GYPSETIN, A NEW INHIBITOR OF ACYL-CoA : CHOLESTEROL ACYLTRANSFERASE PRODUCED BY

Nannizzia gypsea var. incurvata IFO 9228

I. FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY

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A novel inhibitor of acyl-CoA : cholesterol acyltransferase (ACAT), designated gypsetin, was isolated from the cultured broth of Nannizzia gypsea var. incurvata IFO 9228 by solvent extraction, silica gel chromatography and crystallization. Gypsetin inhibited rat liver microsomal ACAT activity competitively with respect to the substrate oleoyl-CoA with an apparent Ki value of $5.5\,\mu M$. In cultured macrophage J774 cells incubated with oxidized low density lipoprotein, gypsetin inhibited cholesteryl ester formation from [^{14}C]oleate by 50% at a concentration of $0.65\,\mu M$ without affecting cell surface binding, uptake and degradation of the lipoprotein.

Acyl-CoA : cholesterol acyltransferase (ACAT), plays an essential role both in intestinal absorption of cholesterol and cholesteryl ester formation in a variety of tissues and cells[1]. Since elevated plasma level of cholesterol is related to an increased risk of coronary heart disease[2] and massive accumulation of cholesteryl esters in macrophage-derived foam cells is a hallmark of the atherosclerotic plaques[3], controlling ACAT activity may be of importance in prevention and treatment of atherosclerosis.

In the search for microbial metabolites which inhibit cholesteryl ester formation in macrophages, we isolated a new inhibitor of ACAT, named gypsetin (Fig. 1). In this paper, we describe the fermentation, isolation, physico-chemical properties and biological activity of gypsetin. Details of the structure elucidation of gypsetin will be reported in the accompanying paper[4].

Fig. 1.   The structure of gypsetin.
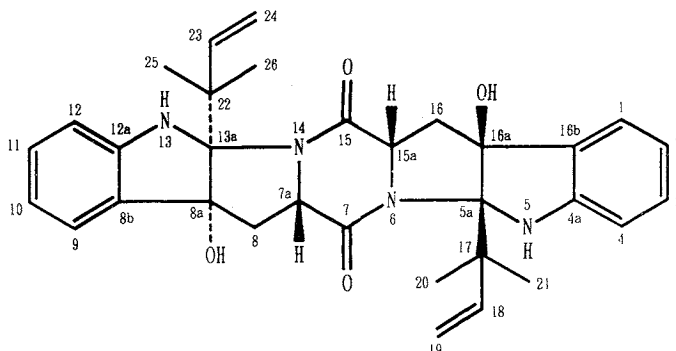
The configuration shown is relative one.

<div align="center">Fermentation</div>

N. gypsea var. *incurvata* IFO 9228 (original deposition; accession number IMI 86518 of the CAB International Mycological Institute, Ferrylane, Kew, England) obtained from the Institute for Fermentation, Osaka, Japan, was aerobically grown at 25°C in a medium containing 3% glucose, 1% soybean meal, 0.3% meat extract, 0.3% Polypepton, 0.3% yeast extract, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.01% CB442 (pH 5.9). The above-mentioned medium (100 ml in a 500-ml Sakaguchi flask) was inoculated with the mycelial scrapings from the slant culture and incubated on a reciprocal shaker (120 rpm) at 25°C for two days. One milliliter portion of this seed culture was inoculated into the same medium (100 ml in a 500-ml Sakaguchi flask). The flask was incubated under the same conditions as above for 7 days. A typical time course of the fermentation is shown in Fig. 2.

<div align="center">Isolation</div>

The cultured broth (3 liters) was filtered to separate the filtrate and mycelial cake. The latter was extracted three times with 1 liter each of acetone. The acetone extract was evaporated to remove the organic solvent and combined with the culture filtrate. After adjusting at pH 8 with NaOH, the combined mixture was extracted with dichloromethane (twice with 3 liters each followed by once with 1 liter). The solvent layer was dried over anhydrous sodium sulfate and concentrated to dryness, giving 1.47 g of a residue.

This material was applied to a silica gel column packed with *n*-hexane (30 × 160 mm, Wakogel C-200). After washing with 1.5 liters of *n*-hexane - acetone (19 : 1), the column was developed with 1.8 liters of *n*-hexane - acetone (9 : 1), and 100-ml fractions were collected. Fractions showing an inhibition of cholesteryl ester formation in macrophages were combined and concentrated to dryness, giving 198 mg of yellowish powder. This powder was dissolved in chloroform - methanol to crystallize gypsetin (138 mg).

