

INHIBITION OF ACYL-CoA: CHOLESTEROL ACYLTRANSFERASE
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The effect was studied of the fungal cyclodepsipeptide antibiotics beauvericin and seven distinct enniatins on acyl-CoA: cholesterol acyltransferase (ACAT) activity. In an enzyme assay using rat liver microsomes, all the compounds were found to inhibit ACAT activity. The drug concentration that caused 50% inhibition (IC_{50} value) of the enzyme activity was determined to be $3.0\ \mu\text{M}$ for beauvericin, indicating that the compound is one of the most potent ACAT inhibitors of microbial origin. Enniatins exhibited much higher IC_{50} values of 22 to $110\ \mu\text{M}$. More hydrophobic enniatins showed more potent inhibitory activity. Furthermore, the ACAT inhibitory activity was evaluated as inhibition of cholesteryl ester formation in a cell assay using J774 macrophages. Calculation of the ratio, CD_{50} value (the drug concentration causing 50% cell damage)/ IC_{50} value of cholesteryl ester formation, indicated that beauvericin shows the highest specificity. These data indicate that beauvericin is one of the most potent and specific ACAT inhibitors of microbial origin.

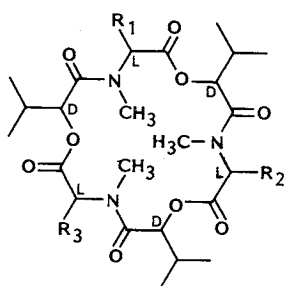
Acyl-CoA: cholesterol acyltransferase (ACAT) [EC 2.3.1.26], which catalyzes the conversion of cellular cholesterol and long chain fatty acyl-CoA to cholesteryl ester, plays an important role in cholesteryl ester accumulation in atherogenesis¹⁾ and in cholesterol absorption from intestines²⁾. Since elevated levels of plasma cholesterol is a well-documented risk factor of atherosclerosis, much attention has been paid to ACAT inhibitors for the treatment and prevention of atherosclerosis and hypercholesterolemia.

A lot of synthetic ACAT inhibitors have been reported having a urea or an amide moiety. We have started to find new ACAT inhibitors and new lead compounds of microbial origin. During our screening program, fungal strains *Fusarium* spp. FO-740 and FO-1305 were found to produce ACAT inhibitors. The active compounds were identified as beauvericin (Fig. 1) from the former strain and seven components of enniatins (A, A1, B, B1, D, E and F, Fig. 1) from the latter strain³⁾. All of these cyclodepsipeptides were classified as ionophore antibiotics, most of which were originally reported as antifungal or insecticidal antibiotics produced by *Beauveria*^{4,5)}, *Paecilomyces*⁶⁾, *Fusarium*^{7,8)} and *Polyporus*⁹⁾ spp. However, it is not clearly understood whether these biological activities are due to the ionophoric activity.

The cyclodepsipeptides were found by the authors to show a new biological activity of ACAT inhibition³⁾. In this study, we have examined the effect of beauvericin and enniatins on cholesterol

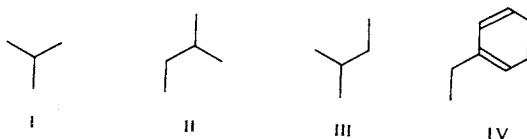
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Fig. 1. Structures of beauvericin and enniatins.



Compound	R ₁	R ₂	R ₃
Enniatin A	III	III	III
Enniatin A1	I	III	III
Enniatin B	I	I	I
Enniatin B1	I	I	III
Enniatin D	I	I	II
Enniatin E	I	II (III)	III (II)
Enniatin F	II	III	III
Beauvericin	IV	IV	IV

Enniatin E is a mixture of enniatins E1 and E2³⁾.



esterification in isolated rat liver microsomes and cultured cells such as J774 and mouse peritoneal macrophages. These cells are postulated to provide a model of foam cell formation since they have been reported to accumulate large quantities of cholesteryl ester when exposed to normal, abnormal modified lipoproteins and cholesterol-rich phospholipid liposomes^{9~12)}. Thus, the macrophages were used to evaluate ACAT inhibitors. The relationships are discussed between the ACAT inhibitory activity, the antimicrobial activity and the hydrophobicity of the ionophore antibiotics.

