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The Novel Gluconeogenesis Inhibitor FR225654 that Originates from *Phoma* sp. No. 00144

I. Taxonomy, Fermentation, Isolation and Physico-chemical Properties

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Abstract FR225654, a novel gluconeogenesis inhibitor, was isolated from the culture broth of *Phoma* sp. No. 00144 and purified by adsorptive resin and reverse-phase column chromatography. This compound is a potent inhibitor of gluconeogenesis and is a promising candidate of anti-diabetic agent.

Keywords gluconeogenesis, hepatocyte, diabetes, FR225654

Introduction

It is estimated that there are about 7.4 million and 135 million patients of diabetes in Japan and in the world, respectively. The populations of diabetic patients are increasing accompanying with lack of exercise and excess of diet. In diabetes, the insulin resistance and/or deficiency causes a loss of glucose utilization in skeletal muscles and adipose tissues and an acceleration of glucose production in liver [1].

Hepatic glucose production is brought by both of gluconeogenesis and glycogenolysis. The hyperproduction of glucose from liver in diabetes is based on a greater upregulation of the gluconeogenesis pathway compared with the glycogenolysis pathway [1]. Consequently, it is possible that inhibitors of gluconeogenesis are effective anti-diabetic drugs. To identify novel gluconeogenesis inhibitors, we used primary cultured rat hepatocytes to screen various microbial products for their ability to inhibit glucose production *in vitro*. During the course of this screening, we discovered a novel inhibitor named FR225654 (Fig. 1).

FR225654 originated from the cultured broth of fungal strain No. 00144, which was isolated from a decayed leaf. This compound had potent inhibitory activity of gluconeogenesis *in vitro* and hypoglycemic activities *in vivo*. In this paper, we describe the taxonomy and the fermentation of fungal strain No. 00144 and the isolation

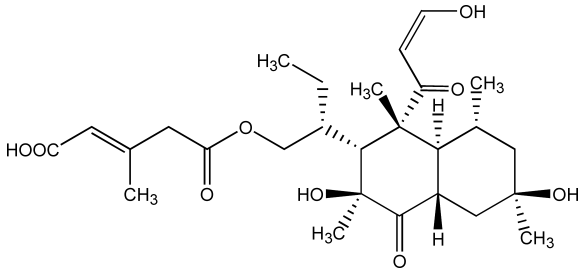

Fig. 1 Structure of FR225654.

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procedure of FR225654. We also characterized the physico-chemical properties of this compound.

Materials and Methods

Taxonomy

The FR225654-producing fungus, strain No. 00144, was originally isolated from a decayed leaf sample that collected in Yamanashi Prefecture, in Japan (Fig. 2). It was grown restrictedly on various culture media, and formed olive brown to dark green colonies. On or in the agar media, the strain produced pycnidial conidiomata. The conidiomata were globose to subglobose, brown, and formed ampulliform conidiogenous cells on their inner walls. Conidia were hyaline, one-celled and globose to subglobose. The strain did not formed teleomorph. Its mycological characteristics were described as follows. These characteristics were observed after 14 days of incubation at 25°C. The color description was based on Methuen Handbook of Colour [2].

HPLC Analysis of FR225654

FR225654 was detected in the fermentation broth and the fractions obtained after purification by HPLC using a reverse-phase column YMC Pack Pro C18 (AS302, 150×4.6 mm i.d., YMC Co., Ltd.). The solvent system was a mixture of 50% aqueous acetonitrile containing 0.05% trifluoroacetic acid. The flow rate was 1.0 ml/minute and the detection wavelength was set at 280 nm.

In Vitro Gluconeogenesis Assay

Primary cultured rat hepatocytes were isolated by the collagenase perfusion method [3] and cultured in William's E medium containing fetal bovine serum 5% (v/v) and penicillin G (100 units/ml), streptomycin (100 μg/ml) at 37°C in a humidified incubator (95% air - 5% CO_2). After 6 hours, the cells were washed with phosphate-buffered saline and incubated with 1×10^{-8} M glucagon and FR225654 in Dulbecco's Modified Eagle's Medium lacking glucose but supplemented with sodium pyruvate 20 mM, fetal bovine serum 1% (v/v), penicillin G (100 units/ml), streptomycin (100 μg/ml). After 14 hours, the glucose concentration in the medium was determined enzymatically (Glucose C-II test Wako, Wako Pure Chemical Industries, Ltd). The gluconeogenesis rate was determined to be the levels of glucose derived from pyruvate in the medium.

