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ORIGINAL ARTICLE

Dinapinones, novel inhibitors of triacylglycerol synthesis in mammalian cells, produced by *Penicillium pinophilum* FKI-3864

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Dinapinone A, a novel biaryl dihydronaphthopyranone, was isolated from the culture broth of *Penicillium pinophilum* FKI-3864 by solvent extraction, silica gel and ODS column chromatography and HPLC. Dinapinone A showed very potent inhibition of triacylglycerol (TG) synthesis in intact Chinese hamster ovary K1 (CHO-K1) cells with an IC_{50} value of 0.097 μM. Dinapinone A was found to be a mixture of stereoisomers, resulting in its separation into dinapinones A1 and A2 by HPLC using a C30 reverse-phase column. Dinapinone A1 did not inhibit TG synthesis in CHO-K1 cells even at 12 μM, and dinapinone A2 showed less potent inhibition (IC_{50}; 0.65 μM) than dinapinone A; however, a mixture of isolated dinapinones A1 and A2 (a 1:1 ratio) recovered the potent TG inhibitory activity (IC_{50}; 0.054 μM). A similar effect of dinapinone on TG synthesis in intact Raji cells was also observed.
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Keywords: atropisomer; dinapinone; dihydronaphthopyranone; fungal metabolites; obesity; *Penicillium pinophilum*; triacylglycerol biosynthesis inhibitor

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 or 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 exerts an effect centrally to inhibit serotonin and noradrenaline uptake,[1,2] and orlistat, which inhibits lipase to interfere with lipid absorption from the small intestine.[3,4] One of the potential strategies for the treatment of obesity is to block TG synthesis;[5] therefore, inhibitors of TG synthesis are considered as therapeutic agents for obesity.[6,7] TG is synthesized by a number of enzymes.[8] Among them, 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 intact CHO-K1 cells. During our screening for the inhibitors of TG synthesis using this cell-based assay, new biaryl dihydronaphthopyranones, named dinapinone (Figure 1), were isolated from the culture broth of *Penicillium pinophilum* FKI-3864. The physico-chemical properties and structure elucidation including the absolute stereochemistry of dinapinones were reported and described elsewhere.[15] In this study, the fermentation, isolation and inhibitory effect on TG synthesis in CHO-K1 cells of dinapinones are described.

RESULTS

Taxonomy of strain FKI-3864

Conidiophores on Czapek yeast-extract agar (CYA) medium arose from basal hyphae, rarely branched, smooth-walled, 50–180×1.0–2.5 μm, with thick walls. Penicilli from condiophores were biverticillate (consisting of metulae and phialides) and symmetrical. Metulae had 2–6 branches, which were usually rather appressed or sometimes slightly divergent with larger size, 7.5–12.5×2.0–2.5 μm. Phialides were acerose, 7.5–12.5×2.0–2.5 μm, and smooth (Figure 2d). Conidia were subglobose to globose, smooth-walled, 2.3–2.8 μm, and produced divergent long chains.

Colonies on CYA after 7 days at 25 °C (Figure 2a) were 34–37 mm in diameter, dense, colliculose, floccose with a smooth and pale-yellow (1 ia) margin. The center of the colony was light olive gray (1 ge) in conidial color, with clear exudate droplets. The reverse side was yellowish-brown (2 pe). Colonies on malt extract agar (MEA) medium (Figure 2b) were 37–39 mm in diameter, less dense than on CYA,

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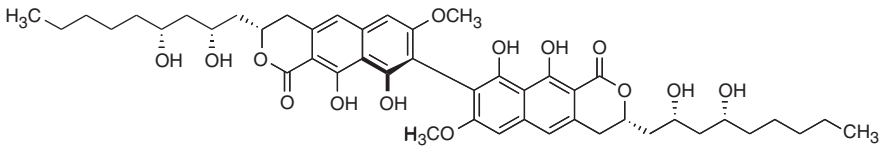

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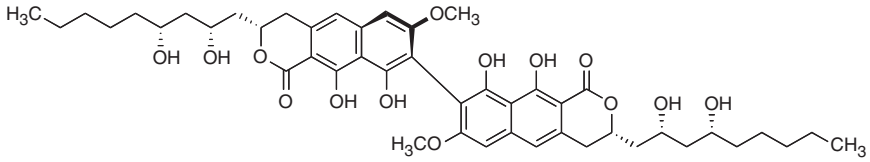

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Figure 1 Structures of dinapinone A1 (**1**) and A2 (**2**).

