INHIBITION OF ACYL-CoA: CHOLESTEROL ACYLTRANSFERASE BY HELMINTHOSPOROL AND ITS RELATED COMPOUNDS

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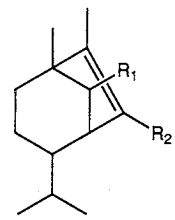
Acyl-CoA : cholesterol acyltransferase (ACAT) (EC 2.3.1.26) is a microsomal enzyme which catalyzes the synthesis of cholesteryl ester from acyl-CoA and cholesterol. This enzyme plays key roles in both intestinal absorption of cholesterol[1] and cholesteryl ester accumulation in macrophage-derived foam cells, which are prominent in atherosclerotic plaques[2]. In the course of the search for microbial metabolites which inhibit cholesteryl ester formation in macrophages, helminthosporol (**1**)[3], helminthosporal-derived acid (**2**)[4] and helminthosporic acid (**3**)[3] (Fig. 1) were isolated as active compounds. These compounds are known as growth promoting substances for plants, while their effect on animal cells have not been reported[5]. In this study, we show that **1**, **2** and **3** inhibit ACAT microsomal activity and cholesteryl ester formation in macrophages.

[1-^{14}C]Oleyl-CoA (52.9 mCi/mmol) was obtained from Amersham and [1-^{14}C]oleate (54.9 mCi/mmol) from ICN Radiochemicals. Human low density lipoprotein (LDL) (d = 1.019 ~ 1.063 g/ml) and fetal carf lipoprotein-deficient serum (d > 1.215 g/ml) were prepared by ultracentrifugation[6]. Oxidized LDL was obtained by incubating LDL with 5 μM $CuSO_4$[7]. Compounds **1**, **2** and **3** were isolated from culture of *Cochliobolus sativus* IFO 7259.

ACAT activity was determined using microsomes prepared from rat liver as described previously[8] with slight modification. Thus, 30 μl of enzyme solution (16.7 mg/ml microsomal protein and 150 mM potassium phosphate, pH 7.4) was preincubated at 37°C for 15 minutes. Then reaction was initiated by adding 20 μl of substrate solution (150 mM potassium phosphate, pH 7.4, 0.25 mM [^{14}C]oleoyl-CoA (10,000 dpm/nmol) and 200 μM fatty acid-free bovine serum albumin). After incubation at 37°C for 70 seconds, reaction was stopped by adding 250 μl of ethanol and then lipids were extracted with 1 ml of *n*-hexane. The extract was evaporated to dryness, dissolved in 40 μl *n*-hexane and submitted to TLC[6]. The cholesteryl [^{14}C]oleate formed was counted for radioactivity. Cholesteryl ester formation from [^{14}C]oleate in macrophages were assayed as described previously[8].

ACAT activity in rat liver microsomes was inhibited by **1**, **2** and **3** (Fig. 2). Among these, compound **1** was most active in inhibiting ACAT.

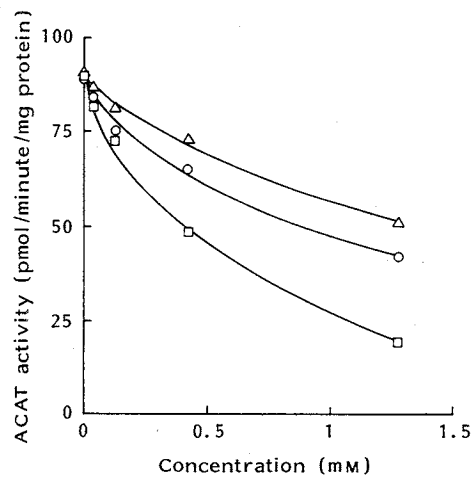
Fig. 1. The structures of helminthosporol (**1**), helminthosporal-derived acid (**2**) and helminthosporic acid (**3**).



	R_1	R_2
1	CH_2OH	CHO
2	COOH	CHO
3	CH_2OH	COOH

Fig. 2. Effect of **1**, **2** and **3** on ACAT activity in rat liver microsomes.

Enzyme mixture (30 μl) was preincubated at 37°C for 15 minutes in the presence of the indicated concentrations of **1** (□), **2** (△) or **3** (○).



Subsequently, substrate mixture (20 μl) was added and ACAT activity was determined. Each value represents the average of duplicate determinations.

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Table 1. Effect of microsomal washing on the ACAT inhibition by **1**.

Time of washing	ACAT activity in microsomes (pmol/minutes/mg protein)		Percent of control
	No addition	**1** (1.27 mM)	
0	98.3	22.8	23.2
1	90.3	82.8	91.7
2	84.9	78.8	92.8

Microsomes (16.7 mg/ml) were incubated at 37°C for 15 minutes in 300 μl of 150 mM potassium phosphate, pH 7.4, in the absence or presence of 1.27 mM of **1**. Subsequently, aliquot (30 μl) was removed to determine ACAT activity in duplicate, and remaining portion (240 μl) was diluted by adding 2.5 ml of buffer containing 150 mM potassium phosphate, pH 7.4, and 80 μM bovine serum albumin, followed by centrifugation at 105,000 × g for 1 hour at 4°C. Microsomal pellet was suspended in 240 μl of 150 mM potassium phosphate, pH 7.4, and aliquot (30 μl) was removed and assayed for ACAT activity in duplicate. Remaining portion was washed centrifugally in a similar way as described above. Each value represents the average of duplicate determinations.

