

ORIGINAL ARTICLE

# Pentaceciliides, new inhibitors of lipid droplet formation in mouse macrophages, produced by *Penicillium cecidicola* FKI-3765-1: I. Taxonomy, fermentation, isolation and biological properties

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New compounds designated pentaceciliides A to C were isolated from the fermentation broth of *Penicillium cecidicola* FKI-3765-1 by solvent extraction, silica gel column chromatography and preparative HPLC. Pentaceciliides A and B dose-dependently inhibited lipid droplet formation in mouse macrophages. Furthermore, pentaceciliides A and B were found to inhibit the synthesis of cholesteryl ester in mouse macrophages with respective IC<sub>50</sub> values of 3.65 and 4.76 μM without any cytotoxic effect, but pentaceciliide C showed almost no activity. The study of the mechanism of action strongly suggested that pentaceciliides A and B inhibit acyl-CoA: cholesterol acyltransferase activity in macrophages.

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**Keywords:** acyl-CoA:cholesterol acyltransferase; fungal metabolite; inhibitor; lipid droplet formation; *Penicillium cecidicola*; pentaceciliide; thailandolide

## INTRODUCTION

In the early stage of atherosclerosis, macrophages penetrate the intima, efficiently take up modified low-density lipoprotein, store cholesterol and fatty acid as a respective form of cholesteryl ester (CE) and triacylglycerol (TG) in cytosolic lipid droplets, and are converted into foam cells, leading to the development of atherosclerosis in the arterial wall. Therefore, inhibition of lipid droplet accumulation in macrophages would be expected to retard the progression of atherosclerosis.<sup>1–4</sup>

In the course of our screening program for microbial inhibitors of lipid droplet formation in mouse macrophages,<sup>5–10</sup> three new compounds designated pentaceciliides A to C (Figure 1), structurally related to known thailandolides A and B,<sup>11</sup> were isolated from the culture broth of a soil-isolated fungus, FKI-3765-1. Thailandolides were originally isolated as fungal metabolites from *Talaromyces thailandiasis*,<sup>11</sup> and were not isolated from the culture broth of strain FKI-3765-1. Pentaceciliides A and B were found to inhibit lipid droplet formation in mouse macrophages, although the biological activity of thailandolides has not been reported. The structure elucidation of pentaceciliides will be described in an accompanying study.<sup>12</sup> In this study, the taxonomy of the producing strain, fermentation, isolation and biological properties of pentaceciliides A to C are described.

## RESULTS

### Taxonomy of strain FKI-3765-1

Colonies on Czapek yeast agar (CYA) after 7 days at 25 °C (Figure 2a) were 44–45 mm in diameter, dense, colliculose, floccose to funiculose, with a smooth margin and white (a). The center of the colony was dark olive (1 pn) in conidial color, exuding clear drops. The reverse side was dark brown (5 pn). Colonies on malt extract agar (Figure 2b) were 49–51 mm in diameter, dense, colliculose, floccose, with a smooth margin, and white (a), without exudate drops. The reverse side was amber (3 lc). Colonies on G25N (Figure 2c) were 5.0–7.0 mm in diameter, pulvinate, floccose, with a smooth margin, and bamboo (2 gc) in color, without exudate drops. The reverse side was bamboo (2 gc). Colonies on CYA after 7 days at 37 °C were dense, colliculose, floccose to funiculose, with a smooth margin, and sand (2 ec) in color. The reverse was cork tan (4 ic). The colony on CYA at 5.0 °C showed no growth.

