A NOVEL HEPATOPROTECTIVE γ-LACTONE, MH-031

I. DISCOVERY, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND STRUCTURAL ELUCIDATION

YUMIKO ITOH, HIROSHI SHIMURA, MAYUMI ITO, NAOHARU WATANABE†, MICHIO YAMAGISHI, MASAHARU TAMAI and KAZUNORI HANADA

Research Center, Taisho Pharmaceutical Co., Ltd.,
1-403 Yoshino-cho, Omiya-shi, Saitama 330, Japan

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A new subspecies of *Streptomyces rishiriensis* A-5969 (FERM BP-1394) was isolated and shown to produce a novel α,β-unsaturated γ-lactone derivative, MH-031, which exhibited hepatoprotective activity in primary cultured rat hepatocytes intoxicated with D-galactosamine.

During the process of screening for novel hepatoprotective compounds, a strain of *Streptomyces rishiriensis* A-5969, isolated from a soil sample collected in Maebashi-shi, Gunma Prefecture, Japan, was found to produce a new compound, MH-031. The chemical structure of MH-031 was elucidated on the basis of spectroscopic evidence as shown in Fig. 1.

In this paper we describe the taxonomy of the producing strain, the fermentation, the isolation procedures, the physico-chemical properties and structural elucidation of MH-031.

Materials and Methods

Taxonomic Study

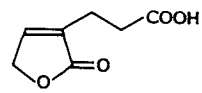
The methods described by SHIRLING and GOTTLIEB[1] were principally employed for this taxonomic study. Morphological observation was made using both light and electron microscopes of cultures grown at 30°C for 14 days on inorganic salts-starch agar, yeast extract-malt extract agar, oatmeal agar, glucose-asparagine agar, glycerol-asparagine agar, sucrose-nitrate agar and tyrosine agar. Cell wall analysis was performed by the methods of BECKER *et al.*,[2] and YAMAGUCHI.[3] The temperature range for growth was determined on yeast extract-malt extract agar using a temperature gradient incubator model TN-3 (Toyo Kagaku Sangyo Co., Ltd., Japan).

Utilization of the carbon sources was examined according to the method of PRIDHAM and GOTTLIEB.[4] The results were determined after 14 days incubation at 30°C.

Fermentation

A sterilized culture medium (100 ml) containing glucose 2%, oatmeal 2%, meat extract 0.3%, NaCl 0.3%, calcium carbonate 0.3%, ferric sulfate 0.04% and manganese chloride 0.04% at pH 7.0 was used. The medium in a 500-ml Erlenmeyer flask was inoculated with a loopful of a slant culture of *S. rishiriensis* A-5969 and incubated at 30°C for 96 hours on a rotary shaker. The culture broth (600 ml) was inoculated into 30 liters of the same medium in a 50-liter jar fermenter. This was cultured at 30°C

Fig. 1. Structure of MH-031.



† Present address: Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422, Japan.

for 65 hours with aeration of 30 liters/minute and agitation at 300 rpm.

Assay of Antihepatotoxic Activity (*In Vitro*)

Antihepatotoxic activity in primary cultured rat hepatocytes was assayed using D-galactosamine (GalN) as a toxin, as previously described,[5] with some modifications. Rat hepatocytes isolated by the perfusion method[6] were incubated for 1.5 hours in WILLIAMS' medium E containing 10% calf serum, 10^{-6} M dexamethasone, 10^{-8} M insulin and antibiotics at a concentration of 5×10^5 cells/ml/4 cm^2 under 5% CO_2-95% air at 37°C in a humidified incubator. Then the medium was changed to 0.5 ml of the medium supplemented with 0.5 mM GalN and a DMSO solution (0.01 ml/ml medium) of the crude materials or test compounds. After 20 hours of incubation, glutamic-pyruvic transaminase activity (GPT) in the medium was measured with a Hitachi 712 automatic analyzer.

