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A New 2,2′-dipyridine and Its Analogues from Endophytic *Streptomyces* sp. KIB H017c with Potent Cytotoxicity

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Abstract: A new 2,2′-dipyridyl compound **1**, along with three known analogues (**2**–**4**), was isolated from the culture broth of endophytic *Streptomyces* sp. KIB H017c derived from *Fragaria ananassa*. Their structures were elucidated by analysis of 1D and 2D NMR as well as HRESIMS data, and comparison with literature data. The biological properties of all isolates were explored for antibacterial, antifungal and cytotoxic activity. All four compounds exhibited weak antifungal activity against *Saccharomyces cerevisiae*, and compound **4** exhibited weak antifungal activity against *Penicillium decumbens* ATCC 10436. Compound **3** displayed moderate cytotoxic activity against HL-60 (human myeloid leukemia), SMMC-7721 (hepatocellular carcinoma), A-549 (lung carcinoma), MCF-7 (breast adenocarcinoma), and SW480 (colon carcinoma) with IC_{50} values of 12.10, 11.15, 23.64, 31.13 and 14.62 μM, respectively. Compound **4** exhibited potent activity against A-549 (lung carcinoma), MCF-7 (breast adenocarcinoma), and SW480 (colon carcinoma) with IC_{50} values 0.88, 0.34 and 0.55 μM, respectively.

Keywords: Endophytic *Streptomyces*; 2,2′-dipyridine; antimicrobial activity; cytotoxicity.

1. Introduction

Endophytic microorganisms are recognized as an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas [1]. Endophytic microorganisms are to be found in virtually every plant on earth. Commonly, several to hundreds of endophyte species can be isolated from a single plant [2]. Noteworthily, nearly 300,000 plant species that exist on the earth, each individual plant is considered to host one or more type of endophytes [3], which could creat an enormous biodiversity. Endophytes have attracted increasing attention from those seeking for new pharmaceutically useful products in recent years. More than 33,500 biologically active compounds have been obtained from

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microbes by the end of 2010. Among them, 40% were produced by actinobacteria, especially the excellent producers in the genus *Streptomyces* [4].

With the aim of discovering new biologically active agents from endophytic actinomycetes [5,6,7], we isolated a *Streptomyces* sp. KIB H017c from a healthy *Fragaria ananassa* collected in Wenshan, Yunnan Province, China in 2014. In this report, we describe the isolation and structure elucidation of a new compound **1** as a 2,2′-dipyridyl derivative, and together with three known analogues (**2**–**4**) from the culture broth of KIB H017c. All isolates were evaluated for their antimicrobial and cytotoxic activity.

2. Materials and Methods

2.1. General

The UV data was detected by Shimadzu UV-2401PC (Shimadzu Corp., Japan). NMR spectra were recorded on Bruker Avance Ⅲ 600 (Bruker Corp., Switzerland) with ^{1}H NMR at 600 MHz and ^{13}C NMR at 150 MHz. Chemical shifts were reported in units of ppm and referenced to solvent residual in both ^{1}H NMR and ^{13}C NMR spectra, coupling constants (*J*) were expressed in Hz. HRESIMS analysis was carried out on an Agilent 1290 UPLC/6540 Q-TOF mass spectrometer, and general ESIMS on a Waters Xevo TQ-S spectrometer. Preparative HPLC separations were performed on a CXTH system, equipped with a UV3000 detector set at 203 nm (Beijing Chuangxintongheng Instruments Co. Ltd., China) and a MCI column (310 mm × 26 mm I. D.) at a column temperature of 28 °C. Semipreparative HPLC was conducted on a Hitachi Chromaster system (Hitachi Ltd., Japan) equipped with an YMC−Triart C18 column (250 × 10 mm i.d., 5 μm), at a column temperature of 28 °C, and detection was performed with a DAD detector. The chromatographic silica gel (200–300 mesh) was supplied by Qingdao Marine Chemical Inc., China and Sephadex LH-20 (25–100 μm) was supplied by Pharmacia Biotech Ltd., Sweden. Thin-layer chromatography (TLC) was performed using precoated silica gel GF254 plates (0.25 mm in thickness, Qingdao Marine Chemical Inc., China) with various solvent systems, and spots were visualized by UV light (254 nm) and colorized using iodine, or by heating after spraying with 10% H_2SO_4 in MeOH.

