GERI-BP002-A, Novel Inhibitor of Acyl-CoA : Cholesterol Acyltransferase Produced by *Aspergillus fumigatus* F93

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A new inhibitor of acyl-CoA : cholesterol acyltransferase (ACAT), designated GERI-BP002-A, was isolated from the culture broth of *Aspergillus fumigatus* F93 by acetone extraction, EtOAc extraction, SiO_2 column chromatography and reverse phase HPLC. Spectroscopic analyses of the compound identified bis (2-hydroxy-3-*tert*-butyl-5-methylphenyl) methane as the structure and its molecular weight and formula to be 340 and $C_{23}H_{32}O_2$, respectively. GERI-BP002-A inhibited ACAT activity by 50% at the concentration of 50 μM in an enzyme assay system using rat liver microsomes.

The enzyme acyl-CoA : cholesterol acyltransferase (EC 2.3.1.26) is responsible for catalyzing the intracellular esterification of cholesterol and acyl-CoA[1,2]. ACAT plays various roles in cholesterol metabolism in mammals. ACAT-mediated cholesterol esterification is believed to play a key role in intestinal absorption of cholesterol, hepatic production of lipoproteins, and the deposition of cholesteryl esters in atherosclerotic lesions. 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 high concentration of cholesteryl esters that are derived from cholesterol by the activity of ACAT. Therefore, ACAT inhibition is an attractive target for prediction and treatment of hypercholesterolemia and atherosclerosis[3].

A large number of synthetic ACAT inhibitors have been reported[4]. They are classified into several groups; fatty-acyl amides, a series of substituted ureas, and a series of triphenyl-imidazole derivatives. During the course of our screening program to find new ACAT inhibitors from microbial sources, GERI-BP002-A (Fig. 1) was isolated from the fermentation broth of *A. fumigatus* F93. Structure elucidation shows that GERI-BP002-A is novel. In this paper, we report the taxonomy of the GERI-BP002-A producing strain, as well as the isolation, physico-chemical characteristics and structure elucidation of GERI-BP002-A.

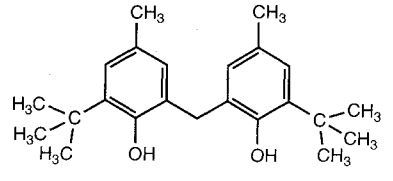
Materials and Methods

Microorganism and Chemicals

A microorganism designated F93 was originally isolated from a soil sample collected at Jangdong, Yusung-ku, Taejon, Korea. Taxonomic studies of the strain were carried out by the method of RAPER and FENNEL[5]. CZAPEK's medium ($NaNO_3$ 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%), MEA medium (malt extract 2.5%, agar 1.5%), CYA medium (yeast extract 0.5%, sucrose 3.0%, $NaNO_3$ 0.3%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $ZnCl_2 \cdot 7H_2O$ 0.001%), and CY20S medium (CYA medium with sucrose increased to 20%) were used in the identification of strain F93. For the evaluation of cultural characteristics, the strain was incubated for 14 days at 25 and 45°C. Morphological observation was carried out using a scanning electron microscope (Philips, SEM515). The color names used were based on the ISCC-NBC Color-Name Chart Illustrated with Centroid Colors.

3-*tert*-Butyl-4-hydroxy-5-methylphenyl sulfide, 4-4'-di-*tert*-butylphenyl, 2,2'-dimethyl bibenzyl, 1,1'-bis(3,4-

Fig. 1. Structure of GERI-BP002-A.



dimethylphentyl)ethane, 2-benzylpyridine, 3-benzylpyridine, diphenylmethane, 2,2-diphenylpropane, *cis*-stillben, *trans*-stillben, bibenzyl, sulfadiazine, sulfisomidine were purchased from the Sigma Chemical Company.

Spectral Analysis

The UV spectrum was recorded on a Shimadzu UV265 UV-Visible spectrophotometer. pH was measured with an Orion research digital pH/millivolt meter 611 and Ross electrode. HPLC was carried out using a Perkin Elmer (Binary LC pump, LC-95) UV/Visible spectrophotometer and ODS packed column (Phenomenex, 22.2 × 250 mm). Preparative HPLC was carried out using a Waters Delta Prep 4000 chromatograph with a photodiode array detector (Waters 991) and an ODS packed column (Waters DELTA PAC C-18-100A, 50 × 300 mm). EI and FAB mass spectra were recorded on a Vacuum Generator spectrometer (VG70-VSEQ). m-Nitrobenzyl alcohol was used in the FAB mode as a matrix. ^{1}H and ^{13}C NMR spectra were obtained on a Varian Unity 500 spectrometer using $CDCl_3$. Chemical shifts are given in ppm using TMS as internal standard.

