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Penicillium griseofulvum F1959, High-Production Strain of Pyripyropene A, Specific Inhibitor of Acyl-CoA: Cholesterol Acyltransferase 2

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Acyl-coenzyme A: cholesterol acyltransferase (ACAT) catalyzes cholesterol esterification and plays an important role in the intestinal absorption of cholesterol, hepatic production of lipoproteins, and accumulation of cholesteryl ester within cells. During the course of screening to find ACAT inhibitors from microbial sources, the present authors isolated pyripyropene A from *Penicillium griseofulvum* F1959. Pyripyropene A, an ACAT2-specific inhibitor, has already been produced from *Aspergillus fumigatus*. Yet, *Aspergillus fumigatus* is a pathogen and only produces a limited amount of pyripyropene A, making the isolation of pyripyropene A troublesome. In contrast, *Penicillium griseofulvum* F1959 was found to produce approximately 28 times more pyripyropene A than *Aspergillus fumigatus*, plus this report also describes the ideal conditions for the production of pyripyropene A by *Penicillium griseofulvum* F1959 and its subsequent purification.

Keywords: Acyl-coenzyme A: cholesterol acyltransferase (ACAT), *Penicillium griseofulvum* F1959, pyripyropene A, atherosclerosis

The enzyme acyl-CoA: cholesterol acyltransferase (ACAT, E.C. 2.3.1.26) is responsible for catalyzing the intracellular esterification of cholesterol and acyl-CoA [5, 6]. ACAT plays various roles in cholesterol metabolism in mammals. ACAT-mediated cholesterol esterification is believed to play a key role in the intestinal absorption of cholesterol, hepatic production of lipoproteins, and deposition of cholesteryl esters in atherosclerotic lesions [8]. The enzyme has been found to be present as two isoforms in mammals, ACAT1 and ACAT2, with separate genes exhibiting a 44–47% low amino acid homology and different tissue distribution, as well as a speculated different membrane topology in the endoplasmic reticulum [9]. ACAT1 plays a critical role in foam cell formation in macrophages, yet a complete deficiency of ACAT1 in LDL-deficient mice has been shown to result in an increase in atherosclerotic lesions [2]. Meanwhile, ACAT2 is in charge of the cholesterol absorption process in intestinal mucosal cells, is localized in tissues that express apoB, and has been suggested to perform a function in the production of cholesterol esters [1]. Therefore, research on atherosclerosis treatment has been focused on ACAT2-specific inhibitors [7].

In the course of searching for ACAT inhibitors from microbial sources, the present authors isolated many fungal strains from soil samples taken in Korea. Among culture broths of these strains, the broth of F1959 showed ACAT inhibitory activity. Strain F1959 was originally isolated from a soil sample collected at Ulsan, Korea. This strain was already deposited in the Korean Collection for Type Cultures (KCTC), Korea, as KCTC 0387 BP. Strain F1959 was identified as *Penicillium*

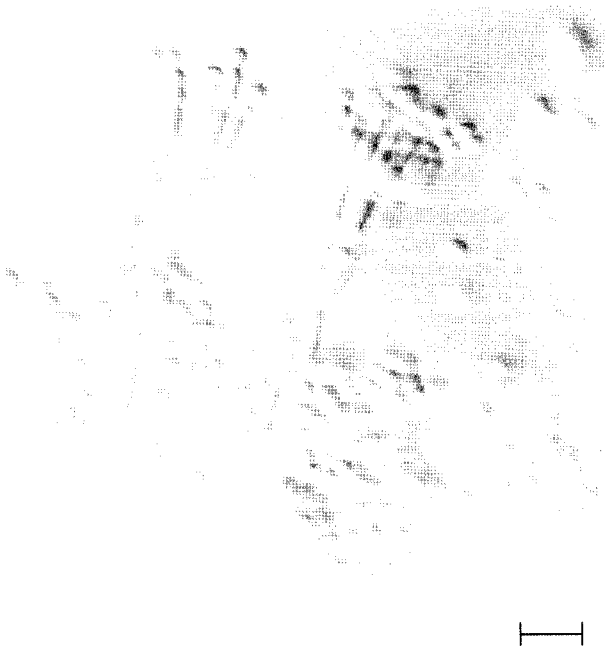

Fig. 1. *Penicillium griseofulvum* F1959.
Bar represents 20 μm.