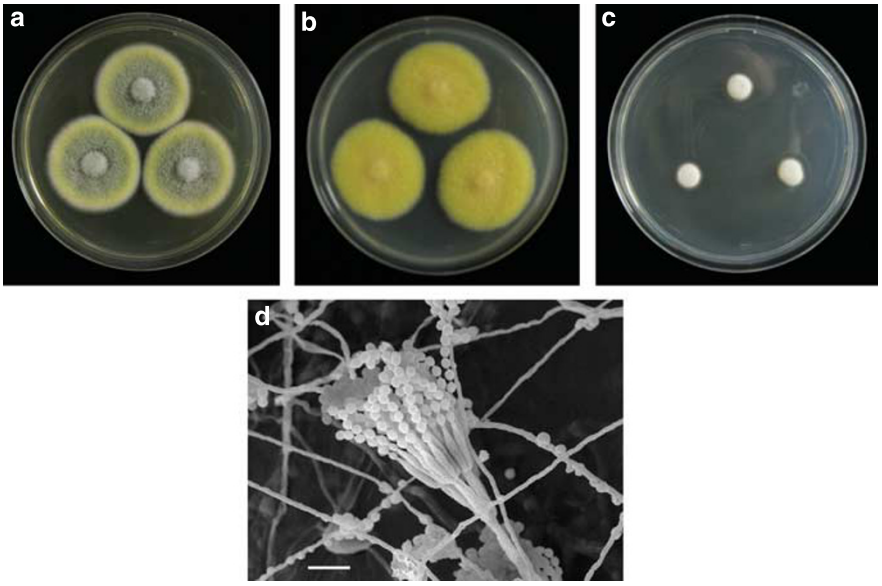

Figure 2 Morphological characteristics of dinapinone-producing strain FKI-3864. (**a**) Micrograph of colonies grown on Czapek yeast-extract agar (CYA) for 7 days. (**b**) Micrograph of colonies grown on malt extract agar (MEA) for 7 days. (**c**) Micrograph of colonies grown on G25N for 7 days. (**d**) Scanning electron micrograph of metulae, phialides and conidia of strain FKI-3864. Bar represents 10 μm.

colliculose, floccose, with a smooth margin, and pale yellow (1 ia), without exudate droplets. The reverse side was yellow (1 1/2 la). Colonies on G25N (Figure 2c) were 9–10 mm in diameter, pulvinate, floccose, with a smooth margin, and white (a), with clear exudate droplets. The reverse side was light yellow (1 1/2 ga). Colonies on CYA after 7 days at 37°C were 49–50 mm in diameter, dense, colliculose, floccose, with a smooth margin, and yellowish gray (2 ca). The reverse was reddish yellow (3 pc). The culture on CYA at 5 °C showed no growth.

From the above morphological characteristics, strain FKI-3864 was considered to belong to the genus *Penicillium* in the subgenus *Biverticillium* section *Simplicia*.[16] Furthermore, the strain was characterized by its pale-yellow mycelial on CYA, rapid growth at 37 °C on CYA and length of conidiophores. In addition, in a Blast search, the ribosomal DNA sequence of internal transcribed spacer (ITS) region (572 bp) determined for strain FKI-3864 showed 99.8% similarity to that of *Penicillium pinophilum* ILLS 54734 (GenBank accession No AF176660). ITS sequence was deposited at The DNA Data Bank of Japan (DDBJ) with accession number AB455516. Therefore the producing strain FKI-3864 was identified as a strain of *Penicillium pinophilum* and was deposited at the International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, Tukuba, Japan as FERM AP-21483.

Isolation of dinapinones

The isolation of dinapinones from the culture broth of *P. pinophilum* FKI-3864 is summarized in Figure 3 and Table 1. Activity-guided isolation was carried out step by step. The culture broth (6 l) was treated with EtOH (6 l) for 30 min and the mixture was filtered to remove the mycelium. After concentration of the mixture to remove EtOH, the aqueous solution was extracted with EtOAc (6 l). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a brown material (4.5 g). The material was dissolved in a small amount of $CHCl_3$ and applied to a silica gel column (i.d. 3.6×30 cm, Silica gel 60, 70–230 mm; Merck KgaA, Frankfurter Strasse 250, 64293 Darmstadt, Germany) previously equilibrated with $CHCl_3$, and the materials were eluted stepwise with $CHCl_3$-MeOH solutions (1 l each, 100:0, 10:1, 1:1 and 0:100). Dinapinones were recovered