Cytotoxicity Test

The cytotoxicity of FR225654 was examined using EL-4 cells. After 3 days incubation with the compound, cell viability was determined colorimetrically at 550 nm and 660 nm as a reference according to the MTT method. Cytotoxicity is expressed as the lowest concentration of the compound needed to reduce EL-4 cell viability by 50%.

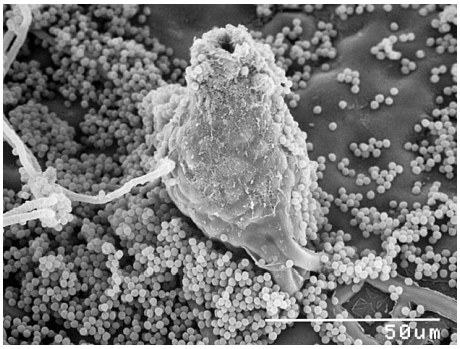

Fig. 2 Micrograph of strain No. 00144.

Results

Identification of the Producing Strain

The growth of the strain on potato dextrose agar was restrictedly, attaining 2.0~3.0 cm in diameter two weeks later at 25°C. This colony surface was plane to raised, felty to cottony, and greenish gray to dark green. Many conidiomata were formed on or in the media. The reverse color was dark gray to reddish gray, and sometimes producing reddish soluble pigments. Colonies on corn meal agar spread more restrictedly than on potato dextrose agar, attaining 1.5~2.5 cm diameter under the same conditions. The surface was plane, felty, exudates, brownish gray to olive brown at the center, and dark gray to dark green at the margin. The reverse was dark gray to dark green. Conidiomata were abundantly formed.

The morphological characteristics were determined from the cultures on a Miura's $_{LC}$A plate [4]. Conidiomata were pycnidial, superficial or immersed, separate and brown to dark brown. Their shape was globose to sybglobose, sometimes papillate, distinctly ostiolate, unilocular, and 60~90 (~110)×55~85 μm in size. Ostioles were 10~30 (~35) μm in diameter. In old culture, a few pycnidia formed 1~3 (~5) setae around the ostiole. The setae were dark brown, smooth, thick-walled, unbranched, somewhat flexuous, acute at the apex, and 14~24×4~6 μm. Pycnidial walles were thin and composed of 1~2 cells layer. The cells of pycnidial walls were thick-walled, brown, irregularly shaped, 3.5~8×2.5~6.5 μm, and formed textura angularis. The inner pycnidial walls were formed directly conidiogenous cells without conidiophores.

The conidiogenous cells were discrete, acrogenous, hyaline, smooth, ampulliform to lageniform, and 3.5～8×2.5～6 μm. The tips of conidiogenous cells were 1.5～2.5 μm wide. Conidia were enteroblastic, phialidic, hyaline, smooth, one-celled, globose to subglobose, with a small projection at the base, and 2.5～3.5×(2～) 2.5～3 μm. Vegetative hyphae were smooth, septate, brown and branched. The hyphal cells were cylindrical and 1.5～5 μm in width. Chlamydospores were not observed.

Strain No. 00144 was able to grow at the temperature range from 5～30°C. These temperature data were determined on potato dextrose agar (NISSUI Pharm. Co., Ltd., Japan).

On the basis of comparing the morphological characteristics with fungal taxonomic criteria by von Arx [5] and by Sutton [6], strain No. 00144 was considered to belong to the coelomycete genus *Phoma* Sacc. 1880 (Sphaeropsidales). Thus, we identified this isolate as one strain of genus Phoma, and named it *Phoma* sp. No. 00144. The strain has been deposited to the National Institute of Bioscience and Human-Technology, Japan, as FERM BP-6360.

