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Anti-*Mycobacterium tuberculosis* Active Metabolites from an Endophytic *Streptomyces* sp. YIM65484

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Abstract: In our screening for antitubercular leading compounds, an endophytic *Streptomyces* sp. YIM65484 was selected by biological assay. Four bioactive metabolites were isolated from this strain. Their structures were determined as (2*E*,4*E*)-5-(3-hydroxyphenyl)- penta-2,4- dienamide (**1**), ergosterol (**2**), ergosterol peroxide (**3**) and halolitoralin B (**4**) by spectral analysis. Compound **1** is a new phenylpentadienamide with its rarely encountered skeleton in natural products. Compounds **1**-**4** showed selectivity for anti-*M. Tuberculosis* activity in comparison with other antimicrobial activity. Compound **4** was first isolated from *Streptomyces*.

Keywords: *Streptomyces* sp.; anti-*M. tuberculosis*; phenylpentadienamide.

1. Introduction

Tuberculosis (TB) is one of the chronic infectious diseases caused by the bacillus *Mycobacterium tuberculosis*, which is responsible for the death of around 2-3 million per year[1]. Tuberculosis is becoming a serious threat for disease control, and its deadly synergy with HIV/AIDS and the prevalence of multidrug resistant (MDRTB) strains have raised concerns for the difficulty of treating TB in the future [2]. Therefore the need for new drugs to extend the variety of TB drug options is urgent. In some recent researches, plants [3-4], marine organisms [5-6], and microbes [7-8] have all been reported as promising sources of antitubercular natural products, which could be considered for further drug research and development [1].

Streptomyces is believed to be a rich source of new and useful compounds, and many active metabolites were found in endophytic actinomycetes[9]. In an effort of searching for antitubercular leading compounds, we assessed a plenty of the endophytic *Streptomyces* for growth inhibitory activity against *M. tuberculosis*. Among them, the extract in fermentation broth of an endophytic *Streptomyces* sp. YIM65484 from *Tripterygium wilfordii* exhibited promising level of anti-*M. tuberculosis* activity with MIC at 32 μg/mL. Four antimicrobial compounds (**1**-**4**) were isolated from the fermentation broth of *Streptomyces* sp. YIM65484. Compounds **1**, **2**, **3** and **4** exhibited anti-*M. tuberculosis* activity with MICs of 128, 64, 32 and 64 μg/mL respectively. Compound **1** was a new compound. The structures of

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compounds **1**-**4** were determined as (2*E*,4*E*)-5-(3-hydroxyphenyl)penta-2,4-dienamide (**1**), ergosterol (**2**) [10], ergosterol peroxide (**3**) [11] and halolitoralin B (**4**) [12] by spectral analysis (Figure. 1). Furthermore, other antimicrobial activity of compounds **1**-**4** against *Candida albicans*, *Escherichia Coli* and *Staphylococcus aureus* were also developed. Compounds **1**-**4** showed some selectivity in anti-*M. Tuberculosis* in comparison with other antimicrobial activity.

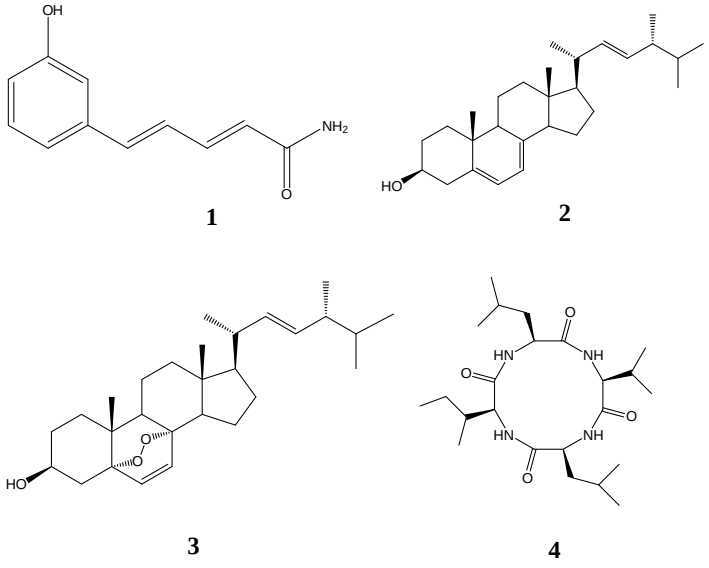

Figure 1. Structures of compounds **1**–**4**.