Materials and Methods

Materials

[1-¹⁴C]Oleoyl-CoA was purchased from New England Nuclear. Oleoyl-CoA, cholesterol, phosphatidylcholine from soybean and bovine serum albumin (BSA; fatty acid-free from fraction V) were obtained from Sigma. Triton WR-1339 was purchased from Nakarai Chemicals, Ltd. Beauvericin was purified from a cultured broth of *Fusarium* sp. FO-740. Enniatins A, A1, B, B1, D, E and F were also purified from *Fusarium* sp. FO-1305. Synthetic ACAT inhibitors (CL 277,082¹³⁾ and CL 283,546¹⁴⁾ were generous gifts of Pfizer Inc., U.S.A. All other reagents and solvents used were commercial products of analytical grade.

ACAT Assay Using Rat Liver Microsomes

Male Wister-Imamichi rats fed a normal diet for 1 week were killed by decapitation and livers were removed. The microsomal fractions of rat livers were prepared as described previously¹⁵⁾ and stored at -80°C.

ACAT activity was measured according to the method of LICHTENSTEIN and BRECHER¹⁵⁾ with some modification. The reaction mixture contained 0.1 M sodium phosphate (pH 7.4), 100~200 μg microsomal protein, 300 μM BSA, 30 μM [1-¹⁴C]oleoyl-CoA (0.02 μCi) and a dispersion of cholesterol in Triton WR-1339 (the amounts of cholesterol and Triton WR-1339 were 20 μg and 600 μg, respectively) in a total volume of 0.2 ml. The cholesterol dispersion was prepared by the method of BILLHEIMER *et al.*¹⁶⁾. The assay medium was preincubated for 30 minutes at 37°C and the reaction was initiated by the addition of [1-¹⁴C]oleoyl-CoA. After a 30-minute incubation the reaction was stopped by an addition of 1.2 ml of chloroform-methanol (1:2) and lipid extracts were prepared according to the method of FOLCH *et al.*¹⁷⁾. The cholesteryl ester was isolated by thin layer chromatography (TLC) on Silica gel 60 plates (F₂₅₄, Merck Co.) using a petroleum ether-diethyl ether-acetic acid (90:10:1) solvent system. The distribution of radioactivity on TLC was analyzed with a radioscaner (radioanalytic imaging system, AMBIS System Inc.) to determine the amount of cholesteryl [1-¹⁴C]oleate.

Assay for Cholesteryl Ester Formation in J774 Macrophages

J774 (J774.1) macrophages¹⁸⁾ were routinely maintained in a growth medium (RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO) and penicillin-streptomycin solution (final concentrations of benzylpenicillin at 50 units/ml and streptomycin at 50 $\mu\text{g/ml}$) in 25 cm^2 tissue culture flasks (Corning Co.) at 37°C in a humidified incubator (95% air - 5% CO_2).

Lipid dispersions having molar ratios of free cholesterol/phosphatidylcholine greater than 2 were prepared as reported previously^{19,20)}. Phosphatidylcholine (4 mg) dissolved in chloroform was dried under N_2 in a 10-ml test tube and free cholesterol (16 mg) in benzene was added. After mixing, the solvent was evaporated under N_2 and the lipids were held in a vacuum desiccator for 2 hours. Then, 8 ml of sterilized 0.3 M glucose was added and sonicated for 5 minutes.

Assay for cholesteryl ester formation in J774 macrophages was carried out according to the method described by McCLOSKEY *et al.*²⁰⁾ with some modification. Two-day cultured J774 macrophages collected by centrifugation (800 rpm, 5 minutes) were resuspended in the growth medium at a concentration of 1.0×10^6 cells/ml. Then 0.74 ml of the cell suspension was transferred into each well of 24-well microplates (Corning Co.). After 2-hour incubation the medium was changed to the same volume of FBS-free growth medium, and then the lipid dispersion (40 μl), [^{14}C]oleic acid (0.25 μCi in 10 μl of 50% aq ethanol) and various amounts of inhibitor (dissolved in 10 μl of ethanol) were added to each well. The cells were incubated at 37°C in a humidified incubator (95% air - 5% CO_2). After 24 hours, 800 μl of 0.1% SDS in PBS was added to each well. Lipids were extracted and separated on TLC as described above. Cell viability was also determined by the trypan blue dye exclusion method²¹⁾ following 24-hour incubation of J774 cells in the FBS-free growth medium with the lipid dispersion and in the presence or absence of different concentrations of inhibitor.

