PYRIPYROPENES, NOVEL INHIBITORS OF ACYL-CoA : CHOLESTEROL ACYLTRANSFERASE PRODUCED BY *Aspergillus fumigatus*

I. PRODUCTION, ISOLATION, AND BIOLOGICAL PROPERTIES

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Aspergillus fumigatus FO-1289, a soil isolate, was found to produce a series of novel inhibitors of acyl-CoA : cholesterol acyltansferase (ACAT). Four active compounds, named pyripyropenes A, B, C and D, were isolated from the fermentation broth of the producing strain by solvent extraction, silica gel column chromatography, ODS column chromatography and preparative HPLC. Pyripyropenes A, B, C and D show very potent ACAT inhibitory activity in an enzyme assay system using rat liver microsomes with IC_{50} values of 58, 117, 53 and 268 nM, respectively.

The enzyme acyl-CoA : cholesterol acyltransferase (ACAT) (EC 2.3.1.26) is believed to play an important role in three events which contribute to the atherosclerotic process: absorption of dietary cholesterol at intestines, lipoprotein synthesis at liver, and accumulation of cholesteryl esters as oil droplets within macrophages and smooth muscle cells of developing arterial lesions.[1~3] Therefore, inhibition of ACAT would be expected to retard the progression of atherosclerosis either by reducing serum cholesterol levels or by directly preventing the accumulation of cholesterol in arterial tissues.

Synthetic ACAT inhibitors, most of which are amide- or urea-related compounds,[4] have not been clinically used to date. We have been interested in microbial metabolites as a new lead of ACAT inhibitors and discovered purpactins,[5] cyclodepsipeptides[6] and glisoprenins[7] from fungal strains. However, these natural products were less potent than a synthetic inhibitor, CL-283,546.[8] From our continuous screening for potent ACAT inhibitors, a soil isolated fungal strain was found to produce novel ACAT inhibitors termed pyripyropenes A, B, C and D (Fig. 1).[9]

In this paper, the taxonomy of the producing strain, fermentation, isolation and biological characteristics of pyripyropenes are described.

Taxonomy of Producing Organism

Strain FO-1289 was originally isolated from a soil sample collected at Jingugaien, Sinjuku-ku, Tokyo, Japan. Taxonomic studies of the strain were carried out by the method of M. A. KLICH and J. I. PITT.[10] CYA (yeast extract 0.5%, sucrose 3.0%, K_2HPO_4 0.1%, $NaNO_3$ 0.3%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $ZnSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4 \cdot 5H_2O$ 0.0005%, agar 1.5%), MEA (malt extract 2.0% , peptone 0.1%, glucose 2.0%, agar 2.0%), and CY 20S (CYA medium with sucrose increased to 20%) media were used for the identification of the fungus. The cultures were incubated at 5, 25 and 37°C for about 14 days. The cultural characteristics of the organism after incubation at 25°C for 7 days are summarized in Table 1.

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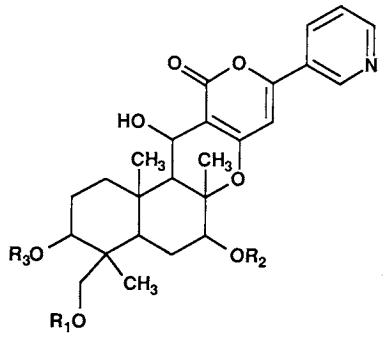
Fig. 1. Structures of pyripyropenes A, B, C and D.



	R_1	R_2	R_3
Pyripyropene A	$-COCH_3$	$-COCH_3$	$-COCH_3$
B	$-COCH_2CH_3$	$-COCH_3$	$-COCH_3$
C	$-COCH_3$	$-COCH_2CH_3$	$-COCH_3$
D	$-COCH_3$	$-COCH_3$	$-COCH_2CH_3$

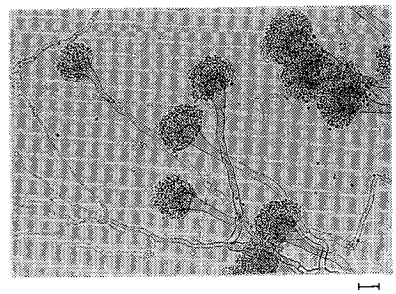
Table 1. Cultural characteristics of strain FO-1289.