Fermentation

One frozen stock vial (1 ml of spore suspension in 10% glycerol, −80°C) of F93 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 autoclaving). Distilled water was used in preparation of seed and production media. The seed culture was incubated at 29°C for 18 hours on a rotary shaker at 150 rpm (radius 7 cm). Twenty ml of the 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 autoclaving). The fermentation was carried out for 144 hours at 29°C on a rotary shaker at 150 rpm. To determine the cell dry weight, mycelia were collected by filtration using Whatman No. 1 filter paper, then washed twice with distilled water and oven dried to a constant weight.

Antimicrobial Activity

Antimicrobial activity was tested using an agar dilution method. Bacteria were grown on Mueller Hinton agar medium. Among them, *Streptococcus pyogenes* was grown on Mueller Hinton agar medium with 10% sheep blood. Antibacterial activity was determined after 24-hour incubation at 37°C.

Antitumor Activity

The cytotoxicity studies were carried out with the sulforhodamine B (SRB) microculture colorimetric assay described previously[6,7]. Briefly, A549 (non-small human lung cell), SKOV-3 (ovarian), HCT-15 (colon), XF-498 (CNS) and SKMEL-2 (melanoma) cells were maintained as stocks in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco). The rapidly growing cells were harvested and counted, and then inoculated at the appropriate concentration ($1 \sim 2 \times 10^4$ cells/well) into 96 well microtiter plates. After incubation for 24 hours, the test samples dissolved in DMSO (final 10%) were added in triplicate to cultured cells and incubated for 48 hours at 37°C under 5% CO_2 atmosphere. The cultures were fixed with cold trichloroacetic acid, and stained by 0.4% sulforhodamine B (dissolved in 1% acetic acid). After solubilizing the bound dye with 10 mM unbuffered tris base by gyro-shaker, the cells were measured at 520 nm and 690 nm in a microplate reader (Dynatech Model MR 700). The absorbance measured at 690 nm was substracted from the absorbance at 520 nm so as to eliminate the effects of non-specific absorbance. The data were transferred and transformed into Lotus-123 format and survival fractions were calculated by comparing the sample with control. All data was represented as the average values for a minimum of three wells.

Results

Taxonomy

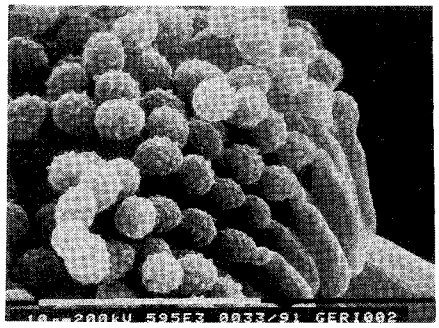
The cultural characteristics of the microorganism after incubation at 25°C for 7 days are summarized in Table 1. As shown in Fig. 2, morphological observation was carried out using a scanning electron microscope. After 14 days on CZAPEK's agar at 25°C, the conidial heads were columnar and green to dark green in color. The reverse side was a gray reddish brown color. Conidiophores with short and smooth walls were $350 \sim 480 \times 2 \sim 3$ μm and pale green in the upper part. Vesicles were club-like to pyriform, $13 \sim 17$ μm i.d. and pale green in color. Metulae were not observed. The upper two-thirds of the vesicle abundantly bore phialides 4 to 8 μm in

Table 1. Cultural characteristics of a fungal strain F 93 on several media.

Media	Temperature/Time	Colony size/ Surface of colony	Color of conidia*	Color of reverse side
CYA	25°C/ 7 days	70 mm/Velvety	Greenish yellow	Dark yellow
MEA	25°C/ 7 days	70 mm/Velvety	Olive green	Greenish yellow
CY20S	25°C/ 7 days	70 mm/Velvety	Olive green	Greenish yellow
Czapek	25°C/14 days	60 mm/Velvety	Olive green	Greenish yellow
	45°C/14 days	70 mm/Velvety	Olive green	Greenish yellow

* ISCC-NBC color-name chart illustrated with centroid colors.

Fig. 2. SEM photomicrograph of *A. fumigatus* F 93.



length and 1 μm in width at the widest part. Conidia were rough-walled globose, 4 ~ 5 μm i.d., and dark green in color. Strain F93 grew well at temperatures up to 45°C. From these characteristics, strain F93 was identified as a strain of *A. fumigatus*. It was deposited in the Korea Collection for Type Cultures (KCTC), Korea, as KCTC 0111BP.

