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Novel 3,5-diaryl pyrazolines as human acyl-CoA:cholesterol acyltransferase inhibitors

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Abstract—A series of pyrazoline derivatives were prepared for evaluating their acyl-CoA:cholesterol acyltransferase activities. 3-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-5-(multi-substituted 4-hydroxyphenyl)-2-pyrazolines **4a**–**i** were shown in vitro inhibitory activity on hACAT-1 and -2.

As the understanding of cellular cholesterol metabolism has advanced, acyl-CoA:cholesterol acyltransferase (ACAT) has been known to play a crucial role in the development of atherosclerosis. It catalyzes the formation of cholesterol esters from cholesterol and long-chain fatty acyl-coenzyme A, and also modulates cholesterol absorption from the intestine, secretion of VLDL from the liver, and steroidogenesis.[1] Recently, 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] In mammals, such as mouse and monkey, ACAT-1 is located in various tissues as a ubiquitous manner, whereas ACAT-2 is found only in liver and intestine. However, in the adult human, ACAT-1 is the major ACAT isoform in the liver, whereas ACAT-2 is the major ACAT isoform in the intestine.[4] ACAT-2 appears to be responsible for cholesterol ester formation in intestine and liver, and ACAT-1 also in peripheral tissue including human liver has a very important role to synthesize cholesterol ester for storage in the cell.

Although many compounds have been reported as potent ACAT inhibitors,[5] thus far, however, ACAT inhibitors with significant preference for one isoform over the other have not been described for the human ACAT. So, we reported recently mass-production of hACAT-1 and hACAT-2 individually from Hi5 cells to screen isoform-specific inhibitors.[6] Also, analogues of pyrazolines have potential antioxidant activities against low-density lipoprotein (LDL).[7] Therefore, pyrazolines were tentatively applied to ACAT enzyme to search a specific inhibitor against hACAT-1 or -2. In mammals, selective inhibition of ACAT-2 would be beneficial for treating hypercholesterolemia and cholesterol gallstones, whereas inhibition of ACAT-1 and -2 might be an useful strategy for atherosclerosis.[8] In this study, we describe the synthesis and in vitro ACAT inhibitory activities of a novel series of pyrazoline derivatives **4a**–**i**.

Pyrazolines **4a**–**i** were prepared according to the methods shown in Scheme 1. Treatment of 1-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethanone (**1**) with 3,5-di- or 2,3,5-tri-substituted 4-hydroxybenzaldehydes **2a**–**i** gave α,β-unsaturated ketones **3a**–**i** by a typical acid-catalyzed aldol condensation in 28–86% yields, as shown in Scheme 1. Reaction of **3a**–**i** and hydrazine monohydrate afforded pyrazolines **4a**–**i** via a one-pot addition–cyclocondensation process in 50–96% yields (Table 1). The structures of **4a**–**i** were determined by their spectroscopic analysis.[9]

The potential of compounds **4a**–**i** was evaluated as an inhibitor of hACAT-1 or -2 that was expressed and characterized from Hi5 cells by recombinant baculoviruses.[6] Then, the rate of incorporation of [1-^{14}C]oleoyl-CoA into cholesteryl ester was determined using the expressed hACAT-1 or -2.[10] The ACAT inhibitory

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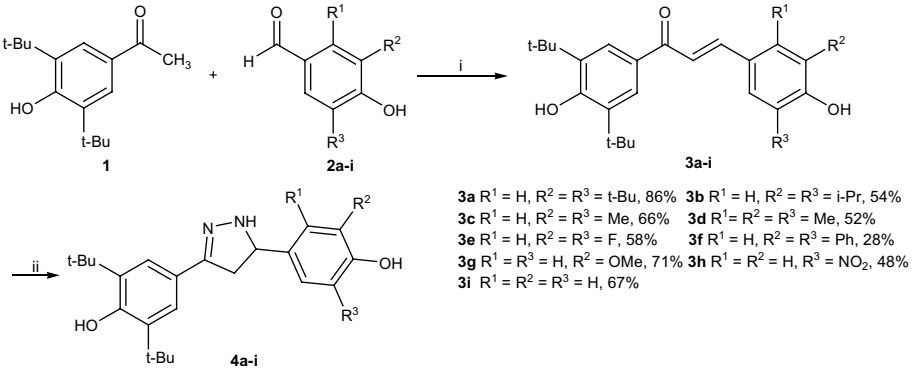
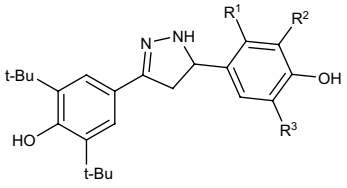


