PURPACTINS, NEW INHIBITORS OF ACYL-CoA:CHOLESTEROL ACYLTRANSFERASE PRODUCED BY *Penicillium purpurogenum*

I. PRODUCTION, ISOLATION AND PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

HIROSHI TOMODA, HIROYUKI NISHIDA†, ROKURO MASUMA, JING CAO, SHIGENOBU OKUDA and SATOSHI ŌMURA*

Research Center for Biological Function, The Kitasato Institute, and School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan

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Penicillium purpurogenum FO-608, a soil isolate, was found to produce a series of new inhibitors of acyl-CoA : cholesterol acyltransferase (ACAT). Three active compounds, designated purpactins A, B and C, were isolated from the fermentation broth of the producing strain by solvent extraction, silica gel column chromatography and HPLC. Purpactins inhibit ACAT activity in an enzyme assay system using rat liver microsomes with IC_{50} values of 121 ~ 126 μM. Purpactin A also inhibited cholesterol ester formation in J 774 macrophages, indicating the inhibition of ACAT activity in the living cells by purpactin A.

Acyl-CoA : cholesterol acyltransferase (ACAT) (EC 2.3.1.26) plays an important role in cholesterol ester accumulation in atherogenesis[1] and in cholesterol absorption from the intestines[2]. Inhibitors of ACAT activity are expected to be effective for treatment of atherosclerosis and hypercholesterolemia. ACAT inhibitors of natural origin have been rarely reported. During the course of our screening program for ACAT inhibitors of microbial origin, we discovered a series of new ACAT inhibitors termed purpactin in a cultured broth of a fungal strain FO-608. Three active compounds, purpactins A, B and C, were isolated from the cultured broth. In this paper, the taxonomy of the producing strain, fermentation, isolation, and physico-chemical and biological characteristics of purpactins A, B and C.

Taxonomy of Producing Organism

Strain FO-608 was orginally isolated from a soil sample. For the identification of the fungus, CZAPEK's agar, potato-glucose agar, malt extract agar, oatmeal agar and YpSs agar were used. A slant culture of the isolate was inoculated onto these media and incubated at 5, 25 and 37°C for about 30 days. The cultural and physiological characteristics of the organism after growth at 25°C for 14 days are summarized in Table 1. The organism grew rather rapidly (40 ~ 65 mm in colony diameter) on various agar media, and formed pea green colonies. The colony surface was velvety to felt. The conidial structures were abundantly produced on various agar media. The reverse color was dark red under almost all the conditions employed.

Morphological observation was done under a scanning microscope (Hitachi, model S-430). When the strain FO-608 was grown on malt extract agar at 25°C for 7 days, the penicillia were biveticillate-symmetrica

† On leave from Pfizer Central Research, Nagoya, Pfizer Pharmaceuticals Inc., 5-2 Taketoyo, Aichi 470-23, Japan.

Table 1. Cultural and physiological characteristics of strain FO-608 at 25°C, 14 days.

	Growth (colony size)	Color of conidia	Reverse	Soluble pigment
CZAPEK's agar	45~50 mm velvety to felt	Pea green	Deep red	Red (faint)
Potato-glucose agar	50~60 mm velvety to felt	Sage green	Dark red	Red (faint)
Malt extract agar	50~65 mm velvety	Pea green	Pale yellow green, partially dark red	None
YpSs agar	50~60 mm velvety	Sage green	Ivory	Red (faint)
Oatmeal agar	40~45 mm velvety	Bright olive green	Dark red	None
Temperature range for growth		15~39°C		
pH range for growth		2~9		

and consisted of 3~6 metulae as shown in Fig. 1. The metulae were 9~11 × 2.0~2.5 μm in size, with whorls of 3~6 phialides. The conidia were ellipse and 2.5~4.0 × 1.8~2.2 μm i.d. and its surface was smooth.