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griseofulvum, and a taxonomic study of the strain was already reported by Rho *et al.* [8].

One frozen stock vial (1 ml of spore suspension in 10% glycerol, -80°C) of *Penicillium griseofulvum* F1959 (Fig. 1) was inoculated into a 1-l baffled flask containing 100 ml of a seed medium (0.5% glucose, 0.7% yeast extract, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.8 before autoclaving). Distilled water was used to prepare seed and production media. The seed culture was incubated at 29°C for 120 h on a rotary shaker at 150 rpm (radius 7 cm). Twenty ml of the seed culture was inoculated into a 10-l baffled flask containing 1 l of a production medium (2% soluble starch, 0.4% soytone, 0.3% Yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.3% $CaCO_3$, and 0.2% NaCl, pH 6.0 before autoclaving). The culture was carried out for 168 h at 29°C on a rotary shaker at 150 rpm. To determine the cell dry weight, the mycelia were collected by filtration using Whatman No. 1 filter paper, and then washed twice with distilled water and oven dried to a constant weight. The *Penicillium griseofulvum* F1959 cell mass leveled off after 5 days of cultivation, while the ACAT inhibitory activity reached a maximum after 7 days. The ACAT activity was determined using [1-^{14}C]oleoyl-CoA and cholesterol as substrates and rat liver microsomes as the ACAT source [4]. A 7-day-old culture broth (10 l) of *Penicillium griseofulvum* F1959 was partitioned three times with the same volume of ethyl acetate. The ethyl acetate layers were combined and concentrated *in vacuo* to yield a brown oily material (5.6 g). This material was chromatographed on a silica gel column (Merck, Kieselgel 60, 230–400 mesh, 60 g) and eluted stepwise with a gradient of chloroform-methanol (100:0, 50:1, 20:1, 10:1, 5:1, 2:1, 1:1; each 300 ml). The active fractions were then combined and concentrated *in vacuo* yielding an oily residue. For further purification, the active fractions (20:1 $CHCl_3$/MeOH fractions, 1.8 g) were subjected to reverse phase column chromatography (YMC-Gel RP-18, 70–230 mesh, 36 g) with step gradient elution using CH_3CN/H_2O (40:60, 50:50, 60:40, 70:30, 80:20, 90:10, 100:0; each 90 ml) to give 35 subfractions. The active fractions (55:45 CH_3CN/H_2O fractions, 400 mg) were successively separated by semipreparative HPLC (YMC J'sphere ODS H-80, 4 μm, ϕ20×150 mm; UV 210 nm; flow rate 6 ml/min) with elution by CH_3CN/H_2O (55:45). The active compound was eluted with a retention time of 29 min (**1**) and concentrated *in vacuo* to yield pure compound **1** (210 mg). The characteristics of the NMR data and other physicochemical data of compound **1** coincided well with pyripyropene A (Fig. 2) [3].

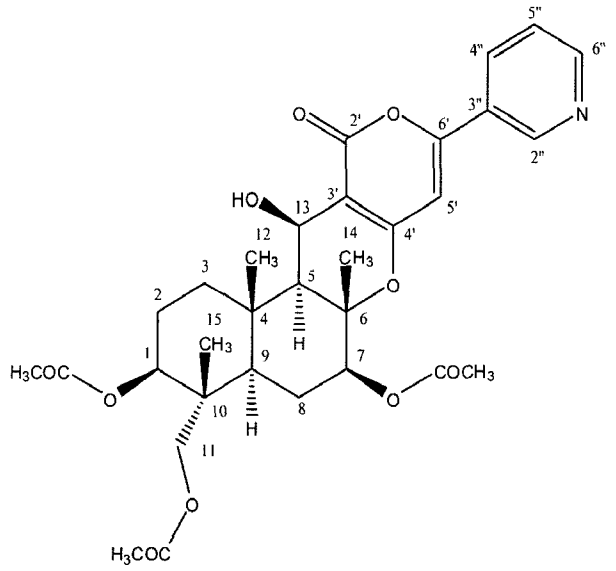

Fig. 2. Structure of pyripyropene A.