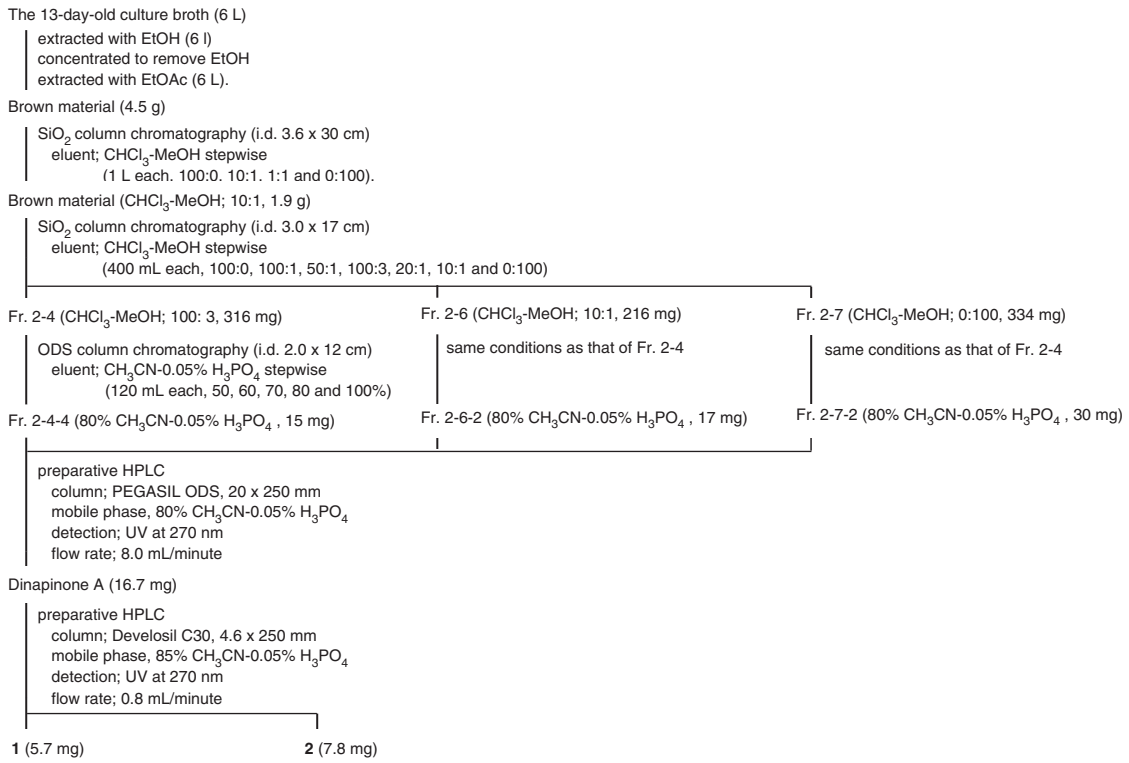

Figure 3 Isolation procedure of dinapinones A1 (**1**) and A2 (**2**).

Table 1 Isolation of dinapinone A from the culture broth of *P. pinophilum* FKI-3864

Step	*Eluting solvent*	*Weight (mg)*	*IC_{50} ($\mu g\,ml^{-1}$)*	*Total activity (Weight/IC_{50})*	*Yield (%)*
EtOAc extract		4500	10.1	446	100
1st-Silica	$CHCl_3$:MeOH				
Fr.2	10:1	1900	6.1	311	69.7
2nd-Silica	$CHCl_3$:MeOH				
Fr.2–4	100:3	316	6.1	51.8	11.6
Fr.2–6	10:1	216	6.1	35.4	7.9
Fr.2–7	0:100	334	9.1	36.7	8.2
ODS open	CH_3CN-0.05% H_3PO_4				
Fr.2–4–4	80%	15	<0.2	>75	>16.8
Fr.2–6–2	80%	17	0.4	42.5	9.5
Fr.2–7–2	80%	30	0.4	75	16.8
HPLC (ODS)					
Dinapinone A		16.7	0.081	206.2	46.2
HPLC (C30)					
Dinapinone A1		5.7	>10	<0.57	<0.13
A2		7.8	0.54	14.4	3.2

