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ORIGINAL ARTICLE

New isochaetochromin, an inhibitor of triacylglycerol synthesis in mammalian cells, produced by *Penicillium* sp. FKI-4942: I. Taxonomy, fermentation, isolation and biological properties

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A new bis-naphtho-γ-pyrone isomer named isochaetochromin A_1 was isolated along with known isochaetochromins B_1 and B_2 from the culture broth of *Penicillium* sp. FKI-4942 by solvent extraction, silica gel column chromatography and HPLC. Among them, isochaetochromin B_1 showed the most potent inhibitory activity of triacylglycerol synthesis with an IC_{50} value of 5.6 μM, followed by isochaetochromins B_2 (IC_{50}, 11 μM) and A_1 (33 μM).
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Keywords: diacylglycerol acyltransferase; fungal metabolites; inhibitor; isochaetochromin; *Penicillium*; triacylglycerol biosynthesis; γ-pyrone

INTRODUCTION

Neutral lipids, triacylglycerol (TG) and cholesteryl ester (CE) are the final storage forms of free long-chain fatty acid and cholesterol in mammals. TG synthesis is important in many biological processes, including lactation, energy storage in adipose tissue and muscle, and fat absorption in the intestine. Excessive accumulation of TG in adipocytes as a result of a fat-rich diet or sedentary lifestyle causes obesity. The condition is also closely related to lifestyle-related diseases and metabolic syndrome. Recently, much attention has been paid to these disorders because of their current importance in health care. Several approaches have been evaluated for the treatment of obesity, and two drugs have been approved for use: sibtramine, which acts centrally to inhibit serotonin and noradrenaline uptake,[1,2] and orlistat, which inhibits lipid lipase and therefore interferes with lipid absorption from the small intestine.[3,4] One potential strategy for the treatment of obesity is to block TG synthesis;[5] therefore, inhibitors of TG synthesis are expected to be therapeutic agents for obesity.[6,7] TG is synthesized by a number of enzymes.[8] Acyl-CoA: diacylglycerol acyltransferase (DGAT)[9] is the final enzyme to synthesize TG, and only a few DGAT inhibitors have been reported by several groups, including ours.[10–14]

We established a high content assay to observe the TG biosynthetic pathway using Chinese hamster ovary (CHO) K1 cells.[15] During our screening for inhibitors of TG synthesis, a new compound named isochaetochromin A_1 was isolated along with known isochaetochromins B_1 and B_2[16] from the culture broth of *Penicillium* sp. FKI-4942 (Figure 1). These compounds are isomers having the same planar structure of bis-naphtho-γ-pyrone.[16] In this study, the taxonomy of producing fungus, the fermentation, isolation and inhibitory activity against TG synthesis in CHO-K1 cells of isochaetochromins are described.

MATERIALS AND METHODS

General experimental procedures

Fungal strain FKI-4942 was originally isolated from a soil sample collected in the Bonin Islands, Tokyo, Japan. This strain was used for the production of isochaetochromins A_1, B_1 and B_2. Silica gel 60 (Merck, Darmstadt, Germany) was used for silica gel column chromatography. HPLC was carried out using the L-6200 system (Hitachi, Tokyo, Japan).

Taxonomic studies of the producing strain FKI-4942

Morphological studies and identification were conducted according to the procedures described by Pitt.[17] For taxonomic studies, the strain was inoculated in three-point cultures on Czapeck yeast extract agar (CYA), malt extract agar (MEA) and 25% glycerol nitrate agar (G25N), and then grown for 7 days at 25 °C (also at 5 °C and 37 °C on CYA) in the dark. Morphological characteristics were observed under a scanning electron microscope (JSM-5600; JEOL, Tokyo, Japan). Color names and hue numbers were determined according to the Color Harmony Manual 4th Edition (Container Corporation of America, Chicago, IL, USA).[18] For sequencing analysis, genomic DNA of the fungal strain FKI-4942 was extracted using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA,

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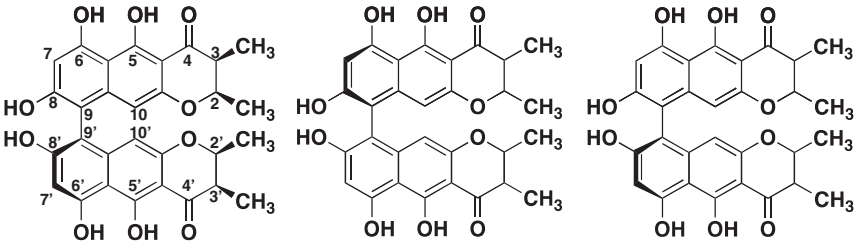

Figure 1 Structures of isochaetochromins A_1, B_1 and B_2.