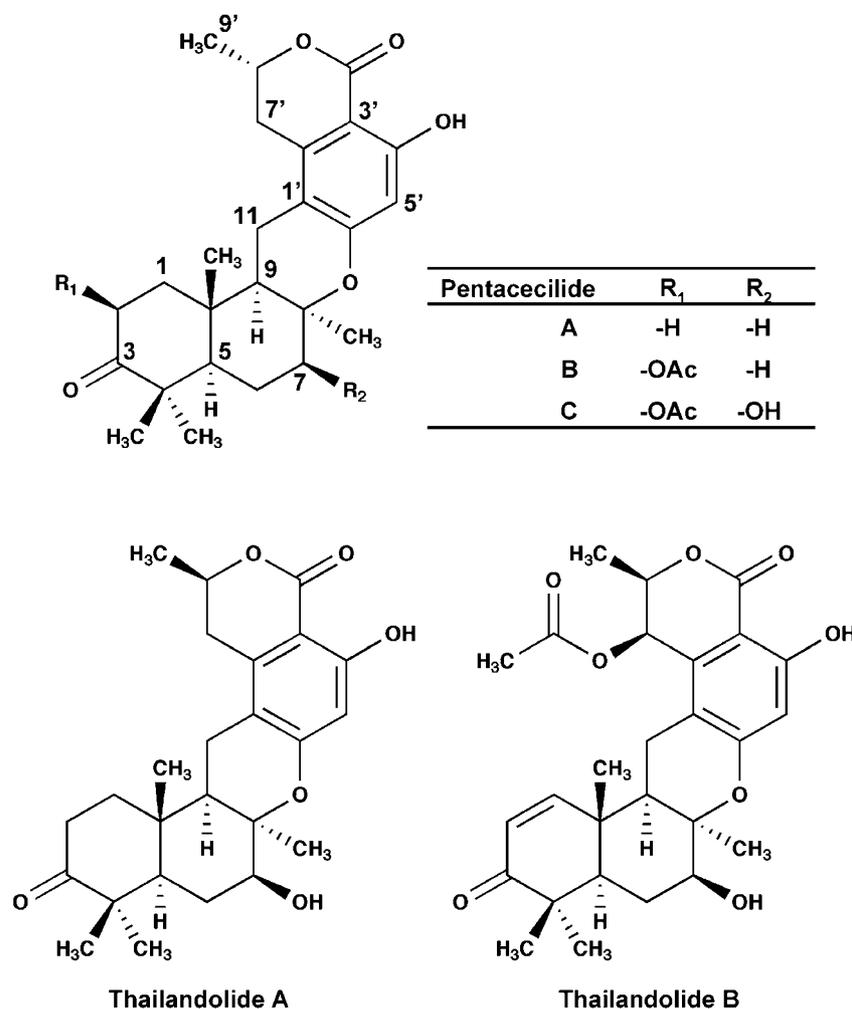
Conidiophores on CYA were from basal hyphae, rarely branching, 17.5–65 × 2.5–3.5 μm, with a smooth wall. Penicilli from conidiophores were biverticillate (consisting of metulae and phialides) and symmetrical (Figure 2d). Metulae were 3–7 branches, which were usually rather appressed or sometimes slightly divergent of larger size, 10–12.5 × 2.5–2.7 μm. Phialides were acerose, 10–15 × 2.5–3.0 μm,

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**Figure 1** Structures of pentacecilibes A to C and thailandolides A and B.

with smooth walls. Conidia were subglobose to globose, smooth-walled, 2.7–3.5 (5.0) × 2.3–3.0 μm in size, and with divergent long chains (Figure 2e).

From the above morphological characteristics, strain FKI-3765-1 was considered to belong to genus *Penicillium* in the subgenus *Biverticillium* Section *Simplicia*.<sup>13</sup> Furthermore, from the characteristics of the mycelial colors on CYA, the rapid growth at 37 °C on CYA and the length of conidiophores, the strain was considered to be *Penicillium cecidicola*. In addition, the ITS rDNA sequence (550 nucleotides) of strain FKI-3765-1 showed 99.4% similarity to that of *Penicillium cecidicola* (strain name) (accession no. AY787844). As a result, the producing strain FKI-3765-1 was identified as *P. cecidicola*.

