Amidepsines, Inhibitors of Diacylglycerol Acyltransferase Produced by *Humicola* sp. FO-2942

I. Production, Isolation and Biological Properties

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Humicola sp. FO-2942, a soil isolate, was found to produce a series of new inhibitors of diacylglycerol acyltransferase (DGAT). Three active compounds, designated amidepsines A, B and C, were isolated from the fermentation broth of the producing strain by solvent extraction, silica gel column chromatography, ODS column chromatography and HPLC. Amidepsines inhibit DGAT activity with IC_{50} values of 10.2~51.6 μ M in an enzyme assay system using rat liver microsomes. Amidepsines also showed specific inhibition of triacylglycerol formation in intact Raji cells, indicating that they inhibit DGAT activity in living cells.

Triacylglycerol is the main storage form of energy, and is believed to be predominantly metabolic. Too much accumulation of triacylglycerol in certain organs and tissues of the body causes high risk conditions of fatty liver, obesity, and hypertriglyceridemia, leading to serious diseases of atherosclerosis, diabetes, metabolic

disorders and functional depression of some organs. Therefore, control of triacylglycerol synthesis is expected to be effective on treatment and prevention for these diseases. Diacylglycerol acyltransferase (acyl-CoA:1,2-diacyl-*sn*-glycerol *O*-acyltransferase, abbreviated as DGAT) [EC 2.3.1.20] catalyzes the reaction of acyl residue transfer from acyl-CoA to diacylglycerol to form triacylglycerol. The reaction is the final step of *de novo* triacylglycerol biosynthesis, and is the only pathway which is exclusively involved in triacylglycerol formation¹⁾. Therefore, DGAT is considered as a potential target of inhibition for specific control of such high triacylglycerol-induced disorders. However DGAT inhibitors have been rarely reported²⁾.

During the course of our screening program for DGAT inhibitors of microbial origin, we discovered and isolated a series of DGAT inhibitors from the culture broth of a fungal strain FO-2942. Four structurally related active compounds, designated as amidepsines A, B, C and D (Fig. 1), were isolated from the culture broth. Amidepsine D was identical with a known lichen tridepside 2,4-di-*O*-methylgyrophoric acid³⁾, but amidepsines A, B and C were found to be new compounds⁴⁾. In this paper, the taxonomy of the producing strain, fermentation, isolation and biological properties of amidepsines are described.

Fig. 1. Structures of amidepsines A, B, C and D (2,4-di-*O*-methylgyrophoric acid).

Materials and Methods

General Experimental Procedures

Fungal strain FO-2942 isolated from a soil sample was used for production of amidepsines. Kieselgel 60 (E. Merck) and SSC-ODS-7515-12 (Senshu Sci. Co.) were used for column chromatography. HPLC was carried out using JASCO system (TRI ROTAR V).

Taxonomic Studies

For the identification of the fungus, potato dextrose agar (Difco), malt extract agar, CZAPEK's agar, corn meal agar (Difco) and YpSs agar (soluble starch 1.5%, yeast extract 0.4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and agar 2.0%, pH 6.0) were used. Morphological observations were done under a microscope (Olympus Vanox-S AH-2) and a scanning electron microscope (Hitachi S-430).

DGAT Assay Using Rat Liver Microsomes

Male Wister-Imamichi or Donryu rats fed a normal diet for 1 week were killed by decapitation and livers were removed. The microsomal fractions of rat livers were prepared as described previously⁵⁾ and stored at $-80^\circ C$.

DGAT activity was measured according to the method of MAYOREK and BAR-TANA²⁾ with some modification. The reaction mixture contained 0.175 M tris-HCl (pH 8.0), 100~200 μg microsomal protein, 14.5 μM BSA, 30 μM [$1-^{14}C$]palmitoyl-CoA (0.02 μCi , Amersham), 8 mM $MgCl_2$, 2.5 mM diisopropyl fluorophosphate and 0.75 mM 1,2-dioleoyl-*sn*-glycerol (Sigma) and a test sample dissolved in 5~10 μl of 50% ethanol in a total volume of 0.2 ml. The assay was initiated by the addition of rat liver microsomal fraction. After a 15-minute incubation at $23^\circ C$ the reaction was stopped by an addition of 1.2 ml of chloroform-methanol (1:2) and lipids were extracted according to the method of FOLCH *et al.*⁶⁾. The triacylglycerol was separated by thin layer chromatography (TLC) on Silica gel 60 plates (F_{254} , Merck Co.) using a petroleum ether-diethyl ether-acetic acid (80:20:1) solvent system. The distribution of radioactivity on TLC was analyzed with a radioscanner (Radioanalytic Imaging System, AMBIS System Inc.) to determine the amount of [^{14}C]triacylglycerol.