Fig. 2. Time course of the production of gypsetin.
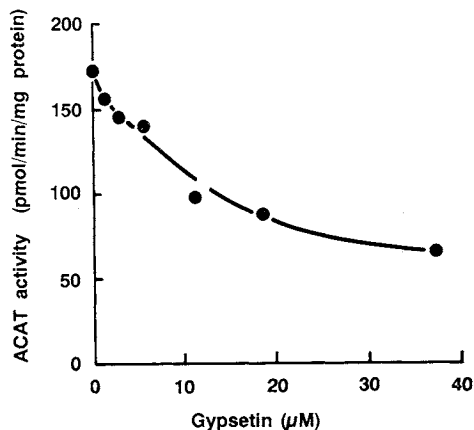
(●) pH, (○) gypsetin.
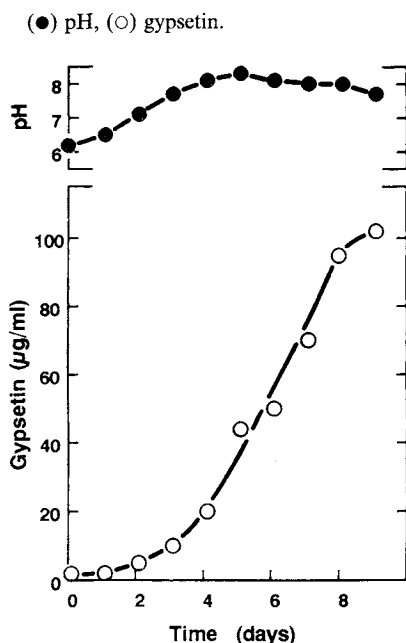
Nannizzia gypsea var. *incurvata* IFO 9228 was grown aerobically as described in the text. One milliliter aliquot of the cultured broth was removed everyday and extracted three times with 1 ml each of dichloromethane after adjusting at pH 8. Portions of concentrated extracts were analyzed by HPLC (column; Inertsil PREP-ODS (6 × 250 mm), mobile phase; 80% aq acetonitrile, detection; absorbance at 220 nm) and quantified using purified gypsetin as a standard.

<div align="center">Physico-chemical Properties</div>

The physico-chemical properties of gypsetin are summarized in Table 1. Molecular formula of

Table 1. Physico-chemical properties of gypsetin.

Appearance	Colorless needle
Melting point	162°C
Molecular formula	$C_{32}H_{36}N_4O_4$
SI-MS (*m/z*)	541 $(M+H)^+$, 563 $(M+Na)^+$
HREI-MS (*m/z*)	
Found:	540.2728 (M^+)
Calcd:	540.2737 (for $C_{32}H_{36}N_4O_4$)
UV λ_{max}^{MeOH} nm (ε)	205 (58,900), 237 (14,900), 296 (4,430)
IR ν_{max} (KBr) cm^{-1}	3500, 3360, 2960, 2930, 2870, 2350, 1670, 1600, 1480, 1460, 1360, 1080

Fig. 3. Inhibition of rat liver microsomal ACAT activity by gypsetin.

Rat liver microsomes, at a concentration of 16.7 mg protein/ml, were preincubated in the absence or presence of the indicated concentrations of gypsetin at 37°C for 15 minutes in 30 μl of 150 mM potassium phosphate, pH 7.4. Subsequently, 20 μl of mixture containing 150 mM potassium phosphate, pH 7.4, 0.2 mM bovine serum albumin and 0.25 mM [14C]-oleoyl-CoA (10,000 dpm/nmol) was added to initiate enzyme reaction. After incubation at 37°C for 70 seconds, the reaction was terminated by the addition of 0.25 ml of ethanol and cholesteryl [14C]oleate formed was determined by TLC followed by scintillation counting[5] of the scraped spot from the plates. Each value represents the average of duplicate determinations.

Fig. 4. Double reciprocal plots for the inhibition of ACAT by gypsetin.
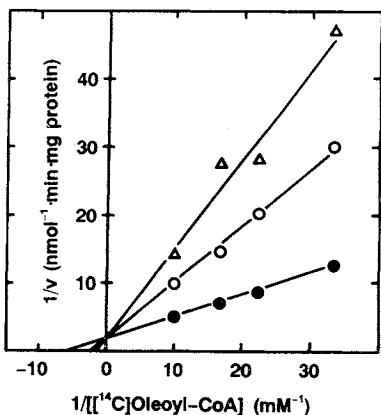

ACAT activity was determined in the presence of 0 (●), 7.4 (○) and 18.5 μM (△) of gypsetin. Other conditions were identical with those in the experiments shown in Fig. 3 except that various concentrations of [14C]oleoyl-CoA were used. Each value represents the average of duplicate determinations.

Table 2. Reversibility of the inhibition of ACAT by gypsetin.