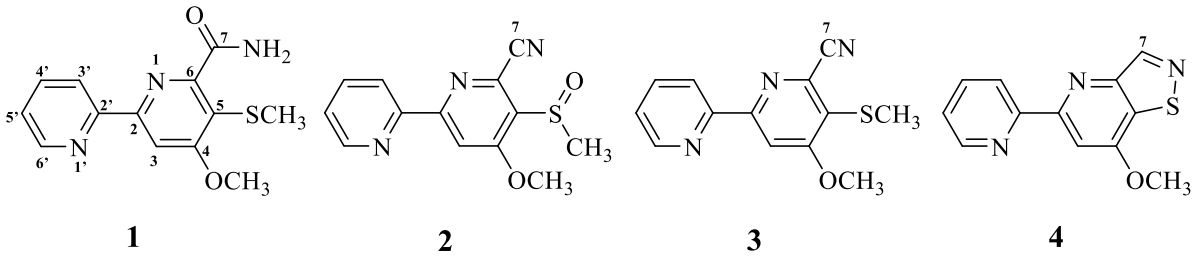
2.2. Biological Material

The Strain KIB H017c was isolated from a healthy *Fragaria ananassa* plant collected at east longitude 104°20′46.0″, north latitude 23°17′39.0″ in Wenshan, Yunnan Province, China in 2014. Its 16S rRNA gene sequence (GenBank accession No. MK049995) of this strain showed a 99% identity to *Streptomyces* sp. S8 (GenBank accession No. CP015362). Pathogenic bacteria strains were provided by North West Agriculture and Forestry University, Xi'an. Strains have been saved in the State Key Laboratory of Phytochemistry and Plant Resources in West China, CAS Center for Excellence in Molecular Plant Sciences, Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Fermentation and Isolation

The seed medium was prepared in 250 mL baffle Erlenmeyer flasks. Each flask was filled with 50 mL of Tryptone Soy Broth (30 g/L, pH was not adjusted) and cultivated for 48 hours at 28 °C on a rotary shaker (200 rpm). Fermentations were carried out in 1000 mL baffle Erlenmeyer flasks. Each flask was filled with 250 mL of medium consisting of Tryptone 0.5%, yeast extract 0.5%, soluble starch 1%, D-glucose 1%, Glycerol 1% and $CaCO_3$ 0.3% in deionized H_2O (pH was adjusted to 7.0). Every fermentation flask was inoculated with 10 mL seed medium and cultivated for 7 days at 28 °C on a rotary shaker (200 rpm). The culture broth (15 L) was centrifuged (4000 rpm, 20 min) and the liquid supernatant was extracted with ethyl acetate (6 × 5 L) and concentrated in vacuo, while the mycelium was extracted with acetone (3 × 0.5 L) and concentrated in vacuo, then partitioned with ethyl acetate twice and concentrated. Both organic parts were combined and evaporated to remove the solvent. The residue (1.5 g) was subjected to silica gel column chromatography. Elution with petroleum ether-ethyl acetate (90:10, 50:50 and 0:100 v/v) and MeOH yielded three fractions A to C.