from the fractions of $CHCl_3$-MeOH (10:1), which were concentrated under reduced pressure to give brown material (1.9 g). The material was dissolved in chloroform and subjected to a second silica gel column chromatography (i.d. 3.0×17 cm), and materials were eluted stepwise with $CHCl_3$-MeOH solutions (400 ml each, 100:0, 100:1, 50:1, 100:3, 20:1, 10:1 and 0:100). The three fractions ($CHCl_3$-MeOH, 100:3 (Fr. 2–4), 10:1 (Fr. 2–6) and 0:100 (fr. 2–7)) containing dinapinones were concentrated to give yellow powder (316, 216 and 334 mg, respectively). They were dissolved in 50% CH_3CN, and were purified by ODS column chromatography (i.d. 2.0×12 cm; Sensyu Scientific Ltd., Tokyo, Japan). Active materials were eluted stepwise with CH_3CN-0.05% H_3PO_4 solutions (120 ml each, 50, 60, 70, 80 and 100%), and were recovered from Fr. 2–4–4, Fr. 2–6–2 and Fr. 2–7–2 in the 80% CH_3CN-0.05% H_3PO_4 solution, which were concentrated under reduced pressure to give a yellow powder (15, 17 and 30 mg, respectively). They were combined and finally purified by HPLC under the following conditions: column, Pegasil ODS (i.d. 20×250 mm, Sensyu Scientific); mobile phase, 80% CH_3CN-0.05% H_3PO_4; flow rate, 8 ml min^{-1}; detection, UV 270 nm. Dinapinone A was eluted as a peak with a retention time of 13 min (Figure 4a). The fraction of the peak was collected and concentrated to remove CH_3CN. The aqueous solution was extracted with EtOAc, and the organic layer was

concentrated to dryness to give dinapinone A (16.7 mg) as a yellow amorphous solid. As described in detail in the accompanying study,[15] the NMR spectral data strongly suggested that dinapinone A is composed of stereoisomers; therefore, we tested various HPLC conditions to isolate these isomers, and dinapinone A was subjected to HPLC under the following conditions: column, Develosil C30 (i.d. 4.6×250 mm, Nomura Chemical, Aichi, Japan); mobile phase, 85% CH_3CN-0.05% H_3PO_4; flow rate, 0.8 ml min^{-1}; detection, UV 270 nm. Dinapinones A1 and A2 were eluted as a peak with retention times of 11 and 13 min, respectively (Figure 4b). These fractions were pooled and concentrated to remove CH_3CN. The aqueous solutions were extracted with EtOAc, and the organic layers were concentrated to dryness to give dinapinones A1 (5.7 mg) and A2 (7.8 mg) as yellow amorphous solids.

Biological activity

Effects of dinapinones on TG synthesis in animal cells. The effect of dinapinones on TG synthesis was evaluated in the cell-based assay to quantify [^{14}C]TG and [^{14}C]CE in CHO-K1 cells and Raji cells. As shown in Figure 5, in CHO-K1 cells, dinapinone A, a mixture of dinapinones A1 and A2, inhibited [^{14}C]TG and [^{14}C]CE synthesis in a dose-dependent manner with IC_{50} values of 0.097 and 0.31 μM, respectively. However, dinapinone A1 isolated from dinapinone A showed no effect on [^{14}C]TG syntheses at 12 μM, and dinapinone A2 showed less potent inhibitory activity than dinapinone A of [^{14}C]TG and [^{14}C]CE synthesis with respective IC_{50} values of 0.65 and 5.24 μM (Table 2).

To elucidate the results, isolated dinapinones A1 and A2 were mixed at various ratios, and the effect of such mixtures on TG synthesis was tested by the same methods using CHO-K1 cells. Interestingly, mixtures with a ratio of 5:1 to 2:1 (A1 > A2) showed almost no TG inhibitory activity, but mixtures of 1:1 to 1:15 (A1 ⩽ A2) recovered their activity (Table 3). In particular, the ratio of 1:1 and 1:2 exhibited the most potent inhibitory activity with IC_{50} values of 0.054 and 0.073 μM, respectively.

The inhibitory effect of dinapinones on TG synthesis was confirmed in the assay using Raji cells. In this assay, the synthesis of only [^{14}C]TG and [^{14}C]PL from [^{14}C]oleic acid was observed.[17] The IC_{50} values are summarized in Table 2. Dinapinone A showed potent inhibition of TG synthesis (IC_{50}; 0.38 μM), but dinapinone A2 had lower activity than dinapinoneA (IC_{50}; 5.42 μM), and dinapinone A1 almost lost the activity. It was also observed that a mixture of dinapinones A1 and A2 (1:1 and 1:2) recovered the potent TG inhibitory activity (IC_{50}; 0.09 and 1.62 μM) (data not shown). These findings are essentially similar to those observed in CHO-K1 cells.