From the above characteristics, strain FO-608 was identified as a strain of *Penicillium purpurogenum* Stoll[3,4], and named *P. purpurogenum* FO-608. This culture was deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology Japan, as FERM P-10776.

Fig. 1. Scanning electron micrograph of penicilla of strain FO-608 grown on malt extract agar at 25°C for 7 days.

Bar represents 10 μm.

Fermentation

A slant culture of strain FO-608 grown on potato-glucose agar was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of a seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypeptone 0.5%, KH_2PO_4 0.1%, agar 0.1%, pH 6.0). The flasks were shaken on a rotary shaker for 2 days at 27°C. Seven hundred ml of the seed culture were transferred into 70 liters of a production medium (glycerol 3.0%, glucose 1.0%, peptone 0.5%, NaCl 0.2%, agar 0.1%, pH 7.0) in a 100-liter jar fermenter. The fermentation was carried out at 27°C. A typical time course of the fermentation is shown in Fig. 2. The production of purpactins was measured by HPLC under the following conditions: column; Chemcosorb 5 ODS-H (4.6 × 150 mm), solvent; 60% aq CH_3CN, detection; UV at 280 nm, flow rate; 1 ml/minute. Under these conditions, purpactin B was eluted first with a Rt at 6.7 minutes, followed by purpactin C at 7.5 minutes, and purpactin A at 10.7 minutes (Fig. 3). Penicillide reported as a root-growth stimulant by SASSA *et al.*[5] was also detected with a Rt at 5.1 minutes, which is structurally related to purpactin A. As shown in Fig. 2, the production of purpactins B and C reached a maximum at 48 hours, then decreased rapidly. On the other hand, purpactin A production increased gradually at 48 hours, reached a maximum at 72 hours and therefore slightly decreased. Later study revealed that unstable purpactin B was converted to purpactin A, as described in the following paper[6].

Fig. 2. Time course of purpactins production in a 100-liter jar fermenter.

● Purpactin A, ○ purpactin B, ■ purpactin C, □ penicillide, ▲ packed cell volume, △ pH.

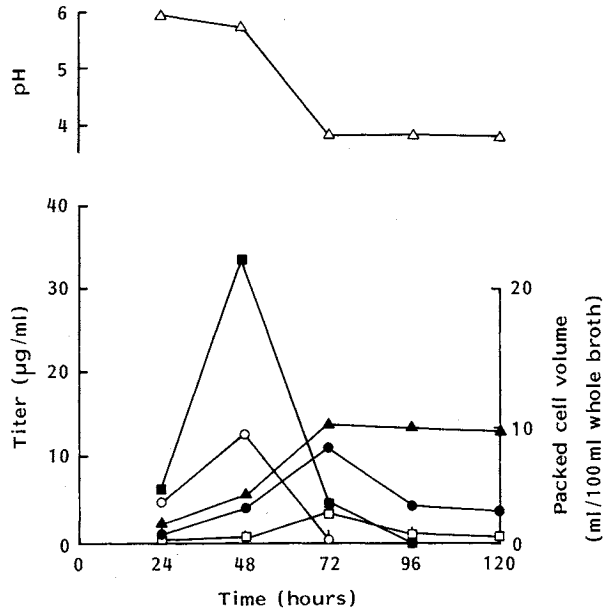
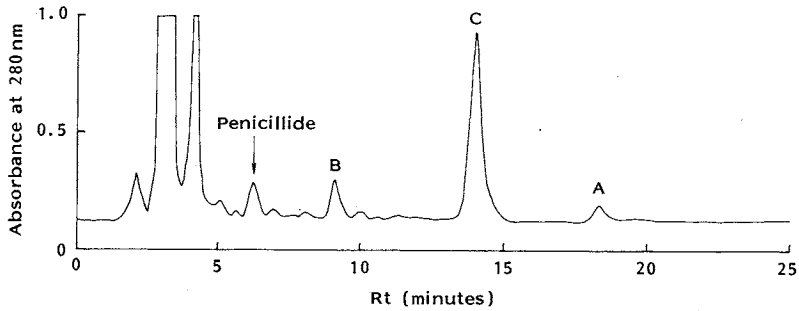


Fig. 3. HPLC chromatogram of a crude mixture of purpactins and penicillide.