Assay for Cholesteryl Ester Formation in Mouse Peritoneal Macrophages

Cholesteryl ester formation in cultured mouse peritoneal macrophages was assayed by the method of NISHIKAWA *et al.*¹²⁾

Results

Inhibitory Effect of Cyclodepsipeptides on ACAT Activity in an Enzyme Assay

The effect of beauvericin and enniatins on ACAT activity was tested in an assay using rat liver microsomes as a source of enzyme. As shown in Fig. 2, all of the compounds inhibited the activity in a dose-dependent manner. Among them beauvericin showed the most potent inhibitory activity with an IC_{50} value of 3.0 μM . Enniatins exhibited relatively weak inhibition (IC_{50} values: 22 to 110 μM). The order of hydrophobicity of enniatins was estimated to be A, F, A1, E, B1, D and B from the order eluted through an ODS-column by HPLC (Table 1), and the data indicated a correlation between hydrophobicity and potency of activity against ACAT. However, beauvericin is not so hydrophobic because it was eluted between enniatin B1 and enniatin E. Under the same conditions, the IC_{50} values of the synthetic ACAT inhibitors CL 283,546 and CL 277,082 were 1.3 and 6.6 μM , respectively.

Effect of Cyclodepsipeptides on Cholesteryl Ester Formation in Macrophages

When J774 macrophages were exposed to the medium containing cholesterol-rich phospholipid dispersions, the formation of both cellular cholesteryl ester and triacylglycerol increased in a time-dependent fashion at least up to 24 hours (data not shown). In the presence of beauvericin or enniatins at various concentrations, the cholesteryl ester and triacylglycerol formations and cytotoxicity were measured. The results of beauvericin and enniatins A and D are shown in Fig. 3, and similar results were obtained from the other enniatins. The cholesteryl ester formation was inhibited in a dose-dependent manner by these antibiotics, suggesting inhibition of ACAT activity in the living cell. The IC_{50} values for cholesteryl ester

Fig. 2. Inhibition of ACAT activity by beauvericin and enniatins in an enzyme assay using rat liver microsomes.

● Beauvericin, ○ enniatin A, △ enniatin A1, ▽ enniatin B, □ enniatin B1, ▼ enniatin D, ■ enniatin E, ▲ enniatin F.

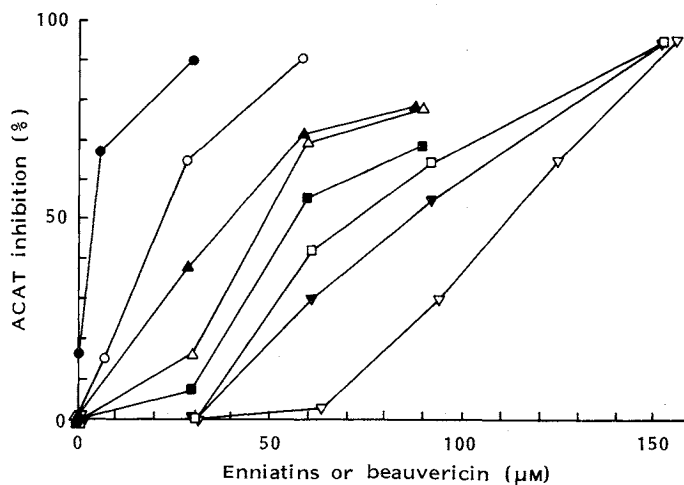


Table 1. Summary of hydrophobicity, ACAT inhibitory activity (IC_{50}) and cytotoxicity (CD_{50}) of cyclodepsipeptides and synthetic inhibitors in assays using rat liver microsomes and J774 macrophages.

