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The Novel Gluconeogenesis Inhibitors FR225659 and Related Compounds that Originate from *Helicomyces* sp. No. 19353

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

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FR225659 and four related compounds are novel gluconeogenesis inhibitors that consist of a novel acyl-group and three abnormal amino acids. They were isolated from the culture broth of *Helicomyces* sp. No. 19353 and can be purified by absorptive resin and reverse-phase column chromatography. They are potent inhibitors of gluconeogenesis in primary cultured rat hepatocytes and thus may be useful as anti-diabetic agents.

The insulin resistance or altered insulin/glucagon ratios in diabetic patients causes hepatic glucose production to accelerate[1]. This hyperproduction leads in turn to the hyperglycemia that is responsible for many of the complications seen in diabetes[2]. This situation can be seen as arising from a greater upregulation of the gluconeogenesis pathway compared to the glycogenolysis pathway[3]. Consequently, it is possible that inhibitors of gluconeogenesis could be effective anti-diabetic drugs. To identify novel gluconeogenesis inhibitors, we used primary 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 that we denoted FR225659. This compound and four of its related compounds, which also potently inhibit gluconeogenesis *in vitro*, share a very unique structure that consists of a novel acyl-group and three abnormal amino acids (Fig. 1). FR225659 originated from the cultured broth of fungal strain No. 19353, which was isolated from a decayed leaf. In this paper, we describe the taxonomy of fungal strain No. 19353, how it is fermented, and how FR225659 and its four related compounds are isolated. We also characterized the physico-chemical properties of FR225659 and its related compounds.

Materials and Methods

Taxonomy

The FR225659-producing fungus, strain No. 19353, was originally isolated from a decayed leaf sample that was collected in Fukushima Prefecture, in Japan. It was grown on various types of agar plates and the colonies were observed after 14 days of cultivation at 25°C. The compositions of malt extract agar and Czapek's solution agar were based on the JCM Catalogue of Strains[4]. The color names used in this study were taken from the Methuen Handbook of Colour[5]. The temperature range of growth was determined on potato dextrose agar. The morphological characteristics were principally determined using cultures grown on the LCA plate described by MIURA and KUDO[6].

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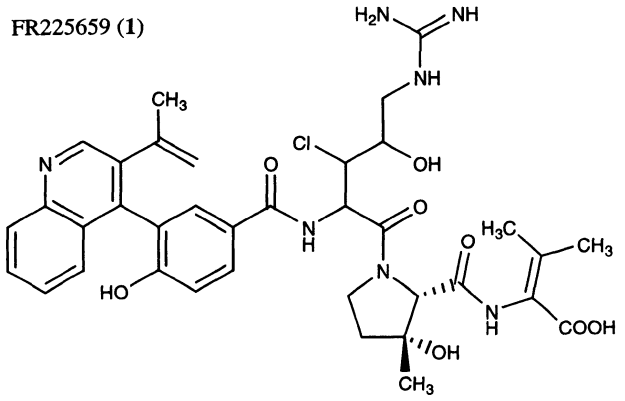
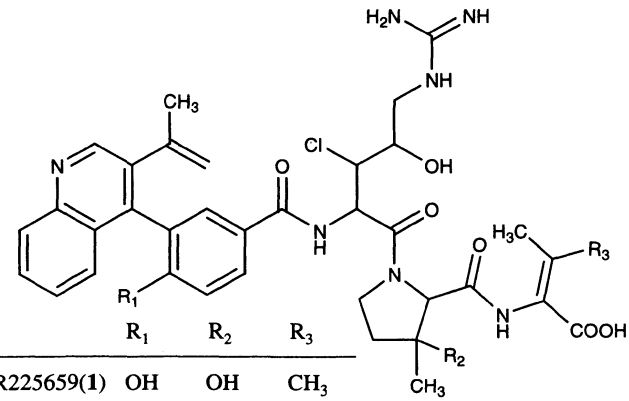
Fig. 1. Structures of FR225659 and related compounds.