Microsomal washing (frequency)	ACAT activity in microsomes (pmol/minute)	
	No gypsetin treatment	Gypsetin treatment
0	81.0	29.1
1	84.9	39.7
2	77.0	53.2

Rat liver microsomes (16.7 mg/ml in 150 mM potassium phosphate, pH 7.4) were incubated at 37°C for 15 minutes in the absence or presence of 61 μM of gypsetin. Subsequently, two 30-μl aliquots were removed for ACAT assay and remaining portion was washed by ultracentrifugation with 150 mM potassium phosphate, pH 7.4, containing 80 μM bovine serum albumin as described previously[5]. ACAT activity was determined as described in the legend to Fig. 3. Each value represents the average of duplicate determinations.

Fig. 5. Effects of gypsetin on the incorporation of [14C]oleate into cholesteryl ester and triacylglycerol in macrophage J774.
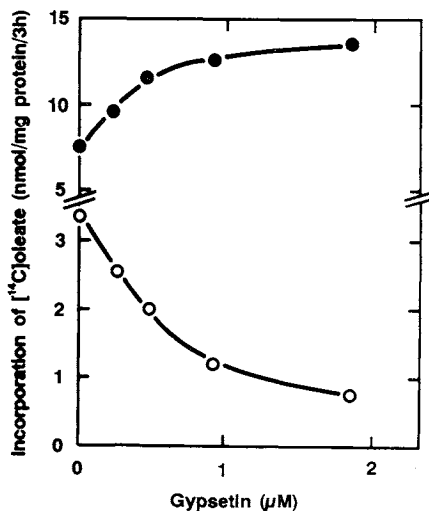
J774 macrophages were grown as described previously[6] and received 100 μg protein/ml of oxidized LDL and 0.1 mM [14C]oleate in complex with albumin. After incubation at 37°C for 3 hours in the absence or presence of the indicated concentrations of gypsetin, [14C]oleate incorporated into cholesteryl ester (○) and triacylglycerol (●) was determined[6]. Each value represents the average of duplicate determinations. The mean control values were 1.57 and 14.5 nmol/3 hours/mg cell protein for the incorporation into cholesteryl ester and triacylglycerol, respectively.

gypsetin was determined to be $C_{32}H_{36}N_4O_4$ by a combination of HREI-MS, SI-MS and ^{13}C NMR analyses. The UV absorption spectrum was essentially unaltered when it was measured either in MeOH - 0.1 M NaOH (60 : 1) or in MeOH - 0.1 M HCl (60 : 1). The IR absorption bands at 3600~3300 and 1670 cm^{-1} are consistent with the presence of hydroxyl and carbonyl groups, respectively.

Biological Activity

ACAT Inhibition

ACAT activity was determined as described previously[5] with slight modifications. Details are shown in the legend to Fig. 3. Gypsetin inhibited microsomal ACAT activity 50% at a concentration of 18 μM (Fig. 3). When the microsomes treated with 61 μM gypsetin were washed with buffer as described previously[5], they recovered from inhibition of ACAT activity (Table 2). Inhibition kinetics were determined by measuring the enzyme activity at the various concentrations of both [^{14}C]oleoyl-CoA and gypsetin. Double reciprocal plots of the data suggested that the inhibition is competitive with respect to the substrate [^{14}C]oleoyl-CoA (Fig. 4). The apparent Ki value was calculated to be 5.5 μM.

Inhibition of Cholesteryl Ester Formation in Macrophages

When incubated with oxidized low density lipoprotein (LDL), macrophages avidly take up and degrade the lipoprotein, resulting in the formation of cholesteryl esters. This activity, as measured by incorporation of [^{14}C]oleate into cholesteryl ester[6], was inhibited 50% by gypsetin at a concentration of 0.65 μM, while [^{14}C]oleate incorporation into triacylglycerol was increased by 1.5-fold (Fig. 5). Under the similar conditions where ^{125}I-labeled oxidized LDL was used in place of oxidized LDL, gypsetin did not inhibit cell surface binding, uptake and degradation of oxidized ^{125}I-LDL (data not shown).

Discussion

A new microbial metabolite, gypsetin, was isolated from the cultured broth of *N. gypsea* var. *incurvata* IFO 9228 as a competitive inhibitor of ACAT. Gypsetin inhibition of cholesteryl ester formation in the cultured macrophages (IC$_{50}$ = 0.65 μM) was 27-fold more potent than that in the cell-free ACAT assay under the conditions used in this study. Since, gypsetin inhibited neither surface binding, uptake nor degradation of oxidized ^{125}I-LDL, its inhibition of cholesteryl ester formation in macrophages may, at least partly, be ascribed to the inhibition of ACAT. The apparent discrepancy in the inhibitory potency of gypsetin between in the macrophage and the cell-free assays may arise from a difference in the conditions where ACAT excerts its activity; for example, availability of the substrate, cholesterol and oleoyl-CoA, may affect the potency of the agent. We and others have observed similar results that several other ACAT inhibitors are 5- to 100-fold more potent in macrophage assays than in cell-free ones[7~9].

Acknowledgment

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