Sespendole, a New Inhibitor of Lipid Droplet Synthesis in Macrophages, Produced by *Pseudobotrytis terrestris* FKA-25

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Abstract Sespendole was isolated as an inhibitor of lipid droplet formation in macrophages from the culture broth of a fungal strain *Pseudobotrytis terrestris* FKA-25. The compound inhibited the synthesis of cholesteryl ester and triacylglycerol by mouse macrophages with IC_{50} values of 4.0 and 3.2 μ M, respectively.

Keywords sespendole, lipid droplet synthesis, fungal metabolites, *Pseudobotrytis terrestris*, indolosesquiterpene

Introduction

Our research group has focused on discovery of biological active compounds from microbial metabolites [1–8]. Lipid droplet synthesis in macrophages is an early event in the process of macrophage foam cell formation, which leads to arteriosclerosis. During the course of screening for leads of anti-arteriosclerotic agents from microorganisms [9], a fungal strain FKA-25, identified as *Pseudobotrytis terrestris* [10, 11], was found to produce an inhibitor of lipid droplet synthesis in macrophages. A novel compound designated sespendole with a unique diprenyl indolosesquiterpene skeleton (Fig. 1) was isolated from the culture broth. Previously indoloditerpene terpendoles [12–14] were discovered as inhibitors of acyl-CoA: cholesterol acyltransferase (ACAT) isolated from the culture broth of a fungus *Albophoma* [15]. The structure elucidation and biosynthesis of sespendole will be reported in the near future. In this paper, fermentation, isolation and biological properties of sespendole are described.

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Fig. 1 Structures of sespendole (1) and terpendole C (2).

Furthermore, the inhibitory effects of sespendole and terpendole on lipid droplet formation in macrophages are compared.

Materials and Methods

Fermentation Media

For production of sespendole, the seed medium was used containing 2.0% glucose, 0.2% yeast extract (Oriental Yeast Co.), 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% Polypepton (Daigo Nutritive Chemicals), 0.1% KH_2PO_4 and 0.1% agar. The pH was adjusted to 6.0 prior to sterilization. The production

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medium was composed of 2.0% sucrose, 1.0% glucose, 0.5% corn steep powder (Iwaki Co., Ltd.), 0.5% meat extract (Kyokuto Pharmaceutical Co. Ltd.), 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3% CaCO_3 and 0.1% agar. The pH was adjusted to 6.0 prior to sterilization.

Quantitative Analysis of Suspendole by HPLC

The amount of suspendole was measured by the analytical HPLC system (HP1100, Hewlett-Packard). Samples dissolved in methanol were analyzed under the following conditions: column, Symmetry C18/3.5 μm (i.d. 2.1×150 mm, Waters); mobile phase, a 20-minute linear gradient from 30% $\text{CH}_3\text{CN}/0.05\%$ H_3PO_4 to 70% $\text{CH}_3\text{CN}/0.05\%$ H_3PO_4 ; flow rate, 0.2 ml/minute; detection, UV at 240 nm. Suspendole was eluted as a peak with a retention time of 17.8 minutes.

Assay for [^{14}C]Neutral Lipid Synthesis in Macrophages

Assay for [^{14}C]cholesteryl ester (CE) and [^{14}C]triacylglycerol (TG) syntheses from [^{14}C]oleic acid in macrophages was described previously [16]. In brief, mouse peritoneal macrophages (5.0×10^5 cells/0.25 ml GIT medium) were cultured in each well of a 48-well plastic microplate (Corning Co.), and then a test sample (2.5 μl in MeOH) and phosphatidylserine-containing liposomes (10 μl) together with [^{14}C]oleic acid (0.05 μCi , 5 μl in 10% EtOH/PBS solution) were added to each culture. Following a 14-hour incubation, the medium was removed, and the cells in each well were washed three times with PBS. The cells were lysed by adding 0.25 ml of PBS containing 0.1% (w/v) sodium dodecyl sulfate, and the cellular lipids were extracted by the method of Bligh and Dyer [17]. After removing the organic solvent, the total lipids were separated on a TLC plate (silica gel F254, 0.5 mm thick, Merck) using hexane-diethyl ether-acetic acid (70:30:1) and were analyzed with a radioscanner (AMBIS Systems, Inc.).

