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A New Heliquinomycin Analogue with Immunosuppressive Activity from *Streptomyces* sp. jys28

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Abstract: Heliquinomycin (**1**) and a new analogue 9′-methoxy-heliquinomycin (**2**) were isolated from the culture broth of *Streptomyces* sp. jys28. The structure of new analogue was elucidated by comprehensive analyses of HR-ESI-MS and NMR spectroscopic data. Among them, Heliquinomycin (**1**) showed immunosuppressive and antibacterial activities.

Keywords: Heliquinomycin; immunosuppressive activity; antibacterial activity.

1. Introduction

Rubromycins are a family of structurally related compounds isolated from Actinomycetes [1]. They exhibit an attractive array of biological activity, such as antimicrobial [2,3], cytotoxicity [4], enzyme inhibition [5,6], and telomerase inhibition [5,7]. Heliquinomycin, a member of rubromycin family containing a rare deoxycymarose, has been reported from *Streptomyces* sp. MJ929-SF2 with selective inhibition of DNA helicase [3,8,9]. During our continuous efforts to discover new or bioactive natural products from characteristic actinomycetes (mainly *Streptomyces*) [10-13], *Streptomyces* sp. jys28, which can produce secondary metabolites with distinctive UV absorptions, attracted our attention. Strain *Streptomyces* sp. jys28 was isolated from the rhizoshere soil of *Pinus yunnanensis*, which was collected from the surrounding area of Fuxian lake, Yunnan Province, China. Two compounds, heliquinomycin (**1**) and its new analogue (**2**), were isolated from the culture broth of *Streptomyces* sp. jys28. In this paper, we describe the isolation, structural elucidation, immunosuppressive and antimicrobial activities of compounds **1** and **2**.

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2. Materials and Methods

2.1. General

Optical rotation was detected using a JASCO P-1020 digital Polarimeter. UV spectrum was measured using Shimadzu UV-2401PC UV-VIS spectrophotometer. IR spectrum was measured in KBr discs using a Bruker Tensor 27 FTIR Spectrometer. 1D and 2D NMR spectra were measured in $CDCl_3$ on a Bruker AVANCE III-600 spectrometer with TMS as an internal standard. The ESI-MS and HR-ESI-MS were taken using a UPLC-IT-TOF mass spectrometer (Shimadzu Biotech, Kyoto, Japan). Column chromatography (CC) was performed on silica gel (200-300 mesh, Qingdao Marine Chemical Inc.) or Sephadex LH-20 (25–100 μm, Pharmacia Biotech Ltd.). Semipreparative HPLC was conducted on a HITACHI Chromaster system equipped with a DAD detector, a YMC-Triart C_{18} column (250 mm × 10 mm i.d., 5 μm, YMC Corp.), at a flow rate of 3.0 mL/min and a column temperature of 25 °C.

2.2. Isolation of Streptomyces sp. jys28 and the Cultivation

The strain *Streptomyces* sp. jys28 was isolated from the rhizosphere soil of *Pinus yunnanensis*. It was identified as one species of the genus *Streptomyces* based on 16S rRNA gene sequence analysis (GenBank No. SUB4300555). This strain was maintained on oatmeal agar plates (Oat 20 g, KNO_3 0.2 g, $MgSO_4$ 0.2 g, K_2HPO_4 0.5 g and agar 20 g in 1 L of water) for 6 d at 30 °C. Then, it was inoculated into 250 mL baffle Erlenmeyer flasks containing 50 mL of sterile seed medium (Tryptone soy broth, 30 g/L) and cultivated for 2 days on a rotary shaker at 200 rpm and 30 °C. 25 L production medium (20 g glucose, 10 g malt extract, 5 g yeast extract in 1 L of water, pH 7.2) was transferred into 1 L Erlenmeyer flasks, each with 250 mL. After sterilized, the seed broth was transferred into production medium at 5% inoculation proportion. After cultured on a rotary shaker at 200 rpm and 30 °C for 7 d, the production medium was collected for later use.