Effect of dinapinones on DGAT activity in microsomes. DGAT in TG synthesis is one possible or one potential target of dinapinones; therefore, the effect of dinapinones on enzyme activity was tested in microsomes prepared from rat livers, CHO-K1 cells and DGAT1- and DGAT2-expressing *Saccharomyces cerevisiae*;[18] however, dinapinones A, A1 and A2 showed no inhibition of DGAT activity, even at 12 μM, indicating that the target molecule of dinapinones is not DGAT.

Cytotoxic activity. The effect of dinapinones on CHO-K1 cells and Raji cells was tested by the MTT assay. No cytotoxic effect was observed at 12 μM within 6 h; however, after 6 h, a cytotoxic effect was observed and almost all cells died gradually by 24 h.

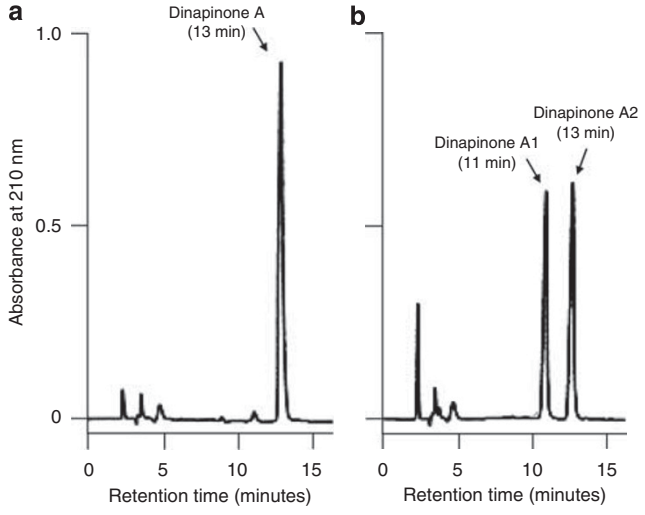

Figure 4 Purification of dinapinones by HPLC. (**a**) Dinapinone A was purified by HPLC with a C18 reverse-phase column. (**b**) Dinapinone A was subjected to HPLC with a C30 reverse-phase column to isolate atropisomers dinapinones A1 (**1**) and A2 (**2**).

Table 2 Effects of dinapinones on lipid synthesis in CHO-K1 and Raji cells

	IC_{50} in CHO-K1 cells (μM)			*IC_{50} in Raji cells (μM)*	
	CE	*TG*	*PL*	*TG*	*PL*
Dinapinone A	0.31	0.097	>1.2	0.38	>1.2
Dinapinone A1	>12	>12	>12	>12	>12
Dinapinone A2	5.2	0.65	>12	5.4	>12

Abbreviations: CE, cholesteryl ester; PL, phospholipid; TG, triacylglycerol.

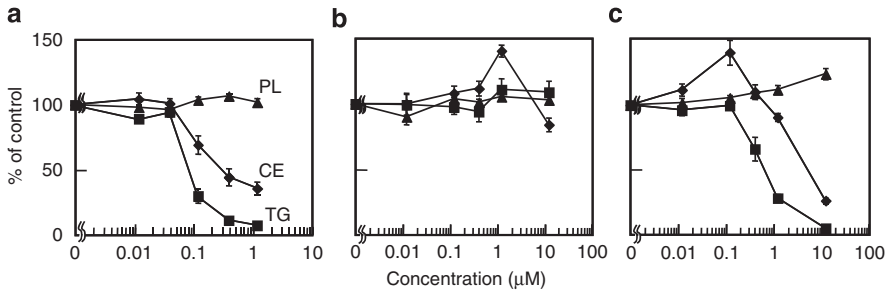

Figure 5 Effects of dinapinones on the synthesis of [^{14}C]TG, [^{14}C]CE and [^{14}C]PL from [^{14}C]oleic acid in CHO-K1 cells. (**a**) Dinapinone A; (**b**) Dinapinone A1 (**1**); and (**c**) dinapinone A2 (**2**). Error bars ± s.d. (n=4).

Table 3 Effect of a mixture of dinapinones A1 and A2 at a various ratio on TG synthesis in CHO-nK1 cells

Ratio A1:A2	*IC_{50} (μM)*
1:0	>12
5:1	>1.2
4:1	>1.2
3:1	>1.2
2:1	>1.2
1:1	0.054
1:2	0.073
1:3	0.16
1:4	0.20
1:5	0.13
1:8	0.25
1:10	0.24
1:15	0.35
0:1	0.65
Dinapinone A	0.097

Anticrobial activities. No antimicrobial activity of dinapinones (10 μg per disk) against the six microorganisms was observed.

