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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Endophytic *Streptomyces* sp. Y3111 from traditional Chinese medicine produced antitubercular pluramycins

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Abstract As part of a search for antitubercular substances from natural sources, we screened a library of endophytic microbes (50 strains and 300 crude extracts in total) isolated from traditional Chinese medicines (TCMs) for growth inhibitory activity against Bacillus Calmette-Guérin (BCG). The crude extract of *Streptomyces* sp. strain Y3111, which was associated with the stems of *Heracleum souliei*, showed good anti-BCG activity with an MIC value of 12.5 μg/mL. Bioassay-guided isolation led to four new pluramycin-type compounds, heraclemycins A–D (**1**–**4**). Their structures were determined by different spectroscopic techniques including HRMSESI, 1D NMR, and 2D NMR. This is the first report of pluramycin analogues produced by TCM endophytic microbes as well as the first example of BCG-selective pluramycins. Heraclemycin C (**3**) showed selective antitubercular activity against BCG with a MIC value of 6.25 μg/mL and a potential new mode of action.

Keywords Pluramycins · Heraclemycins · *Heracleum souliei* · Traditional Chinese medicine · Endophytes · Anti-BCG

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Introduction

Bacterial infections constitute an important cause of morbidity and mortality in human populations all over the world. The emergence of acquired resistance to antimicrobial drugs has been observed in almost all pathogenic bacteria (Planson et al. 2011). Tuberculosis (TB) is a contagious airborne disease caused by infection with *Mycobacterium tuberculosis* (Mtb) with a long human history (WHO 2009). Multidrug-resistant tuberculosis as well as TB-HIV coinfection has become a great threat to global health (Nayyar and Jain 2005; WHO 2013; Spigelman 2007; Matteeli et al. 2007). However, the last truly novel drug that was approved for the treatment of TB was discovered 40 years ago. To overcome this problem of antimicrobial resistance, the search for new effective anti-TB drugs with novel molecular scaffolds has never been more intensive.

Natural products derived from microbes have been an important source of TB therapeutics. In order to minimize rediscovery of known compounds and access new and novel molecular scaffolds for drug discovery, rare and previously undiscovered microbial taxa sourced from extreme environments and specialized niches are needed. Plant endophytes represent one such source. Plants have developed

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highly diverse mechanisms to cope with their environment, and it is remarkable that all plants on earth harbor endophytes (Strobel et al. 2003). Many of them are capable of synthesizing bioactive compounds that can be used by the plant for defense against pathogenic fungi and bacteria. Endophytes represent a large variety of microbial adaptations that have developed in special and sequestered environments, and their diversity and specialized habitat make them an exciting field of searching for new medicines (Owen and Hundley 2004).

In responding to this challenge, our laboratory is committed to the study of new antibiotics especially those that are effective against drug-resistant pathogens (Song et al. 2010; Ashforth et al. 2010; Zhang et al. 2007; Liu et al. 2012; Zhuo et al. 2010). With the aim of identifying new anti-TB natural products, we screened a library of endophytic microbes (50 strains and 300 crude extracts in total) isolated from TCMs for growth inhibitory activity against Bacillus Calmette-Guérin (BCG) (Wang et al. 2010), an attenuated strain of the bovine tuberculosis bacillus *Mycobacterium bovis* (Fig. 1). The crude extract of *Streptomyces* sp. Y3111 (Fig. S25), isolated from the stems of TCM *Heracleum souliei*, showed good anti-BCG activity with an MIC value of 12.5 μg/mL. Bioassay-guided isolation yielded four new pluramycin-type compounds named heraclemycins A (**1**), B (**2**), C (**3**), and D (**4**) along with two known compounds, β-indomycinone (**5**) and saptomycin A (**6**). Herein, we report the isolation and structural elucidation of the new metabolites as well as their biological activities against different pathogens.