	R_1	R_2	R_3
FR225659(1)	OH	OH	CH_3
2	OH	OH	CH_2CH_3
3	OH	H	CH_3
4	OCH_3	OH	CH_2CH_3
5	OCH_3	OH	CH_3

HPLC Analysis of FR225659 and Its Related Compounds

FR225659 and its related compounds were detected in the fermentation broth and the fractions obtained after purification by HPLC using a reverse-phase column YMC·Pack Pro C18 (AS303, 250×4.6 mm i.d., YMC Co., Ltd.). The solvent system was a mixture of 30% aqueous acetonitrile containing sodium-phosphate buffer 50 mM, pH 5.8 and SDS 5 mM. The column temperature was 70°C. The flow rate was 1.0 ml/minute and the detection wavelength was set at 210 nm.

Results

Identification of the Producing Strain

Cultural characteristics of strain No. 19353 on various agar media are summarized in Table 1. The growth of the strain on various culture media was restricted or very restricted and the strain tended to form dark brown or grayish colonies. Culture on potato dextrose agar grew restrictedly, attaining 1.5~2.5 cm in diameter. This colony surface was convex, felty, radiately sulcate or wrinkly, partly hygroscopic, brownish gray to grayish brown, and white to yellowish white at the margin. The reverse color was brownish gray to grayish brown, and pale gray to yellowish gray at the margin. Conidial structures were not observed. The growth rates on corn meal agar and potato dextrose agar were similar. The surface of the corn meal agar-grown colony was plane, thin, submerged, and hygroscopic, dark gray at the center, and brownish gray to olive brown at the margin. The reverse color was olive gray to olive. Some conidial structures were observed. This strain was able to grow at the temperature range from 5 to

Table 1. Culture characteristics of strain No. 19353.

Medium	Cultural characteristics
Malt extract agar	G: Restrictedly, 1.5-2.5 cm S: Circular, plane, thin, hygroscopic, formed no anamorphs, brown (6E5) to dark brown (6F5) at the center and yellowish gray (4B2) at the margin R: Olive brown (4E4-4F4) at the center and yellowish gray (4B2) to grayish yellow (4B3) at the margin
Potato dextrose agar (Difco 0013)	G: Restrictedly, 1.5-2.5 cm S: Circular, convex, felty, radiately sulcate or wrinkly, partly hygroscopic, formed no anamorph, brownish gray (6F2) to grayish brown (6F3) at the center and white to yellowish white (4A2) at the margin R: Brownish gray (5F2) to grayish brown (5F3) at the center and pale gray (1B1) to yellowish gray (4B2) at the margin
Czapek's solution agar	G: Very restrictedly, 1.0-1.5 cm S: Circular, submerged, thin, plane, formed no anamorph, yellowish gray (4B2) to grayish yellow (4C3) R: Yellowish gray (4B2) to grayish yellow (4C3)
Sabouraud dextrose agar (Difco 0190)	G: Restictedly, 1.5-2.5 cm S: Circular, raised, felty, radiately sulcate, formed no anamorphs, dark brown (6F3-6F4) at the center and white to yellowish white (4A2) at the margin R: Light brown (6D3) to dark brown (6F3) at the center and yellowish gray (4B2) at the margin
Emerson Yp Ss agar (Difco 0739)	G: Restrictedly, 1.5-2.5 cm S: Circular, plane, felty, wrinkly, hygroscopic, formed no anamorph, grayish brown (5F3) to brown (5E4) at the center and yellowish gray (4B2) or reddish brown (9E4) at the margin R: Yellowish gray (4B2) to brownish gray (4D2)
Corn meal agar (Difco 0386)	R: Restrictedly, 1.5-2.5 cm S: Circular, thin, plane, submerged, hygroscopic, formed no anamorph, dark gray (1F1) at the center and brownish gray (4F2) to olive brown (4F3) at the margin R: Olive gray (2F2) to olive (2E3)
MY20 agar	G: Very restrictedly, 1.0-1.5 cm S: Circular, raised, felty to cottony, formed no anamorph, brownish gray (6E2-7E2) R: Dark gray (1F1)
Oatmeal agar (Difco 0552)	G: Very restrictedly, 1.0-1.5 cm S: Circular, plane, felty, formed no anamorphs, blackish blue (19F5-20F5) at the center and dark brown (6F8) at the margin
Abbreviations	G: growth, measuring colony size in diameter S: colony surface R: reverse

31°C, with the growth optimum at 24 to 27°C.

