mice were randomly divided into two groups of 10 animals and fed on a high cholesterol diet (CRF-1 supplemented with 1.25% cholesterol, 6% fat, and 0.5% Na–cholate, Oriental Yeast Co. Ltd., Japan), the first group without supplementation (control), the second group supplemented with 3.5 mg/kg diet of saucerneol B (**1**). The diet and water were given ad libitum. After treating the test compounds for six weeks, the mice were anesthetized with ethyl ether, and the blood was obtained from the retro-orbital sinus using a heparinized capillary tube. Then, the blood was centrifuged at 8000*g* for 10 min, and the plasma was collected. The concentration of plasma total cholesterol was measured with an automatic blood chemical analyzer (Hitachi 7020, Japan). To evaluate statistical significance between control and experimental groups, student's *t* test was performed, and a *p* value of <0.05 was considered to be statistically significant.

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