2.3. Extraction and Isolation

The production medium was separated by centrifugation and filtration. The supernatant was extracted three times using an equal volume of EtOAc. The combined EtOAc phase was concentrated on a rotary evaporator to yield the crude extract. This extract was subjected to a 200−300 mesh silica gel column chromatography using a gradient petroleum ether/ EtOAc solvent system (1:0, 20:1, 10:1, 3:1, 1:1, 1:3, 1:10, 0:1) to afford eight fractions. Fraction of petroleum ether/ EtOAc phase 1:3 (1.02 g) was then separated by Sephadex LH-20 column (MeOH−$CHCl_3$, 1:1) to give four subfractions. The second subfraction was further separated by semipreparative HPLC eluting with CH_3CN/H_2O (57:43, v/v) to afford compounds **1** (20.5 mg, t_R = 12.9 min) and **2** (2.1 mg, t_R = 10.6 min).

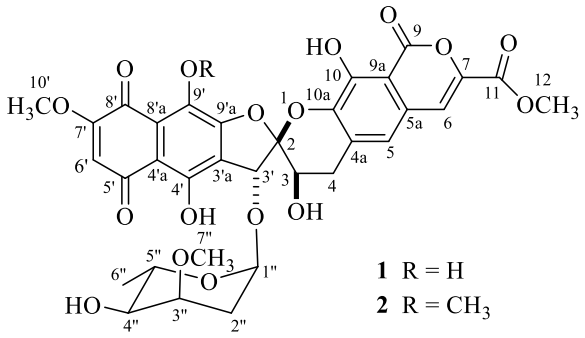

Figure 1. Structure of compounds **1** and **2**

2.4. Immunosuppressive Activity Test

Immunosuppressive activity test was constructed according to the reported protocol [14]. The T cells were isolated from PBMCs of healthy donors. Plate-bound anti-CD3 and soluble anti-CD28 were used to activate the naive T cells. Subsequently, they were incubated with or without different concentrations of the tested compounds for 72 h. The proliferation of the T cells was measured by flow cytometry. The negative control was the cells without stimulation or drug, and the cells with the stimulation but without the drug was the positive control. Cell viability assay was performed as follows: human naive T cells were treated with different concentrations of the tested compounds or vehicle for 72 h. The viability of cells were assessed using the CCK-8 assay kit though the OD values at 450 nm in a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale,CA, USA).

GraphPad Prism 6 (Graph Pad, San Diego, CA, USA) was used to calculate 50% inhibitory concentrations (IC_{50}) for T cell proliferation and 50% cytotoxic concentration (CC_{50}) of the tested compounds.

2.5. Antibacterial Activity Test

Tested strains were *Escherichia coli* ATCC 8099, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 6538. Minimum inhibitory concentration (MIC) was tested using 96-well plate microdilution method [12]. 160 μL of the LB medium was dispensed in the first well, while the other wells were dispensed 100 μL of LB medium. 10 μL of 20 mg/mL of the tested compounds dissolved in dimethyl sulfoxide (DMSO) and 30 μL of sterile water were dispensed in the first well and mixed with a micropipette, then 100 μL of this dilution was transferred to next well to yield two-fold serial dilution. This process was repeated twenty-three times and 100 μL of mixed liquid in the last well was discarded, finally resulting in twenty-four concentrations of the tested compounds. For the positive control kanamycin, 40 μL of 5 mg/mL of kanamycin dissolved in water was dispensed in the first well, and then was also serially diluted as the tested compounds. 25% DMSO aqueous solution was the negative sample. 100 μL of tested strain (an OD_{600} of 0.02) was dispensed to each of the wells included the positive and negative control. The microplates were incubated at 37°C for 16 hours. After the incubation, bacterial growth was observed by the naked eye, and the lowest concentration at which the medium was clear was taken as the MIC value.