DISCUSSION

Dinapinones were the first inhibitors of TG synthesis discovered in our cell-based assay using CHO-K1 cells. Initially we had considered that dinapinone A, efficiently isolated from a culture broth of the fungus (Table 1), was a pure compound with rather selective TG inhibitory activity; however, from the spectral analyses described in the accompanying study,[15] dinapinone A was found to be a mixture of stereoisomers, leading to the purification of atropisomers dinapinones A1 and A2 by HPLC using a C30 reverse-phase column (Figure 3). Unfortunately, dinapinone A2 showed less potent inhibition than dinapinone A (respective IC_{50}, 0.65 vs 0.097 μM for TG synthesis) (Table 2), although its selectivity toward TG inhibition increased (8.1 vs 3.2), and dinapinone A1 almost lost its inhibitory activity. Interestingly, a mixture of pure dinapinones A1 and A2 at a ratio of 1:1 or 1:2 recovered as potent activity as dinapinone A (Table 3). The same results were observed in the Raji cell assay (Table 2). Namely, dinapinone A showed potent inhibition of TG synthesis (IC_{50}, 0.38 μM). Dinapinone A2 (not A1) had lower activity (IC_{50}, 5.4 μM) and dinapinone A1 (not A2) almost lost the activity (IC_{50}, >12 μM); however, a mixture of dinapinones A1 and A2 at a ratio of 1:1 or 1:2 recovered the activity. These findings strongly suggested that the inhibitory activity of dinapinone A2 against TG synthesis is potentiated by dinapinone A1. One of the potential targets in TG synthesis is DGAT. We have reported several DGAT inhibitors of natural origin;[12–14] however, dinapinones showed no effects on DGAT activity in our assay systems.[19] Thus, the target molecule of dinapinones remains to be elucidated.

METHODS

Taxonomic studies of the producing strain FKI-3864

Fungal strain FKI-3864 was isolated from soil collected in Hilo, Hawaii, USA. Morphological studies and identification were conducted following the procedures described by Pitt.[16] For taxonomic studies, CYA, MEA and 25% glycerol nitrate agar (G25N) were used. Morphological characteristics were observed under a light microscope (Vanox-S AH-2; Olympus, Tokyo, Japan) and a scanning electron microscope (JSM-5600; JEOL, Tokyo, Japan). Color names and hue numbers were determined according to the Color Harmony Manual.[20]

For molecular phylogenetic study, genomic DNA was extracted using the PrepMan Ultra Sample Preparation Reagent (AB Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The rDNA internal transcribed spacer (rDNA ITS) regions, including the 5.8S rDNA gene, were amplified by PCR using primers ITS1 and ITS4.[21] Amplifications were carried out in a PCR Thermal Cycler Dice mini Model TP100 (Takara Bio, Shiga, Japan). The amplified PCR products were purified by the QIAquick PCR DNA Purification Kit (Qiagen, Valencia, CA, USA). Sequencing reactions were directly performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and the products were purified with a DyeEX 2.0 Spin Kit (Qiagen). DNA sequences were read on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) and assembled using the programs SeqMan and SeqBuilder from the Lasergene7 package (DNAStar, Madison, WI, USA). The ITS sequence was compared with database of the National Canter for Biotechnology Information.[22]

Fermentation

A slant culture of strain FKI-3864 grown on LCA medium (0.10% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4$. $7H_2O$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% yeast extract and 1.5% agar, adjusted to pH 6.0 before sterilization) was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the seed medium (2.0% glucose, 0.5% polypeptone, 0.05% $MgSO_4{\cdot}7H_2O$, 0.2% yeast extract, 0.1% KH_2PO_4 and 0.10% agar, adjusted to pH 6.0 before sterilization). The flask was incubated on a rotary shaker (270 rpm) for 3 days at 27 °C to obtain the seed culture. The production culture was initiated by transferring 2-ml seed culture into a 1-l Roux flask containing production medium (3.0% sucrose, 3.0% soluble starch, 1.0% malt extract, 0.3% Ebios (Asahi Food & Helthcare, Tokyo, Japan) 0.5% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, adjusted to pH 6.0 before sterilization) and fermentation was carried out under static conditions at 27 °C for 13 days.