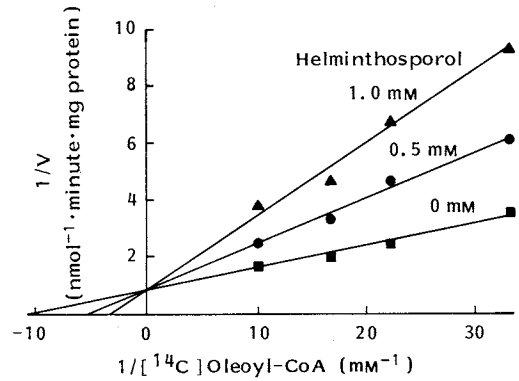
Thus, the inhibition by **1** was 50% at a concentration of 0.5 mM, while concentrations required for 50% inhibition by **2** and **3** were 1.5 and 1.0 mM, respectively. Reversibility of ACAT inhibition by **1** was determined by ultracentrifugal washing of microsomes. As shown in Table 1, ACAT activity in unwashed microsomes that had been treated with 1.27 mM of **1** was 25% of the activity in untreated microsomes. After washing 1~2 times, ACAT activity in **1**-treated microsomes recovered to 92~93% of control value. Lineweaver-Burk plots for the ACAT inhibition by **1** showed that the inhibition is competitive with respect to the substrate [^{14}C]oleoyl-CoA (Fig. 3). The apparent *Ki* value was calculated to be 0.64 mM.

Macrophage J774 cells synthesize large amounts of cholesteryl ester upon incubation with oxidized LDL. This activity, as determined by measuring incorporation of [^{14}C]oleate into cholesteryl ester, was inhibited 50% by **1**, **2** and **3** at concentrations of 90, 210 and 145 μM, respectively (Fig. 4). On the other hand, [^{14}C]oleate incorporation into triacylglycerol was slightly elevated by these compounds (Fig. 4).

Helminthosporol-related compounds are known as plant growth-promoting agents, while mechanism of this activity has not been elucidated. In the present study, we have shown that some of these compounds, **1**, **2** and **3**, inhibit ACAT activity in

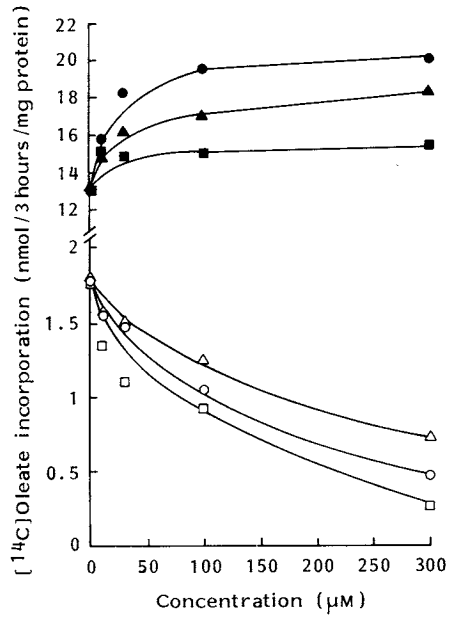
Fig. 3. Lineweaver-Burk plots for the inhibition of ACAT by **1**.

Concentrations of **1** were 0 (■), 0.5 (●) and 1.0 mM (▲). Each value represents the average of duplicate determinations.



Experimental conditions were identical to those in Fig. 2, except that concentration of [^{14}C]oleoyl-CoA was varied as indicated.

Fig. 4. Effects of **1**, **2** and **3** on the incorporation of [^{14}C]oleate into cholesteryl ester and triacylglycerol in J774 macrophages.



J774 macrophages were incubated with 100 μg protein/ml oxidized LDL and 0.1 mM [^{14}C]oleate (10,000 dpm/nmol) in complex with albumin at 37°C for 3 hours in the presence of the indicated concentrations of **1** (squares), **2** (triangles) or **3** (circles). Subsequently, [^{14}C]oleate incorporated into cholesteryl ester (open symbols) and triacylglycerol (closed symbols) was determined. Each value represents the average of duplicate determinations.

rat liver microsomes. However, it is not clear that this inhibitory activity of these compounds is related to the growth-promoting effect in plants.

Compound **1** was two and three times more active than **3** and **2**, respectively, in inhibiting microsomal ACAT activity. R_1 and R_2 of **1** (Fig. 1) are hydroxymethyl and formy groups, respectively. These positions are substituted respectively by carboxyl and formyl groups in **2**, and hydroxymethyl and carboxyl groups in **3**. These observations suggest that R_1 and R_2 substituents in helminthosporol-related compounds play an important role in ACAT inhibition.

Acknowledgments

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