USA) according to the manufacturer's instructions. The rDNA internal transcribed spacer regions, including the 5.8S rDNA were amplified by PCR using primers ITS1 and ITS4.[19] Amplifications were performed using a PCR Verity 96-well thermal cycler (Applied Biosystems). PCR products were purified using a QIAquick PCR DNA Purification kit (Qiagen, Valencia, CA, USA). Sequencing reactions were directly performed in both directions using primers ITS1, ITS2, ITS3 and ITS4 with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and the products were purified by ethanol/EDTA precipitation. DNA sequences were read on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) and assembled using forward and reverse sequences with the programs SeqMan and SeqBuilder from the Lasergene8 package (DNAStar, Madison, WI, USA). The ITS sequence of the strain FKI-4942 was deposited in DDBJ (DNA Data Bank of Japan) with accession number AB588822.

Fermentation media

For the production of isochaetochromins, the seed medium contained 2.0% glucose, 0.50% polypeptone, 0.050% $MgSO_4{\cdot}7H_2O$, 0.20% yeast extract, 0.10% KH_2PO_4 and 0.10% agar, adjusted to pH 6.0 before sterilization. The production medium was composed of 3.0% sucrose, 3.0% soluble starch, 1.0% malt extract, 0.30% Ebios (Asahi Food & Healthcare, Tokyo, Japan), 0.50% KH_2PO_4 and 0.050% $MgSO_4{\cdot}7H_2O$, adjusted to pH 6.0 before sterilization.

Fermentation

A stock culture of strain FKI-4942 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml seed medium and incubated on a rotary shaker (210 r.p.m.) at 27°C for 3.0 days. The production culture was initiated by transferring 1.0 ml seed culture into a 500-ml Erlenmeyer flask containing 100 ml production medium, and fermentation was carried out on a rotary shaker (210 r.p.m.) at 27°C for 3.0 days and then under static conditions at 27°C for 10 days.

Cell culture

CHO-K1 cells were kind gifts from Dr K Hanada (National Institute of Infectious Diseases, Tokyo, Japan). Cells were maintained at 37 °C in 5.0% CO_2 in Ham's F-12 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum by the method described previously.[15]

Assay for TG synthesis activity using CHO-K1 cells

Assays for TG and CE synthesis using CHO-K1 cells were carried out using established methods[20] with some modifications. CHO-K1 cells (1.25×10^5 cells per 250 µl) were cultured in a 48-well plastic microplate. A sample (2.5 µl in methanol) and [1-^{14}C]oleic acid (1 nmol, 1.85 kBq, 5.0 µl in 10% ethanol/phosphate-buffered saline solution) were added to each well of the culture. The cells were cultured at 37 °C in 5.0% CO_2. After 6-h incubation, cells in each well were washed twice with phosphate-buffered saline. The cells were lysed by adding 0.25 ml of 10 mM Tris–HCl (pH 7.5) containing 0.10% (w/v) sodium dodecyl sulfate, and the cellular lipids were extracted by Bligh and Dyer's method.[21] The total lipids were separated on a TLC plate (silica gel F254, 0.50 mm thick; Merck,) and analyzed with a bioimaging analyzer (BAS 2000; Fujifilm, Tokyo, Japan) to measure the amount of [^{14}C]lipids. Lipid synthesis activity (%) was defined as ([^{14}C]lipid-sample/[^{14}C]lipid-control)×100. The IC_{50} value was defined as the drug concentration causing 50% inhibition of lipid synthesis.