Fermentation

As shown in Fig. 3, the F93 cell mass reached a maximum after 2 days of fermentation while the ACAT inhibition activity leveled off after 6 days. ACAT activity was determined using [1-^{14}C]oleoyl-CoA and cholesterol as substrates and rat liver microsomes as the ACAT source.

Isolation

A six-day old fermentation broth of *A. fumigatus* F93 (14 liters) was centrifuged at 10,800 $\times g$ (8,000 rpm using a GS-3 rotor with a Sorvall RC5C Refrigerated Superspeed Centrifuge) for 15 minutes at 4°C. After removing the supernatant, the resulting mycelial cake was extracted twice with 3 liters of acetone. The acetone extracts were filtered with Whatman No. 2 filter paper. The extracts were concentrated *in vacuo* about 500 ml and then partitioned between ethyl acetate and water with two 500 ml portions of ethyl acetate. The ethyl acetate layers were combined and concentrated *in vacuo* to yield a brown oily material (6 g). This material was chromatographed on 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* yielding an oily residue. The residue was dissolved in methanol and subjected to preparative HPLC using an ODS column

Fig. 3. Time course of GERI-BP002-A production by *A. fumigatus* F 93.

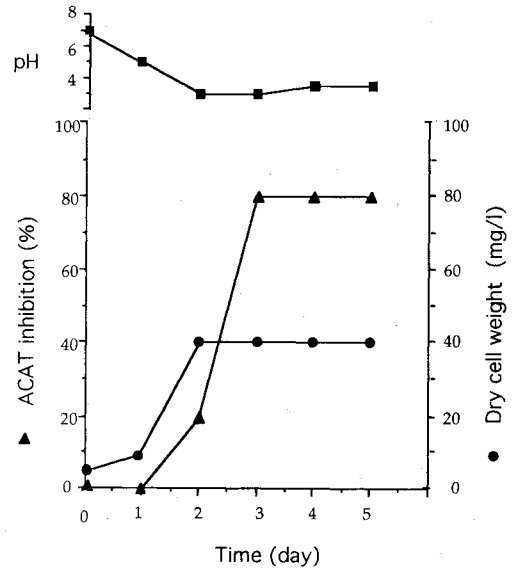


Table 2. Physico-chemical properties of GERI-BP002-A.

Appearance	Pale yellow powder
Molecular formula	$C_{23}H_{32}O_2$
HREI-MS (*m/z*)	
Found	340.2418
Calcd.	340.2404
EI-MS (*m/z*)	340 $(M)^+$
	284 $(M-tert\text{-Butyl}+H)^+$
	228 $(M-2tert\text{-Butyl}+2H)^+$
	57 $(tert\text{-Butyl})^+$
UV (nm) in MeOH	212, 281
Solubility	
Soluble	MeOH, $CHCl_3$, EtOAc
Insoluble	H_2O, *n*-Hexane
Color reaction	
Positive	50% aq H_2SO_4

(Waters, DELTA PAK C18-100 Å, 50 × 300 mm; mobile phase: CH_3CN/water = 8 : 2, flow rate: 30 ml/minute; detection: 3 dimensional photodiode array detector). Active fractions were concentrated *in vacuo* giving a yellow powder. Further purification of the ACAT inhibitor was carried out by HPLC using an Ultracarb 10 ODS-30 column (21.2 × 250 mm, Phenomenex). The column was eluted with CH_3CN - water (8 : 2) at a flow rate of 5 ml/minute. GERI-BP002-A was eluted as a peak with a retention time of 33 minutes and detected by UV absorption at 210 nm with a Perkin-Elmer LC 90 UV spectrophotometric detector. Fractions were collected and concentrated *in vacuo* to yield pure GERI-BP002-A (30 mg) as a pale yellow powder.

