GERI-BP001 Compounds, New Inhibitors of Acyl-CoA : Cholesterol Acyltransferase from *Aspergillus fumigatus* F37

I. Production, Isolation, and Physico-chemical and Biological Properties

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(Received for publication July 29, 1994)

GERI-BP001 compounds, new inhibitors of acyl-CoA : cholesterol acyltransferase (ACAT), were isolated from a culture broth of *Aspergillus fumigatus* F37 by acetone extraction, EtOAc extraction, SiO_2 column chromatography, and reverse phase HPLC. GERI-BP001 M, A, and B inhibit ACAT activity in an enzyme assay system using rat liver microsomes by 50% at concentrations of 42, 94, and 40 μM, respectively.

Acyl-CoA : cholesterol acyltransferase (EC 2.3.1.26) plays an important role in the control of intracellular free cholesterol content *via* its cholesterol esterifying activity[1,2]. In both experimental and clinical atherosclerosis, the formation of foam cells derived from macrophages and smooth muscle cells is an important event because foam cells contain a large amount of cholesteryl esters that are derived from cholesterol by the activity of ACAT. Since the propagation of foam cells in the arterial walls is directly related to the increment of ACAT activity, the development of ACAT inhibitors as potential antiatherosclerotic agents is desirable[3].

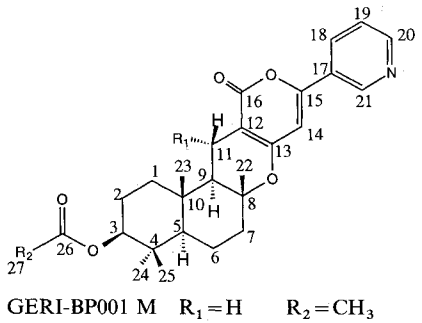
In the course of a screening program for ACAT inhibitors from microbial sources, GERI-BP001[4] (GERI-BP001 M; M is a major fraction), GERI-BP001 A, and GERI-BP001 B (Fig. 1) were isolated from the fermentation broth of a fungal strain, *Aspergillus fumigatus* F37. Structure elucidation shows that GERI-BP001 compounds are new ones which are structurally related to pyripyropenes[5]. This paper describes the taxonomy of the GERI-BP001 producing strain, fermentation, isolation, and physico-chemical and biological characteristics of GERI-BP001 compounds. Structure elucidation studies of GERI-BP001 M, A, and B will be reported in a separate paper.

Taxonomy of Producing Organism

A fungal strain F37 was originally isolated from a soil sample collected at Mountain Dukyou, Cheonbuk Province, Korea. Taxonomic studies of the strain were carried out by the method of RAPER and FENNEL[6]. Czapek's media ($NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, sucrose 3%, agar 1.5%) and MEA media (malt extract 2.5%, agar 1.5%) were used for the identification of strain F37. The cultures were incubated at 25 and 45°C for about 14 days. The cultural characteristics of microorganism after incubation at 25°C for 7 days are summarized in Table 1.

As shown in Fig. 2, morphological observation was done under a microscope (Carl Zeiss, Jenalumar). On Czapek's agar at 25°C after 7 days, the conidial heads were columnar and green to dark green in color. The reverse side was a gray reddish brown color. Conidiophores with short and smooth walls were 200~250 × 3~4 μm and pale green in the upper part. Vesicles were

Fig. 1. Structures of GERI-BP001 M, A, and B.



GERI-BP001 M R_1=H R_2=CH_3
GERI-BP001 A R_1=OH R_2=CH_3
GERI-BP001 B R_1=H R_2=CH_2CH_3

Table 1. Cultural characteristics of a fungal strain F37.

	Media	
	Czapek	MEA
Temperature/Time	25℃ / 7 days	25℃ / 7 days
Colony size/Surface of colony	50mm/Velvety	50mm/Velvety
Color of conidia	Gray olive green	Gray olive green
Color of reverse side	Gray reddish brown	Moderate yellow green

Fig. 2. Photomicrograph of *Aspergillus fumigatus* F37.
Bar represents 20 μm.