Compound	Hydrophobicity retention time (minutes) ^a	Rat liver microsomes	J774 macrophages		
		IC_{50} (μM)	IC_{50} (μM)	CD_{50} (μM)	CD_{50}/IC_{50}
Enniatin A	17.5	22	0.44	2.6	5.9
A1	13.6	49	1.1	2.6	2.3
B	8.7	113	0.81	> 10	> 12
B1	10.8	73	0.44	> 5.0	> 11
D	10.0	87	0.70	8.0	11
E	12.5	57	0.28	2.7	9.6
F	15.7	40	0.43	2.9	6.7
Beauvericin	12.0	3.0	0.17	11	65
CL 283,546	NT ^b	1.3	0.074	> 22	> 300
CL 277,082	NT	6.6	0.30	7.8	26

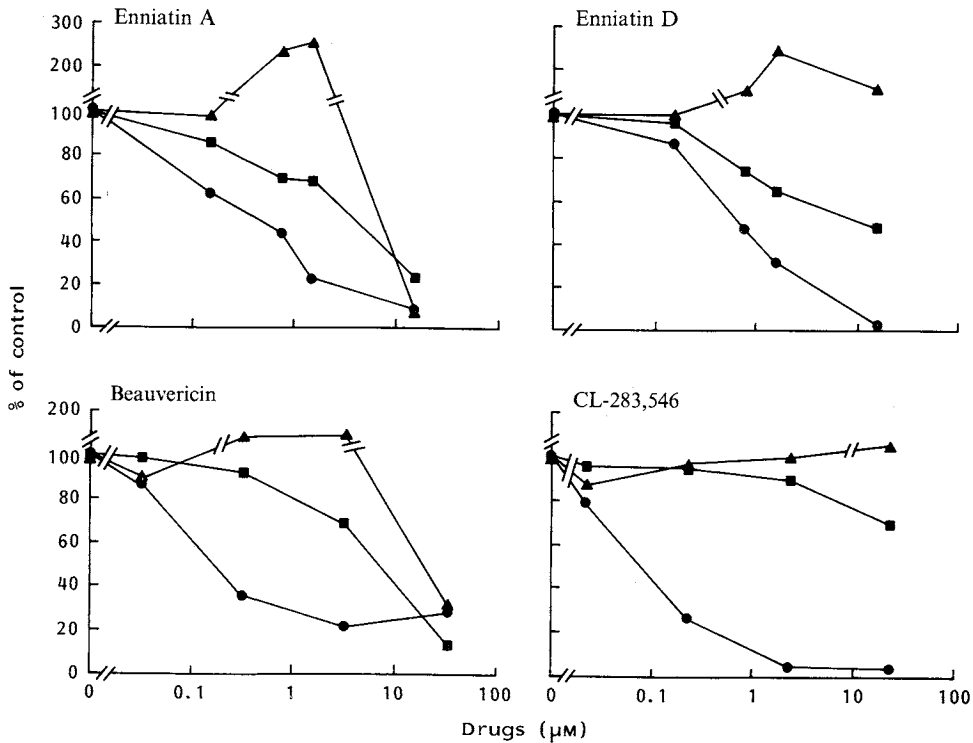
^a Retention time when eluted through an ODS column (Chemcosorb 50DS-UH, 4.6 × 150 mm) under following conditions: solvent; 75% aq CH_3CN , flow rate; 1.0 ml/minute.

^b Not tested.

formation and cytotoxicity (CD_{50} values: drug concentration causing 50% cell damage) are summarized in Table 1. Beauvericin showed the most potent inhibitory activity against cholesteryl ester formation in this cell assay with an IC_{50} value of 0.17 μM . The IC_{50} values of enniatins ranged between 0.28 ~ 1.1 μM . The CD_{50}/IC_{50} ratios indicated that beauvericin shows much higher specificity for ACAT inhibition than enniatins (Table 1). Of all the compounds tested, CL 283,546 demonstrated the highest specificity (> 300), followed by beauvericin (65) and CL 277,082 (26). Interestingly, triacylglycerol formation was 1.5- to 4-fold enhanced at drug concentrations of cyclodepsipeptides where the formation of cholesteryl ester was inhibited. Such high enhancement was not observed for CL-283,546 (Fig. 3).

Fig. 3. Effects of beauvericin, enniatins A and D and CL-283,546 on cholesteryl ester (CE) and triacylglycerol (TG) formation and on viability of J774 macrophages.

● Cholesteryl ester, ▲ triacylglycerol, ■ cell viability.