Assay for Triacylglycerol Formation in Raji Cells

To investigate the specificity of DGAT inhibition, triacylglycerol formation was measured in an intact cell assay using Raji cells as reported previously⁷⁾. In brief, Raji cells (4×10^5 cells) were incubated in the presence of [$1-^{14}C$]oleic acid (0.02 μCi , ICN, 54.9 mCi/mmol) with or without amidepsines in 200 μl of PBS at $37^\circ C$ for 20 minutes, then [^{14}C]oleic acid incorporation into lipid fractions was calculated by the radioscanner after lipids were separated on TLC.

Antimicrobial Activity

Antimicrobial activity was tested using paper disks (i.d. 6 mm, ADVANTEC). Bacteria were grown on Müller-Hinton agar medium (Difco), and fungi and yeasts were grown on potato broth agar medium. Antimicrobial activity was observed after 24-hour incubation at $37^\circ C$ for bacteria and after 48-hour incubation at $27^\circ C$ for fungi and yeasts.

Results

Characteristics of the Producing Strain

The strain FO-2942 was isolated from a soil sample collected at Nagasaki, Japan. On potato-dextrose agar, corn meal agar, malt extract agar and yeast extract-soluble starch (YpSs) agar, this strain grew rapidly to form white to gray colonies with diameter of 60~80 mm after incubation for 14 days at $25^\circ C$. At $37^\circ C$, growth is nil. Conidia, abundantly born on the colony surface, were solitary, and were produced directly on the vegetative hyphae or on short branches (Fig. 2). They were smooth, dark brown, unicellular, thin-walled, globose to subglobose, and 10~18 μm in size. The above characteristics indicated that the strain FO-2942 belongs to the genus *Humicola*⁸⁾. The strain was deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as *Humicola* sp. FO-2942 with the accession number FERM-P 14671.

Fermentation

A slant culture of the strain FO-2942 grown on YpSs agar was used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of a seed medium (glucose 2.0%, yeast extract 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, Polypepton 0.5%, KH_2PO_4 0.1% and agar 0.1%, pH 6.0). The flasks were shaken on a rotary shaker for 4 days at $27^\circ C$. Five hundred milliliters of the seed culture was transferred into 30 liters of the production medium (sucrose 2.0%,

Fig. 2. Photomicrograph of conidia of strain FO-2942.

Bar represents 20 μm .

glucose 1.0%, corn steep liquor 1.0%, meat extract 0.5%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, trace elements containing in g/liter: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 1.0, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.0, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1.0 and $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$ 1.0 (300 ml), CaCO_3 0.3% and agar 0.1%, pH 6.0) in a 50-liter jar fermenter. The fermentation was carried out at 27°C. The production of amidepsines was measured by HPLC under the same conditions described below. A typical time course of the fermentation is shown in Fig. 3. The production of amidepsines started on day 2 and the concentration reached a maximum on day 3 after inoculation. Amidepsine B was the most abundant component in the culture broth with a maximum concentration of 6 µg/ml at day 3.

Isolation

The 96-hour old whole broth (30 liters) was extracted with 36 liters of acetone. After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with 36 liters of ethyl acetate. The extracts were dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a brownish oil (19.2 g). The material was dissolved in chloroform and applied on a silica gel column (Kieselgel 60, 1000 ml) previously equilibrated with chloroform. The materials were eluted stepwise with chloroform-methanol solutions (5000 ml, 100:0, 98:2, 95:5, and 0:100, v/v), and each 500 ml of the elution was collected. The active (11th~28th) fractions were pooled and concentrated *in vacuo* to give a brownish powder (550 mg). Then, the residue, suspended in 10% aq acetonitrile, was subjected to an ODS column (Senshu SSC-ODS-7515-12, 56 ml). The materials were eluted stepwise with aq acetonitrile solutions (300 ml, 10, 30, 50 and 70% aq CH_3CN), and each 50 ml of the elution was collected. The 8th~16th fractions were collected,

concentrated and extracted with ethyl acetate to give a brownish powder (124 mg). The resulting active powder was purified by preparative HPLC (YMC-pack D-ODS-5, 20 × 250 mm; a linear gradient from 50% CH_3CN in 0.05% H_3PO_4 to 100% CH_3CN for 40 minutes; UV at 215 nm; 6.0 ml/minute). Under this conditions, amidepsines A, B, C and D were eluted with retention times of 22.5, 24.5, 28.0 and 32.0 minutes, respectively (Fig. 4), each of which was concentrated and extracted with ethyl acetate to give amidepsines A (6.4 mg), B (6.8 mg), C (3.5 mg) and D (9.0 mg) fractions. Finally, each fraction was purified by gel filtration using Sephadex LH-20 (4 × 190 mm; methanol; 1.0 ml/fraction). Amidepsine A was recovered from the 12th to 14th fractions, amidepsins B and C were both from the eleventh to 14th fractions, and amidepsine D was from the 13th to 19th fractions. The four fractions were concentrated to give pure amidepsines A (2.6 mg), B (4.6 mg) and C (1.7 mg) as pale yellow powders and D (4.6 mg) as white powder.