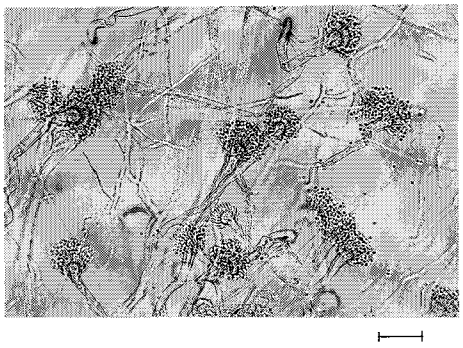
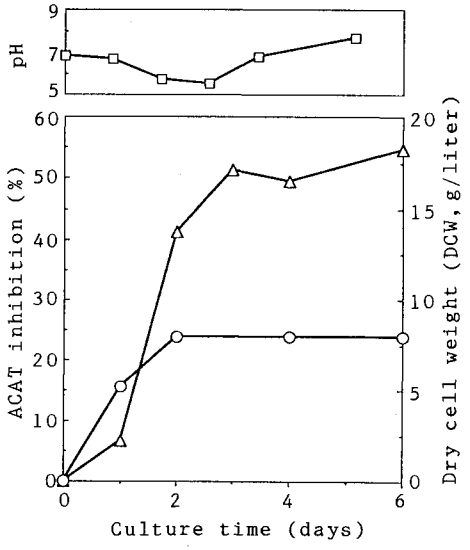


Fig. 3. Typical time course of GERI-BP001 production.
△ ACAT inhibition (%), ○ DCW (g/liter).



club-like in form to pyriform, 10~15 μm i.d. and pale green in color. Metulae were not observed. The upper two-thirds of the vesicle abundantly beared phialides 6 to 8 μm in length and 1 to 2 μm in width at the widest part. Conidia were rough-walled globose, 2~3 μm i.d., and dark green in color. Strain F37 grew well at temperature up to 45°C. In chemotaxonomic studies, nuclear DNA (G+C content) was 49.2% and quinone type was Q10. From the above characteristics, strain F37 was identified as a strain of *Aspergillus fumigatus*. It was deposited at the Korea Collection for Type Cultures (KCTC), Korea, as KCTC 0087BP.

Fermentation

One frozen stock vial (1 ml of spore suspension in 10% glycerol, −80°C) of F37 was inoculated into a 1-liter baffled flask containing 100 ml of seed medium (glucose 0.5%, soluble starch 1.5%, yeast extract 0.2%, polypeptone 0.5%, KH_2PO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 5.8 prior to autoclave). Seed culture was incubated at 29°C for 18 hours on a rotatory shaker at 150 rpm (radius 7 cm). Twenty ml of seed culture was inoculated into a 5-liter baffled flask containing 1 liter of production medium (soluble starch 3%, soytone 1%, pH 7.0 before autoclave). The fermentation was carried out for 144 hours at 29°C on the rotary shaker at 150 rpm. As shown in Fig. 3, cell mass reached to a maximum level after 2 days of fermentation while the ACAT inhibitory activity leveled off after 6 days of culture. To determine the dry cell weight, mycelia were collected by filtration on Whatman no. 1 filter, then washed twice with distilled water and dried in oven to a constant weight. ACAT activity was determined using [1-^{14}C]-oleoyl-CoA and cholesterol as substrates and rat liver microsomes as an enzyme source.

Isolation

The isolation procedures are summarized in Fig. 4. A six-day old fermentation broth of *Aspergillus fumigatus* F37, 1 liter, was centrifuged at 10,800 × *g* (8,000 rpm in GS-3 rotor on a Sorvall RC5 C Refrigerated Superspeed Centrifuge) for 15 minutes. After removing the supernatant, the resulting mycelial cake was extracted twice with 0.3 liter acetone. The acetone-extracts were filtered with a Whatman no. 2 filter paper. The extracts were concentrated *in vacuo* to yield about 100 ml and then partitioned between ethyl acetate and water with two 100 ml portions of ethyl acetate. The ethyl acetate layers were combined and concentrated *in vacuo* to yield a brown

oily material (370 mg). This material was charged to a silica gel column (E. Merck, Kieselgel 60, 230 ~ 400 mesh, 50 ml) and eluted stepwise with a gradient of *n*-hexane - ethyl acetate (1 : 1, 1 : 2, 1 : 3 and 1 : 5, 200 ml each). The active fractions were combined and concentrated *in vacuo* resulting in an oily residue. The residue was dissolved in methanol and applied to a preparative HPLC using an ODS column (Waters, DELTA PAK C18-100 Å, 50 × 300 mm; mobile phase: MeOH - water 9 : 1; flow rate: 30 ml/minute; detection: 3 dimensional photodiode array detector). Active fractions were concentrated *in vacuo* to obtain pale yellow powder. Further purification of ACAT inhibitors was carried out by HPLC using an Ultracarb 10 ODS-30 column (21.2 × 250 mm, Phenomenex, U.S.A.). The column was eluted with MeOH - water (9 : 1) at a flow rate of 5 ml/minute. The compounds

Fig. 4. Isolation and purification procedures of GERI-BP001 compounds.