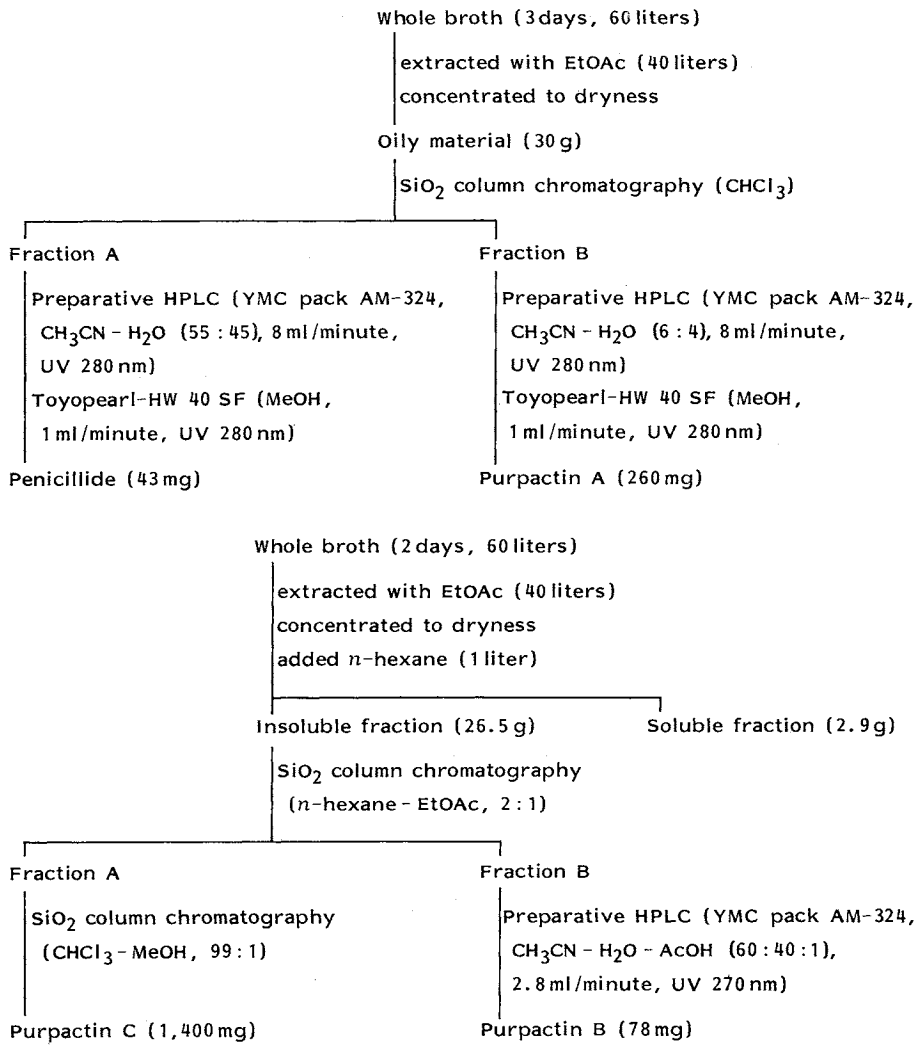
Column: Chemcosorb 5 ODS-H (4.6 × 150 mm); mobile phase: CH_3CN-H_2O (6:4), 1 ml/minute; detection: 280 nm.