Media	Temperature/Time	Colony size/ Surface of colony	Color of conidia	Color of reverse side
CYA	25°C/7 day	>60 mm/Velvety	Blue green	Pale yellow
MEA	25°C/7 day	55 mm/Velvety	Pale yellow	Pale yellow
CY20S	25°C/7 day	55 mm/Velvety	Blue green	Pale yellow

Morphological observation (Fig. 2) was done under a microscope (Olympus, NEW VANOX, model AH2-NIC). On CYA at 37°C after 7 days, the conidial heads were columnar and green to dark green in color. Conidiophores having smooth walls were $100\sim350\times2\sim5\,\mu m$ and pale green in color. Vesicles were flask-shaped to pyriform, $10\sim25\,\mu m$ i.d. and pale green in color. Mutalae were not produced. Phialides produced directly in upper half to two-thirds of the vesicle were $5\sim9\times2\sim3\,\mu m$ in size and pale green in color. Conidia were globose to subglobose, $2\sim3\,\mu m$ i.d., and deep green in color. No teleomorphs and hulle cells were observed. From the above characteristics, strain FO-1289 was identified as a strain of *Aspergillus fumigatus*. It was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM P-12194.

Fig. 2. Photomicrograph of *Aspergillus fumigatus* FO-1289.

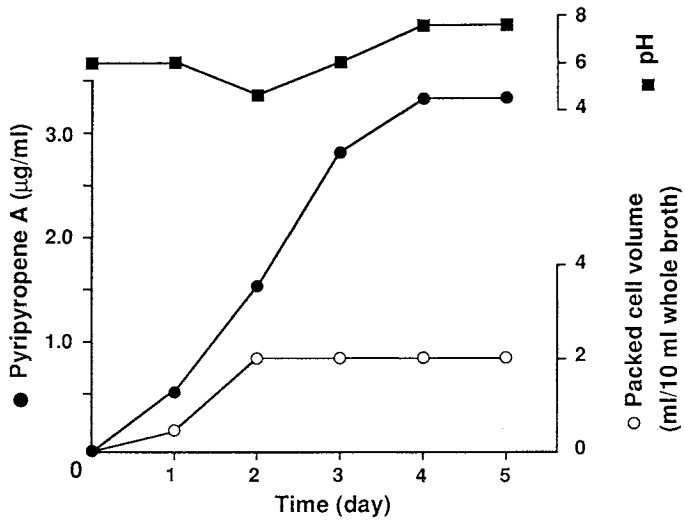
Bar represents 20 μm.



Fermentation

A slant culture of strain FO-1289 grown on yeast extract-soluble starch (YpSs) agar was inoculated into test tubes (20×200 mm) containing 10 ml of a seed medium (glucose 2%, yeast extract 0.2%, Polypepton 0.5%, $MgSO_4\cdot7H_2O$ 0.05%, KH_2PO_4 0.1% and agar 0.1%, adjusted to pH 6.0 prior to

Fig. 3. Time course of pyripyropenes production by *Aspergillus fumigatus* FO-1289.



sterilization). Seed tubes were incubated at 27°C for 48 hours on a reciprocal shaker at 300 strokes per minute. One per cent of seed culture was transferred into 500-ml Erlenmeyer flasks containing 100 ml of seed medium, which were incubated as described above. After 48-hour incubation, 1% of the second seed culture was inoculated into a 30-liter jar fermentor charged with 15 liters of a production medium consisting of glucose 1%, tryptone 0.5%, yeast extract 0.3%, malt extract 0.3%, agar 0.1% and antifoam (Nissan disfoam CB-442 (polyoxyalkyleneglycol derivative), Nippon Oil & Fats Co., Ltd.) 0.03%. The fermentation was carried out for 120 hours with an agitation rate of 200 rpm and an aeration rate of 0.5 v/v/minute and at 27°C. A typical time course of the fermentation is shown in Fig. 3. The packed cell volume was determined after centrifugation of 10 ml of culture broth at 3,000 g for 10 minutes. To determine the production of pyripyropene A, the fermentation broth (10 ml) was extracted twice with the same volume of ethyl acetate. The combined extracts were concentrated under reduced pressure and the residue was dissolved in 1 ml of MeOH. The resulting methanol solution (10 μl) was analyzed by HPLC (JASCO model TRI ROTAR-V, column: Senshu pak ODS, 4.6×150 mm; mobile phase: 50% aq CH_3CN; flow rate: 1 ml/minute; detection: UV at 320 nm). Pyripyropene A was eluted with a retention time of 5.9 minutes. The concentration of pyripyropene A reached a maximum at 96~120 hours, then decreased slowly.