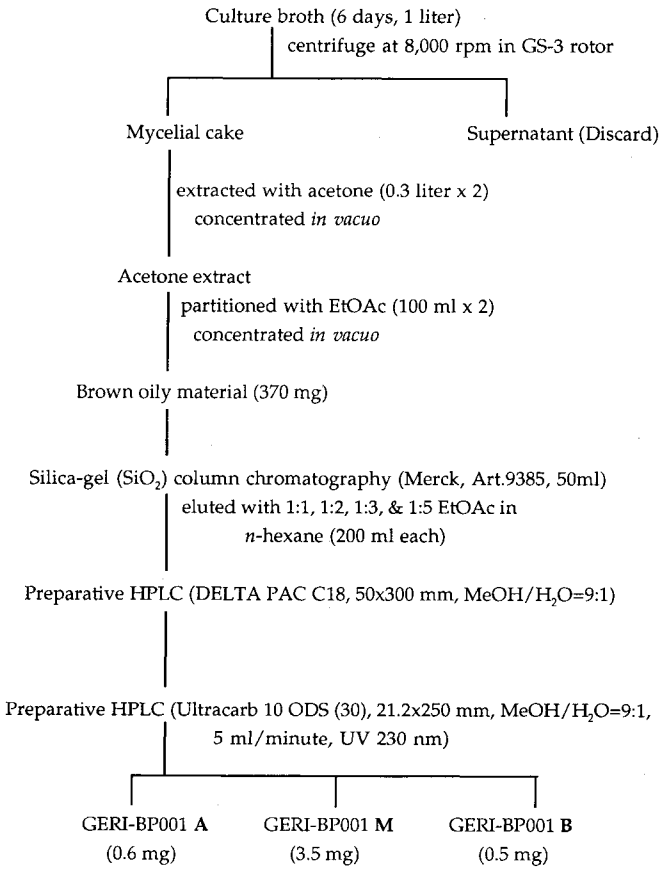
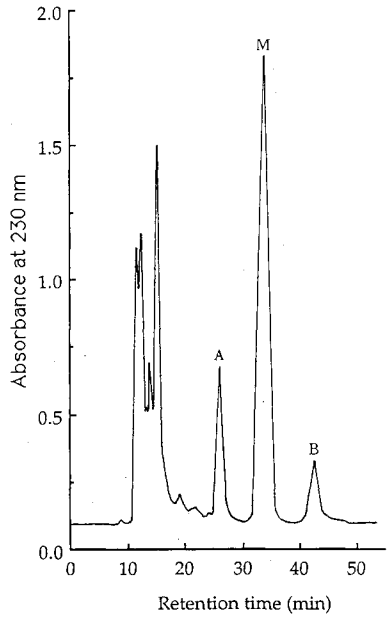


Fig. 5. HPLC chromatography of crude GERI-BP001 compounds.



Column: Ultracarb 10 ODS-30 (21.2 × 250 mm, Phenomenex, U.S.A.); mobile phase MeOH - H_2O (9 : 1); flow rate 5 ml/minute; detection 230 nm.

Table 2. Physico-chemical properties of GERI-BP001 compounds.

	GERI-BP001 M	GERI-BP001 A	GERI-BP001 B
Appearance	White powder	White powder	White powder
$[\alpha]_D^{18}$ (c 0.5, $CHCl_3$)	+146.2	+39.6	+59.5
Molecular formula	$C_{27}H_{33}NO_5$	$C_{27}H_{33}NO_6$	$C_{28}H_{35}NO_5$
HREI-MS (m/z)			
Calcd:	451.2358	467.2307	465.2515
Found:	451.2347	467.2354	465.2492
UV λ_{max}^{MeOH} (nm)	232 (ε=19,200)	232 (ε=8,400)	232 (ε=20,200)
	322 (ε=11,100)	322 (ε=3,600)	322 (ε=11,500)
IR ν_{max} (CCl_4) cm^{-1}	2947, 1716, 1246	3560, 2927, 1732, 1249	2927, 1732, 1102
Solubility			
Soluble:	MeOH, $CHCl_3$, EtOAc	MeOH, $CHCl_3$, EtOAc	MeOH, $CHCl_3$, EtOAc
Insoluble:	*n*-Hexane	*n*-Hexane	*n*-Hexane
Color reaction			
Positive:	50% H_2SO_4, Anisaldehyde	50% H_2SO_4, Anisaldehyde	50% H_2SO_4, Anisaldehyde
Negative:	Ninhydrin	Ninhydrin	Ninhydrin

eluted were detected by UV absorption at 230 nm with a Perkin-Elmer LC 90 UV Spectrophotometric Detector. GERI-BP001 A was eluted with a retention time of 26 minutes, followed by GERI-BP001 M at 34 minutes and GERI-BP001 B at 43 minutes (Fig. 5). They were collected and concentrated *in vacuo* to yield pure GERI-BP001 A (0.6 mg), M (3.5 mg), and B (0.5 mg) as white powder.