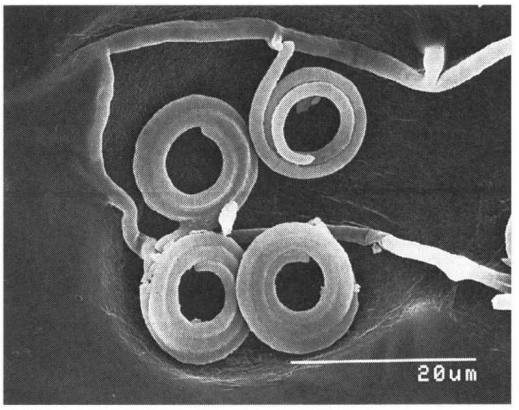
Strain No. 19353 formed helical conidia directly from vegetative hyphae (Fig. 2) but did not produce telemorphs. The conidia were borne on sessile denticles of the vegetative and aerial hyphae, or on short lateral branches. Conidiogenous cells were acrogenous or intercalary, monoblastic or polyblastic, pale brown, smooth, cylindrical, and denticulate. The denticles were 2~5×1~2 μm in size. The conidia were holoblastic, solitary, hyaline, smooth, helical, coiled 2~3 times in two dimensions, planate, and 13~18 (~25) μm in diameter. They were sometimes uncoiled and formed filamentous shapes. The conidial filaments were 5~12 septate and 1~2 μm thick. Chlamydospores were formed intercalary. They tended to be solitary but aggregated in old cultures. They were pale brown, smooth, subglobose to pyriform, and 5~9 μm in diameter. The vegetative hyphae were smooth, septate, pale brown and branched, while the hyphal cells were cylindrical and 2~4 μm in width.

We compared the above morphological characteristics with the fungal taxonomic criteria described by VON ARX[7] and GOOS[8]. As a consequence, strain No.19353 was considered to belong to the hyphomycete genus *Helicomyces* Link 1809. Thus, we identified this isolate as one strain of the genus *Helicomyces*, and named this strain as *Helicomyces* sp. No. 19353. The strain has been deposited in the International Patent Organism Depositary in the National Institute of Advanced Industrial Science and Technology, Japan, as FERM BP-6358.

Fermentation of Strain No. 19353

A loopful of strain No. 19353 was inoculated from a slant culture into 30 ml of sterilized seed medium consisting of sucrose 4%, glucose 1%, soluble starch 2%, cottonseed flour 3%, KH_2PO_4 1%, $CaCO_3$ 0.2%, Adekanol LG-109 (Asahi Denka Co., Ltd.) 0.05% and Silicone KM-70 (ShinEtsu Chemical Co., Ltd.) 0.05% and placed in a 100 ml Erlenmeyer flask. Six such flasks were prepared. The flasks were incubated at 25°C for 5 days on a rotary shaker (220 rpm, 5.1 cm-throw) and then inoculated (5%) into 160 ml of the same sterilized seed medium in each of sixteen 500 ml Erlenmyer flasks. These flasks were then incubated at 25°C for 2 days on a rotary shaker (220 rpm, 5.1 cm-throw). The resulting seed culture was inoculated (3%) into 20 liters of sterilized production medium in each of four 30-liter jar fermenters. The production medium was composed of glucose 1%, modified starch 6%, cottonseed flour 1%, soybean flour 1%, KH_2PO_4 1.6%, $Na_2HPO_4 \cdot 12H_2O$ 1.2%, Adekanol LG-109 0.05%, and Silicone KM-70 0.05%. The fermentation was carried out at 25°C for 5 days under aeration of 20 liters/minute and agitation of 250 rpm. The amount of FR225659 in the fermentation broth reached about 50 μg/ml at 5 days.

Fig. 2. Micrograph of the conidial structures of strain No. 19353 (on the LCA plate, 25°C, 14 days).