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was isolated as a red powder, its ESI-MS spectrum showed a molecular ion peak at *m/z* 697 $[M-H]^-$. ^1H and ^{13}C NMR spectroscopic data of compound **1** were summarized in Table 1. Compound **1** was proved to be heliquinomycin by direct comparison of these data with those from the literature [8].

Compound **2**, $[\alpha]_D^{25}$ +24.36 (*c* 0.34, $CHCl_3$), UV ($CHCl_3$) λ_{max} nm (log ε): 455.5 (3.59), 363.0 (3.89), 350.5 (3.92), 311.0 (4.15) and 242 (4.37), was isolated as a saffron yellow powder. Its ESI-MS spectrum showed a pseudo-molecular ion peak at *m/z* 711 $[M - H]^-$ and molecular formula was determined as $C_{34}H_{32}O_{17}$ by HRESI-MS at *m/z* 711.1567 $[M - H]^-$ (calcd for $C_{34}H_{32}O_{17}$, 711.1564). The IR spectrum of **2** showed characteristic absorption bands of hydroxyl and ketone groups at 3446 and 1726 cm^{-1}, respectively. The NMR data of **2** were similar to those of **1**, except for the presence of an additional methoxy group signal at δ_H 3.77 and δ_C 61.3 (Table 1) in **1**. The HMBC spectrum of **2** showed long-range correlations between 9'-OCH_3 and C-9', which suggested that C-9' of **2** was connected with a methoxy group. The NMR data of **2** showed that it contains a cymarose as **1**. The ^1H NMR peak of cymarose moiety in the $CDCl_3$ solvent is not splitted well. We then measured the ^1H NMR in pyridine-d_5 solvent. From the ^1H NMR in pyridine-d_5, we got the *J* value of H-1″ is 3.7 Hz. In the ROESY spectrum, significant cross peaks between H-2″/H-4″ and H-4″/H-6″ were observed.

From the above all analyses, it can be indicated that the relative configuration of the cymarose in the **2** was the same as in the heliquinomycin. Thus, the structure of **2** was identified as shown in Figure 1, and named 9′-methoxy-heliquinomycin.

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150MHz) data of compounds **1** and **2** in $CDCl_3$

Position	1		2	
	δc (ppm)	**δ_H (ppm, *J* in Hz)**	**δc (ppm)**	**δ_H (ppm, *J* in Hz)**
2,2'	112.1		111.6	
3	61.7	4.55 (1H, s, H-3)	61.8	4.54 (1H, br s)
		5.34 (1H, br s) (3-OH)		5.31 (1H, br s) (3-OH)
4	30.4	3.08 (1H, br d, *J* = 16.8)	30.4	3.10 (1H, dd, *J* = 17.0, 1.8)
		3.55 (1H, br d, *J* = 16.8)		3.50 (1H, dd, *J* = 17.0, 3.3)
4a	130.4		130.3	
5	119.2	6.95 (1H, s)	119.1	6.96 (1H, s)
5a	128.1		128.0	
6	113.8	7.43 (1H, s)	113.7	7.44 (1H, s)
7	141.4		141.3	
9	165.2		165.1	
9a	106.9		106.9	
10	150.4		150.4	
10-OH		11.00 (1H, s)		11.00 (1H, s)
10a	140.7		140.7	
11	160.7		160.7	
12	53.2	3.94 (3H, s)	53.1	3.95 (3H, s)
3'	76.9	5.80 (1H, s)	76.8	5.82 (1H, s)
3'a	123.0		120.7	
4'	160.1		157.2	
4'-OH		13.54 (1H, s)		13.68 (1H, s)
4'a	106.8		109.8	
5'	183.8		189.9	
6'	110.6	6.20 (1H, s)	108.4	6.02 (1H, s)
7'	160.0		161.1	
8'	179.3		178.1	
8'a	114.2		124.6	
9'	150.6		141.0	
9'-OH		12.13 (1H, s)		
9'a	156.2		159.1	
10'	57.0	3.94 (3H, s)	56.9	3.88 (3H, s)
9'-OCH_3			61.3	3.77 (3H, s)
1"	94.3	5.57 (1H, s)	94.3	5.59 (1H, br s)
				5.92 (1H, dd, *J* = 3.7)[a]
2"	31.0	1.87 (1H, br d, *J* = 15.0)	30.9	1.87 (1H, br d, *J* = 15.0)
		2.32 (1H, br d, *J* = 15.0)		2.32 (1H, br d, *J* = 15.0)
3"	76.5	3.70 (1H, br s)	76.5	3.70 (1H, br d, *J* = 2.6)
4"	71.9	3.33 (1H, br s),	71.8	3.34 (1H, br s)
4"-OH		2.06 (1H, br s)		2.20 (1H, br s)
5"	66.0	3.93 (1H, dd, *J* = 9.3, 6.0)	66.0	3.92 (1H, dd, *J* = 9.3, 6.0)
6"	18.4	1.40 (3H, d, *J* = 6.0)	18.3	1.41 (3H, d, *J* = 6.0)
7"	57.6	3.50 (3H, s)	57.5	3.51 (3H, s)