2. Materials and Methods

2.1. General

Melting points (mp) were determined on a XRC-1 Melting Point Apparatus and uncorrected. UV spectra were recorded on a Shimadzu UV-VIS 2550 spectrometer. IR spectra were obtained on a Nicolet Magna-IR 550 spectrometer. 1D and 2D NMR spectra were obtained on a Bruker Avance III 400 MHz instruments with TMS as internal standard. MS spectra were recorded with Agilent G3250AA. Thin layer chromatography (TLC) was performed on plates precoated with silica gel GF-254 (10-40 μm, Qingdao Marine Chemical Inc.). Column chromatography (CC) was performed over silica gel (200-300 mesh, Qingdao Marine Chemical Inc.) and Sephadex LH-20 (GE Healthcare Co.).

2.2. Microorganism Material

The bacterial strain YIM65484 was isolated from the plant of *T. wilfordii* which was collected from Dali, Yunnan Province of China. Its 16S rRNA gene sequence shows high similarities to *Streptomyces microflavus* NBRC 13062^T (GenBank accession no. HQ995517), and it was identified as *Streptomyces* sp. YIM65484. The strain has been preserved at Yunnan Institute of Microbiology, Yunnan University, China.

2.3 Fermentation and Isolation

This bacterium was cultivated on 50 L scale using 1 L Erlenmeyer flasks containing 250 mL of the seed medium (yeast extract 0.4%, glucose 0.4%, malt extract 1.0%, decavitamin 0.01%, pH 7.2) and the fermentation medium (soluble starch 2.4%, beef extract 0.3%, glucose 0.1%, peptone 0.3%, yeast extract 0.5%, $CaCO_3$ 0.4%, pH 7.0) at 28 °C on rotary shaker (250 rpm). After 7 days of growth, the mycelia were removed from the cultures (50 L) by filtration. The filtrate was extracted with ethyl acetate (EtOAc, 3 × 50 L), and the solvent was removed under vacuum to give a crude extract (14.0 g).

The EtOAc extract (14.0 g) was separated into four fractions (Fr 1 to Fr 4) by a chromatographic column on silica gel (200-300 mesh) eluted with stepwise $CHCl_3$/MeOH gradient ($CHCl_3$, $CHCl_3$/MeOH = 30:1 *v*/*v*, $CHCl_3$/MeOH = 10:1 *v*/*v*, MeOH, 1.5 L each). The Fr 1 (4.8 g, eluted with $CHCl_3$) was placed in a silica gel column and eluted with petroleum ether/ethyl acetate mixture (20:1) to ethyl acetate, then MeOH, which gave three fractions (Fr 1-1 to Fr 1-3). Fr 1-1 (0.9 g) was separated by a silica gel column with $CHCl_3$/MeOH (50:1 to 10:1) to afford **2** (21.6 mg). Fr 1-2 (1.0 g) was subjected to further elution on a silica gel column with $CHCl_3$/MeOH (40:1 to 9:1) and then separated by a chromatographic column on Sephadex LH-20 (MeOH) to give **3** (5.4 mg). The Fr 2 (3.6 g, eluted with $CHCl_3$/MeOH = 30:1 *v*/*v*) was separated by a silica gel column eluted with stepwise $CHCl_3$/MeOH gradient (from 40:1 to 9:1) to produce three fractions (Fr 2-1 to Fr 2-3). Fr 2-2 (0.7 g) was further purified by a chromatographic column on Sephadex LH-20 (MeOH) to afford **4** (4.1 mg). Fr 2-3 (0.5 g) was further purified by a chromatographic column on Sephadex LH-20 (MeOH) to afford **1** (3.9 mg).

(2E,4E)-5-(3-hydroxyphenyl)penta-2,4-dienamide (**1**): colorless needles (MeOH); mp 203-206 °C; UV (MeOH) λ_{max} (log ε) 251 (3.58), 341 (4.10) nm; IR (KBr) ν_{max} 3337, 3219, 1649, 1560 cm^{-1}; ^1H and ^{13}C NMR data: see Table 1; HR-ESI-MS *m/z* 190.0862 $[M+H]^+$ (calcd for $C_{11}H_{12}NO_2$: 190.0868).