Scheme 1. Reagents and conditions: (i) H_2SO_4, MeOH, reflux; (ii) N_2H_4, EtOH, rt—reflux.

activities of the compounds **4a**–**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.[6] Compounds **4a**–**d** containing alkyl moieties (*t*-Bu, *i*-Pr, and Me) at the 2,3,5-position of phenol ring showed similar degree of inhibitory activities in both hACAT-1 and hACAT-2 in vitro assays, whereas compounds **4e**–**i** having weak inhibitory activities relative to **4a**–**d** showed selectivity of hACAT-1 over hACAT-2. According Rudel et al.'s results,[11] 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 substrate binding site but also at lipophilicity of inhibitors, which are through ER membrane. Therefore, these results may be rationalized that more specificity of compounds **4e**–**i** against hACAT-1 may be due to hydrogen bonding effect between hydrophilic groups (fluoro, methoxy, phenyl, and nitro) and enzyme. In the case of **4i**, it also may conclude that **4i** includes less hindered phenol to lead to hydrogen bonding with enzyme, when compared to hindered alkyl groups that were substituted at 2,3,5-position of phenol ring of **4a**–**d**. From the results of inhibitory activities of **4a**–**i**, it appeared that the activity was insensitive to the size of alkyl groups at the 2,3,5-position on 5-phenol ring, whereas it was influenced by electron density of substituents on the 5-phenol ring to show specificity against hACAT-1 and -2.

In conclusion, we have discovered a novel class of hACAT-1 and hACAT-2 enzyme inhibitors, 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-5-(di- or tri-substituted 4-hydroxyphenyl)-2-pyrazolines **4a**–**i**. Furthermore, the structure–activity relationship (SAR), stereospecific inhibitory activity, and pharmacological studies of this novel series of pyrazolines will be the subject of future publications.

Table 1. ACAT inhibitory activities of pyrazoline compounds **4a**–**i**



Compound	R^1	R^2	R^3	Yield (%)[a]	IC_{50} (μM)[b]	
					hACAT1	hACAT2
4a	H	*t*-Bu	*t*-Bu	50	12.8	14.7
4b	H	*i*-Pr	*i*-Pr	50	12.4	16.4
4c	H	Me	Me	84	12.8	19.9
4d	Me	Me	Me	79	27.0	31.5
4e	H	F	F	95	35.6	164.3
4f	H	Ph	Ph	40	23.3	127.4
4g	H	OMe	OMe	96	32.1	64.5
4h	H	H	NO_2	96	65.4	125.8
4i	H	H	H	96	32.4	68.5

[a] Isolated yield from **3a**–**i**.

[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.

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9. Physical and spectroscopic data: **4a**: colorless prisms, mp 243–244 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (18H, s), 1.46 (18H, s), 3.00 (1H, dd, $J = 10.5$, 16.2 Hz), 3.43 (1H, dd, $J = 10.2$, 15.9 Hz), 4.83 (1H, t like, $J = 10.5$ Hz), 5.20 (1H, s, –OH), 5.37 (1H, s, –OH), 5.84 (1H, br, –NH), 7.23 (2H, s), 7.53 (2H, s); ^{13}C NMR (75 MHz, $CDCl_3$) δ 30.2, 30.3, 34.37, 34.4, 42.2, 64.9, 123.1, 123.3, 124.4, 133.1, 136.0, 136.1, 152.6, 153.3, 154.7.
10. ACAT activity assay: Microsomal fractions of Hi5 cells containing baculovirally expressed ACAT-1 or -2 were used as the sources of enzymes.[6] The activity of the hACAT-1 and hACAT-2 was measured according to the method of Brecher and Chan[12] with slight modification.[13] The reaction mixture, containing 4 μL of microsomes (8 mg/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.
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