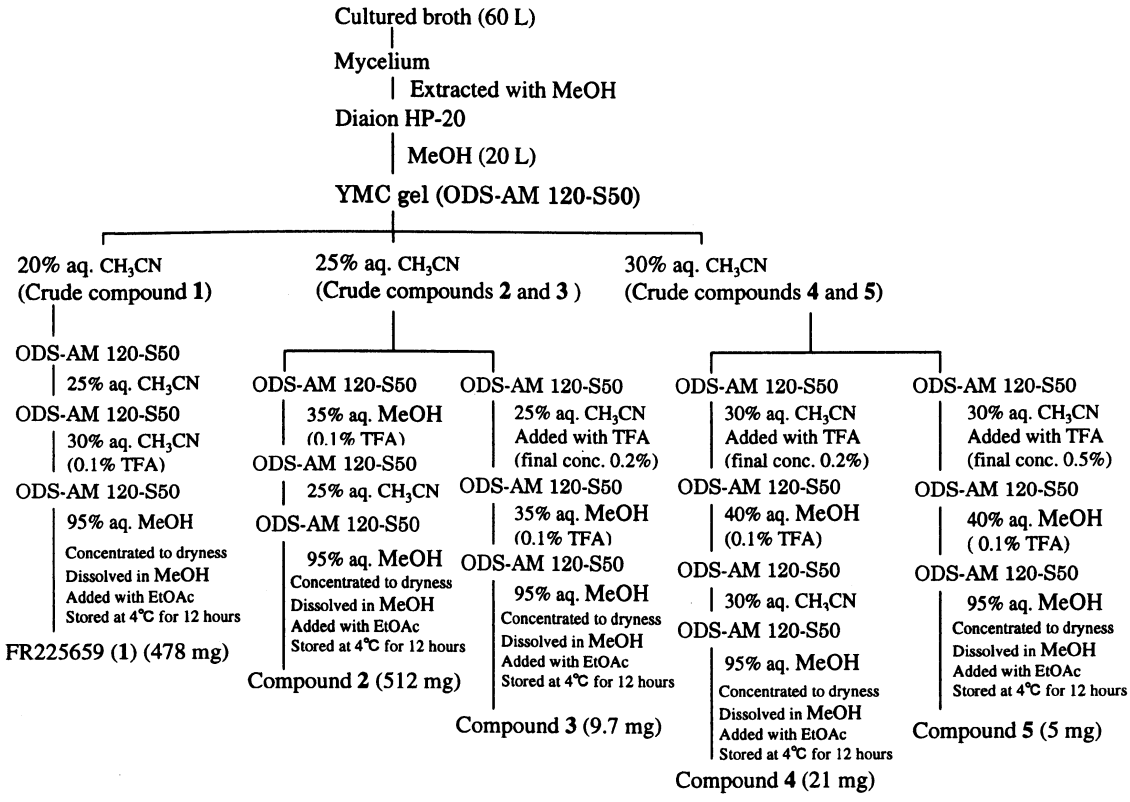


Isolation and Purification of FR225659 and Related Compounds

The procedures used to isolate FR225659 and its related compounds are summarized in Fig. 3. After the cultivation was completed, the culture broth (60 liters) was filtered with the aid of diatomaceous earth. Fifty liters of methanol was then added to the mycelium and the mixture was allowed to stand at room temperature for about 2 hours with stirring. The resulting mixture was again filtered with the aid of diatomaceous earth and the filtrate was diluted with an equal volume of water and passed through a column (5 liters) of DIAION HP20 (Mitsubishi Chemical Co., Ltd.) packed with water. The column was washed with 50% aqueous methanol (15 liters) and eluted with methanol (20 liters). The eluate was concentrated *in vacuo* to an aqueous solution and 3 liters of water was added to the solution. This solution was passed through a column (2 liters) of YMC-GEL (ODS-AM120-S50, YMC Co., Ltd.) packed with water. The column was serially eluted with 20% (13 liters), 25% (7.5 liters) and 30% aqueous acetonitrile (5.5 liters). The eluates were monitored by analytical HPLC as described in Materials and Methods.

The 20% aqueous acetonitrile fractions containing FR225659 were combined and diluted with an equal volume of water and passed through a column (2 liters) of

Fig. 3. Isolation procedure of FR225659 (**1**) and related compounds.