Isolation

Five-day old culture broth was used for isolation of pyripyropenes. The whole broth (60 liters) was extracted with 40 liters of ethyl acetate. The extracts were concentrated *in vacuo* to dryness to yield a brown oily material (40 g). After *n*-hexane treatment, the residue was applied to a silica gel column (E. Merck, Kieselgel 60, 230~400 mesh, 700 ml). The materials were eluted with a stepwise gradient of $CHCl_3$-CH_3OH (100:0, 99:1, 98:2, 95:5 and 90:10, each 2 liters). Active fractions ($CHCl_3$-CH_3OH, 99:1) were concentrated *in vacuo* to yield an oily material (10 g). The residue was applied to an ODS column (Senshu SS 1020T, 200 ml) using a stepwise gradient of CH_3CN-H_2O (3:7, 4:6, 5:5, 7:3 and 10:0, each 500 ml) as a solvent. Active fractions (CH_3CN-H_2O, 7:3) were concentrated to an aqueous solution. The solution was extracted twice with the same volume of ethyl acetate. The extracts were

combined and concentrated. Further purification of pyripyropenes was carried out by HPLC using a preparative ODS column (Senshu ODS-5, 30×250 mm; mobile phase: 70% aq CH_3CN; detection: UV at 230 nm; flow rate: 10 ml/minute). Pyripyropene A was eluted with a retention time of 18.5 minutes, followed by pyripyropene B at 21 minutes, and pyripyropenes C and D with an identical retention time of 23 minutes. Each fraction of pyripyropenes A and B was concentrated and extracted with ethyl acetate, and the solvent was removed to yield pure pyripyropenes A and B. Final purification of pyripyropenes C and D was carried out by HPLC using a silica gel column (YMC pak Si-60, 20×250 mm; mobile phase: *n*-hexane-tetrahydrofuran-isopropyl alcohol, 65:15:20; flow rate: 8 ml/minute; UV at 320 nm). Pyripyropene C was eluted with a retention time of 14.0 minutes followed by pyripyropene D at 15.5 minutes. Pure pyripyropenes A (45 mg), B (4.6 mg), C (4.5 mg) and D (4.1 mg) were obtained as white powders.

Biological Properties

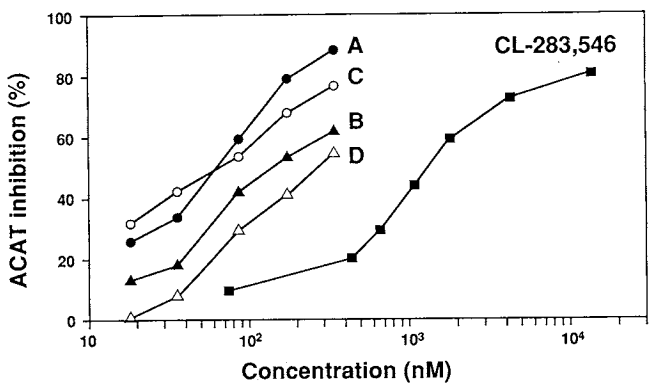
Effect of Pyripyropenes on ACAT Activity in an Enzyme Assay

ACAT activity was assayed as reported previously.[11] In brief, the reaction mixture containing 0.1 M potassium phosphate buffer (KPB) pH 7.4, 300 μM bovine serum albumin, 260 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 methanol) 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: final concentration 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 (1:2). Total lipids extracted by the method of FOLCH *et al.*[12] were separated by TLC (F_{254}, Merck Co.) using petroleum ether-diethyl ether-acetic acid (90:10:1) as a solvent. The radioactivity of the cholesteryl [^{14}C]oleate region on TLC was measured with a Radioactive Image Analyzer (AMBIS). ACAT inhibitory activity (inhibition %) 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 pyripyropenes A, B, C and D is shown in Fig. 4. The concentrations required for 50% ACAT inhibition (IC_{50}) of pyripyropenes A, B, C and D were calculated to be 58, 117,

Fig. 4. ACAT inhibitory activity by pyripyropenes and CL-283,546 in an enzyme assay using rat liver microsomes.

