HS071, A New Furan-type Cytotoxic Metabolite from *Streptomyces* sp. HS-HY-071

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Abstract Investigation of the bioactive metabolites from the soil-derived actinomycete, *Streptomyces* sp. HS-HY-071, a new furan-type antibiotic, HS071 (**1**), was isolated. Its structure was established as methyl 4-hydroxymethyl-2-pentylfuran-3-carboxylate on the basis of extensive 1D and 2D NMR and MS spectral analysis. It exhibited *in vitro* activity against HCT-116 cell with an IC_{50} of 18.2 μ g/ml.

Keywords *Streptomyces* sp. HS-HY-071, HS071, cytotoxic

Introduction

In order to search for new natural antitumor bioactive metabolites, we have screened thousands of microbial strains isolated from the soil samples collected from Xishuangbanna in Yunnan Province. Among them, 257 strains were found having good activities against some lines of tumor cell. In the further chemical study of a strain HS-HY-071, a new furan-type cytotoxic antibiotic, HS071 (**1**) was isolated. This paper reports the basic taxonomy and fermentation of the strain HS-HY-071 and the isolation, structure elucidation and biological property of **1**.

Materials and Methods

Microorganism

The producing organism designated HS-HY-071 was isolated from a soil sample collected at Xishuangbanna, Yunnan, China. Strain HS-HY-071 was deposited in the Pharmaceutical Research Culture Collection, Zhejiang Hisun Group Co., Ltd., with the name of *Streptomyces* sp. HS-HY-071 under accession No: HSAS071.

Taxonomy

The International *Streptomyces* Project (ISP) media recommended by Shirling and Gottlieb [1] were used to investigate the culture and physiological characteristics. Cultures were routinely observed after the incubation for two weeks at 28°C. All carbon-sources for carbon-utilization tests were filter-sterilized and tested at the concentrations recommended by Shirling and Gottlieb [1] and Williams *et al.* [2]. Cell wall analysis was performed by the method of Hasegawa and Takizawa [3]. Extraction of genomic DNA and 16S rDNA gene amplification were carried out according to Pitcher *et al.* [4] 16S rDNA sequences were compared with those of other bacterial 16S rDNA sequences available in the GenBank database.

Fermentation

Strain HS-HY-071 was inoculated into a 500-ml flask containing 100 ml of seed culture medium consisting of (%) soluble starch 1.5, glucose 0.5, peptone 0.5, yeast extract 0.5, NaCl 0.05, $MgSO_4 \cdot 7H_2O$ 0.05, $CaCO_3$ 0.1, pH 7.2. Incubation was carried out at 28°C for 3 days on a rotary shaker operating at 250 rpm. Then a 7.0% of seed culture broth was cultured in a 20-liter of fermentation at 28°C for 6 days, the medium is consisted of (%) soluble starch 4.0, glucose 0.5, soybean meal 2.5, yeast extract 0.5,

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Table 1 Cultural characteristics of strain HS-HY-071

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Tryptone-yeast extract agar (ISP-1)	Very good	Light gray	Blue grey	None
Yeast extract-malt agar (ISP-2)	Very good	Purple black	Purple black	None
Oatmeal agar (ISP-3)	Very good	White	Light brown	None
Inorganic salt-starch agar(ISP-4)	Good	Light yellow	Pale yellow	None
Glycerol-asparagine agar (ISP-5)	Good	Pale yellow	Yellow green	None
YMS	Very good	Light gray	Black brown	None

NaCl 0.05, MgSO₄·7H₂O 0.05, CaCO₃ 0.1, pH 7.2.

Isolation and Physico-chemical Characterization

After fermentation for 6 days, the cultured broth was centrifugated to separate mycelial cake and supernatant. The mycelial cake was extracted with MeOH and the supernatant was extracted with Diaion HP-20 and eluted with 95% EtOH. The combined methanol soluble and the EtOH eluates were evaporated to give the crude extract. Fractionations of the crude extract by silica gel column chromatography, Sephadex LH-20 and reversed-phase HPLC successively gave compound **1**.

General

UV spectra was obtained on a Varian CARY 300 BIO spectrophotometer; IR spectra was recorded on a Nicolet Magna FT-IR 750 spectrometer ($\nu_{\max}$ in cm⁻¹); ¹H- and ¹³C-NMR spectra were measured with a Bruker DRX-400 (400 MHz for ¹H and 100 MHz for ¹³C) spectrometer. Chemical shifts are reported in parts per million (δ), using residual CHCl₃ (δ_{H} 7.26 ppm) and CDCl₃ (δ_{C} 77.0 ppm) as internal standards, with coupling constants (J) in Hz. ¹H- and ¹³C-NMR assignments were supported by ¹H-¹H COSY, HMQC and HMBC experiments. The ESI-MS and HRESI-MS spectra were taken on a Q-TOF Micro LC-MS-MS mass spectrometer. RP-HPLC was conducted on an Agilent 1100 series. Commercial silica gel (Qing Dao Hai Yang Chemical Group Co., 100~200 and 200~300 mesh) was used for column chromatography.