Pyripyropene A (**1**): $C_{31}H_{37}NO_{10}$; a white powder; UV (MeOH): λ_{max}=232 and 322 nm; FAB-MS: *m/z* 584.23 $[M+H]^+$; ^{1}H-NMR (300 MHz, CD_3OD): δ 0.88 (3H, *s*, H-15), 1.38, 1.82 (2H, *m*, H-3), 1.90, 2.13 (2H, *m*, H-2), 1.43 (3H, *s*, H-12), 1.54 (1H, *m*, H-5), 1.63(1H, *m*, H-9), 1.69 (3H, *s*, H-14), 1.78 (2H, *m*, H-8), 2.04 (3H, *s*, 1-CH_3), 2.08 (3H, *s*, 7-CH_3), 2.15 (3H, *s*, 11-CH_3), 2.93 (1H, *br. s*, 13-OH), 3.70 (2H, *dd*, *J*=11.7, 12.0 Hz, H-11), 4.79 (1H, *dd*, *J*=4.8, 5.4 Hz, H-1), 5.02 (1H, *m*, H-7), 6.45 (1H, *s*, H-5'), 7.40 (1H, *dd*, *J*=4.8 Hz, H-5"), 8.09 (1H, *dd*, *J*=1.8 Hz, H-3"), 8.68 (1H, *d*, *J*=4.5 Hz, H-6"), 9.00 (1H, *br. s*, H-2").

Aspergillus fumigatus only produces a minimal amount of pyripyropene A (**1**), and exposure to *Aspergillus fumigatus* can lead to aspergillosis, a pulmonary infection, in immunocompromised individuals. In contrast, *Penicillium griseofulvum* F1959 is known to be a safe strain and was found to produce a 7-fold higher yield of pyripyropene A than that produced by *Aspergillus fumigatus* [10].

As ACAT2 is a potential drug target for the prevention and treatment of atherosclerosis, the acquisition of pyripyropene A as an ACAT2-specific inhibitor is important. Pyripyropene A is the most powerful ACAT2-specific inhibitor among ACAT inhibitors from natural products and its synthesis is

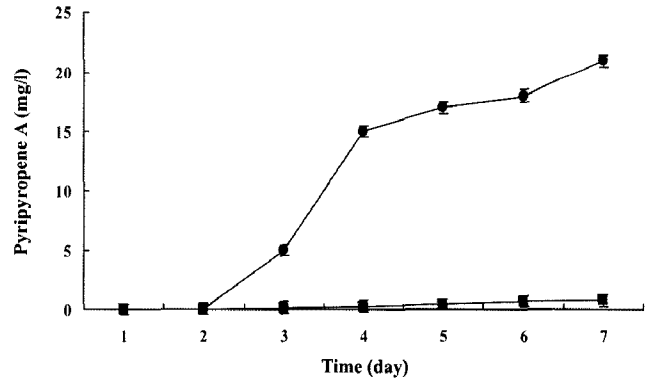

Fig. 3. Time course of pyripyropene A production.
Amount of pyripyropene A produced by *Penicillium griseofulvum* F1959 (solid circles) and *Aspergillus fumigatus* (solid squares). The experiment was performed three times.

complicated and expensive. Therefore, *Penicillium griseofulvum* F1959 that can produce a high yield of pyripyropene A can be a useful tool for the design of new ACAT2-specific inhibitors.

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