2.4. Antimicrobial assay

Antimicrobial assays were performed in 96-well sterilized microplates using a microdilution method. Briefly, 4-day-old spores from *C. albicans* (grown on PDB medium: potato 20%, glucose 2% g), and the test concentration was 1×10^3 spores/mL. The 18-hour-old bacterial cultures from *M. tuberculosis* (grown on yeast extract medium: yeast extract 0.4%, mall extract 1%, glucose 0.4%, pH 7.3), *E. Coli* and *S. aureus* (grown on LB medium: yeast extract 0.5%, tryptone 1%, NaCl 1%, pH 7.0) to reach 1×10^5 colony-forming units/mL. The test samples were dissolved in DMSO, and their final concentrations ranged from 512 to 0.5 μg/mL by using a 2-fold serial dilution method. The final concentration of DMSO did not exceed 5%. The wells containing test strains and diluted samples were incubated at 28 °C (4 days) for fungi and 37 °C (24 h) for bacteria. The wells containing a culture suspension and DMSO were run as negative controls. As a positive control, nystatin (Taicheng Pharmaceutical Co., Ltd., Guangdong, China) had antifungal activity against *C. albicans* with a MIC of 16 μg/mL, kanamycin (Yunke Biotechnology, Kunming, China) showed antibacterial acitivity against *M. tuberculosis*, *E. Coli* and *S. aureus* with MICs of 8, 8 and 4 μg/mL, respectively. All experiments were repeated three times. The growth of test strains was observed with a CX21BIM-SET5 microscope (Olympus Corp.). MICs were determined as the lowest concentrations that produce complete growth inhibition of the tested microorganisms.

3. Results and Discussion

3.1. Structure elucidation

Compound **1** was obtained as colorless needles, and shown to have a molecular formula of $C_{11}H_{11}NO_2$ on the basis of HR-ESI-MS, and confirmed by ^{13}C NMR data. Exhaustive analyses of the NMR data for **1** (Table 1) indicated the presence of eight olefinic methines, three quaternary carbons (one for carbonyl group). As shown by the bold line in Figure 2, the COSY correlations between H-2 and H-3, H-3 and H-4, H-4 and H-5, together with $J_{2,3}$ = 15.2 Hz and $J_{4,5}$ = 16.3 Hz in ^1H NMR for both H-2, 3 and H-4, 5 at *trans*, indicated the presence of (2*E*, 4*E*)-2.4-pentadiene residue. The *m*-disubstituted benzene moiety was deduced by the COSY correlations between H-9 and H-10, H-10 and H-11, together with the HMBC correlations from H-7 and H-11 to C-5 and C-6, from H-7 and H-9 to C-8 (δ 157.5) which was attached to a hydroxy group, the ^1H NMR for H-7 at δ 6.92 (s) were also confirmed this deduction. The amide group connected to C-2 was elucidated by the HMBC correlations from H-2 and H-3 to C-1. So compound **1** was established as (2*E*,4*E*)-5-(3-hydroxyphenyl)- penta-2,4-dienamide.

Table 1. NMR Data for compound **1** (MeOD, 400 MHz)

position	δ_C	δ_H(*J* in Hz)	position	δ_C	δ_H(*J* in Hz)
1	169.9		7	113.0	6.92 (1H, *s*)
2	123.0	6.14 (1H, *d*, *J* = 15.2)	8	157.5	
3	141.6	7.31 (1H, *dd*, *J* = 15.2, 9.8)	9	115.6	6.75 (1H, *d*, *J* = 8.0)
4	126.0	6.90 (1H, *dd*, *J* = 16.3, 9.8)	10	129.4	7.16 (1H, *t*, *J* = 8.0)
5	139.5	6.89 (1H, *d*, *J* = 16.3)	11	118.4	6.98 (1H, *d*, J = 8.0)
6	137.7				

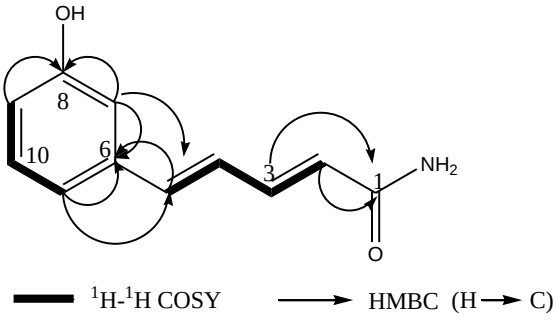

Figure 2. ^{1}H-^{1}H COSY correlations and the selected HMBC correlations of compound **1**

3.2 Antimicrobial activity

The isolates (**1**-**4**) were tested for their activity against *M. tuberculosis* as well as *C. albicans*, *E. coli*, and *S. aureus*. The results were showed in Table 2. **1** and **4** also showed activity against *E. Coli* with MICs of 64 and 32 μg/mL. All compounds showed lower antifungal activity against *C. albicans* than other antibacterial activity.

Table 2. The minimum inhibitory concentrations (MICs in μg/mL) of compounds **1**-**4**.

	M. tuberculosis	*C. albicans*	*E. Coli*	*S. aureus*
1	128	128	64	256
2	64	256	>256	>256
3	32	128	256	128
4	64	128	32	256
Kanamycin	8	-	8	4
Nystatin	-	16	-	-

Supporting Information

Supporting Information accompanied with this paper on http://www.acgpubs.org/RNP

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