#### Isolation

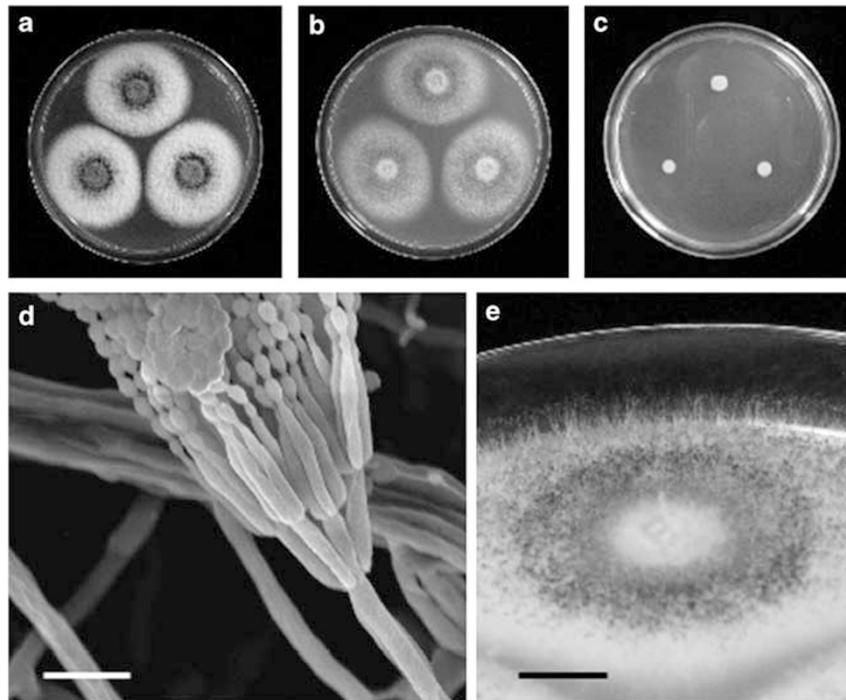
The 15-day-old whole broth (250 g) was extracted with 0.50 l of acetone. After the acetone extracts were filtered and concentrated to remove acetone, the aqueous solution was extracted with ethyl acetate. The extracts were dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo* to dryness to yield a red brown material (438 mg). The material was dissolved in hexane-ethyl acetate (2:1, v/v), applied to a silica gel column (10 g), and eluted stepwise with 2:1 (v/v) of hexane-ethyl acetate and 100:0, 10:1, 5:1, 1:1 and 0:100 (v/v) of CHCl<sub>3</sub>-CH<sub>3</sub>OH (100 ml for each solvent). Active fractions from a 5:1 solvent contain-

ing pentacecilibes were concentrated *in vacuo* to dryness to give a red brown oily material (171 mg). The material was finally purified by preparative HPLC (column; PEGASIL ODS (Senshu Scientific Co. Ltd, Tokyo, Japan), 20 × 250 mm; solvent, 70% CH<sub>3</sub>CN; detection, UV at 210 nm; flow rate, 8.0 ml min<sup>-1</sup>). Under these conditions, pentacecilibes A to C were eluted as a peak with a retention time of 30.5, 27.3 and 12.7 min, respectively (Figure 3). The fractions were concentrated *in vacuo* to dryness to give pure pentacecilibes A (4.47 mg), B (6.58 mg) and C (24.2 mg) as a white crystal.

#### Biological properties

**Inhibition of lipid droplet formation in macrophages.** In the control assay (no drug), mouse peritoneal macrophages accumulated a massive number of lipid droplets in the cytosol. In the presence of pentacecilibes A and B at 0.21–24.3 μM, the size and number of lipid droplets in macrophages fell in a dose-dependent manner (data not shown). No morphological changes or cytotoxic effects were observed on macrophages even at the highest dose (21.3–24.3 μM), indicating that pentacecilibes A and B specifically inhibit lipid droplet formation in mouse macrophages. Pentacecilibes C showed almost no effect on lipid droplet formation in macrophages.

**Inhibition of CE synthesis in macrophages.** In the control assay (no drug), approximately 40% of exogenously added [<sup>14</sup>C]oleic acid was



**Figure 2** Morphological characteristics of pentacecicide-producing *Penicillium cecidicola* FKI-3765-1. (a) Micrograph of colonies grown on Czapek yeast agar (CYA) after 7 days. (b) Micrograph of colonies grown on malt extract agar (MEA) after 7 days. (c) Micrograph colonies grown on G25N after 7 days. (d) Scanning electron micrograph of conidiophores grown on MEA. Scale bar, 10  $\mu\text{m}$ . (e) Micrograph of synnemata grown on MEA. Scale bar, 10 mm.

incorporated into [ $^{14}\text{C}$ ]CE (approximately 25%) and [ $^{14}\text{C}$ ]TG (approximately 15%), which are the main constituents of lipid droplets in macrophages.<sup>14</sup> As shown in Figure 4, pentacecicides A and B inhibited [ $^{14}\text{C}$ ]CE synthesis in a dose-dependent manner with  $\text{IC}_{50}$  values of 3.65 and 4.76  $\mu\text{M}$ , respectively, but showed almost no inhibition of [ $^{14}\text{C}$ ]TG synthesis even at the highest dose (20.6 and 21.3  $\mu\text{M}$ ), indicating that pentacecicides A and B selectively inhibit CE synthesis in macrophages. In contrast, pentacecicide C did not inhibit [ $^{14}\text{C}$ ]CE, [ $^{14}\text{C}$ ]TG and [ $^{14}\text{C}$ ]PL synthesis. These data are comparable with the results of lipid droplet formation in macrophages, as described above.