Assay for DGAT activities in CHO-K1 microsomes

The assay for TG synthesis activity using CHO-K1 microsomes was carried out by our established method.[14,15] Briefly, the reaction mixture contained 175 mM Tris–HCl (pH 8.0), 50 µg protein of microsomal fraction, 120 µM BSA, 14 µM palmitoyl-CoA, 1.7 µM [1-^{14}C]palmitoyl-CoA (0.74 kBq GE Healthcare UK, Buckinghamshire, England), 8.0 mM $MgCl_2$, 2.5 mM diisopropyl fluorophosphates and 150 µM 1,2-dioleoyl-*sn*-glycerol (Sigma-Aldrich) and a test sample (5.0 µl in MeOH) in a total volume of 0.20 ml. The assay was initiated by the addition of a microsomal fraction. After 15-min incubation at 23 °C, the reaction was stopped by the addition of $CHCl_3$-MeOH (1:2, 1.2 ml), and lipids were extracted by the Bligh and Dyer's method.[21] The total lipids were separated on a TLC plate and analyzed with bioimaging to measure the amount of [^{14}C]TG. DGAT activity (%) was defined as ([^{14}C]TG-sample/[^{14}C]TG-control)×100. The IC_{50} value was defined as the drug concentration causing 50% inhibition of DGAT activity.

Cytotoxic assays

Cytotoxicity of samples to CHO-K1 cells was measured by the colorimetric assay using 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich). CHO-K1 cells (5.0×10^5 cells in 100 µl) were added to each well of a 96-well micro plate. A sample (1.0 µl in MeOH) was added to each well to make a final concentration of 0 to 92 µM. The cells were incubated for 6.0 h at 37 °C. MTT (10 µl of 5.5 mg ml^{-1} stock solution) and lysis solution (90 µl, 40% *N*,*N*-dimethylformamide, 20% sodium dodecyl sulfate, 2.0% CH_3COOH and 0.030% HCl) were added to each well, and the microplate was shaken for 2.0 h. The OD of each well was measured at 540 nm using a microtiter-plate reader (Elx 808; BioTek Instruments, Winooski, VT, USA).

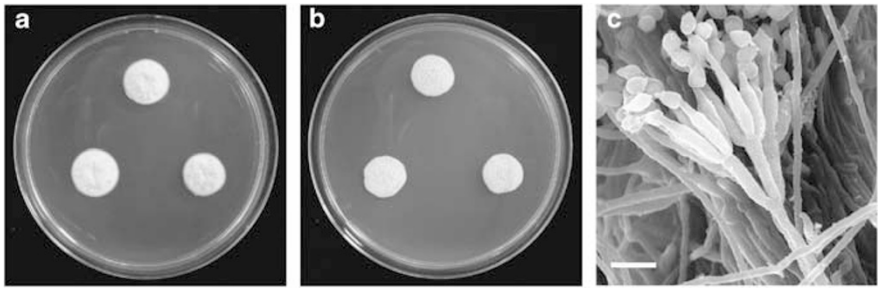

Figure 2 Morphological characteristics of the isochaetochromins-producing strain FKI-4942. (**a**) Photograph of colonies grown on CYA for 7.0 days. (**b**) Photograph of colonies grown on MEA for 7.0 days. (**c**) Scanning electron micrograph of conidiophore of strain FKI-4942. Bar represents 10 μm.

RESULTS

Taxonomy of strain FKI-4942

Colonies on CYA after 7 days at 25 °C (Figure 2a) were 15–17 mm in diameter, radially sulcate, floccose, colliculose, with aerial mycelium and white (hue number; a) in color. The center of the colony was lemon yellow (hue number; 1 ia) in conidial color, without exudate drops. The reverse side was gold (hue number; 2 lc) with an entire margin, without soluble pigment. Colonies on MEA after 7 days at 25 °C (Figure 2b) were 13–15 mm in diameter, less dense than on CYA, colliculose, with floccose aerial mycelium, and light yellow (hue number; 1 ea) in color, exuding sparse clear drops. The reverse side was golden yellow (2 kb) with an entire margin, without soluble pigment. Colonies on G25N at 25 °C showed no growth. Colonies on CYA at 5 °C and 37 °C showed no growth.