Instrumental Analyses

IR and UV spectra were recorded on a Perkin-Elmer 1760 FT-IR and Hitachi 220A spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were taken using a Jeol JNM-GX400 FT NMR spectrometer at 400 MHz and 100 MHz, respectively. Chemical shifts were converted to δ values in ppm downfield from TMS as an internal standard. FD-MS and SI-MS were measured on Jeol DX-303 GC-MS and Hitachi M-80A GC-MS spectrometers, respectively.

Results

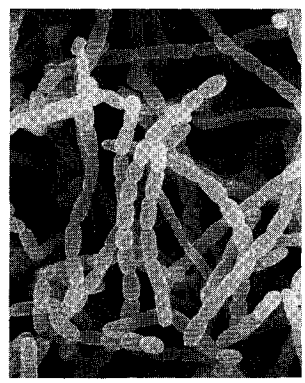
Taxonomy of Strain A-5969

The producing strain A-5969 was isolated from a soil sample from Maebashi-shi, Gunma Prefecture, Japan. The mature spores developed in chains of more than 10 spores forming spirals or sometimes retinaculiaperti. The spores were determined by electron microscopy to be ellipsoidal and measured $0.45 \sim 0.55 \times 1.01 \sim 1.06$ μm in size. Spore surfaces were smooth (Fig. 2). Neither fragmentation of hyphae nor formation of spores occured in the substrate mycelium. Sporangia, sclerotia and flagella spores were not observed.

Analysis of whole cell hydrolysates showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain is classified as type I. These results suggest that strain A-5969 belongs to the genus *Streptomyces*.

A summary of the physiological properties of strain A-5969 were shown in Table 1. The temperature range for growth was from 12 to 38°C, with an optimum temperature of from 27 to 30°C. Hydrolysis of starch and milk peptonization were positive. Gelatin liquefaction was negative. Production of melanoid pigment was observed on tyrosine agar. As shown in Table 2, this strain efficiently utilized D-glucose, D-xylose, raffinose and L-arabinose, but inefficiently utilized sucrose, D-fructose, L-rhamnose and inositol. Mannitol was not ulilized.

Fig. 2. Electron micrograph of spore chains of strain *Streptomyces rishiriensis* A-5969.



Based on taxonomic studies of strain A-5969, it was concluded to classify it as *S. rishiriensis* which is described in BERGEY's Manual and International Streptomyces Project (ISP).[7~9] The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology,

Table 1. Physiological properties of *Streptomyces rishiriensis* A-5969.

Conditions	Characteristics
Temperature range for growth	12~38°C
Optimum temperature for growth	27~30°C
Starch hydrolysis	Positive
Milk coagulation	Negative
Milk peptonization	Positive (slow)
Production of melanoid pigment	Positive
Gelatin liquefaction	Negative

Table 2. Carbon utilization of *Streptomyces rishiriensis* A-5969.

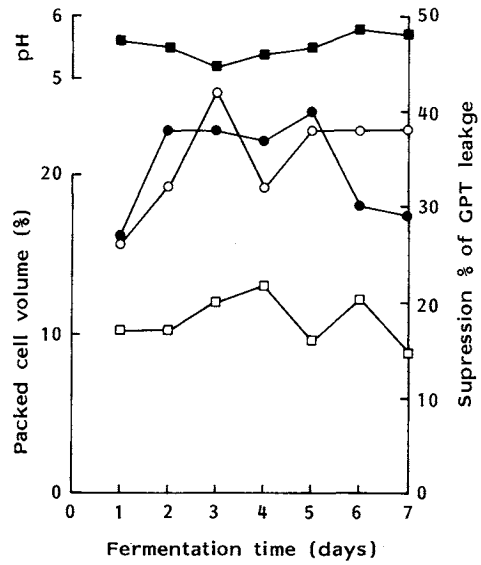
Compounds	Growth	Compounds	Growth
D-Glucose	+	Raffinose	+
Sucrose	±	L-Arabinose	+
D-Xylose	+	Inositol	±
D-Fructose	±	Mannitol	−
L-Rhamnose	±		

+: Utilization, ±: poor utilization, −: unutilized.