Cell culture

CHO-K1 cells (a generous gift from Dr Kentaro Hanada, National Institute of Infectious Diseases, Tokyo, Japan) were maintained at 37 °C in 5% CO_2 under Ham's F-12 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated FBS using the method described previously.[17] Raji cells were routinely maintained at 37 °C under 5% CO_2 in RPMI-1640 (Sigma-Aldrich) culture medium supplemented with 10% heat-inactivated FBS.[23]

Assay for TG synthesis in intact CHO-K1 and Raji cells

Assays for TG, CE and phospholipid (PL) synthesis using CHO-K1 cells[24] and TG and PL synthesis using Raji cells[23] were carried out using established methods with some modifications. CHO-K1 cells (1.25×10^5 cells/250 μl) or Raji cells (Dainippon Sumitomo Pharma, Osaka, Japan) (1.25×10^5 cells/250 μl) were cultured in a well of a 48-well plastic microplate. A sample (2.5 μl in methanol) and [^{14}C]oleic acid (1 nmol, 1.85 KBq, 5.0 μl in 10% ethanol/PBS solution) were added to each well of the cell culture. The cells were cultured at 37°C in 5% CO_2. After 6-h incubation, cells in each well were washed twice with PBS. The cells were lysed by adding 0.25 ml of 10 mM Tris-HCl (pH 7.5) containing 0.1% (w/v) SDS, and the cellular lipids were extracted by the method of Bligh and Dyer.[19] The total lipids were separated on a TLC plate (silica gel F254, 0.5-mm thick, Merck KGaA) and the TLC plate was 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–drug/[^{14}C]lipid–control)×100. The IC_{50} value was defined as the drug concentration causing 50% inhibition of lipid synthesis.

Assay for DGAT activity

DGAT activity in microsomes prepared from mouse liver, CHO-K1 cells and DGAT1- and DGAT2-expressing *Saccharomyces cerevisiae* was assayed using established methods.[14,18] Briefly, the reaction mixture contained 175 mM Tris-HCl (pH 8.0) 50–200 μg protein of each microsomal fraction, 120 μM BSA, 14 μM palmitoyl-CoA, 1.7 μM [^{14}C]palmitoyl-CoA (0.02 μCi; GE Healthcare UK, Little Chalfont, Buckinghamshire, England), 8 mM $MgCl_2$, 2.5 mM diisopropyl fluorophosphates and 150 μM 1,2-dioleoyl-sn-glycerol (Sigma-Aldrich) and a test sample (5 μl in MeOH) in a total volume of 0.2 ml. The

assay was initiated by the addition of a microsomal fraction. After a 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 method of Bligh and Dyer.[19] 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.

Antimicrobial activity

Antimicrobial activity of a sample against six species of microorganisms was measured by the agar diffusion method using paper disks. Media for microorganisms were as follows: nutrient agar for *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* PCI 1001, *Escherichia coli* NIHJ JC-2 IFO 12734 and *Xanthomonas oryzae*, and a medium composed of 1.0% glucose, 0.5% yeast extract and 0.8% agar for *Mucor racemosus* IFO 4581 and *Candida albicans* KF 1. A paper disk (i.d. 6 mm; Toyo Roshi Kaisha, Tokyo, Japan) containing a sample (10 μg) was placed on the agar plate. Bacteria except *X. oryzae* were incubated at 37 °C for 24 h. *X. oryzae* was incubated at 27 °C for 24 h. *C. albicans* and *M. racemosus* were incubated at 27 °C for 48 h. Antimicrobial activity was expressed as the diameter (mm) of the inhibitory zone.

Cytotoxic activity

Cytotoxicity of a sample to CHO-K1 cells or Raji cells was measured by the colorimetric assay on 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich).[25] CHO-K1 cells (5×10^4 cells in 100 μl) or Raji cells (5×10^4 cells in 100 μl) were added to each well of a 96-well microplate. A sample (1 μl in MeOH) was added to each well to make a final concentration of 0 to 12 μM. The cells were incubated for 6 or 24 h at 37 °C. MTT (10 μl of 5.5 mg ml^{-1} stock solution) and a cell lysate solution (90 μl, 40% N,N-dimethylformamide, 20% sodium dodecyl sulfate, 2% CH_3COOH and 0.03% HCl) were added to each well, and the microplate was shaken for 2 h. The OD of each well was measured at 540 nm using a microtiter-plate reader (Elx 808; BioTek Instruments, Winooski, VT, USA).