Conidiophores on MEA were borne on a basal felt or directly from the agar, and stipes were simple or rarely branching, (15–)20–65×1.7–3.7 μm, with a heavy wall. Penicilli were typically biverticillate (Figure 2c). Metulae were in whorls of 2–4, individually more or less cylindrical across the top, and about 11.6–18.6×(1.1–)1.5–2.5 μm in size. They were usually rather appressed, or sometimes slightly divergent when forced apart by larger whorls. Phialides were ampulliformed 11.4–19.6×1.2–3.0 μm in size, and conidiogenous were apertured 0.5–1.5 μm wide. Conidia were subglobose to ellipsoidal or fusiform, slightly roughened, (1.9–)2.7–3.8×(1.2–)1.5–2.7 μm in size, and with chains.

The total length of the rDNA ITS (including 5.8S rDNA) of FKI-4942 was 580 bp. In a BLAST search using blastn from the National Center for Biotechnology Information (NCBI),[22] the sequence of FKI-4942 had a 94.8% match (30 nucleotide sequence difference) with that of *Penicillium variabile* FRR 1290 (GenBank accession number AY373936).

From the results of morphological characteristics and BLAST search, the producing strain FKI-4942 was considered to belong to the genus *Penicillium*.

Isolation of isochaetochromins

The 13-day-old culture broth (2.0 l) was extracted with 2.0 l of acetone. After acetone extracts were filtered and concentrated to remove acetone, the aqueous solution was extracted with 2.0 l of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a yellow brown material (1.1 g). The material was dissolved in a small amount of $CHCl_3$ and applied to a silica gel column (100 g) previously equilibrated with $CHCl_3$ and eluted stepwise with $CHCl_3$-MeOH solutions (200 ml×2 each, 100:0, 100:1, 50:1, 10:1, 1:1, 0:100). The second fraction of 50:1 ($CHCl_3$-MeOH) was concentrated *in vacuo* to dryness to yield a yellow brown material (81 mg), which was finally purified by preparative HPLC (column, PEGASIL ODS, 20×250 mm; Senshu Scientific, Tokyo, Japan; solvent, 55% MeCN containing 0.050% trifluoroacetic acid; detection, UV at 210 nm; flow rate, 8.0 ml min^{-1}). Under these conditions, isochaetochromins A_1, B_1 and B_2 were eluted as peaks with retention times of 57, 62 and 74 min, respectively (Figure 3). The fractions of the peaks were collected and concentrated to remove MeCN. The aqueous solution was extracted with EtOAc, and the organic layer was concentrated to dryness to give isochaetochromins A_1 (2.5 mg), B_1 (14 mg) and B_2 (26 mg) as yellow powders.

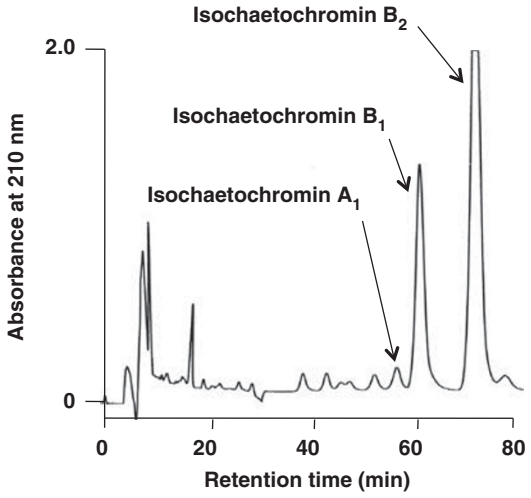

Figure 3 Chromatographic profile of isochaetochromin isolation by preparative HPLC. (HPLC conditions: column, PEGASIL ODS, 20×250 mm; Senshu Scientific; solvent, 55% MeCN containing 0.050% trifluoroacetic acid; detection, UV at 210 nm; flow rate, 8.0 ml min^{-1}).

Effects of isochaetochromins on TG and CE synthesis in CHO-K1 cells

Effects of all isochaetochromins on TG and CE synthesis were evaluated using a cell-based assay to quantify [^{14}C]TG and [^{14}C]CE from [^{14}C]oleic acid in CHO-K1 cells. As shown in Figure 4, isochaetochromins A_1, B_1 and B_2 inhibited [^{14}C]TG synthesis with IC_{50} values of 33, 5.6 and 11 μM, respectively, and [^{14}C]CE synthesis with IC_{50} values of 50, 58 and 44 μM, respectively. Isochaetochromins A_1 and B_1 showed no cytotoxic effect up to 92 μM, whereas only isochaetochromin B_2 weakly inhibited cell growth with an IC_{50} value of 60 μM. In this cell-based assay, isochaetochromin B_1 is the most selective inhibitor of TG synthesis.