Japan, under acess No. FERM BP-1394.

Fig. 3. Time course of MH-031 production.

□ Packed mycelium volume, ■ pH of culture broth, ○ hepatoprotective activity (supression % of GPT leakage) in mycelial extract, ● hepatoprotective activity in culture broth.



Fermentation

The time course of the production of MH-031 by *S. rishiriensis* A-5969 in a 50-liter jar fermenter is shown in Fig. 3. The organism reached a stationary phase of growth after 96-hour of incubation. The production of antihepatotoxic compounds began at 24 hours after inoculation, and maximum accumulation was observed after a 72-hour incubation period.

Isolation Procedure

A flow diagram of the isolation procedure described below is shown in Fig. 4. The culture broth (25 liters) was filtered through diatomaceous earth, and the mycelial cake was extracted with 80% aqueous acetone. After removal of the acetone, the aqueous solution was combined with the broth filtrate. It was passed through a column (column bed volume: Vt=3,000 ml) of Diaion HP-20 (Mitsubishi Chemical Ind. Ltd., Japan). After being washed with water and 50% aqueous methanol, the active components were eluted with methanol. After removal of methanol *in vacuo*, the concentrated aqueous solution, was extracted with ethyl acetate at pH 4 (3 times). The organic layer was evaporated *in vacuo* to give crude antihepatotoxic materials (16.3 g). It was chromatographed on a column (Vt=850 ml) of silica gel (Wakogel C-200, Japan) packed with chloroform and developed with the solvent system of chloroform-methanol (stepwisely increasing the latter from 99:1 to 80:20). Fractions containing MH-031 were collected and concentrated *in vacuo* to afford a brownish material (1.1 g). For further purification, it was applied to a TLC plate (Merck Kieselgel 60 F_{254} with a concentration zone: 20×20 cm), which was developed with a solvent mixture of chloroform-methanol-acetic acid (10:0.5:0.1). Visualized bands resulting from exposure to iodine vapor were scraped off and fractions were extracted with ethyl acetate. A fraction at Rf 0.14~0.25 which showed positive reaction to BCG reagent and antihepatotoxic activity was concentrated *in vacuo* giving a purified material. Crystallization from a mixture of ethyl acetate and *n*-hexane gave MH-031 as

Fig. 4. Isolation procedure of MH-031.