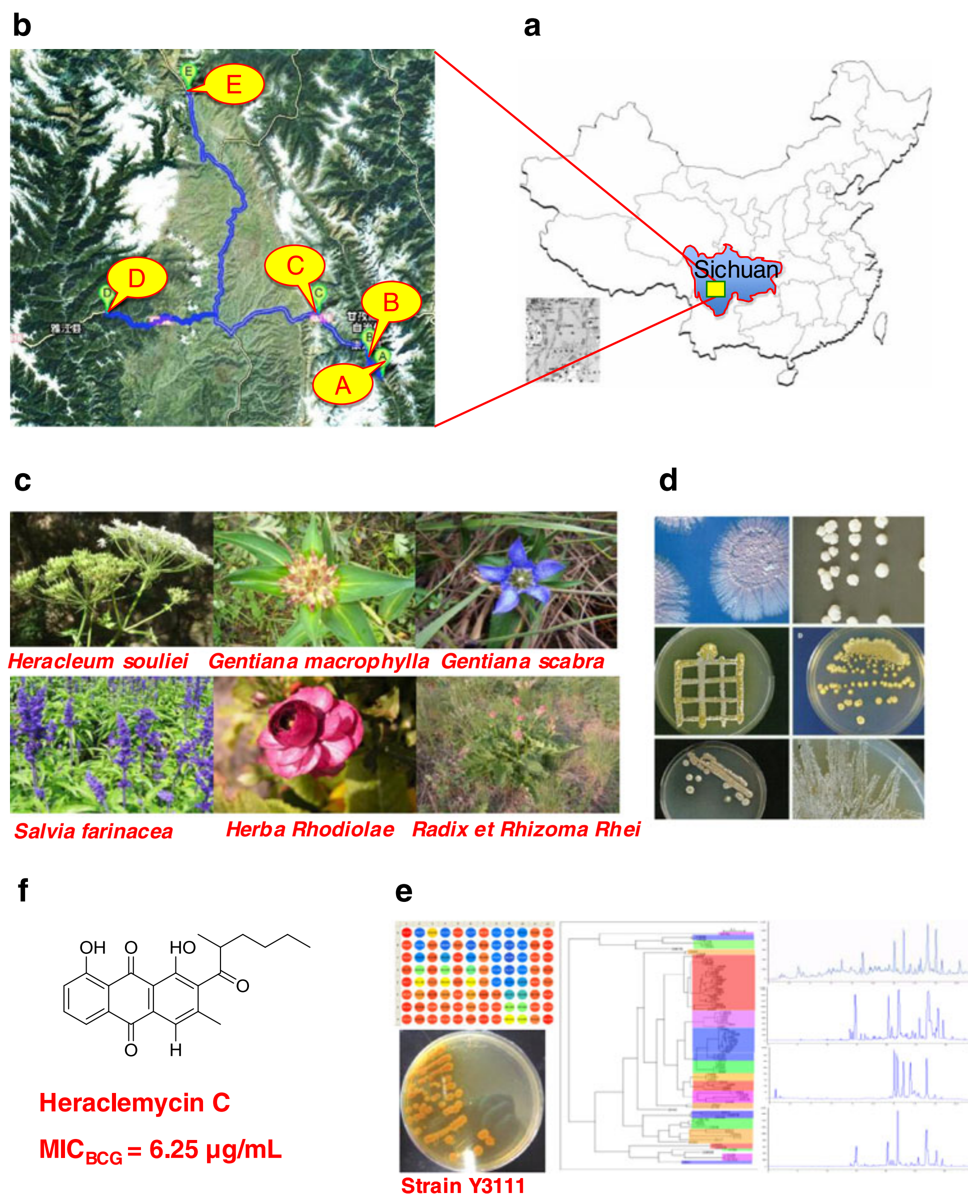

Fig. 1 Overview of systematic searching anti-BCG compounds from endophytic microbes library from TCMs. **a** The location of Sichuan Province in China; **b** the sampling locations of studies endophytic microbes from TCMs on Sichuan plateau, *A* Yajiageng, Kangding County, *B* Laoyulin, Kangding county, *C* Zheduo Mountain, Kangding County, *D* Bajiaolou, Yajiang County, and *E* Longdeng, Daofu County; **c** 11 selected TCMs (Table S5); **d** diversified microbes isolated; **e** evaluation of the constructed natural product library including activity screening, microbes identification, and HPLC analysis. A promising strain Y3111 was selected for further investigation subsequently; **f** bioassay-guided isolation and structure elucidation

Materials and methods

General experimental procedures

Optical rotations were measured on a Perkin-Elmer Model 343 polarimeter. UV spectra were obtained on a Cary 50 spectrophotometer. CD spectra were recorded on a JASCO J-815 spectropolarimeter. NMR spectra were obtained on a Bruker Avance DRX600 spectrometer. Chemical shifts were calibrated internally against the residual signal of the solvent in which the sample was dissolved ($CDCl_3$, δ_H 7.26 and δ_C 77.0). HRESIMS measurements were obtained on a Brukermicro TOF mass spectrometer. Resin HP-20 (Diaion, Japan) and ODS-A (YMC, Japan) were used for purification. RP-HPLC was carried out using an Agilent Chromatorex Zorbax SB C3 (5 μm) semi-preparative column (9.4× 250 mm) on an Agilent 1100 Series separations module equipped with Agilent 1100 Series diode array and/or multiple wavelength detectors controlled using ChemStation Rev.9.03A and Purify version A.1.2 software.