Physico-chemical Properties

The physico-chemical properties of GERI-BP001 M, A, and B are summarized in Table 2. White powder of GERI-BP001 compounds are readily soluble in methanol, chloroform, and ethyl acetate while insoluble in water and *n*-hexane. UV spectra of GERI-BP001 M, A, and B have two maximum peaks at 232 and 322 nm in methanol (Fig. 6). The molecular formula of GERI-BP001 compounds are determined as $C_{27}H_{33}NO_5$ for GERI-BP001 M, $C_{27}H_{33}NO_6$ for GERI-BP001 A, and $C_{28}H_{35}NO_5$ for GERI-BP001 B on the basis of high resolution electron impact mass (HREI-MS) spectral analysis. Intensive NMR work of GERI-BP001 compounds reveals that sesquiterpene, α-pyrone, and pyridine are present in all of the components (Fig. 1).

Biological Properties

Effect on ACAT Activity in an Enzyme Assay System

Microsomes prepared from rat liver[7] were used as a source of the enzyme. The activity of the microsomal ACAT was measured according to the method of P. BRECHER *et al.*[8] with modifications. The reaction mixture, containing 4 μl of microsomes (10 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 30 minutes at 37°C. The reaction was initiated by the addition of 8 μl of [1-^{14}C]oleoyl-CoA solution (0.05 μCi, final conc. 10 μM). After 15 minutes 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 assay buffer (prepared by diluting 0.5 M assay buffer 1:5 in water) was then added to the terminated reaction mixture. The above solution was mixed and allowed to phase separation under gravity for 2 minutes. 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 a 7 ml liquid scintillation vial with 4 ml of scintillation cocktail (Lipoluma, Lumac Co.) using a liquid scintillation counter (Packard Delta-2000). Background values were obtained by preparing heat inactivated microsomes.

This assay system is designed to test the activity of acyl-CoA : cholesterol acyltransferase suitable for an easy

Fig. 6. UV spectrum of GERI-BP001 M (MeOH).

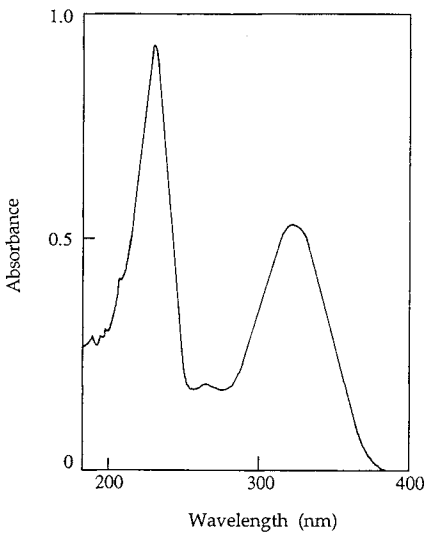
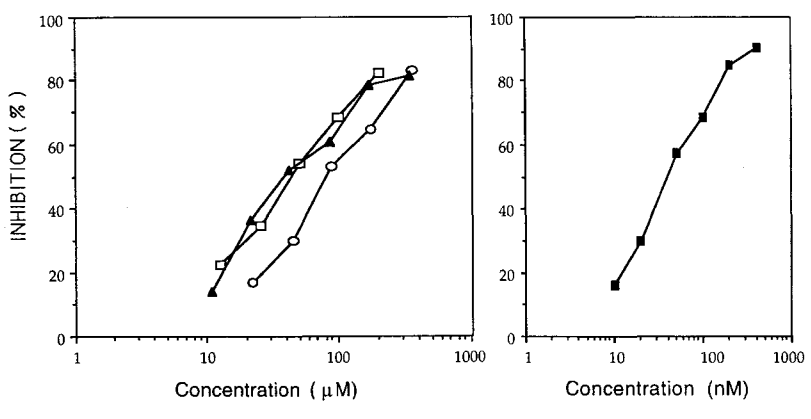


Fig. 7. ACAT inhibition activities by GERI-BP001 M, A, and B and pyripyropenes A in a rat liver microsomal assay system.
□ GERI-BP001 M, ○ GERI-BP001 A, ▲ GERI-BP001 B, ■ Pyripyropene A.