Physico-chemical Properties and Structure Elucidation

The physico-chemical properties of GERI-BP002-A are summarized in Table 2. GERI-BP002-A was readily soluble in methanol, chloroform and ethyl acetate while insoluble in water and *n*-hexane. The UV spectrum (in methanol) of GERI-BP002-A exhibited two absorption maxima at 211 and 280 nm. The molecular formula was determined to be $C_{23}H_{32}O_2$ on the basis of fast atom bombardment mass spectra (FAB-MS) and high resolution electron impact (HREI) mass spectra (found *m/z* 340.2418, calcd *m/z* 340.2404). In the EI-MS of GERI-BP002-A (Fig. 4), the presence of two *tert*-butyl residues was revealed from the fragment ion peaks at *m/z* 284 $(M-$*tert*-butyl$+H)^+$, 228 $(M-2$-*tert*-butyl$+2H)^+$, and 57 $(tert\text{-butyl})^+$. The ^1H NMR spectrum exhibited signals at δ_H 6.96, δ_H 5.85 (OH), 3.88, 2.25 and 1.38 with relative intensities of 2, 1, 1, 3, 9, respectively. Since the presence of two butyl moieties was revealed from MS, these were interpreted to be four aromatic, two hydroxy, two benzylic methylene, two methyl and two *tert*-butyl protons, respectively. The ^{13}C NMR spectrum showed 10 carbon signals including those of 5 proton bearing carbons. A combination of Distortionless enhancement by polarization transfer (DEPT) and heteronuclear multiple quantum coherence (HMQC) experiments precisely matched all of the proton bearing carbons and their protons. Since the singlet proton signal at δ_H 3.88 corresponded to the benzylic methylene protons, the dimeric nature of GERI-BP002-A was revealed. Gross structure of this compound was deduced by a combination of heteronuclear multiple bond connectivity (HMBC) optimized for couplings of 8 Hz

Fig. 4. Mass fragmentation of GERI-BP002-A.

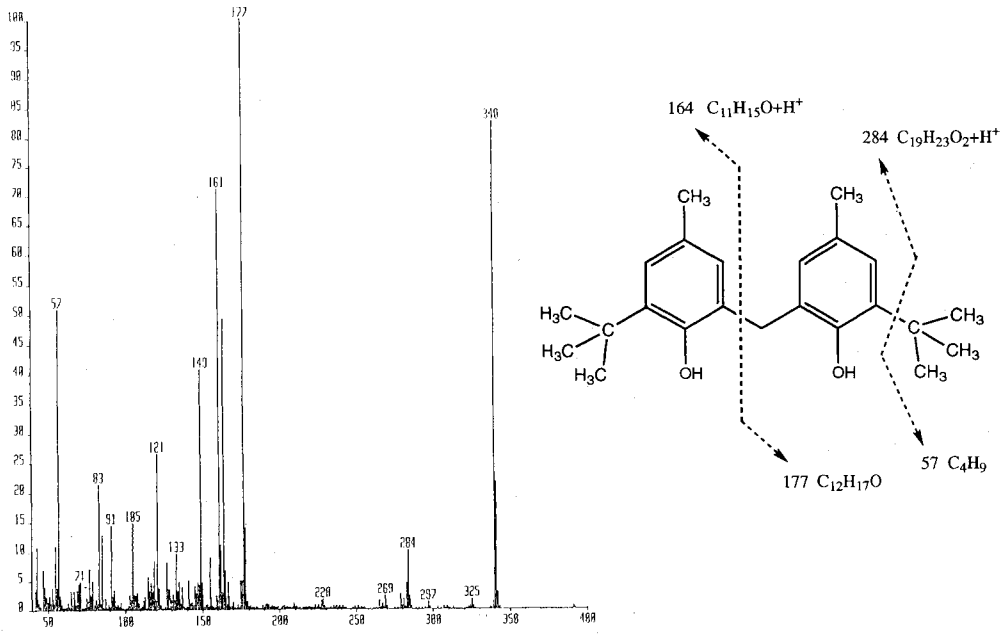


Table 3. ^1H and ^{13}C NMR chemical shifts of GERI-BP002-A.

Carbon No.	^{13}C	^1H	NOE	HMBC (8 Hz)
1	150.65			H-3, H-7
2*	136.88			
3	127.02	6.96	Methyl, *tert*-Butyl	Methyl
4*	130.40			Methyl
5	129.53	6.96	H-7, Methyl(*t*-Bu)	H-3, H-7
6	127.35			H-7
7	32.73	3.88		H-5
tert-Butyl	34.99, 30.64	1.38	H-3 (H-5)	
Methyl	21.56	2.25	H-3 (H-5)	H-3 (H-5)

* All of the information for C-2 and C-4 is interchangeable depending on the structures (I and II).
NOE differences are the number of protons enhanced.
HMBC are the correlated protons.

and ^1H nuclear overhauser enhancement difference spectroscopy (NOEDS) experiments (Table 3). Long-range coupling of the benzylic protons at δ_H 3.88 with carbons at δ_C 150.65 and 129.53 revealed an ortho substitution between the benzylic and hydroxyl groups. Another coupling between the methyl protons at δ_H 2.25 and proton bearing aromatic carbons at δ_C 127.02 assigned the connectivity of the methyl group to an ortho position of a ring proton. Meta-substitution of the butyl, methylene, and methyl protons were determined by NOEDS in which irradiation of these protons significantly enhanced aromatic protons at δ_H 6.96 only. A combination of these results suggested two possible structures for GERI-BP002-A. The absence HMBC correlation between the C-1 and *tert*-butyl protons revealed that structure I was correct (Fig. 5) (Table 3). This interpretation was supported by the calculation of chemical shifts of aromatic carbons based on the empirical parameters of substituted benzenes (Table 4). This was further supported by a chemical transformation of GERI-BP002-A. Treatment with diazomethane converted GERI-BP002-A to a monomethoxy derivative. NOE irradiations of the methoxy protons (δ_H 3.95) and the butyl protons (δ_H 1.37) significantly enhanced each other.