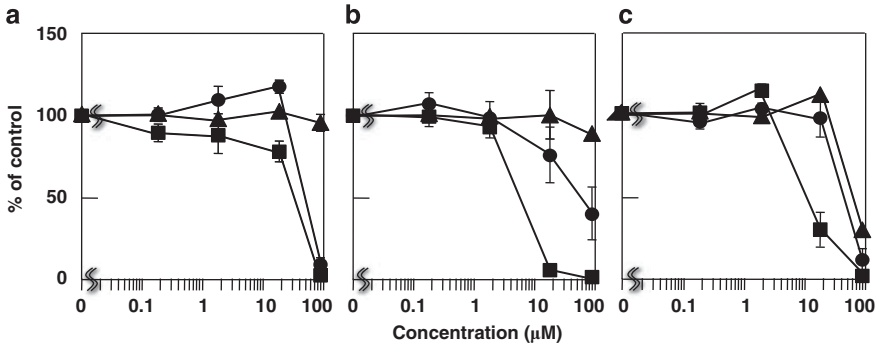

Figure 4 Effects of isochaetochromins on TG synthesis, CE synthesis and toxicity in CHO-K1 cells. (**a**) isochaetochromin A_1, (**b**) isochaetochromin B_1 and (**c**) isochaetochromin B_2. [^{14}C]TG (■), [^{14}C]CE (●) and cytotoxicity (▲). Error bars: ± s.d. (n=4)

Table 1 Effects of isochaetochromins on TG synthesis, CE synthesis and cytotoxicity in CHO-K1 cells, and DGAT activity in microsomes prepared from CHO-K1 cells

	IC_{50} (μM)			
Compound	*TG*	*CE*	*Cytotoxicity*	*DGAT*
Isochaetochromin A_1	33	50	>92	320
Isochaetochromin B_1	5.6	58	>92	190
Isochaetochromin B_2	11	44	60	310

Abbreviations: CE, cholesteryl ester ; DGAT, diacylglycerol acyltransferase; TG, triacylglycerol.

Effect of isochaetochromins on DGAT activity

DGAT is the enzyme catalyzing the final step of TG synthesis.[9] DGAT was considered a potential target of isochaetochromins in the inhibition of TG synthesis; therefore, the effect of isochaetochromins A_1, B_1 and B_2 on DGAT activity was evaluated in microsomes prepared from CHO-K1 cells. As a result, isochaetochromins A_1, B_1 and B_2 were found to inhibit DGAT activity in a dose-dependent manner, but the IC_{50} values were rather high (Table 1).

DISCUSSION

As described in the accompanying paper,[23] these compounds have the same planar bis-naphtho-γ-pyrone structure and are different in the stereochemistry of 2/2′ and 3/3′ methyl residues and the *aS*/*aR*-type axis, although not specified in Figure 1. These subtle differences appeared to affect the TG synthesis, CE synthesis and cytotoxicity in CHO-K1 cells (Table 1). Namely, isochaetochromin B_1 showed the highest selectivity in TG synthesis inhibition (IC_{50}, 5.6 μM) when compared with CE synthesis inhibition (IC_{50}, 58 μM). Isochaetochromin A_1 was found to be a moderate dual inhibitor of TG and CE synthesis in this assay. Isochaetochromin B_2 seemed rather selective in TG synthesis inhibition, but showed a cytotoxic effect on CHO-K1 cells at higher concentrations; therefore, although it is difficult to explain the biological activity of this compound, this planar structure might have intrinsic potential to affect CE synthesis, and the *aS* axis structure (only isochaetochromin B_1) might be responsible for the selective inhibition of TG synthesis in this assay. Regarding the inhibition of TG synthesis, DGAT is considered one of the potential targets; however, DGAT inhibitory activity of isochaetochromins was very weak in microsomes of CHO-K1 cells although they showed dose-dependent inhibition. It might be that isochaetochromins have distinct or multiple targets to exhibit TG/CE synthesis inhibition.

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