Biological Assays

Human colon carcinoma cell line HCT-116 was routinely cultured in DMEM containing 10% calf serum at 37°C for 4 hours, in a humidified atmosphere of 5% CO₂ incubator. The adherent cells at their logarithmic growth stage were digested, and were inoculated onto 96-well culture plate at a density of 1.0×10⁴/well for the determination of proliferation. Test samples were added to the medium, and incubation was continued for 72 hours. Coloration

substrate, cell counting kit-8 (CKK-8, Dojindo), was added to the medium followed by further incubation for 3 hours. Absorbance at 450 nm with a 600 nm reference was measured thereafter. Media and DMSO control wells, in which compound was absent, were included in all the experiments in order to eliminated the influence of DMSO. The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition (\%)} = [\text{OD}_{\text{control}} - \text{OD}_{\text{treated}}] / \text{OD}_{\text{control}} \times 100$$

The cytotoxicity of compound on tumor cells was expressed as IC₅₀ values (the drug concentration reducing by 50% the absorbance in treated cells, with respect to untreated cells) and was calculated by LOGIT method.

Results and Discussion

Taxonomy of the Producing Strain

The strain HS-HY-071 is a Gram-positive actinomycete which formed well-developed and branching substrate mycelium and aerial mycelium, but fragmentation of the substrate mycelium was not observed. Good growth was observed on ISP-1, ISP-2, ISP-3 and YMS. The best medium for the culture of this strain was ISP-2, on which it grew abundantly (Table 1). The long spore chains were of the *Spirales*-type with smooth surface. Mature spore were oval with a diameter of (0.5~0.7)×(0.7~0.9) μm. Physiological characteristics and carbon utilization of the strain are summarized in Table 2. Diaminopimelic acid (DAP) analysis showed the presence of LL-DAP in the peptidoglycan [5]. Furthermore, a BLAST search of 16S rDNA sequences in the Genbank database showed the highest similarity of *Streptomyces minoensis*. The above results support the identification of this strain as a species of the genus *Streptomyces*, and named *Streptomyces* sp. HS-HY-071.

Fermentation and Isolation

Streptomyces sp. HS-HY-071 was grown at 28°C in a 20-

Table 2 Morphological and physiological characteristics and carbon utilization of strain HS-HY071

Spore chain morphology	Spirales
Spore surface	Smooth
Spore dimensions (μm)	(0.5~0.7)×(0.7~0.9)
Temperature range for growth ($^{\circ}\text{C}$)	15~36
Optimal temperature for growth ($^{\circ}\text{C}$)	26~30
Production of H_2S	+
Liquefaction of gelatin	—
Degradation of casein	—
Hydrolysis of starch	+
Reduction of nitrate	+
Carbon utilization	
D-Glucose	+
D-Xylose	+
L-Arabinose	+
L-Rhamnose	+
D-Fructose	—
D-Mannitol	+
D-Sucrose	+
D-Galactose	±

liter of fermentation. After 6 days, the cultured broth (9.0 liters) was centrifugated to separate mycelial cake and supernatant. The mycelial cake was extracted with MeOH (3.0 liters) and the supernatant was extracted with Diaion HP-20 and eluted with 95% EtOH. The combined MeOH soluble and the EtOH eluates were evaporated to give 11.5 g of crude extract. This crude extract was subjected to a silica gel column and eluted with petrol ether- Me_2CO from 19:1~1:1. The active fractions showing inhibitory activity against HCT-116 cell were collected and evaporated to afford 550 mg of a residue. The residue was also subjected to a Sephadex LH-20 column and eluted with MeOH and the active fraction was further separated by semi-preparative HPLC using a reversed-phase column (Zorbax SB-C18, 5.0 μm , 250×9.4 mm i.d) and a solvent of 75% MeOH/ H_2O with a flow rate of 1.5 ml/minute at a room temperature. The eluates were monitored with a photodiode array detector at 220 nm. Compound **1** was eluted at 12.3 min to give pure **1** (9 mg) as a viscous colorless oil.