YMC-GEL packed with water. The column was eluted with 25% aqueous acetonitrile (8 liters). The fraction containing FR225659 was diluted with an equal volume of water and passed through a column (1 liter) of YMC-GEL packed with water. The column was washed with water and eluted with 30% aqueous acetonitrile containing 0.1% trifluoroacetic acid. The fraction containing FR225659 was diluted with an equal volume of water and passed through a column (180 ml) of YMC-GEL packed with water. The column was washed with water and eluted with 95% aqueous methanol. The eluate was concentrated *in vacuo* to dryness and dissolved in a small amount of methanol. About twice this amount of ethyl acetate was added to the solution and the mixture was allowed to stand at 4°C for about 12 hours. The FR225659 substance was obtained as a white powder (478 mg).

Compounds **2** and **3** were present in the 25% aqueous acetonitrile eluates at the first ODS column chromatographic step, while compounds **4** and **5** were present in the 30% aqueous acetonitrile eluates. These four compounds were further purified by ODS column chromatography as shown in Figure 3.

Physico-chemical Properties of FR225659 and Related Compounds

The physico-chemical properties of FR225659 and related compounds are summarized in Table 2. All compounds except for **3** are readily soluble in methanol, acetic acid and DMSO, and insoluble in *n*-hexane, ethyl acetate and water. Compound **3** showed poor solubility in methanol and DMSO. The IR spectra exhibited characteristic signals from an amide (1630 cm^{-1}) and a C–Cl bond (770 cm^{-1}) while the UV absorption at 233 nm suggested the presence of the quinoline chromophore. The details of the structural elucidation of these compounds will be described accompanying paper[10].

Discussion

In animals, glucose is mainly produced by the liver[10]. In diabetes mellitus, hepatic gluconeogenesis is constantly upregulated[2], resulting in the high glucose concentrations that cause many of the complications of this disease.

Table 2. Physico-chemical properties of FR225659 and related compounds.

	FR225659 (1)	2	3	4	5
Appearance	White powder	White powder	White powder	White powder	White powder
Molecular formula	$C_{36}H_{42}ClN_7O_8$	$C_{37}H_{44}ClN_7O_8$	$C_{36}H_{42}ClN_7O_7$	$C_{38}H_{46}ClN_7O_7$	$C_{37}H_{44}ClN_7O_8$
Molecular weight	736	750	720	764	750
ESI-MS (*m/z*)	734 $(M-H)^-$	748 $(M-H)^-$	718 $(M-H)^-$	762 $(M-H)^-$	748 $(M-H)^-$
	736 $(M+H)^+$	750 $(M+H)^+$	720 $(M+H)^+$	764 $(M+H)^+$	750 $(M+H)^+$
HRESI-MS (*m/z*)					
Found	736.2861	750.3033	720.2932	764.3158	750.3022
Calc. For M+H	736.2862	750.3018	720.2912	764.3175	750.3018
m.p.	225-230℃ (dec.)	230℃ (dec.)	225-230℃ (dec.)	210-215℃ (dec.)	230-235℃ (dec.)
[α]D (23℃, DMSO)	+40° (*c* 0.5)	+37° (*c* 0.5)	+22° (*c* 0.06)	+31° (*c* 0.3)	+35° (*c* 0.1)
UV λ max (nm, MeOH)	233	232	232	232	228
ε	54000	63000	47000	49000	43000
Solubility					
Soluble	MeOH, AcOH, DMSO	MeOH, AcOH, DMSO	AcOH	MeOH, AcOH, DMSO	MeOH, AcOH, DMSO
Slightly soluble			DMSO, MeOH		
Insoluble	EtOAc, n-hexane	EtOAc, n-hexane	EtOAc, n-hexane	EtOAc, n-hexane	EtOAc, n-hexane
TLC Rf [a]	0.5	0.6	0.6	0.6	0.6
HPLC [b]	5.0	5.6	8.0	13.9	12.0
IR ν_{max} (cm^{-1}, KBr)	3390, 2980, 2940, 1630, 1540, 1500, 1440, 1380, 1280, 1200, 1100, 1040, 1010, 770	3400, 2940, 1650, 1645, 1635, 1540, 1500, 1440, 1380, 1280, 1200, 1160, 1140, 910, 770	3350, 2950, 1640, 1540, 1500, 1450, 1290, 1110, 910, 770	3370, 2970, 1640, 1540, 1490, 1440, 1270, 1020, 770	3370, 2970, 1640, 1490, 1440, 1270, 1200, 1140, 1020, 770