Isolation

The isolation procedures were summarized in Fig. 4. Three-day cultured broth was used for isolation of purpactin A and penicillide. The whole broth (60 liters) was extracted with 40 liters of ethyl acetate. The extracts were concentrated *in vacuo* to dryness to yield a red oily material (30 g). The material was applied to a silica gel column (E. Merck, Kieselgel 60, 600 ml). The active components were eluted with 5 liters of chloroform, and each 500 ml was successively collected. The 5th fraction enriched with purpactin A and the 7th with penicillide were concentrated *in vacuo* to give red oily materials (1.3 and 1.4 g, respectively). Further purification of purpactin A was carried out by HPLC (column; YMC pack ODS-5 AM-343 20 × 250 mm, solvent; 60% aq CH_3CN, detection; UV at 280 nm, flow rate; 8 ml/minute). The fractions of purpactin A were concentrated and extracted with ethyl acetate to give a white powder (320 mg).

Fig. 4. Isolation procedures of purpactins and penicillide.



The powder was finally purified by Toyopearl-HW 40 SF column chromatography (column size; 50 × 300 mm, solvent; methanol, flow rate; 1 ml/minute). Pure purpactin A (260 mg) was obtained as a white powder. Purification of penicillide was also carried out by HPLC (solvent; 55% aq CH_3CN, the other conditions were the same as those for purpactin A) and by Toyopearl cloumn chromatography under the same conditions as above to give pure penicillide as a white powder (43 mg).

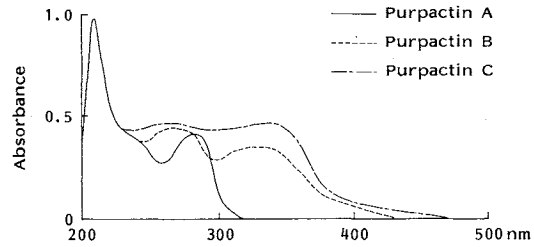
Purpactins B and C were isolated from 2 day-cultured broth. The whole broth (60 liters) was extracted with ethyl acetate (40 liters). The extracts were concentrated *in vacuo* to dryness to yield a yellow oily material. The material was treated with *n*-hexane to precipitate the active components. The precipitated material (26.5 g) was applied to silica gel column (E. Merck, Kieselgel 60, 520 g). The active components were eluted with 13 liters of *n*-hexane - ethyl acetate (2 : 1), and each 260 ml was pooled for fractionation. The 13 th to 17 th fractions enriched with purpactin C were combined and concentrated *in vacuo* to give a yellow powder (3.1 g), and the 23th to 37th with purpactin B were also combined to give a yellow oil

Table 2. Physico-chemical properties of purpactins.

	Purpactin A	Purpactin B	Purpactin C
Appearance	Colorless powder	Yellow oil	Yellow powder
$[\alpha]_D^{18}$ (c 1, $CHCl_3$)	−57.6°	+355.6°	+476.2°
Molecular formula	$C_{23}H_{26}O_7$	$C_{23}H_{26}O_7$	$C_{23}H_{24}O_7$
HREI-MS (m/z)			
Calcd:	414.1678	414.1678	412.1522
Found:	414.1653	414.1687	412.1529
UV λ_{max}^{EtOH} (nm)	280	270, 330	270, 330
IR ν_{max} (CCl_4) cm^{-1}	1750, 1600	1740, 1712 (sh), 1680, 1610, 1608 (sh)	1742, 1710 (sh), 1700, 1680, 1610, 1600 (sh)
Solubility			
Soluble:	MeOH, $CHCl_3$	MeOH, $CHCl_3$	MeOH, $CHCl_3$
Insoluble:	n-Hexane, H_2O	n-Hexane, H_2O	n-Hexane, H_2O
Color reaction			
Positive:	50% H_2SO_4	50% H_2SO_4	50% H_2SO_4
Negative:	$FeCl_3$	$FeCl_3$	$FeCl_3$

(1.4 g). The powder of purpactin C was finally purified by the second silica gel column chromatography (180 g, solvent; chroloform - methanol (99 : 1) to give pure purpactin C (1.4 g). The yellow oil of purpactin B was finally purified by HPLC (column; YMC pack ODS-5 AM-324 10 × 300 mm, solvent; 60% aq CH_3CN in 1% acetic acid, detection; UV at 270 nm, flow rate; 2.8 ml/minute). Pure purpactin B was obtained as a yellow oil (78 mg).