Physico-chemical Characterization and Structure Determination

Compound **1** (Fig. 1) was obtained as a viscous colorless oil with UV (EtOH) λ_{max} nm 203 (ϵ 6619) and 249 (ϵ 5643). It is soluble in MeOH, CH_3CN , Me_2CO , CH_2Cl_2 , and CHCl_3 , but is insoluble in H_2O . The physico-chemical properties of **1** are shown in Table 3. Its molecular formula

Table 3 Physico-chemical properties of HS071 (**1**)

Appearance	Colorless oil
Molecular formula	$\text{C}_{12}\text{H}_{18}\text{O}_4$
Molecular weight	226
ESI-MS (m/z) Positive	249 $[\text{M}+\text{Na}]^+$
HRESI-MS (m/z) Found	249.1092 $[\text{M}+\text{Na}]^+$
Calculated	249.1103
UV λ_{max} nm (EtOH)	203 (ϵ 6619) and 249 (ϵ 5643)
IR ν_{max} cm^{-1} (MeOH)	3430, 2925, 1720, 1450

Fig. 1 The structure of HS071 (**1**)**Table 4** ^1H - and ^{13}C -NMR data of HS071 (**1**) in CDCl_3

No.	^{13}C (mult)	^1H
2	165.0 (s)	
3	112.0 (s)	
4	125.8 (s)	
5	138.3 (d)	7.24 (1H, s)
1'	28.2 (t)	2.93 (2H, t, $J=7.5$ Hz)
2'	27.6 (t)	1.66 (2H, m)
3'	31.3 (t)	1.31 (2H, m)
4'	22.3 (t)	1.33 (2H, m)
5'	13.9 (q)	0.90 (3H, t, $J=7.5$ Hz)
CH_2OH	55.7 (t)	4.56 (2H, br s)
CO	165.8 (s)	
CH_3O	51.7 (q)	3.88 (3H, s)

was established as $\text{C}_{12}\text{H}_{18}\text{O}_4$ by HRESI-MS m/z 249.1092 $[\text{M}+\text{Na}]^+$, calc for 249.1103. The IR spectrum of **1** revealed an ester carbonyl absorption at 1720 cm^{-1} and a hydroxy absorption at 3430 cm^{-1} , respectively. The ^1H -NMR spectrum of **1** showed an olefinic proton at δ 7.24 (1H, s), a methoxy signal at δ 3.88 (3H, s), and an aliphatic methyl at δ 0.90 (3H, t). Its ^{13}C -NMR and DEPT 135 data (Table 4) indicated four sp^2 quaternary carbons at δ 165.8, 165.0, 125.8, 112.0, a methylene bonding with oxygen at δ 55.7, and four aliphatic methylenes at δ 31.3, 28.2, 27.6 and 22.3, respectively.

Fig. 2 The ^1H - ^1H COSY and HMBC correlations observed in compound **1**

The gross structure of **1** was established mainly based on the ^1H - ^1H COSY and HMBC correlations (Fig. 2). In the ^1H - ^1H COSY (spectrum), the crossing signals between δ_{H} 2.93 and 1.66, 1.66 and 1.31 connected from C-1'-C-3', and the correlation of δ_{H} 0.90 and 1.33 connected from C-4'-C-5'. Furthermore, the three bonds HMBC correlation from H₃-5' to C-3' assigned the pentyl moiety from C-1'-C-5'. In the HMBC spectrum, the observed correlated signals of δ_{H} 7.24/ δ_{C} 125.8 (s), 55.7 (t), 112.0 (s), 165.0 (s) indicated the presence of a furan ring, and the correlations of δ_{H} 4.56 and δ_{C} 138.3 (d), 125.8 (s), 112.0 (s) showed a hydroxymethyl bonding at position 4 of the furan ring. The pentyl group connected to C-2 confirmed by the HMBC signals between δ_{H} 2.93 and δ_{C} 165.0 (s), 112.0 (s). The HMBC correlation of the methoxy signal at δ_{H} 3.88 and δ_{C} 165.8 showed the δ_{C} 165.8 was an ester carbonyl and it was

placed at C-3. Thus, the structure of **1** was completely elucidated and it was methyl 4-hydroxymethyl-2-pentylfuran-3-carboxylate.

Biological Activities

We examined the inhibitory activity of compound **1** against the growth of human colon carcinoma cell line HCT-116 using the CCK-8 colorimetric method as described in the materials and methods section. Compound **1** dose-dependently inhibited the growth of HCT-116 cells with an IC_{50} value of 18.2 $\mu\text{g}/\text{ml}$.

References

1. Shirling EB, Gottlieb D. Methods for characterization of *Streptomyces* species. *Int J Syst Bacteriol* 16: 313–340 (1966)
2. Williams ST, Goodfellow M, Alderson G. Genus *Streptomyces* Waksman and Henrici 1943. In *Bergey's Manual of Systematic Bacteriology*. Vol. 4, Ed., Williams ST., pp. 2452–2492, Williams & Wilkins, Baltimore (1989)
3. Hasegawa T, Takizawa M, Tanida S. A rapid analysis for chemical group of aerobic actinomycetes. *J Gen Appl Microbiol* 29: 319–322 (1983)
4. Pitcher DD, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidinium thiocyanate. *Lett Appl Microbiol* 8: 151–156 (1989)
5. Lechevalier MP, Lechevalier H. Chemical composition as a criterion in the classification of aerobic actinomycetes. *Int J Syst Bacteriol* 20: 435–443 (1970)