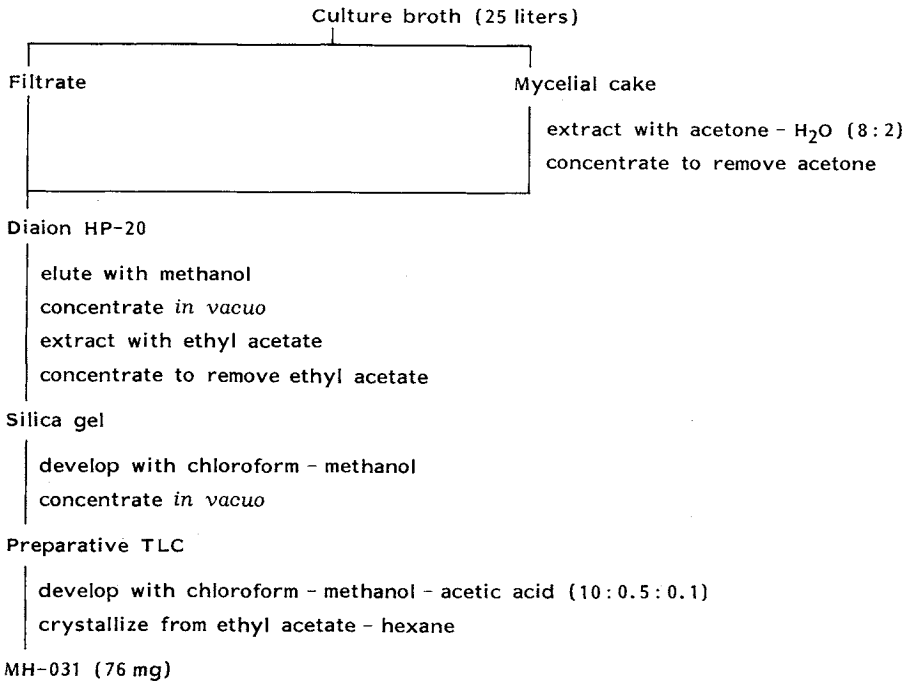


Table 3. Physico-chemical properties of MH-031.

Appearance	Colorless needles	Solubility	Soluble in MeOH, Me_2CO, EtOAc, water
MP	119~120°C		Slightly soluble in $CHCl_3$, benzene
Molecular formula	$C_7H_8O_4$		Insoluble in *n*-hexane
Mass spectrum	SI-MS *m/z* 157 $(M+H)^+$	^1H NMR (400 MHz, CD_3OD used TMS as an internal standard) δ	2.56 (2H, m), 2.60 (2H, m), 4.82 (2H, dt, J=1.8, 1.6 Hz), 7.38 (1H, tt, J=1.8, 1.0 Hz)
	FD-MS *m/z* 157 $(M+H)^+$		
Elemental analysis			
Calcd for:	C 53.65, H 5.03		
Found:	C 53.76, H 5.07		
UV λ_{max}^{MeOH} nm (ε)	210 (9,790)		
IR ν_{max} (KBr) cm^{-1}	3400, 3200, 1750, 1715, 1260, 1210	^{13}C NMR (100 MHz, CD_3OD used TMS as an internal standard) δ	21.8 (t), 32.5 (t), 72.2 (t), 133.1 (s), 148.4 (d), 176.0 (s), 176.6 (s)

colorless needles (76 mg).

Structural Elucidation

The physico-chemical properties of MH-031 are summarized in Table 3. Color reactions are as follows: positive to BCG, phosphorous molybdate and iodine vapor reagents; and negative to ninhydrin and ferric chloride reagents. The Rf value of MH-031 on silica gel TLC developed with a mixture of chloroform - methanol - acetic acid (10 : 0.5 : 0.1) was 0.19.

The molecular formula of MH-031 was established on the basis of the mass spectral data and elemental analysis to be $C_7H_8O_4$. Absorption bands at 1750 and 1715 cm^{-1} in the IR spectrum, positive color reaction to BCG reagent, UV absorption maximum at 210 nm and ^{13}C signals at 176.0 and 176.6 ppm suggested the presence of a carboxyl group and an α,β-unsaturated γ-lactone. The ^1H and ^{13}C NMR

Table 4. ^{13}C NMR chemical shift (ppm) of MH-031 in CD_3OD and in CD_3OH.

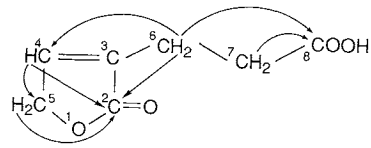
Carbon	CD_3OD	CD_3OH	$\Delta\delta$
2 C=O	176.55	176.53	0.02
3 =C	133.14	133.15	−0.01
4 =CH	148.35	148.32	0.03
5 CH_2O	72.17	72.16	0.01
6 CH_2	21.80	21.83	−0.03
7 CH_2	32.50	32.61	−0.11
8 C=O	176.02	176.18	−0.16

Table 5. Summary of LSPD data of MH-031.