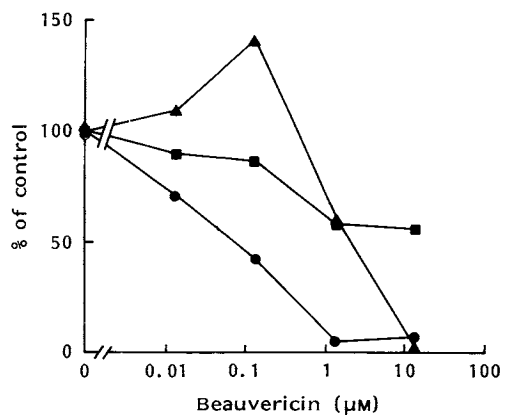
The specificity of beauvericin for ACAT inhibitory activity was further examined in another cell assay using primary cultures of mouse peritoneal macrophages. The effects on cholesteryl ester and triacylglycerol formations and cell viability were essentially similar (Fig. 4) to those in J774 cells, resulting in high specificity with a CD_{50}/IC_{50} ratio of >130 . Enhancement of triacylglycerol formation was also observed to some extent (about 1.5-fold) in this cell assay.

Discussion

The cyclodepsipeptides beauvericin and enniatins behave as ionophore antibiotics in forming complexes with alkali metals. They exhibit various biological activities such as antimicrobial, insecticidal and cytotoxic activities. However, it seems still unclear whether these biological activities are actually derived from the ionophoric activity. In this paper, the cyclodepsipeptides were shown to exhibit a new biological activity of ACAT inhibition.

Fig. 4. Effects of beauvericin on cholesteryl ester and triacylglycerol formation and on viability of mouse peritoneal macrophages.

● Cholesteryl ester, ▲ triacylglycerol, ■ cell viability.



A good correlation was demonstrated between the ACAT inhibition by enniatins in an enzyme assay (Fig. 2) and the hydrophobicity (Table 1). More hydrophobic enniatins produced more potent ACAT inhibition. However, the inhibitory activity of enniatins was not so strong. Since ACAT is a membrane-bound enzyme²²⁾, hydrophobic compounds can associate with membranes and disturb the enzyme activity possibly in a non-specific manner. On the other hand, beauvericin of lower hydrophobicity exhibited the most potent ACAT inhibitory activity (IC_{50} : $3.0 \mu M$), suggesting possible specific ACAT inhibition by beauvericin. To our knowledge, beauvericin is one of the most potent ACAT inhibitors of microbial origin in an enzyme assay. They are structurally different in that beauvericin contains L-phenylalanine instead of the branched alkyl amino acids of enniatins (Fig. 1), indicating that the aromatic group in the structure may contribute to the ACAT inhibition.

The cell assay using J774 macrophages also demonstrated that beauvericin showed the most potent ACAT inhibition (IC_{50} : $0.17 \mu M$). As for J774 cytotoxicity, hydrophobic enniatins A, F, A1 and E (CD_{50} : 2.6 to $2.9 \mu M$) were more cytotoxic than other enniatins and beauvericin (CD_{50} : approximately $10 \mu M$). This suggests that the hydrophobicity affects the cell viability, but not ACAT activity in the cell assay. As a result, beauvericin showed much higher specificity than enniatins. In comparison with those of CL 283,546 and CL 277,082 under the same conditions, CL 283,546 is the most potent and specific ACAT inhibitor followed by beauvericin. Such high specificity of beauvericin for ACAT inhibition was also observed in another cell assay using mouse peritoneal macrophages (Fig. 4). Furthermore, triacylglycerol formation was enhanced for all the cyclodepsipeptides at the drug concentrations where cholesteryl ester formation was inhibited in both cell assays. This suggests that the substrate acyl-CoA accumulated by inhibiting ACAT is utilized predominantly for triacylglycerol synthesis. A similar triacylglycerol accumulation by the ACAT inhibitor 58-035 was reported in CaCo-2 cells by KAM *et al.*²³⁾ However, CL 283,546 had no effect on the triacylglycerol formation (Fig. 3). Intracellular transport of acyl-CoA may also be affected by the compound.

Taken together, beauvericin, regardless of its lower hydrophobicity and weaker antimicrobial activity, showed the most potent ACAT inhibition in both enzyme and cell assays and the highest specificity in cell assays among the cyclodepsipeptides. The data indicate that the ACAT inhibition by beauvericin may be independent of the ionophoric activity.

Acknowledgments

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