Fraction B (1.0 g) was further separated using preparative HPLC on an MCI column into fractions B-1 to B-4, by elution with MeOH (25%, 50%, 75% and 100% in water) using a flow rate of 12 mL/min. Fraction B-3 was further separated by Sephadex LH-20 chromatography (MeOH) into fractions B-3-1 to B-3-3. Fraction B-3-2 was subjected to semipreparative HPLC (Hitachi HPLC system, YMC−Triart C18 column, 250 × 10 mm, DAD detector) using a flow rate of 3 mL/min and isocratic elution with 35% MeOH in H_2O, and yielded **1** (4.0 mg) and **2** (3.0 mg). Fraction B-4 was soluble in chloroform and methanol (1:1, v/v), and yielded colourless crystals after evaporation of the solvent. Repeated washing of the crystals with ethyl acetate and MeOH, yielded compound **3** (10 mg). The residue of fraction B-4 was subjected to semipreparative HPLC with isocratic elution with 75% MeOH in H_2O at a flow rate of 3 mL/min, yielded compound **4** (3.0 mg) (see supporting information for detailed procedure).



1 **2** **3** **4**

Figure 1. Chemical structure of compounds **1**–**4**

2.4. Antimicrobial Activity Test

For plate diffusion assays [8], 20 μg of the isolated compounds were dissolved in acetone and dropped on paper disks (Ø 5 mm, thickness 0.5 mm). These were dried under sterile conditions and placed on agar plates inoculated with the testing organism (two fungi strains, *Saccharomyces cerevisiae* and *Penicillium decumbens* ATCC 10436, two bacteria strains, *Escherichia coli* ATCC 8099 and *Staphylococcus aureus* ATCC 6538). The plates were cultivated at 37 °C (bacteria) for 12 h or 28 °C (fungi) for 48 h, and the inhibition zones were measured. Kanamycin (10 μg/disk) and nystatin (10 μg/disk) were used as positive control for bacteria and fungi, respectively. Each test was performed four times.

2.5. Cytotoxicity Activity Test

The cytotoxicity of compounds **1**–**4** against the tumor cell lines HL-60 (human myeloid leukemia), SMMC-7721 (hepatocellular carcinoma), A-549 (lung carcinoma), MCF-7 (breast adenocarcinoma), and SW480 (colon carcinoma) were assessed using the MTS method [9]. Cisplatin (Sigma, 99% purity) and Paclitaxel (Taxol, Sigma, 97% purity) were used as positive controls. Briefly, all cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT, USA), which were supplemented with 10% fetal bovine serum (Hyclone, USA) at 37 ℃ in a humidified atmosphere containing 5% CO_2. Cells were seeded at 1×10^4 cells per well into 96-well cell culture plates and then incubated at 37 ℃ for 12~24 h. The tested compounds of different concentrations (0.064, 0.32, 1.6, 8.0, 40 μM) were added into the 96-well plates and cultivated for 48 h. Then 20 μL of MTS was added to each well and the incubation continued for 4 h at 37 ℃. Finally, the optical density was measured at 492 nm using a Multiskan FC plate reader (Thermo Scientific, USA). The IC_{50} value of each compound was calculated by the Reed and Muench's method [10].

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was obtained as white powder, and assigned a molecular formula of $C_{13}H_{13}N_3O_2S$ from its HRESIMS data (*m/z* 276.0808 [M+H$^+$], calcd for 276.0801), requiring 9 degrees of unsaturation. Analysis of the ^{13}C NMR data and HSQC spectrum for **1** revealed six quaternary carbons, five methine carbons, and two methyl carbon including a methoxy group (δ_H 4.05, δ_C 56.4) and a methylthio group (δ_H 2.33, δ_C 17.6). The H-H COSY spectrum of **1** indicated a sequence correlation of 3′-H (δ 8.39), 4′-H (δ 7.95), 5′-H (δ 7.48) and 6′-H (δ 8.70), and the HMBC spectrum indicated correlations from 3′-H, 4′-H and 6′-H to 2′-C (δ 154.4), revealing the presence of a 2-pyridyl group. Further comparison of NMR spectra with SF2738A [11] indicated that compound **1** has a similar 2,2′-dipyridyl skeleton, and the only difference was the substituent at 6. As shown in Figure 2 and Table 1, the HMBC experiments (correlations from H-3′ to C-2, from H-3 to C-2′, from δ_H 4.05 (4-OCH_3) to C-4 and from δ_H 2.33 (5-SCH_3) to C-5) revealed that **1** consists of a tetra-substituted pyridine with 2-pyridyl, methoxyl, methylthio and one additional group at positions 2, 4, 5 and 6, respectively. The remaining group was deduced to be an amide group by HMBC correlations from δ_H 7.94 and δ_H 7.57 to δ_C 168.9 (C-7), and δ_H 7.57 (1H, s) to δ_C 158.2 (C-6) in Figure 2. From the foregoing evidence, compound **1** was established as shown in Figure 1.