Bacterial material

The endophytic strain Y3111 was isolated from stems of the TCM *H. souliei* collected in Zheduo Mountain of Kangding, Southwest of China (N30°03′, E101°49′, 4006 m) using modified Gause II media (Yuan et al. 2008). The isolation process was as described by Zhao et al. (2011). It was identified as a *Streptomyces* sp. using 16S rRNA gene sequence analysis (Fig. S25). The DNA sequence has been deposited in GenBank (accession no. JQ724543). This strain has been preserved at the China General Microbiological Culture Collection Center (accession no. 4.7122).

Fermentation, extraction and isolation

The strain Y3111 was cultivated on ISP2 agar plates at 28 °C for 7 days. A 250 mL Erlenmeyer flask pre-culture of Y3111, containing 50 mL of M001 medium, was inoculated and incubated with pieces of well-grown agar cultures of the strain at 28 °C (220 rpm) for 48 h. The pre-culture was used to inoculate ten 250 mL Erlenmeyer flask (each with 5 mL of pre-culture and 100 mL M001 medium) and incubated at 28 °C (220 rpm) for 3 days to obtain seed cultures for large-scaled fermentation. Each of the seed cultures was aseptically transferred to 80 250 mL Erlenmeyer flasks containing 100 mL of M001 medium and 5 g of HP20 resin and was harvested after 5 days incubation at 28 °C 220 rpm. The yellow broth was centrifuged to yield supernatant and a mycelial cake with resin. The mycelial cake with resin was extracted three times with 5 L of MeOH and EtOAc, respectively. The MeOH and EtOAc solution were combined and concentrated to give crude extract (2.59 g). The crude extract was then subjected to a solvent partition scheme with *n*-hexane, methylene chloride, and ethyl acetate. The dichloromethane-soluble crude extract (389.9 mg) was further fractionated by ODS-MPLC using gradient elution from 5 to 100 % aqueous MeOH in 80 min to provide eight fractions (1–8). Fraction 2 (12.2 mg) was purified by RP-HPLC using a Zorbax SB C3 (9.4×250 mm) column eluting at a flow rate of 2.5 mL/min with 50 % aqueous acetonitrile to obtain saptomycin A (**6**) (1.2 mg, t_R=27.0 min). Fraction 4 (43.8 mg) was then purified by RP-HPLC using Zorbax SB C3 (9.4× 250 mm) column eluting at a flow rate of 2.5 mL/min with 60 % aqueous acetonitrile to obtain β-indomycinone (**5**) (6.6 mg, t_R=16.6 min). The hexane-soluble fraction (976.2 mg), showing anti-BCG activity with an MIC value of 12.5 μg/mL, was chromatographed over Sephadex LH20 (CH_2Cl_2-MeOH=1:1) to yield 9 subfractions (A–I). Fraction C (29.7 mg) was purified by reversed-phase HPLC on a Zorbax SB C3 (9.4×250 mm) column, yielding eight fractions (C1–C8). Fraction C5 was purified by RP-HPLC using a Zorbax SB C3 (9.4×250 mm) column eluting at a flow rate of 2.5 mL/min with 85 % aqueous acetonitrile to obtain compound **1** (4.0 mg, t_R=10.4 min). Fraction C6 (36.5 mg) was chromatographed by RP-HPLC using a Zorbax SB C3 (9.4×250 mm) column eluting at a flow rate of 2.5 mL/min with 80 % aqueous acetonitrile to yield compound **3** (1.0 mg, t_R=11.5 min) and compound **2** (2.3 mg, t_R=14.0 min). Fraction C7 was purified by RP-HPLC using a Zorbax SB C3 (9.4×250 mm) column eluting at a flow rate of 2.5 mL/min with 70 % aqueous acetonitrile to obtain compound **4** (1.8 mg, t_R=26.2 min).