Biological Properties

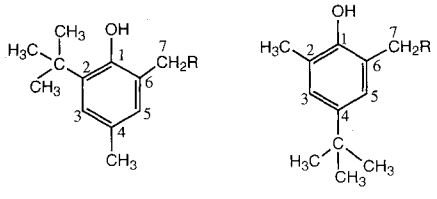
Effect on ACAT Activity in an Enzyme Assay System

Microsomes prepared from rat liver[8] were used as the enzyme source. The activity of microsomal ACAT was measured according to a modified method of P. BRECHER *et al.*[9]. The assays were carried out in 12×75 mm borosilicate tubes. The reaction mixture, containing 10 μl of microsomes (10 mg/ml protein), 20 μl of 1 M potassium phosphate buffer (pH 7.4, 10 mM dithiothreitol), 10 μl of bovine serum albumin (fatty acid free, 180 mg/ml), 2.0 μl of cholesterol in acetone (20 mg/ml, added last), 130 μl of water, and 10 μl of test sample in a total volume of 190 μl, was preincubated for 30 minutes at 37°C. The reaction was initiated by the addition of 10 μl of [1-^{14}C]oleoyl-CoA solution (0.05 μCi, final conc. 10 μM). After 30 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 0.2 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. This was mixed for 2 minutes and allowed to separate into phases. Cholesterol oleate was recovered in the upper (heptane) phase (total volume 0.9 ~ 1.0 μl). 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. Percent inhibition of ACAT activity was 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-BP002-A inhibited ACAT activity by 50% at 50 μM. Some chemicals which were structurally related compounds were purchased from Sigma Chemical Company (see Materials and Methods). None of the purchased compounds inhibited ACAT activity at concentrations up to 300 μM.

Fig. 5. Possible structures of the compound GERI-BP002-A.



I II

Table 4. Differences between the calculated and observed carbon chemical shifts.

Carbon	Model I			Model II		
	Calc.	Obsd.	Δ	Calc.	Obsd.	Δ
1	149.0	150.7	−1.7	152.9	150.7	2.2
2	138.2	136.9	1.3	125.2	130.4	−5.2
3	125.6	127.0	−1.4	125.6	127.0	−1.4
4	130.4	130.4	0	141.4	136.9	4.5
5	127.4	129.5	−2.1	124.1	129.5	−5.4
6	131.5	127.4	4.1	131.5	127.4	4.1

Other Biological Activities

GERI-BP002-A had no *in vitro* antimicrobial activity against *Streptococcus pyogenes*, *Staphyloccus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Klebsiella oxytoca*, *Klebsiella aerogenes*, and *Enterobacter cloacae* at concentrations up to 200 μg/ml, whereas it exhibited *in vitro* cytotoxicity against various cell lines. ED_{50}s of GERI-BP002-A were 5.45 μg/ml in A549, 8.83 μg/ml in SKOV-3, 5.79 μg/ml in HCT-15, 7.30 μg/ml in XF-498 and 5.39 μg/ml in SKMEL-2. Acute toxic effects were not recognized when GERI-BP002-A was subcutaneously injected into an ICR mouse at 500 mg/kg.

Discussion

Many synthetic compounds containing urea or amide moieties have been reported to inhibit ACAT. In addition, microbially produced ACAT inhibitors; purpactins[10], cyclodepsipeptides[11], glisoprenins[12], acaterin[13], pyripyropene[14], AS-183[15], helminthosporol[16], gypsetin[17] and GERI-BP001[18] have been reported. All of these compounds were produced by fungi with the exception of acaterin. We discovered a novel ACAT inhibitor, GERI-BP002-A, in the culture broth of *A. fumigatus* F93. The pyripyropenes and GERI-BP001 were also produced by *A. fumigatus*. It is noteworthy that the structure of GERI-BP002-A is not similar to those of other ACAT inhibitors reported previously. This type of compound appears to be a good model for the design of new ACAT inhibitors and antitumor agents. The structural modifications and synthesis of GERI-BP002-A and analogues are now in progress.

Acknowledgments

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