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150MHz) data of compound **1** in DMSO-d_6

Position	δc (ppm)	δ_H (ppm, *J* in Hz)
2	155.5 (C)	
3	103.4 (CH)	8.02 (1H, s)
4	166.7 (C)	
4-OCH_3	56.4 (CH_3)	4.05 (3H, s)
5	118.5 (C)	
5-SCH_3	17.6 (CH_3)	2.33 (3H, s)
6	158.2 (C)	
7	168.9 (C)	
7-NH_2		7.94 (1H, m)
		7.57 (1H,s)
2'	154.4 (C)	
3'	121.1 (CH)	8.39 (1H, d, *J* = 7.8 Hz)
4'	137.5 (CH)	7.96 (1H, td, *J* = 7.8, 1.2 Hz)
5'	124.8 (CH)	7.48 (1H, ddd, *J* = 7.2, 4.8, 1.2 Hz)
6'	149.3 (CH)	8.70 (1H, d, *J* = 4.2 Hz)

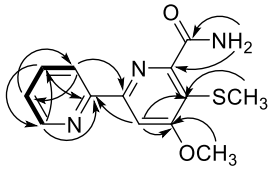


Figure 2. Key HMBC correlations of compound **1**

Additionally, three known compounds were obtained (Figure 1). Compound **2** was isolated as a white powder, its ESI-MS spectrum showed a molecular ion peak at *m/z* 274 [M+H]$^+$. Compound **3** was isolated as colourless needle crystal ($CHCl_3$/CH_3OH 1:1, v/v), its ESI-MS spectrum showed a molecular ion peak at *m/z* 258 [M+H]$^+$. Compound **3** was isolated as white powder, its ESI-MS spectrum showed a molecular ion peak at *m/z 244* [M+H]$^+$. ^{1}H NMR and ^{13}C NMR spectroscopic data of compound **2-4** were summarized in Table 2. Compound **2-4** were proved to be pyrisulfoxin B [12], SF2738D and SF2738F [11] by direct comparison of the data with those from the literature.

Table 2. ^{1}H (600 MHz) and ^{13}C NMR (150MHz) data of compound **2** in $CDCl_3$ and compounds **3-4** in DMSO-d_6.

Position	Pyrisulfoxin B (2)		SF2738D (3)		SF2738F (4)	
	δc (ppm)	**δ_H (ppm, *J* in Hz)**	**δc (ppm)**	**δ_H (ppm, *J* in Hz)**	**δc (ppm)**	**δ_H (ppm, *J* in Hz)**
2	161.2 (C)		157.9 (C)		158.7 (C)	
3	106.6 (CH)	8.31 (1H, s)	106.3 (CH)	8.16 (1H, s)	100.0 (CH)	8.15 (1H, s)
4	164.6 (C)		167.1 (C)		160.2 (C)	
4-OCH_3	57.0 (CH_3)	4.17 (3H, s)	57.0 (CH_3)	4.09 (3H, s)	56.8 (CH_3)	4.19 (3H, s)
5	131.8 (C)		127.1 (C)		135.3 (C)	
5-XCH_3	39.9 (5-$SOCH_3$)	3.11 (3H, s, 5-$SOCH_3$)	17.4 (5-SCH_3)	2.55 (3H, s, 5-SCH_3)		
6	137.4 (C)		137.9 (C)		153.9 (C)	
7	114.5 (C)		116.6 (C)		157.1 (CH)	9.35 (1H, s)
2'	152.8 (C)		153.0 (C)		154.6 (C)	
3'	122.2 (CH)	8.52 (1H, d, *J* = 7.8 Hz)	121.2 (CH)	8.31 (1H, d, *J* = 7.8 Hz)	121.2 (CH)	8.50 (1H, d, *J* = 8.4 Hz)
4'	137.4 (CH)	7.88 (1H, t, *J* = 7.2 Hz)	136.2 (CH)	7.98 (1H, td, *J* = 7.8, 1.8 Hz)	137.8 (CH)	8.02 (1H, td, *J* = 7.8, 1.8 Hz)
5'	125.6 (CH)	7.42 (1H, dd, *J* = 6.6, 5.4 Hz)	125.5 (CH)	7.53 (1H, ddd, *J* = 7.8, 4.8, 1.2 Hz)	125.2 (CH)	7.56 (1H, ddd, *J* = 7.8, 4.8, 1.2 Hz)
6'	149.2 (CH)	8.68 (1H, d, *J* = 4.2 Hz)	149.6 (CH)	8.72 (1H, ddd, *J* = 4.8, 1.8, 1.2 Hz)	149.4 (CH)	8.75 (1H, d, *J* = 4.2 Hz)