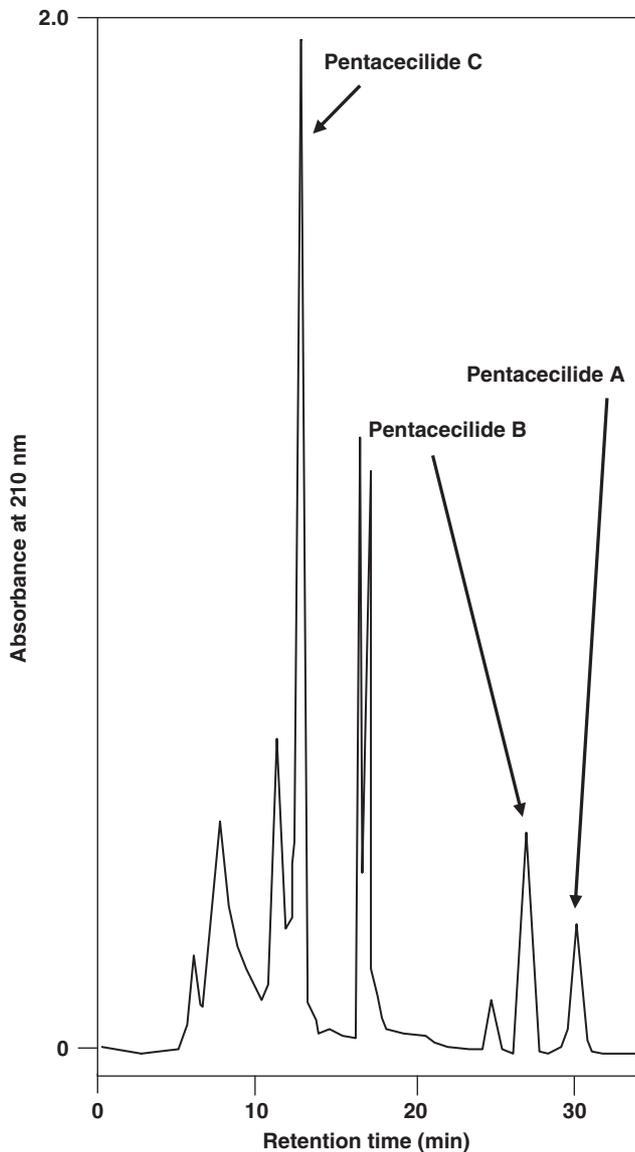
**Inhibition of post-lysosomal cholesterol metabolism in macrophages.** To gain insight into the target molecule of pentacecicides in the inhibition of lipid droplet formation in macrophages, the effects of pentacecicides on the post-lysosomal process of cholesterol metabolism was studied. When macrophages were incubated with [ $^{14}\text{C}$ ]cholesterol-supplemented liposomes in the presence of 10  $\mu\text{M}$  pregnenolone, [ $^{14}\text{C}$ ]CE formation was almost completely suppressed, and unesterified [ $^{14}\text{C}$ ]cholesterol accumulated in the lysosomes of macrophages.<sup>15</sup> After pregnenolone was removed by washing the cells with buffer, the metabolism of lysosomal [ $^{14}\text{C}$ ]cholesterol to [ $^{14}\text{C}$ ]CE was restarted in the presence or absence of pentacecicides. As shown in Figure 5a, pentacecicides A and B inhibited [ $^{14}\text{C}$ ]CE synthesis in a dose-dependent manner with  $\text{IC}_{50}$  values of 5.17 and 9.12  $\mu\text{M}$ , respectively, which are within similar ranges to those for the synthesis of [ $^{14}\text{C}$ ]CE from [ $^{14}\text{C}$ ]oleic acid (Figure 4). In contrast, pentacecicide C showed almost no effect, even at 24.3  $\mu\text{M}$ . These data indicate that the inhibition site of pentacecicides A and B lies within the post-lysosomal steps in cholesterol metabolism.