screening of fermentation broths and natural product extracts. Separation of cholesteryl [1-^{14}C]oleate from residual substrate, [1-^{14}C]oleoyl-CoA, and side-products was performed by solvent partitioning between an upper heptane phase and a lower isopropanol-water phase. Since some of the radioactivity recovered in the upper phase was due to enzymatic incorporation of radioactive fatty acid into other products than cholesteryl oleate, the partitioned heptane phase were separated by TLC (E. Merck, silica gel 60 F_{254}) using a solvent system of *n*-hexane-diethyl ether-acetic acid (90:10:1) to verify inhibition of ACAT. Cholesteryl ester standard (Sigma C9253, cholesteryl oleate) was applied on several lanes accross each plate so that the cholesteryl ester region can be identified. Regions corresponding to the cholesteryl ester (Rf 0.64~0.71) were scraped and the radioactivity of the cholesteryl [1-^{14}C]oleate was measured as described above.

Percent inhibition of ACAT activity is calculated by substracting the background values from both control and test sample values.

$$\% \text{ Inhibition} = 100 \times \left[1 - \frac{\text{Sample (cpm)} - \text{Background (cpm)}}{\text{Control (cpm)} - \text{Background (cpm)}}\right]$$

GERI-BP001 M, A, and B inhibit the ACAT activity in TLC-based assay by 50% at concentrations of 42, 94, and 40 μM, respectively (Fig. 7). Under the same conditions pyripyropene A, a known potent ACAT inhibitor[5], showed the IC_{50} value of 43 nM.

Other Biological Activities

GERI-BP001 M had no *in vitro* antimicrobial activities against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Klebsiella oxytoca*, *Klebsiella aerogenes*, *Enterobacter cloacae* at concentrations up to 200 μg/ml. GERI-BP001 M showed no antiviral activities against Mock infected cells, HSV-1 infected cells, and HSV-2 infected cells at concentrations up to 100 μg/ml. Cytotoxicity was not observed to Vero host cells at 100 μg/ml. Acute toxic effects were not recognized when GERI-BP001 M was subcutaneously injected to ICR mouse at 500 mg/kg.

Discussion

Many synthetic ACAT inhibitors having urea or amide moieties have been reported[9] and about 15 ACAT inhibitors are isolated from microbial sources[10~12]. We discovered a series of ACAT inhibitors, which were called GERI-BP001 compounds, in the culture broth of the fungal strain, *Aspergillus fumigatus* F37.

GERI-BP001 compounds described in this paper are new ones of sesquiterpene derivatives with moderate inhibitory activities against ACAT. GERI-BP001 compounds consist of pyridine, α-pyrone, and sesquiterpene moieties. The structures of GERI-BP001 compounds are very similar with pyripyropenes isolated by ŌMURA *et al.*[5] However, GERI-BP001 compounds contain one *O*-acetyl or propionyl residues in the sesquiterpene moieties while three *O*-acetyl or propionyl residues are present in pyripyropenes. It led us to find a couple of interesting points by comparing the structures of pyripyropenes and GERI-BP001 M, A, and B in relation to inhibitory activities of ACAT. The IC_{50} values of pyripyropenes are at nanomolar levels (53~268 nM)[5], however, the IC_{50} values of GERI-BP001 compounds are at micromolar levels (40~94 μM). There is about 1,000-fold difference in inhibitory activity between pyripyropenes and GERI-BP001 compounds in despite of their structural similarities. It is supposed that the inhibition activities of these types of compounds against ACAT greatly depend on the presence of *O*-acyl groups at C-4 and/or C-7 positions in the sesquiterpene moieties. The stereochemistry of hydroxyl group at C-11 position might be also considerable to determine the ACAT inhibitory activity, because the stereochemistry of hydroxyl group at C-11 is opposite between pyripyropenes[13] and GERI-BP001 A. Those two types of compounds are expected to be a good example for design of new sesquiterpene type ACAT inhibitors. The structural modifications and synthesis of GERI-BP001 compounds are now in progess.

Acknowledgments

We are grateful to Korea Ministry of Science and Technology for financial support. We kindly thank Dr. SATOSHI ŌMURA for supplying of pyripyropene A. We thank Dr. KYUNG-SOOK BAE for characterization of the producing strain. We thank JOUNG-SUK CHO for her generous support for isolation experiments and WOO-SANG LEE, YONG-KOOK KWON and JI-HYUP KIM for their assistances with the fermentation studies.

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