[a] Silica Gel 60 F_{254} (made by E. Merck), n-butanol : acetic acid : water = 4 : 1 : 2
[b] Retention time obtained by YMC Pack Pro C18 (AS303, 250 x 4.6 mm i.d., YMC Co., Ltd.), 30% CH_3CN-50 mM phosphate buffer (pH 5.8)-5 mM SDS, flow rate 1ml/min.

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. There are two reasons we chose this *in vitro* assay system for screening. First, the cell-based assay allows us to screen a wide range of compounds for their activity on unknown targets. In this case, the unknown targets are molecules that participate in the gluconeogenetic pathway. Second, the primary cultured hepatocytes used in the *in vitro* assay system here resemble the liver in the living body better than other established hepatocyte cell lines, as nearly all of these cell lines show either a decrease or a complete loss in original functions such as drug metabolism or glucose production. In contrast, primary cultured hepatocytes show good maintenance of liver functions, which suggests that the compounds identified by this cell system to be effective gluconeogenesis inhibitors *in vitro* are likely to affect this function *in vivo* as well. We found that all four molecules potently inhibit glucose production by cultured primary rat hepatocytes. For example, the 50% inhibitory concentration (IC_{50}) of FR225659 was 0.19 μM. Thus, FR225659 and its four related compounds are novel acyl-tripeptides produced by the *Helicomyces* sp. No. 19353 fungal strain that can act as gluconeogenesis inhibitors *in vitro*. We describe in our accompanying paper whether FR225659 can also affect gluconeogenesis *in vitro* and *in vivo*[11].

References

1) PARRILLA, M.; M. N. GOODMAN & C. J. TOEWS: Effect of glucagon: insulin ratios on hepatic metabolism. Diabetes 23: 725~731, 1974
2) ROSSETTI, R. A.; A. GIACCARI & R. A. DEFRONZO: Glucose toxicity. Diabetes Care 13(6): 610~630, 1990
3) CONSOLI, A.: Predominant role of gluconeogenesis in increased hepatic glucose production in NIDDM. Diabetes Care 15(3): 430~441, 1992
4) NAKASE, T.: JCM Catalogue of Strains, 6th edition. Japan Collection of Microorganisms, the Institute of Physical and Chemical Research (RIKEN), Toppan, 1995
5) KORNERUP, A. & J. H. WANSCHER: Methuen Handbook of Colour, 3rd edition. Methuen, London, 1978

6) MIURA, K. & M. KUDO: An agar-medium for aquatic Hyphomycetes. Trans. Mycol. Soc. Japan 11: 116~118, 1970
7) ARX, J. A. VON: The Genera of Fungi-Sporulating in Pure Culture, 3rd ed., J. Cramer, Vaduz, 1974
8) GOOS, R. D.: Fungi with a twist: The helicosporous Hyphomycetes. Mycologia 79: 1~22, 1987
9) ZENKOH, T.; Y. OHTSU, S. YOSHIMURA, N. SHIGEMATSU, S. TAKASE & M. HINO: The novel gluconeogenesis inhibitors FR225659 and FR225656 from *Helicomyces* sp. No. 19353. III. Structure determination. J. Antibiotics 56: 694~699, 2003
10) OWEN, O. E.; P. FELIG, A. P. MORGAN, J. WAHREN & G. F. CAHILL Jr.: Liver and kidney metabolism during prolonged starvation. J. Clin. Invest. 48: 574~583, 1969
11) OHTSU, Y.; H. SASAMURA, T. SHIBATA, H. NAKAJIMA, M. HINO & T. FUJII: The novel gluconeogenesis inhibitors FR225659 and its related compounds that originate from *Helicomyces* sp. No. 19353. II. Biological profiles. J. Antibiotics 56: 689~693, 2003