Fermentation of Strain No.00144

An aqueous seed medium (30 ml) containing sucrose 4%, glucose 1%, soluble starch 2%, Pharmamedia 3%, soybean flour 1.5%, KH_2PO_4 1%, $CaCO_3$ 0.2% was placed in a 100 ml Erlenmeyer flask and was sterilized at 120°C for 30 minutes. A loopful of strain No. 00144 was inoculated in a seed flask. The inoculated flask was shaken on a rotary shaker (220 rpm, 5.1 cm-throw) at 25°C for 4 days, and 3.2 ml of the seed culture was transferred to 160 ml of the same sterile seed medium in the 500 ml Erlenmeyer flasks. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm-throw) at 25°C for 4 days, and 480 ml (three flasks) of second seed culture was inoculated to 20 liters of sterile production medium containing of glucose 1%, pinedex (malt dextrin, Matsutani Chemical Industry Co., Ltd.) 3%, wheat germ 1%, KH_2PO_4 1%, Adecanol LG-109 (defoaming agent, Asahi Denka Co.,Ltd.) 0.05%, Silicone KM-70 (defoaming agent, Shin-Etsu Chemical Co., Ltd.) 0.05% in a 30-liter jar fermentor. Fermentation was carried out at 25°C for 7 days under aeration of 20 liters/minute and agitation of 400 rpm. The amount of FR225654 in the fermentation broth reached about 90 μg/ml at 7 days.

Isolation and Purification of FR225654

The procedure used to isolate FR225654 is summarized in Fig. 3. The cultured broth (20 liters; containing 1.8 g of FR225654) was filtered with an aid of diatomaceous earth. The filtered mycelium was extracted with 20 liters of acetone by intermittent mixing for 1 hour. The acetone extract was filtered and diluted with twice volume of deionized water. The diluted filtrate was passed through a column (2 liters) of Diaion HP-20 (Mitsubishi Chemical Co., Ltd.). The column was washed with 50% aqueous methanol and eluted with 80% methanol. The eluate (4 liters) was concentrated *in vacuo* to one liter and added with 3 liters of 0.07% aqueous trifluoroacetic acid, and then applied on a column (2 liters) of YMC GEL ODS-AM 120-S-50 (YMC Co., Ltd.) packed with 25% aqueous acetonitrile containing 0.05% trifluoroacetic acid. The column was washed with 30%, 40% aqueous acetonitrile containing 0.05% trifluoroacetic acid and eluted with 50% aqueous acetonitrile containing 0.05% trifluoroacetic acid. The eluates were monitored by analytical HPLC as described in Materials and Methods.

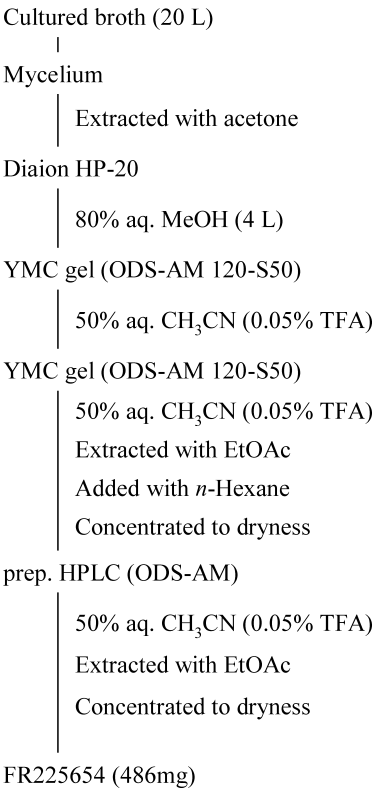

Fig. 3 Isolation Procedure of FR225654.

The fractions containing the FR225654 substance were combined and applied on a column (1 liter) of YMC-GEL (ODS-AM 120-S-50, YMC Co., Ltd.) packed with 25% aqueous acetonitrile containing 0.05% trifluoroacetic acid. The column was washed with 30%, 40% aqueous acetonitrile containing 0.05% trifluoroacetic acid and eluted with 50% aqueous acetonitrile containing 0.05% trifluoroacetic acid. The fractions containing the FR225654 substance were combined and concentrated *in vacuo* to give