Biological Properties

Effect of Amidepsines on DGAT Activity in Microsomes

As shown in Fig. 5, amidepsines inhibited DGAT activity dose-dependently in the enzyme assay. Amidepsine A showed the most potent inhibitory activity with an IC_{50} value of 10.2 µM, followed by D (IC_{50} : 17.5 µM), B (19.2 µM) and C (51.6 µM) (Table 1).

Effect of Amidepsines on Triacylglycerol Formation in Raji Cells

To confirm the DGAT inhibition, the effect of amidepsines was studied on the triacylglycerol formation

Fig. 3. A typical time course of amidepsines production by *Humicola* sp. FO-2942.

Fig. 4. A chromatographic profile of amidepsines separated by preparative HPLC.

YMC-pack D-ODS-5 (20 × 250 mm); a linear gradient 50% CH_3CN in 0.05% H_3PO_4 to 100% CH_3CN for 40 minutes; UV at 215 nm; 6.0 ml/minute.

in an intact cell assay using Raji cells according to our established method⁷⁾. Furthermore, not only triacylglycerol but phosphatidylcholine and phosphatidylethanolamine formations were measured at the same time in this cell assay, allowing an evaluation of the specificity for inhibition of triacylglycerol formation. When Raji cells (4×10^5 cells/200 μ l) were incubated in the presence of various concentrations of the drugs (0~80 μ M) at 37°C for 20 minutes, the incorporation of [14 C]oleic acid into

the triacylglycerol fraction was inhibited by all the amidepsines in a dose-dependent fashion. The IC_{50} values for triacylglycerol formation were calculated to be 15.5, 3.35, 17.2 and 2.82 μ M for amidepsines A, B, C and D, respectively (Table 1). The inhibition specificity of amidepsine B and D is shown in Fig. 6. Both showed similar inhibitory profiles against triacylglycerol formation. However, at higher concentrations amidepsine B had only slight effect on phosphatidylcholine and phosphatidylethanolamine formations, but on the other hand, amidepsine D inhibited phosphatidylcholine and phosphatidylethanolamine formations by 30~60%. These findings indicated that amidepsine B inhibits the triacylglycerol formation specifically. Amidepsines showed no cytotoxic effect on Raji cells under the assay concentrations used.

Fig. 5. DGAT inhibition by amidepsines in rat liver microsomes.

Table 1. DGAT inhibitory activity (IC_{50}) of amidepsines in assays using rat liver microsomes and Raji cells.

Compound	IC_{50} (μ M)	
	Rat liver microsomes	Raji cells
Amidepsine A	10.2	15.5
Amidepsine B	19.2	3.35
Amidepsine C	51.6	17.2
Amidepsine D	17.5	2.82

Other Biological Activities

Antimicrobial activity of amidepsines was tested at 1 mg/ml by paper disk method. Amidepsines A, B, C and D showed antimicrobial activity against *Bacillus subtilis* ATCC 6633 (diameter of inhibition zone: 11, 7, 9 and 8 mm, respectively). But no antimicrobial activity was observed against other microorganisms such as *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces sake*, *Pyricularia oryzae*, *Mucor racemosus* and *Aspergillus niger*.

Discussion

As the structure elucidation of amidepsines will be described in detail in the following paper⁴⁾, amidepsines A, B and C belong to a new type of fungal metabolite consisting of a tridepside and an amino acid. Many

Fig. 6. Effects of amidepsines on triacylglycerol (—■—), phosphatidylethanolamine (—○—) and phosphatidylcholine (—●—) formations Raji cells.

Amidepsine B

Amidepsine D

tridepsides have been isolated from lichens⁹⁾ and fungi as prostaglandin synthesis inhibitors^{10,11)}, phospholipase A2 inhibitors¹²⁾, monoamine oxidase inhibitors¹³⁾ and agents with neurogenic activity¹⁴⁾. However, amidepsines A, B and C appear to be the first tridepside linked to an amino acid. Interestingly, amidepsine D, which was identical with 2,4-di-*O*-methylgyropholic acid originally isolated from a lichen *Parmelia damaziana*³⁾, was also found from the fungus *Humicola* sp. FO-2942.

As summarized in Table 1, amidepsines inhibited DGAT activity in rat liver microsomes (Fig. 5), which was confirmed by inhibition of triacylglycerol formation in Raji cells (Fig. 6). In the cell assay, the inhibitory activity against triacylglycerol formation was similar between amidepsines B and D. Interestingly, amidepsine D showed moderate inhibitory effects on phosphatidylcholine and phosphatidylethanolamine formations but amidepsine B had only a very weak effect, suggesting that the amino acid in amidepsine B is important for specific DGAT inhibition in the cells. As described above, these tridepsides show various biological activities. Therefore, further biological characteristics of amidepsines remain to be evaluated.

Acknowledgments

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