NMR information of compounds 5 and 6

β-Indomycinone (**5**): ^{1}H NMR ($CDCl_3$) δ 1.60 (3H, d, *J*=6.8 Hz), 1.69 (3H, s), 2.76 (1H, dd, *J*=14.4, 8.3 Hz), 2.90 (1H, dd, *J*=14.4, 7.0), 3.00 (3H, s), 5.39 (1H, m), 5.69 (1H, m), 6.55 (1H, s), 7.35 (1H, d, *J*=8.4 Hz), 7.67 (1H, t, *J*=7.8 Hz), 7.81 (1H, d, *J*=7.2 Hz), 12.85 (1H, s); ^{13}C NMR ($CDCl_3$) δ 13.20, 24.30, 26.26, 38.35, 73.83, 109.80, 116.92, 119.65, 123.28, 125.57, 125.91,126.44, 129.84, 132.44, 136.68, 150.18, 156.28, 162.85, 171.91, 181.93, 187.61.

Saptomycin A (**6**): ^{1}H NMR ($CDCl_3$) δ 1.44 (3H, d, *J*=7.2 Hz), 1.67 (3H, d, *J*=1.2 Hz), 2.96 (1H, dq, *J*=4.6, 7.1 Hz), 3.00 (3H, s), 5.00 (1H, t, *J*=3.6 Hz), 5.48 (1H, m), 5.63 (1H, m), 6.26 (1H, s), 7.35 (1H, d, *J*=7.8 Hz), 7.67 (1H, t, *J*=7.8 Hz), 7.80 (1H, d, *J*=7.2 Hz), 8.06 (1H, s), 12.64 (1H, s); ^{13}C NMR ($CDCl_3$) δ 13.01, 13.55, 24.42, 45.11, 68.92, 112.36, 116.84, 119.67, 125.72, 126.58, 128.18, 130.25, 136.18, 136.85, 150.30, 156.63, 162.89, 170.99, 179.15, 181.89, 187.79.

Media

ISP-2: yeast extract 0.4 %, malt extract 1 %, dextrose 0.4 %, agar 2 %; pH 7.2 was adjusted prior to sterilization.

M001: starch 2 %, peptone 0.4 %, yeast extract 0.8 %, $CaCO_3$ 0.1 %; pH 7.2 was adjusted prior to sterilization.

Anti-BCG assay

Identification of inhibitors was performed in an aerobic, logarithmic growth screen of BCG as previously reported (Wang et al. 2010). The BCG used was a *M. bovis* BCG 1173P2 strain transformed with green fluorescent protein (GFP) constitutive expression plasmid pUV3583c with direct readout of fluorescence as a measure of bacterial growth. BCG was grown at 37 °C to mid log phase in Middle brook 7H9 broth (Becton Dickinson) supplemented with 10 % OADC enrichment (Becton Dickinson) 0.05 % tween-80 and 0.2 % glycerol, which then adjusted to OD_{600}=0.025 with culture medium as bacterial suspension. Aliquots (80 μL) of the bacterial suspension were added to each well of the 96-well microplates (clear flat-bottom), followed by adding compounds (2 μL in DMSO), which were serially twofold diluted. Isoniazid served as positive control and DMSO as negative control. The plate was incubated at 37 °C for 3 days, and GFP fluorescence was measured with Multi-label Plate Reader using the bottom read mode, with excitation at 485 nm and emission at 535 nm. MIC is defined as the minimum concentration of drug that inhibits more than 90 % of bacterial growth reflected by fluorescence value.

MIC determination

Antimicrobial assays were performed according to the Antimicrobial Susceptibility Testing Standards outlined by the Clinical and Laboratory Standards Institute (CLSI) (NCCLS 1999) using the bacteria *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633), and methicillin-resistant *S. aureus* (MRSA). For each organism, a loopful of glycerol stock was streaked on an LB agar plate and incubated overnight at 37 °C. A single bacterial colony was picked and suspended in Mueller-Hinton Broth to approximately 1×10^4 CFU/mL. A twofold serial dilution of each compound to be tested (4,000 to 31.25 μg/mL in DMSO) was prepared, and an aliquot of each dilution (2 μL) was added to a 96-well flat-bottom microtiter plate (Greiner). Vancomycin and ciprofloxacin were used as positive controls and DMSO as the negative control. An aliquot (78 μL) of bacterial suspension was then added to each well (to give final compound concentrations of 100 to 0.78 μg/mL in 2.5 % DMSO), and the plate was incubated at 37 °C aerobically for 16 h. Finally, the optical density of each well at 600 nm was measured with an EnVision 2103 Multi-label Plate Reader (Perkin-Elmer Life Sciences). MIC values were defined as the minimum concentration of compound that inhibited visible bacterial growth. All the experiments were performed in triplicate.