**Inhibition of ACAT activity in macrophage microsomes.** The results using pregnenolone indicated that the inhibition site of pentacecicides is between the point of cholesterol departure from lysosomes and the point of cholesterol esterification in the endoplasmic reticulum. As epicochlioquinone, whose carbon skeleton and configuration are very similar to those of pentacecicides, is known to inhibit acyl-CoA: cholesterol acyltransferase (ACAT) activity,<sup>16</sup> the effect of pentacecicides on ACAT activity was studied. For this purpose, microsomes prepared from mouse macrophages were used as an enzyme source. Pentacecicides A and B inhibited ACAT activity with  $\text{IC}_{50}$  values of 11.2 and 4.15  $\mu\text{M}$ , respectively (Figure 5b). In contrast, pentacecicide C showed 50% inhibition at 24.3  $\mu\text{M}$ .

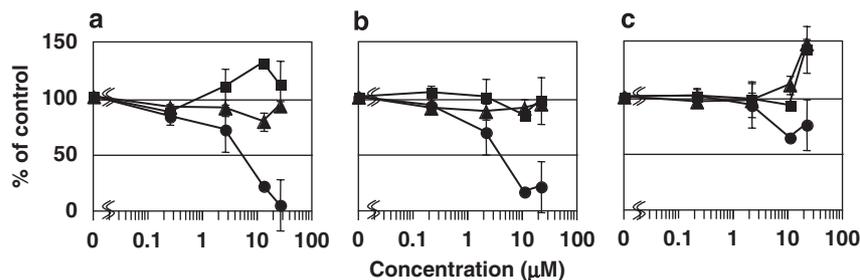
**Inhibition of CE synthesis in ACAT1- and ACAT2-CHO cells.** From the macrophage assays, the potential target of pentacecicides A and B is deduced to be ACAT; therefore, the effect of pentacecicides on ACAT1 and ACAT2 isozymes was evaluated in ACAT1- and ACAT2-CHO cells. As shown in Figure 6, pentacecicides A and B inhibited both ACAT1 and ACAT2 with similar potency. The respective  $\text{IC}_{50}$  values against ACAT1 and ACAT2 were 1.09 and 0.69  $\mu\text{M}$  for pentacecicide A and 10.8 and 3.97  $\mu\text{M}$  for pentacecicide B. These data indicated that pentacecicides A and B are dual inhibitors of ACAT1 and ACAT2.

## DISCUSSION

As described in this study, three pentacecicides (Figure 1) were isolated from the culture broth of *P. cecidicola* FKI-3765-1; however, pentacecicides A and B were found to inhibit lipid droplet formation in mouse macrophages, whereas pentacecicide C showed no activity. These findings suggested that the hydroxy group at C-7 does not favor the inhibitory activity of lipid droplet formation in mouse macrophages.



**Figure 3** A chromatographic profile of pentacecilide purification by preparative HPLC. Column, PEGASIL ODS (20×250 mm); solvent 70% aqueous acetonitrile; detection, UV at 210 nm; flow rate, 8.0 ml min<sup>-1</sup>; sample, 35 mg of active materials (obtained through silica gel column chromatography) dissolved in 700 μl methanol.



**Figure 4** Effect of pentacecilides on the synthesis of [<sup>14</sup>C]JCE, [<sup>14</sup>C]TG and [<sup>14</sup>C]JPL from [<sup>14</sup>C]oleic acid by macrophages. Macrophage monolayers obtained from 5×10<sup>5</sup> cells per well in a microplate were incubated in 0.25 ml medium with a phospholipid/cholesterol liposome composed of phosphatidylcholine, phosphatidylserine, dicetylphosphate and cholesterol at a molar ratio 10:10:2:15 and [<sup>14</sup>C]oleic acid in the absence or presence of the indicated amounts of pentacecilides A (a), B (b) or C (c). After 14-h incubation, cholesteryl [<sup>14</sup>C]oleate (●), [<sup>14</sup>C]TG (■) and [<sup>14</sup>C]JPL (▲) were separated on TLC, determined with a radioscanner as described in Methods. The results are plotted as a percent of the control (without a drug).

Thailandolides (Figure 1), structurally related to pentacecilides, were not isolated from the culture broth of strain FKI-3765-1. As they have a hydroxy group at C-7 as well as pentacecilide C, thailandolides might show no activity of lipid droplet formation in macrophages.