Table 1 Physico-chemical properties of FR225654

	FR225654
Appearance	White powder
Molecular formula	$C_{27}H_{40}O_9$
Elementary analysis	Calcd for $C_{27}H_{40}O_9 \cdot 1/2\ H_2O$: C 62.65, H 7.98 (%)
	Found: C 62.22, H 7.97 (%)
Molecular weight	508.61
ESI-MS (negative):	*m/z* 507 $(M-H)^-$
mp.	85~89°C (dec)
$[\alpha]_D$ (23°C, chloroform)	−16°C (c=0.2)
UV λ_{max} (MeOH)	275 nm (ε=8,000)
Solubility: Soluble	CH_3CN, EtOAc, CH_3Cl, DMSO
Slightly soluble	*n*-hexane
Insoluble	water
TLC Rf[a]	0.5
HPLC[b]	7.9 min.
IR ν_{max} cm^{-1}	3480, 2980, 2930, 1730, 1710, 1650, 1620, 1460, 1380, 1160, 1140

[a] Silica Gel 60 F254 (made by E. Merck), *n*-hexane : EtOAc : AcOH=50 : 50 : 1

[b] Retention time obtained by YMC-Pack Pro C18 (AS-302, 150×4.6 mm i.d., YMC Co., Ltd.), 50% aq. CH_3CN containing 0.05% TFA, flow rate 1 ml/minute.

residual water. The residual water was twice extracted with equal volume of ethyl acetate. The extract was concentrated *in vacuo* to small volume and added with several volumes of *n*-hexane, and then concentrated *in vacuo* to give 810 mg of crude FR225654 substance as a powder.

This crude FR225654 powder was dissolved in small amount of acetonitrile and subjected to preparative HPLC, YMC-packed column (ODS-AM SH-343-5AM S-5, 250×20 mm I.D., YMC Co., Ltd.) with 50% aqueous acetonitrile containing 0.05% trifluoroacetic acid as a mobile phase and flow rate of 10 ml/minute. The portion corresponding to the purified FR225654 substance was concentrated *in vacuo* to give residual water. This residue was twice extracted with equal volume of ethyl acetate and concentrated *in vacuo* to small volume. The concentrated extract was added with several volumes of *n*-hexane and dried *in vacuo* to give 486 mg of purified FR225654 substance as a white powder.

Physico-chemical Properties of FR225654

The physico-chemical properties of FR225654 are summarized in Table 1. FR225654 is an acidic substance and readily soluble in acetonitrile, chloroform, ethyl acetate and DMSO, and insoluble in water. The Rf value of FR225654 on silica gel TLC (silica gel 60, E. Merck) developed with *n*-hexane : ethyl acetate : acetic acid (50 : 50 : 1, v/v) was 0.25.

In Vitro Gluconeogenesis Inhibitiory Activity and Cytotoxicity of FR225654

The ability of FR225654 to inhibit gluconeogenesis *in vitro* was measured using primary cultured rat hepatocytes. This compound strongly inhibited glucose production from pyruvate present in the medium (IC_{50}=0.11 μM).

The amount of FR225654 which inhibits EL-4 cell viability by 50% was 81 μM.

Discussion

Glucose is exogenously supplied from foods and endogenously produced from liver [7]. In diabetes, hepatic gluconeogenesis is constantly upregulated by insulin resistance or deficiency [1]. Therefore, we speculated that inhibitors of hepatic gluconeogenesis may be useful as antidiabetic agents and consequently screened for gluconeogenesis inhibitors using primary cultured rat hepatocytes.

Gluconeogenesis is the *de novo* glucose synthetic system from glycerol, alanine, lactate and pyruvate. Also gluconeogenesis is controlled by a large variety of regulators (*e.g.* fructose-2,6-bisphosphate, glucocorticoid, insulin, glucagon). So we used the cell-assay system employing pyruvate as gluconeogenic precursor because we aimed to screen a wide range of compounds affect to a variety of targets. Therefore, although we had already

reported novel compounds with same screening system [8], we could obtain a novel compound, FR225654, which had completely different chemical and biological profiles from that.

FR225654 has a highly oxygenated *trans*-decalin ring and a β-keto-enol in its main part, and has a characteristic side chain possessing a conjugated carboxylic acid and a tri-substituted olefin [9]. We describe in our accompanying paper for its *in vitro* properties and *in vivo* activities [10].

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