Carbon	Irradiated ^1H	Change in $^{2,3}J$
2 C=O	5,6-H	br d→sharp d
2 C=O	4-H	br d→sharp dt
2 C=O	3-H	br d→br s
4 =CH	5,6-H	m →br t
5 CH_2O	3-H	br d→s
6 CH_2	3-H	m →sharp t
8 C=O	5,6-H	m →s

spectra and decoupling experiments showed the existence of $-CH_2-CH_2-$ (6-H: 2.56, 7-H: 2.60, C-6: 21.8, C-7: 32.5 ppm), $>C=CH-$ (4-H: 7.38, C-3: 133.1, C-4: 148.4 ppm), $-CH_2-O$ (5-H: 4.82, C-5: 72.2 ppm), and two carbonyl groups (C-2: 176.6, C-8; 176.0 ppm) in the molecule. The observation of $-CH=$ signals (4H, C-4) at lower fields in the NMR spectra suggested that this proton is attached to the β-carbon of the double bond to a lactonic carbonyl group. These chemical shift assignments were supported by the data of *iso*-shiphonodin which has 3-substituted-2-(5*H*)-furanone, and by its geometrical isomer shiphonodin.[10] In order to confirm the position of a carboxyl function, the following experiments were carried out: In the ^{13}C NMR spectra of MH-031 taken in each equimolar solution of CD_3OD and CD_3OH, the down field shifts in the latter solution were observed at a carbonyl carbon (C-8: 176.18 ppm) and a methylene carbon (C-7: 32.61 ppm), as shown in Table 4. Thus, the existence of a partial structure of CH_2CH_2COOH was confirmed. The proposed structure of MH-031 (Fig. 1) can be deduced from the connection of an α,β-unsaturated lactone, and the partial structure containing carboxyl moiety. The structure was confirmed by long-range selective proton decoupling (LSPD) experiments as shown in Table 5 and Fig. 5. The results of ^1H-^1H and ^{13}C-^1H COSY analyses were in good agreement with the full assignments of the protons and carbons in the molecule.

Fig. 5. Assignment of LSPD experiments.



Biological Properties of MH-031

MH-031 in doses on 3~30 μg/ml had dose-related ameliorative effects on the release of GPT against GalN-induced cytotoxicity in primary cultured rat hepatocytes. At a dose concentration of 30 μg/ml, the release of GPT was inhibited by 27%.

MH-031 did not show any antimicrobial activity against bacteria, fungi or yeasts tested by the conventional paper disc method on agar plates at a concentration of 2 mg/ml.

Discussion

A number of hepatoprotective compounds from the plant kingdom have been reported.[5,11~16] Some of these compounds, such as glycyrrhizin, gomisin and silymarin, are clinically used for the treatment of chronic hepatitis. However, there has been no report on hepatoprotective compounds of microbial origin.

As a result of our screening for antihepatotoxic compounds from microbial metabolites, MH-031 has been isolated from a fermentation broth of *S. rishiriensis* A-5959. MH-031 belongs to a family of α,β-unsaturated γ-lactones. In natural products, the lactone is often found in cardioglycosides and many

microbial products, the majority being 4-substituted-2-(5A)- or 3,4-disubstituted furanones. For example, scobinolide,[17,18] a 4-substituted butenolide, and seiridin and isoseiridin,[19] 3,4-substituted butenolides have been reported as microbial products a having simple structure similar to MH-031.

On the other hand, the reports on 3-substituted-2-(5)-furanones are not so numerous. Isosiphonodin[10] isolated from small ermine moth has the structure of 3-hydroxymethyl-2-(5A)-furanone. None of the biological properties of isosiphonodin have been reported though siphonodin, a geometrical isomer of isosiphonodin[20] is reported to possess cytotoxic activity. Among the compounds belonging to the family of α,β-unsaturated γ-lactone, MH-031 is the first compound showing hepatoprotective activity to be reported. It is suggested that the agent which has 3-substituted-2-(5A)-furanone moiety in the molecule may show such activity. Chemical synthesis of the furanone family will be necessary for further evaluation of anti-hepatotoxicity and other biological properties.

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