Assay for ACAT Activity

ACAT activity in mouse liver microsomes was assayed by the method previously described [18] with some modifications. Briefly, an assay mixture containing 12.5 mg/ml BSA in 104 mM Tris-HCl (pH 7.8) and [^{14}C]oleoyl-CoA (170 μM , 0.1 μCi) together with a test sample (5.0 μl in methanol to make a final concentration of 0~20 μM), and the microsomal fractions (200 μg of protein) prepared from mouse livers in a total volume of 100 μl was incubated at 37°C for 15 minutes. The reaction was stopped by adding 0.5 ml of ethanol, hexane (1.5 ml) was then added, and the solutions were mixed well. The hexane layer (1.0 ml) containing the product cholesteryl

[^{14}C]oleate was evaporated, and the residue was separated on a TLC plate using a petroleum ether-diethyl ether-acetic acid (90:10:1) solvent. The amount of cholesteryl [^{14}C]oleate on TLC was measured with a radioscanner (AMBIS Systems, Inc.).

Assay for Acyl-CoA Synthetase

Acyl-CoA synthetase (ACS) was assayed by using NEFA C-test kit (Wako Pure Chemical) according to the method described previously [19].

Antimicrobial Activity

Antimicrobial activity against 14 species of microorganisms was measured by a paper disk method. Media for growth of microorganisms were as follows: GAM agar (Nissui Seiyaku Co.) for *Bacteroides fragilis*; Waksman agar for *Mycobacterium smegmatis*; Bacto PPLO agar (Difco) supplemented with 15% horse serum, 0.1% glucose, 0.2% phenol red (5 mg/ml) and 1.5% agar for *Acholeplasma laidlawii*; nutrient agar for *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Xanthomonas oryzae*; a medium composed of 1.0% glucose, 0.5% yeast extract and 0.8% agar for *Pyricularia oryzae*, *Aspergillus niger*, *Mucor racemosus*, *Candida albicans* and *Saccharomyces cerevisiae*. A paper disk (i.d. 6 mm, Advantic) containing 10 μg of a sample was placed on the agar plate. Bacteria except *Xanthomonas oryzae* were incubated at 37°C for 24 hours. Yeasts and *X. oryzae* were incubated at 27°C for 24 hours. Fungi were incubated at 27°C for 48 hours. Antimicrobial activity was expressed as diameter (mm) of inhibitory zone.

Results

Fermentation

A stock culture of strain FKA-25 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium and incubated on a rotary shaker at 27°C for 4 days. The main culture was initiated by transferring 200 ml of the seed culture into a 30-liter jar fermenter containing 20 liters of the production medium, and the fermentation was carried out at 27°C with agitation at 250 rpm. A typical time course of the production is shown in Fig. 2. The concentration of suspendole reached a maximal level (23 $\mu\text{g}/\text{ml}$) on day 4 after inoculation.

Isolation

The isolation procedure for suspendole is summarized in Fig. 3. The 4-day old culture broth (20 liters) was treated with acetone (20 liters) for 30 minutes and the mixture was

Fig. 2 A typical time course of spondole production by *Pseudobotrytis terrestris* FKA-25.

centrifuged to obtain the supernatant. After concentration by removing acetone, the aqueous solution was extracted with ethyl acetate (20 liters). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a brown oil (4.6 g). The oil dissolved in a small amount of chloroform was applied on a silica gel column (i.d. 2.5×16 cm, Silica gel 60, $70 \sim 230 \mu\text{m}$, Merck) previously equilibrated with chloroform, and materials were eluted stepwise with chloroform-methanol solutions (1 liter each, 100:0, 50:1, 9:1, 5:1, 1:1 and 0:100). Spondole was recovered from the fractions of chloroform-methanol (9:1), which were concentrated under reduced pressure to give a brown powder (759 mg). The powder was dissolved in chloroform and subjected to a second silica gel column chromatography (i.d. 2.0×20 cm, Silica gel 60, $230 \sim 400 \mu\text{m}$, Merck), and materials were eluted stepwise with chloroform-methanol solutions (200 ml each, 100:0, 100:1, 50:1, 10:1, 5:1 and 0:100). The fractions (chloroform-methanol, 10:1) containing spondole were concentrated to give a yellow powder (131.5 mg). Spondole was finally purified by HPLC under the following conditions: column, Pegasil ODS (Sensyu Scientific Co., Ltd., i.d. 20×250 mm); mobile phase, 60% aq CH_3CN ; flow rate, 8 ml/minute; detection, UV 240 nm. Spondole was eluted as a peak with a retention time of 16 minutes. The peak was collected and the fraction was concentrated to dryness to give pure spondole (13.9 mg) as a colorless amorphous solid.