3.2 Antimicrobial and Cytotoxicity Activity

The isolated compounds **1**–**4** were evaluated for antimicrobial activity. All isolates at the concentration of 20 μg/disk exhibited minor antifungal activity against *Saccharomyces cerevisiae*, and compound **4** exhibited minor antifungal activity against *Penicillium decumbens* ATCC 10436. The diameters of the inhibition zones ranged between 10 and 11 mm, all compounds (20 μg/disk) were significantly less potent than the positive control, kanamycin (10 μg/disk) and nystatin (10 μg/disk) (Table 3).

Table 3. Antimicrobial activity of compounds **1**–**4**[a, b] (mm)

Test organism	**1** (20μg/disk)	**2** (20μg/disk)	**3** (20μg/disk)	**4** (20μg/disk)	Kanamycin (10μg/disk)	Nystatin (10μg/disk)
Saccharomyces cerevisiae	11	10	10	11	–	14
Penicillium decumbens ATCC 10436	–	–	–	10	–	10
Escherichia coli ATCC 8099	–	–	–	–	15	–
Staphylococcus aureus ATCC 6538	–	–	–	–	15	–

[a]Diameter of inhibition zones in the plate diffusion assay in mm. "–" No activity
[b]Data given were from four duplicates.

Investigation of antiproliferative activity of compounds **1**–**4** was carried out using an MTS assay on five human cancer cell lines: HL-60 (human myeloid leukemia), SMMC-7721 (hepatocellular carcinoma), A-549 (lung carcinoma), MCF-7 (breast adenocarcinoma) and SW480 (colon carcinoma). Compounds **1** and **2** exhibited no obvious antiproliferative activities against the five cancer cell lines (IC_{50} > 40 μM). Compound **3** exhibited moderate inhibitory activity against HL-60, SMMC-7721, A-549, MCF-7 and SW480 cells with IC_{50} values of 12.10, 11.15, 23.64, 31.13 and 14.62 μM, respectively. However, compound **4** displayed significant antiproliferative activity against

A-549, MCF-7 and SW480 cell lines with IC_{50} values of 0.88, 0.34 and 0.55 μM, respectively (Table 4).

Table 4. Cytotoxic activity (IC_{50}, μM) of compounds **1**–**4** against five tumor cell lines: HL-60, SMMC-7721, A-549, MCF-7, and SW480.

Compound	HL-60	SMMC-7721	A-549	MCF-7	SW480
1	> 40	> 40	> 40	> 40	> 40
2	> 40	> 40	> 40	> 40	> 40
3	12.10	11.15	23.64	31.13	14.62
4	> 40	> 40	0.88	0.34	0.55
Cisplatin[a]	2.29	11.87	12.10	11.37	16.42
Paclitaxel[a]	<0.008	<0.008	<0.008	<0.008	<0.008

[a]Positive control.

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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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