Time-kill studies

Time-kill analyses were performed according to CLSI method M26-A.20 (1999). Bacillus Calmette-Guérin Pasteur 1173P2 was cultured for 6 days at 37 °C in 7H9 broth. Cells were diluted in medium to an initial OD_{600} of 0.025 as bacterial suspension. Either compound heraclemycin C, adjusted to final concentrations of 0.5, 2, and 8 times of the MIC, or heraclemycin D, adjusted to final concentrations of 0.5, 2, and 4 times the MIC, or heraclemycins A and B, adjusted to final concentrations of 100, 25, and 6.25 μg/mL, or DMSO was then added. Aliquots (80 μL) of the cultures were removed at 0, 12, 24, 36, 48, 60, 72, 84, 96, and 108 h of incubation, and GFP fluorescence was measured with Multi-label Plate Reader using the bottom read mode, with excitation at 485 nm and emission at 535 nm. Rates of killing were determined by measuring the reduction in GFP fluorescence of viable bacteria (log_{10} RFU/mL) at each time point with fixed concentrations of the compound. Experiments were performed in triplicate.

Results

An endophyte-derived microbial library was constructed from TCMs in the plateau area, Sichuan province, China. A total of 50 endophytic microbes were fermented in six different media (AM2, M001, M12, MPG, M21, and M9) which yielded 300 crude extracts. The anti-BCG activity of these crude extracts was evaluated, and 14 (4.6 %) active extracts showed activity with dilution factors larger than eight (Fig. S26). The bioactivity and chemical diversity evaluation was selected for identification of constituents as its crude extract showed good anti-BCG activity with an MIC value of 6.25 μg/mL. The strain Y3111, which was isolated from the stems of TCM *H. souliei*, was identified as a member of *Streptomyces* according to its 16 s rRNA gene sequence.

The strain Y3111 was grown in 250 mL Erlenmeyer flask containing 100 mL of M001 medium in the presence of HP20 resin for 5 days incubation at 28 °C, 220 rpm, after first-stage seeding in 50 mL for 48 h, 100 mL of M001 for 3 days, upon which cell mass and resin were collected by centrifugation and then extracted with MeOH and EtOAc, respectively. Preliminary analysis of the combined crude extract by HPLC exhibited the presence of several major peaks of highly aromatic compounds (**1**–**6**) that were subsequently isolated via solvent partitioning and chromatography.

Compound **1** was isolated as a yellow powder. The molecular formula was deduced as $C_{24}H_{20}O_5$ from its HR-

ESI-MS (Fig. S1) (m/z 389.1421 $[M+H]^+$, calcd. for 389.1389), which possessed 15° of unsaturation. The UV spectrum of **1** showed maximum absorbance at $\lambda_{max}(\log \varepsilon)$ 198.0 (5.6), 201.0 (5.6), 206.0 (5.6), 239.0 (5.4), 263.0 (5.1), and 417.0 (4.6)nm in MeOH.

The ^1H and ^{13}C NMR spectra in combination with 2D NMR data (COSY, HMQC, and HMBC) (Table 1 and Fig. S2–S6) revealed the presence of three carbonyl groups C-10, C-5, and C-12 (δ_C 187.4, 182.1, and 179.4) conjugated with double bonds, three spin–spin coupling aromatic protons in an ABX system H-6, H-7, and H-8 [δ_H/δ_C 7.80 (d, J=7.8 Hz)/119.4, 7.66 (t, J=7.8 Hz)/136.4, and 7.34 (d, J=7.8 Hz)/125.4], three methyl groups CH_3-20, CH_3-16, and CH_3-11 [δ_H/δ_C 1.60 (d, J=6.7 Hz)/13.0, 1.44 (d, J=6.9 Hz)/17.8, and 3.00 (s)/24.3], four olefinic carbons, including two isolated olefins CH-18 and CH-19 [δ_H/δ_C 5.41 (dt, J=10.8, 8.8Hz)/126.8, 5.54 (dq, J=10.8, 6.7 Hz)/126.7], and an α,β-unsaturated ketone carbon CH-13 and C-14 [δ_H/δ_C 6.23 (s)/111.4 and δ_C 172.6]. One sharp hydrogen resonance at δ_H 12.92 lacked correlation in the HMQC spectrum of **1** (Table 1) and was therefore determined to be hydroxyl group substituted at the aromatic C-9 (δ_C 162.7) based on the HMBC correlations of OH-9 to C-9, C-8, and C-9a.