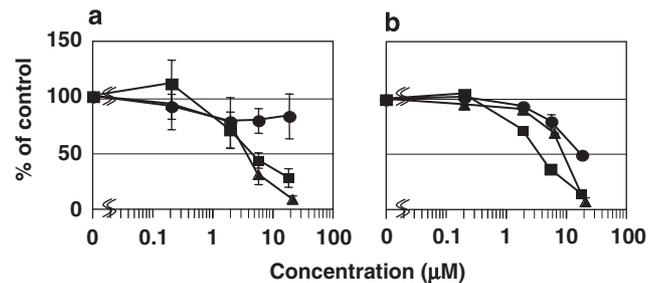
From a study of the action mechanism (Figures 4 and 5), we concluded that pentacecilides A and B inhibit ACAT activity in mouse macrophages to selectively block CE synthesis, leading to the inhibition of lipid droplet formation in mouse macrophages. Pentacecilide C showed almost no activity in these assays.

Pentacecilides A and B gave almost the same IC<sub>50</sub> values in the cell-based assays (Figures 4 and 5a), whereas pentacecilide C showed no effect in the cell-based assays (Figures 4 and 5a). However, pentacecilide C showed weakly inhibitory activity in the microsomal assay (Figure 5b). The discrepancy of IC<sub>50</sub> values between the assays might be due to the permeability of pentacecilide C to intact cells membrane, as our previously reported in the study of piperine.<sup>17</sup> Furthermore, pentacecilides A and B are dual inhibitors of ACAT1 and ACAT2 isozymes.

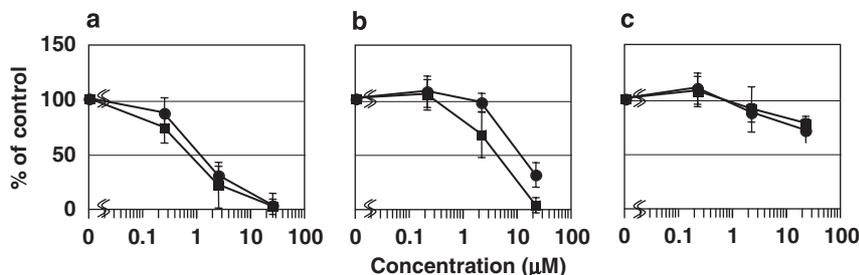
## METHODS

### General experimental procedures

Fungal strain FKI-3765-1 was originally isolated from a soil sample collected in Hilo, Hawaii, USA. This strain was used for the production of pentacecilides A



**Figure 5** Effect of pentacecilides on [<sup>14</sup>C]CE synthesis from lysosomal [<sup>14</sup>C]cholesterol in intact macrophages and on acyl-CoA: cholesterol acyltransferase (ACAT) activity in microsomes of mouse macrophages. (a) The amounts of [<sup>14</sup>C]CE synthesized from lysosomal [<sup>14</sup>C]cholesterol were determined in the presence of pentacecilides A (▲), B (■) and C (●) as described in Methods. These results are plotted as a percent of control (without a drug). (b) ACAT activity in microsomes prepared from mouse macrophages was tested in the presence of pentacecilides A (▲), B (■) and C (●) as described in Methods. These results are plotted as a percent of the control (without a drug).



**Figure 6** Effect of pentaceciliides on [<sup>14</sup>C]JCE synthesis from [<sup>14</sup>C]oleic acid in ACAT1- or ACAT2-CHO cells. ACAT1- (●) or ACAT2-CHO (■) cell monolayers ( $1.25 \times 10^5$  cells per well) in a microplate were incubated in the absence or presence of the indicated amounts of pentaceciliides A (a), B (b) or C (c). After a 6-h incubation, [<sup>14</sup>C]JCE was separated by TLC, and quantified with a bioimage analyzer, as described in Methods. The amounts of [<sup>14</sup>C]JCE signals were plotted as a percent of the control (without drugs).

to C. Kieselgel 60 (Merck KGaA, Darmstadt, Germany) was used for silica gel column chromatography. HPLC was carried out using the L-6200 system (Hitachi Ltd, Tokyo, Japan). To determine the amounts of pentaceciliides A to C in culture broths, the samples (ethyl acetate extracts) dissolved in methanol were analyzed by the HP1100 system (Hewlett-Packard Company, Palo Alto, CA, USA) under the following conditions: column, Symmetry (2.1 × 150 mm; Waters Corporation, Milford, MA, USA); flow rate, 0.2 ml min<sup>-1</sup>; mobile phase, a 20-min linear gradient from 60% CH<sub>3</sub>CN to 100% CH<sub>3</sub>CN containing 0.05% H<sub>3</sub>PO<sub>4</sub>; detection, UV at 210 nm. Under these conditions, pentaceciliides A to C were eluted with a retention time of 9.01, 8.71 and 4.67 min, respectively.