A, pyripyropene A; B, pyripyropene B; C, pyripyropene C; D, pyripyropene D.



53 and 268 nM, respectively. Under the same conditions, CL-283,546, a known chemically synthetic ACAT inhibitor,[8] showed the IC_{50} value of 1.3 μM.

Other Biological Activities

Pyripyropenes showed no *in vitro* antimicrobial activity at a concentration of 1,000 μg/ml (1.77 mM) against *Bacillus subtilis*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces sake*, *Pyricularia oryzae*, *Mucor racemosus* and *Aspergillus niger*. Activities of protein farnesyltransferase and diacylglycerol acyltransferase were not affected by pyripyropene A even at the high concentration of 88 μM. No cytotoxic effect was observed to Vero cells when any of the pyripyropenes was added at 177 μM to the culture medium. No acute toxicity was observed when pyripyropene A was intraperitoneally injected into ddY mice at 200 mg/kg.

Discussion

We have discovered three kinds of ACAT inhibitors of microbial origin, namely, purpactins,[5] cyclodepsipeptides[6,11] and glisoprenins,[7] all of which were produced by fungal strains. Recently, other research groups also reported microbial ACAT inhibitors, acaterin,[13] AS-183[14] and *epi*-cochlinoquinone A.[15] Among them, a known cyclodepsipeptide, beauvericin, and AS-183 showed potent ACAT inhibition with IC_{50} values of 3.0 and 0.94 μM, respectively. Pyripyropenes reported in this paper were much more potent than these two inhibitors with one to two magnitude lower IC_{50} values in an enzyme assay. To our knowledge, pyripyropene C is one of the most potent ACAT inhibitors reported to date.

Pyripyropenes showed the same UV spectrum (maxima at 231 and 320 nm), indicating the presence of the same chromophore. As described in the following paper,[16] pyripyropenes are comprised of three common parts, that is, pyridine, α-pyrone and sesquiterpene moieties, to form a steroid-like structure (Fig. 1). Several steroid analogs such as progesterone, 7-ketocholesterol (SC-31769)[17] and polyoxyethylated cholesterol[18] were reported to inhibit ACAT activity *in vitro*. Among the microbial ACAT inhibitors described above, only *epi*-cochlinoquinone A had a steroid-like structure. However, its ACAT inhibitory activity is much less potent than that of pyripyropenes. Researchers have focused on synthetic ACAT inhibitors, most of which are classified into two groups of fatty acylamides and di- or trisubstituted ureas.[3] In this sense, pyripyropenes having a steroid-like structure are expected to provide a novel type of lead compounds as potent ACAT inhibitors.

Acknowledgments

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References

1) BELL, F. P.: Arterial cholesterol esterification by acyl-CoA : cholesterol acyltransferase, its possible significance in atherogenesis and its inhibition by drugs. *In* Pharmacology Control of Hyperlipidemia. *Ed.*, F. R. RROUS, pp. 409~422, Science Publishers, 1986
2) HEIDER, J. G.: Agent which inhibit cholesterol esterification in the intestine and their potential value in the treatment of hypercholesterolaemia. *In* Pharmacology Control of Hyperlipidemia. *Ed.*, F. R. RROUS, pp. 423~438, Science Publishers, 1986
3) SLISKOVIC, D. R. & A. D. WHITE: Therapeutic potential of ACAT inhibitors as lipid lowing and anti-atherosclerotic agents. Trend Pharmcol. Sci. 12: 194~199, 1991
4) KATHAWALA, F. G. & J. C. HEIDER: Acyl CoA : cholesterol acyltransferase inhibitors and lipid lipoprotein metabolism. *In* Antilipidemic Drugs. *Eds.*, D. T. WITIAK, H. A. I. NEWMAN & D. R. FELLER, pp. 159~195, Elsevier, 1992