[a] ^{1}H (600 MHz) NMR for compound **2** in pyridine-d_5.

3.2. Immunosuppressive and Antibacterial Activities

Immunosuppressive activity of the two isolates were evaluated. Compound **1** showed inhibition against T cell proliferation with IC_{50} value of 7.8 μM and low cytotoxicity with CC_{50} value of 65.7 μM, implying that compound **1** inhibited T cell proliferation through immunosuppressive activity, but not cytotoxicity. The inhibitory activities of the two isolates were also tested *in vitro* against *E. coli*, *B.subtilis*, and *S. aureus*. The results (Table 2) showed that compound **1** had potent antibacterial activity.

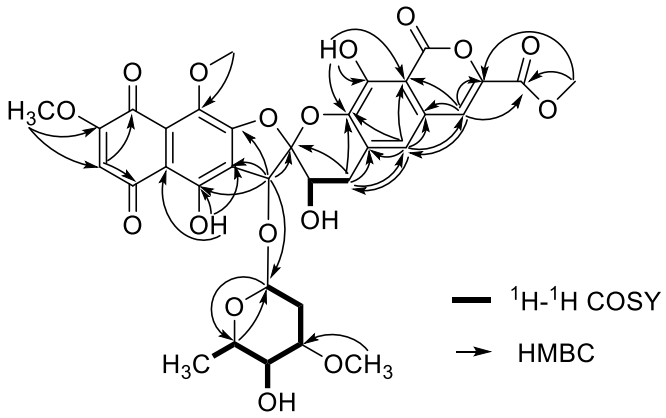

Figure 2. Key ^{1}H-^{1}H COSY and HMBC correlations of compound **2**

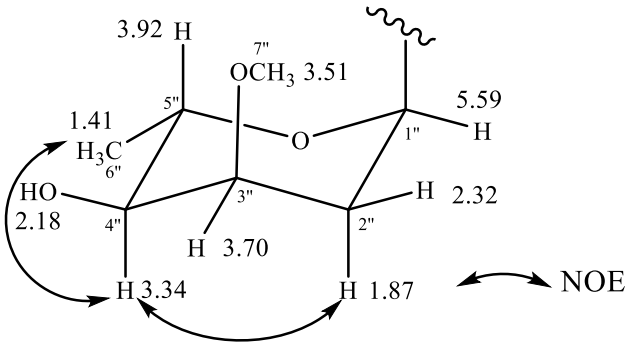

Figure 3. Key ROESY correlations for stereochemistry assignment of the sugar moiety showing the carbon numbering and ^{1}H NMR chemical shift

Table 2. MIC of compounds **1** and **2** (μg/mL)

Test bacterial	1	2	Kanamycin
E. coli	0.49	7.81	7.81
B. subtilis	0.12	62.50	0.49
S. aureus	0.49	15.63	7.81

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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