Biological Properties

Effect of Spondole on [^{14}C]Neutral Lipid Synthesis from [^{14}C]Oleic Acid in Macrophages

From the morphological assay, spondole was found to inhibit the number and size of cytosolic lipid droplets

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Culture broth (20 L)
|
| treated with acetone
| filtered
|
Filtrate
|
| concentrated under reduced pressure
| extracted with EtOAc
|
EtOAc extract (4.6 g)
|
| Silica gel column (70~230 mesh)
| eluted with  $\text{CHCl}_3$ -MeOH (9:1)
|
Crude material (759.3 mg)
|
| Silica gel column (230~400 mesh)
| eluted with  $\text{CHCl}_3$ -MeOH (10:1)
|
Crude spondole (131.5 mg)
|
| Preparative HPLC
| mobile phase:  $\text{CH}_3\text{CN}$ - $\text{H}_2\text{O}$  (3:2)
|
Spondole (13.9 mg)

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Fig. 3 Purification procedure of spondole from the culture broth of *Pseudobotrytis terrestris* FKA-25.

observed in mouse macrophages (Fig. 4). Therefore, the biochemical assay was done to confirm the finding. When macrophages were incubated with the liposomes in the presence of [^{14}C]oleic acid, the [^{14}C]fatty acid was incorporated into the neutral lipid of [^{14}C]CE and [^{14}C]TG fractions (over 40% of [^{14}C]oleic acid added), main constituents of the lipid droplets. As shown in Fig. 5, spondole inhibited [^{14}C]CE and [^{14}C]TG syntheses in a dose-dependent fashion with IC_{50} values of 4.0 and $3.2 \mu\text{M}$, respectively, while the compound showed no effect on [^{14}C]phospholipids (PL, mainly phosphatidylcholine) synthesis and viability of macrophages at $20 \mu\text{M}$. On the other hand, structurally related terpendole C (Fig. 1), discovered as an ACAT inhibitor [11], inhibited [^{14}C]CE synthesis specifically (IC_{50} ; $3.0 \mu\text{M}$), and enhanced [^{14}C]TG and [^{14}C]PL syntheses at $20 \mu\text{M}$ (Fig. 5).

Effect of Spondole on ACS and ACAT Activities

Since the compound inhibited the both [^{14}C]CE and [^{14}C]TG syntheses in macrophages, ACS was suggested as a possible target of inhibition by spondole. However, spondole showed no ACS inhibition at $150 \mu\text{M}$. Furthermore, the compound showed almost no effect on ACAT (IC_{50} ; $550 \mu\text{M}$) either.

Antimicrobial Activity

Spondole showed weak antimicrobial activity against *B. subtilis* (inhibition zone, 7.0 mm,) and *M. smegmatis* (9.5 mm) at $10 \mu\text{g}/6\text{mm}$ disk among the 14 microorganisms tested.

Fig. 4 Effects of sespendole on lipid droplet accumulation in macrophage.

Fig. 5 Effects of sespendole and terpendole C on [^{14}C]CE, [^{14}C]TG and [^{14}C]PL syntheses in macrophages. TG (●), CE (■) and PL (▲).

Discussion

A number of indoloditerpene compounds have been reported as fungal metabolites [20]. However, sespendole is the first fungal metabolite having an indolosesquiterpene skeleton to our knowledge. The structure elucidation will be reported in the near future. We previously discovered terpendoles, members of indoloditerpenes, as inhibitors of ACAT. Therefore, the inhibitory effect on lipid droplet formation in macrophages was compared between sespendole and terpendole C (Fig. 5). Terpendole C specifically inhibited CE synthesis in macrophages due to its ACAT inhibition, while sespendole inhibited both CE and TG syntheses, suggesting that the inhibition site of sespendole is different from that of terpendole C. In fact, sespendole showed almost no effect on ACAT. Furthermore, the compound showed no effect on ACS, either, though predicted from the finding that it inhibited both neutral lipids to similar extents like triacsins [21]. The target molecule of sespendole in lipid droplet formation by macrophages remains to be defined.

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