5) TOMODA, H.; H. NISHIDA, R. MASUMA, J. CAO, S. OKUDA & S. ŌMURA: Purpactins, new inhibitors of acly-CoA : cholesterol acyltransferase produced by *Penicillium purpurogenum*. I. Production, isolation and phsico-chemical and biological properties. J. Antibiotics 44: 136～143, 1991
6) TOMODA, H.; H, NISHIDA, X.-H. HUANG, R. MASUMA, Y. K. KIM & S. ŌMURA: New cyclodepsipeptides, enniatins D, E and F produced by *Fusarium* sp. FO-1305. J. Antibiotics 45: 1207～1215, 1992
7) TOMODA, H.; X.-H. HUANG, H. NISHIDA, R. MASUMA, Y. K. KIM & S. ŌMURA: Glisoprenins, new inhibitors of acyl-CoA : cholesterol acyltransferase produced by *Gliocladium* sp. FO-1513. I. Production, isolation and physico-chemical and biological properties. J. Antibiotics, 45: 1202～1206, 1992
8) KATOCS, Jr. A. S.; C.-H. WANG & E. E. LARGIS: The hypercholesterolemic activity of the ACAT inhibitor CL-283,546 in rat, rabbit and monkey, FASEB J. 2: A1219, 1988
9) ŌMURA, S.; H. TOMODA, Y. K. KIM & H. NISHIDA: Pyripyropenes, highly potent inhibitors of acyl-CoA : cholesterol acyltransferase produced by *Aspergillus fumigatus*. J. Antibiotics 46: 1168～1169, 1993
10) KLICH, M. A. & J. I. PITT: A laboratory guide to the common *Aspergillus* species and their teleomorphs. Published by Commonwealth Scientific and Industrial Research Organization, Division of Food Processing, 1988
11) TOMODA, H.; X.-H. HUANG, J. CAO, H. NISHIDA, R. NAGAO, S. OKUDA, H. TANAKA, S. ŌMURA, H. ARAI & K. INOUE: Inhibition of acyl-CoA : cholesterol acyltransferase activity by cyclodepsipeptide antibiotics. J. Antibiotics 45: 1626～1632, 1992
12) FOLCH, J.; M. LEES & G. M. SLOANE-STANLEY: A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497～509, 1957
13) NAGANUMA, S.; K. SAKAI, K. HASUMI & A. ENDO: Acaterin, a novel inhibitor of acyl-CoA : cholesterol acyltransferase produced by *Pseudomonas* sp. A92. J. Antibiotics 45: 1216～1221, 1992
14) KURODA, K.; M. YOSHIDA, Y. UOSAKI, K. ANDO, I. KAWAMOTO, E. OISHI, H. ONUMA, K. YAMADA & Y. MATSUDA: AS-183, a novel inhibitor of acyl-CoA : cholesterol acyltransferase produced by *Scedosporium* sp. SPC-15549. J. Antibiotics 46: 1196～1202, 1993
15) HAMANO, K.; Y. TSUJITA, T. KOGA, H. HARUYAMA, S. SATO, T. KAGASAKI & G. HOSOYA (Sankyo): *epi*-Cochlinoquinone A. Jpn. Kokai 334383 ('92), Nov. 20, 1992
16) KIM, Y. K.; H. TOMODA, H. NISHIDA, T. SUNAZUKA, R. OBATA & S. ŌMURA: Pyripyropenes, novel inhibitors of acyl-CoA: cholesterol acyltransferase produced by *Aspergillus fumigatus*. II. Structure elucidation of pyripyropenes A, B, C and D. J. Antibiotics 47: 154～162, 1994
17) GOLDSTEIN, J. L.; J. R. FAUST, J. H. DYGOS, R. J. CHORVAT & M. S. BROWN: Inhibition of cholesteryl ester formation in human fibroblasts by an analogue of 7-ketocholesterol and by progesterone. Proc. Natl. Acad. Sci. U.S.A. 75: 1877～1881, 1978
18) FUNG, C. H. & A. K. KHACHADURIAN: Suppression of synthesis and esterification of cholesterol and stimulation of low density lipoprotein receptor activity by polyoxyethylated cholesterol in cultured human fibroblasts. J. Biol. Chem. 255: 676～680, 1980