Fig. 5. UV spectra of purpactins A, B and C (EtOH).



Physico-chemical Properties

The physico-chemical properties of purpactins A, B and C are summarized in Table 2. The molecular formula of purpactins A and B were determined both to be $C_{23}H_{26}O_7$ and that of purpactin C was to be $C_{23}H_{24}O_7$ on the basis of HREI-MS. Similar UV spectra of purpactins B and C were observed with two maximum peaks at 270 and 330 nm in EtOH (Fig. 5).

These data indicated that purpactins are new compounds. Their structures are described in the following paper[6].

Biological Properties

Effect on ACAT Activity in a Rat Microsomal Enzyme System

ACAT activity was assayed as described elsewhere[7]. In brief, the reaction mixture routinely contained 0.1 M potassium phosphate buffer (KPB) (pH 7.4), 300 μM bovine serum albumin (BSA), 30 μM cholesterol (added as a dispersion of cholesterol - Triton WR 1339, 30 : 1, w/w in 20 μl of 0.1 M KPB, pH 7.4), 100 μg of rat liver microsomal protein and a sample (dissolved in 10 μl of 50% aq ethanol) in a total volume of 180 μl was preincubated at 37°C for 30 minutes. Then, the reaction was initiated by the addition of 20 μl of [^{14}C]oleoyl-CoA solution (0.02 μCi: a final concentration of 30 μM) and the mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 2 ml of chroloform - methanol (2 : 1), then lipids were extracted by the method of FOLCH *et al.*[8]. Total lipid extracts were separated on TLC (F254, Merck Co.) using petroleum ether - diethy ether - acetic acid (90 : 10 : 1) as a developing solvent.

Following visualization with iodine vapor, the cholesteryl oleate region (Rf=0.75) was scraped to determine the radioactivity. Percent inhibition of ACAT activity was calculated as [1−(the radioactivity of cholesteryl oleate region with a sample)/(the radioactivity of cholesteryl oleate region without a sample)] × 100.

ACAT inhibitory activity of purpactins A, B and C are summarized in Table 3. All the purpactins showed similar IC_{50} values in the range of 121~126 μM. However, penicillide structurally related to purpactin A (acetylpenicillide) exhibited very weak ACAT inhibitory activity.

Effect on Cholesterol Ester Formation in J774 Macrophages

The detail assay method for cholesterol ester formation in J774 macrophages will be described elsewhere[7]. Briefly, J774 cells cultured in RPMI-1640 medium (Gibco Co.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Co.) and 1% penicillin-streptomycin solution (PS) (final concentrations of 50 units/ml for benzylpenicillin and 50 μg/ml for streptomycin) for 2 days were collected and washed with RPMI-1640 containing 1% PS. The cells were suspended in the same medium at a concentration of 2×10^6 cells/ml. Then, 0.74 ml of the cell suspension was transferred in each well of 24-well microplates (Corning Co.). The cholesterol rich liposomes (80 μg cholesterol and 40 μg phosphatidylcholine in 40 μl of 0.3 M glucose/well), [^{3}H]oleate (0.05 μCi: in 10 μl of 50% aq ethanol/well) and various amounts of purpactin A in 10 μl of ethanol were added to each well. The cells were incubated under a humidified condition of 5% CO_2. After 12 hours, 90 μl of 1% SDS was added to each well to solubilize cells. Extraction and separation of lipids were carried out and the cholesteryl [^{3}H]oleate formation was measured as described above. The cell viability was also measured by trypan blue dye exclusion method after 12-hour incubation of J774 cells in the medium with or without purpactin A.