#### Taxonomic studies of the producing strain FKI-3765-1

Morphological studies and identification were conducted according to the procedures described by Pitt.<sup>13</sup> For taxonomic studies, CYA, malt extract agar and 25% glycerol nitrate agar (G25N) were used. Morphological characteristics were observed under a light microscope (Vanox-S AH-2, Olympus Corporation, Tokyo, Japan) and scanning electron microscope (JSM-5600, JEOL Ltd, Tokyo, Japan). Color names and hue numbers were determined according to the Color Harmony Manual.<sup>18</sup> For molecular phylogenetic study, genomic DNA was extracted using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocol. The rDNA internal transcribed spacer (rDNAITS) regions, including the 5.8S rDNA gene, were amplified by PCR using primers ITS1 and ITS4.<sup>19</sup> Amplifications were performed using a PCR Thermal Cycler Dice mini Model TP100 (Takara Bio Inc., Shiga, Japan). The amplified PCR products were purified using a QIAquick PCR DNA Purification Kit (Qiagen Inc., Valencia, CA, USA). Sequencing reactions were directly performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc.), and the products were purified with a DyeEX 2.0 Spin Kit (Qiagen Inc.). DNA sequences were read on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Inc.) and assembled using the programs SeqMan and SeqBuilder from the Lasergene7 package (DNASTar Inc., Madison, WI, USA). The ITS region rDNA sequence was compared with the database of the National Center for Biotechnology Information, Japan. ITS was deposited in DDBJ with accession number AB457008.

#### Fermentation

A slant culture of strain FKI-3765-1 grown on LCA (glycerol 0.10%, KH<sub>2</sub>PO<sub>4</sub> 0.08%, K<sub>2</sub>HPO<sub>4</sub> 0.02%, MgSO<sub>4</sub> 7H<sub>2</sub>O 0.02%, KCl 0.02%, NaNO<sub>3</sub> 0.20%, yeast extract 0.02%, agar 1.5%, pH 6.0) was inoculated into a 50-ml tube containing 10 ml of the seed medium (glucose 2.0%, polypeptone 0.50%, MgSO<sub>4</sub> 7H<sub>2</sub>O 0.05%, yeast extract 0.20%, KH<sub>2</sub>PO<sub>4</sub> 0.10%, agar 0.10%, pH 6.0). The tube was shaken on a reciprocal shaker for 3 days at 27 °C. A 1 ml portion of the seed culture was then inoculated into a 500-ml Erlenmeyer flask containing the production medium (50 g Italian rice, Japan Europe Trading Co. Ltd, Tokyo, Japan). Fermentation was carried out at 27 °C for 15 days under stable conditions.

#### Assay for lipid droplet formation in mouse macrophages

The assay for lipid droplet formation in mouse macrophages was carried out according to our established method.<sup>20</sup>

#### Assay for lipid synthesis by mouse macrophages

An assay for the synthesis of CE, TG and phospholipids by mouse macrophages was carried out according to the method described earlier.<sup>20</sup>

#### Assay for lysosomal cholesterol metabolism in mouse macrophages

The metabolism of lysosomal [<sup>14</sup>C]cholesterol in mouse macrophages was measured as described earlier.<sup>14</sup>

#### Assay for ACAT activity in mouse macrophage microsomes

Acyl-CoA: cholesterol acyltransferase activity was assayed according to our established method.<sup>21</sup>

#### Culture of ACAT1- and ACAT2-CHO Cells

Two cell lines, CHO cells expressing acyl-CoA: cholesterol acyltransferase 1 (ACAT1) and 2 (ACAT2) isozymes of African green monkey (ACAT1- and ACAT2-CHO cells, respectively)<sup>15</sup> were kind gifts from Dr LL Rudel (Wake Forest School of Medicine, NC). Cells were maintained as described earlier.<sup>22</sup>

#### Assay for ACAT1 and ACAT2 activities using ACAT1- and ACAT2-CHO cells

An assay for ACAT1 and ACAT2 activities in ACAT1- and ACAT2-CHO cells was carried out by our established method.<sup>22</sup>

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