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1 Bray, G. A. *et al.* A double-blind randomized placebo-controlled trial of sibutramine. *Obes. Res.* **4,** 263–270 (1996).
2 Rinaldi, C. M. *et al.* SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.* **350,** 240–244 (1994).
3 Davidson, M. H. *et al.* Weight control and risk factor reduction in obese subjects treated for 2 years with orlistat: a randomized controlled trial. *J. American. Med. Assoc.* **281,** 235–242 (1999).
4 Kopelman, P. *et al.* Cetilistat (ATL-962), a novel lipase inhibitor: a 12-week randomized, placebo-controlled study of weight reduction in obese patients. *Internatl. J. Obes.* **31,** 494–499 (2007).
5 Smith, S. J. *et al.* Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat. *Nat. Genet.* **25,** 87–90 (2000).
6 Tomoda, H. & Ōmura, S. Potential therapeutics for obesity and atherosclerosis: inhibitors of neutral lipid metabolism from microorganisms. *Pharmacol. Ther.* **115,** 375–389 (2007).
7 Matsuda, D. & Tomoda, H. DGAT inhibitors for obesity. *Curr. Opin. Investigat. Drug* **8,** 836–841 (2007).
8 Coleman, R. A. & Lee, D. P. Enzymes of triacylglycerol synthesis and their regulation. *Prog. Lipid. Res.* **43,** 134–176 (2004).
9 Cases, S. *et al.* Identification of a gene encoding an acyl CoA: diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proc. Natl. Acad. Sci. USA* **95,** 13018–13023 (1998).
10 Casaschi, S., Rubio, B. K., Maiyoh, G. K. & Theriault, A. G. Inhibitory activity of diacylglycerol acyltrasferase (DGAT) and microsomal triglyceride transfer protein (MTP) by the flavonoid, taxifolin, in HepG2 cells: potential role in the regulation of apolipoprotein B secretion. *Atherosclerosis* **176,** 247–253 (2004).
11 Lee, S. W. *et al.* New polyacetylenes, DGAT inhibitors from the roots of *Panax ginseng*. *Plan. Med.* **70,** 197–200 (2004).
12 Ōmura, S., Tomoda, H., Tabata, N., Ohyama, Y., Abe, T. & Namikoshi, M. Roselipins, novel fungal metabolites having a highly methylated fatty acid modified with a mannose and an arabinitol. *J. Antibiot.* **52,** 586–589 (1999).
13 Tabata, N., Ito, M., Tomoda, H. & Ōmura, S. Xanthohumols, diacylglycerol acyltransferase inhibitors, from *Humulus lupulus*. *Phytochemistry* **46,** 683–687 (1997).
14 Tomoda, H. *et al.* Amidepsines, inhibitor of diacylglycerol acyltransferase produced by *Humicola* sp. FO-2942. I. Production, isolation, and biological properties. *J. Antibiot.* **48,** 937–941 (1995).
15 Uchida, R. *et al.* Structures and Absolute Stereochemistry of Dinapinones A1 and A2 and Monapinones A and B, Novel Inhibitors of Triacylglycerol Synthesis, Produced by *Penicillium pinophilum* FKI-3864. (Unpublished).
16 Pitt, J. I. *The Genus Penicillium, and its Teleomorphic States Eupenicillium and Talaromyces* 1–634 (Academic Press, London, 1979).
17 Lada, A. T. *et al.* Identification of ACAT1- and ACAT2-specific inhibitors using a novel, cell-based fluorescence assay: individual ACAT uniqueness. *J. Lipid Res.* **45,** 378–386 (2004).
18 Inokoshi, J. *et al.* Expression of two acyl-CoA: diacylglycerol acyltransferase isozymes in yeast and selectivity of microbial inhibitors toward the isozymes. *J. Antibiot.* **62,** 51–54 (2009).
19 Bligh, E. G. & Dyer, W. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37,** 911–917 (1959).
20 Jacobson, E., Granville, W. C. & Foss, C. E. *Color Harmony Manual* 4th edn, (Container of America, Chicago, 1958).
21 White, T. J., Bruns, T., Lee, S. & Taylor, J. W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols a Guide to Methods and Applications* (eds Innis MA, Gelfand RH, Sninsky JJ, White TJ). 315–332 (Academic Press, New York, 1990).
22 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410 (1990).
23 Tomoda, H., Igarashi, K., Cyong, J. C. & Ōmura, S. Evidence for an essential role of long chain acyl-CoA synthetase in animal cell proliferation. Inhibition of long chain acyl-CoA synthetase by triacsins caused inhibition of Raji cell proliferation. *J. Biol. Chem.* **266,** 4214–4219 (1991).
24 Ohshiro, T., Rudel, L. L., Ōmura, S. & Tomoda, H. Selectivity of microbial acyl-CoA: cholesterol acyltransferase inhibitors toward isozymes. *J. Antibiot.* **60,** 43–51 (2007).
25 Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and citotoxicity assays. *J. Immunol. Methods.* **16,** 55–63 (1983).