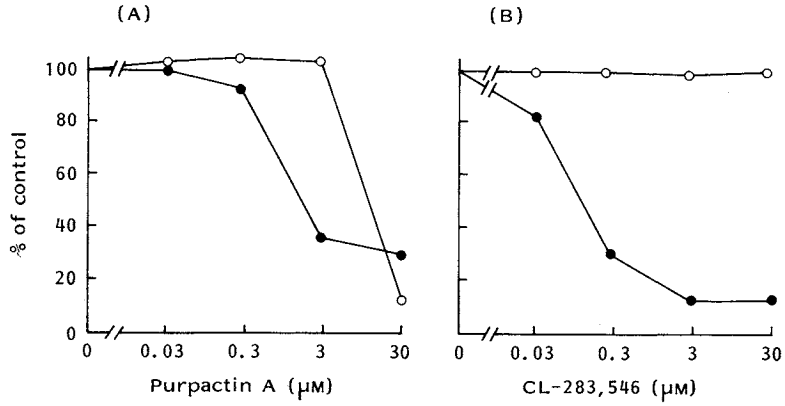
The activity of cholesteryl [^{3}H]oleate formation in J774 macrophages was inhibited by purpactin A (Fig. 6). The IC_{50} value was measured to be 1.2 μM, 100-fold more potent than in an *in vitro* enzyme

Table 3. Summary of ACAT inhibitory activity and cytotoxicity of purpactins.

Compound	IC_{50} (μM)		
	Microsomal assay	J774 macrophage assay	
	CE	CE	Cytotoxicity
Purpactin A	121	1.2	9.7
Purpactin B	121	NT	NT
Purpactin C	126	NT	NT
Penicillide	36% at 270	30% at 33.6	0% at 33.6
CL-283,546	83% at 2.1	0.18	0% at 52.5

CE: Cholesterol ester formation.
NT: Not tested.

Fig. 6. Inhibition of cholesterol ester formation in J774 macrophages by purpactin A and CL-283,546.
(A) Purpactin A, (B) CL-283,546. ● Cholesterol ester formation, ○ cytotoxicity.



assay. Higher concentrations of purpactin A cause cytocidal effect on J774 cells. Penicillide showed only weak ACAT inhibitory activity in this cell assay as well. Under the same conditions, CL-283,546, a known synthetic ACAT inhibitor[9], showed potent inhibitory activity against cholesterol ester formation (IC_{50}: 180 nM) and no cytocidal effect on J774 cells even at 50 μM.

Other Activities

None of the purpactins showed *in vitro* antimicrobial activity at a concentration of 100 μg/ml against *Bacillus subtilis*, *Mycobacterium smegmatis*, Pseudomonas aeruginosa, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces sake*, *Piricularia oryzae*, *Mucor racemosus*, and *Aspergillus niger*.

No toxic effect was observed when purpactin A was intraperitoneally injected to *dd*Y mice at 100 mg/kg.

Discussion

Purpactins B and C showed a similar UV spectrum, indicating the presence of the same chromophore. But the UV spectrum of purpactin A is different from those of purpactins B and C (Fig. 5). The time course of the purpactins production showed purpactins B and C were produced first and then purpactin A was produced (Fig. 2). Purified purpactin B was found to be unstable in neutral and basic conditions with conversion to purpactin A. Thus, it is likely that purpactin B produced first was rearranged to purpactin A under the fermentation conditions probably nonenzymatically. On the other hand, purpactin C is rather stable. It is converted to the purpactin A-type structure only in a basic condition[6]. Consequently, such a rearranged structure of purpactin C could not be isolated from the culture broth and purpactin C was degraded rapidly after 48 hours of fermentation (Fig. 2). The purpactin B-type of precursor of penicillide might be produced first during the fermentation since penicillide is structurally related to purpactin A (acetylpenicillide).

Purpactins were found to inhibit ACAT activity in an enzyme assay using rat liver microsomes. Furthermore, purpactin A exhibited potent inhibitory activity against cholesterol ester formation in J774 cell assay system. To our knowledge, purpactins are the first novel ACAT inhibitors of microbial origin.

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