A Dictionary of Natural Products database search using information from combined UV and 1D NMR data indicated the likelihood of a pluramycin skeleton. Compound **1** exhibited great similarity with the β-indomycinone (**5**), a pluramycin class of antibiotics sourced from a deep-sea actinomycete *Streptomyces* sp. (Brockmann 1968), except that the oxygenated quaternary carbon in β-indomycinone was replaced by a methine carbon C-15 [δ_H/δ_C 2.85 (m)/39.1]. This indicated that **1** was a new member of the pluramycins and its structure was established as shown in Fig. 2. All of the signals were assigned unambiguously based on 2D NMR

Table 1 ^1H (600 MHz) and ^{13}C (150 MHz) NMR Data of 1–4 in $CDCl_3$

Pos.	1		2		3		4	
	δ_C, mult	δ_H, mult (J in Hz)	δ_C, mult	δ_H, mult (J in Hz)	δ_C, mult	δ_H, mult (J in Hz)	δ_C, mult	δ_H, mult (J in Hz)
1	156.8, C		156.9, C		159.8, C		159.7,C	
2	126.6, C		126.7, C		136.4, C		126.4,C	
3	149.8, C		149.8, C		146.4, C		146.5,C	
4	125.6, CH	8.02 s	125.6, CH	8.05 s	122.4, CH	7.70 s	122.4,CH	7.70 s
4a	136.1, C		136.1, C		133.2, C		133.5, C	
5	182.1, C		182.2, C		181.7, C		181.6, C	
5a	132.4, C		132.4, C		133.6, C		133.6, C	
6	119.4, CH	7.80 d (7.8)	119.4, CH	7.81 d (7.8)	120.4, CH	7.85 d (7.8)	120.4,CH	7.84 d (7.2)
7	136.4, CH	7.66 t (7.8)	136.4, CH	7.67 t (7.8)	137.5, CH	7.70 t (7.8)	137.5,CH	7.70 t (7.8)
8	125.4, CH	7.34 d (7.8)	125.4, CH	7.36 d (7.8)	125.0, CH	7.32 d (8.4)	125.0,CH	7.32 d (7.8)
9	162.7, C		162.7, C		162.7, C		162.7, C	
9a	117.0, C		117.0, C		116.0, C		115.5, C	
10	187.4, C		187.4, C		186.3, C		186.8, C	
10a	119.9, C		120.0, C		114.2, C		114.2, C	
11	24.3, CH_3	3.00 s	24.4, CH_3	3.02 s	20.7, CH_3	2.38 s	20.7, CH_3	2.38 s
12	179.4, C		179.5, C		209.1, C		206.4, C	
13	111.4, CH	6.23 s	111.3, CH	6.25 s	47.0,CH	3.17 m	47.2, CH	3.23 m
14	172.6, C		173.3, C		15.4,CH_3	1.19 d (7.2)	15.3,CH_3	1.20 d (7.2)
15a	39.1, CH	2.85 m	39.1, CH	2.79 m	31.8,CH_2	1.81 dt (9.7,7.4)	29.2,CH_2	2.52 m
15b								2.34 m
16	17.8, CH_3	1.44 d (6.9)	18.5, CH_3	1.43 d (6.6)	22.9,CH_2	1.30–1.36 m	127.5,CH	5.40 dt (17.6,6.6)
17a	31.7, CH_2	2.72 dq (14.5,7.3)	34.2, CH_2	1.96 m	29.5, CH_2	1.30–1.36 m	126.4, CH	5.55 dq (17.6, 6.6)
17b		2.50 dq (14.5,7.3)		1.70 m				
18	126.8, CH	5.41 dt (10.8, 8.8)	29.6, CH_2	1.31–1.37 m	14.1, CH_3	0.90 t (6.6)	24.9, CH_3	1.62 d (6.6)
19	126.7, CH	5.54 dq (10.8, 6.7)	22.7, CH_2	1.31–1.37 m	–		–	
20	13.0, CH_3	1.60 d (6.7)	14.1, CH_3	0.90 t (7.2)	–		–	
1-OH	–		–			12.34 s		12.37 s
9-OH		12.92 s		12.93 s		12.00 s		11.99 s

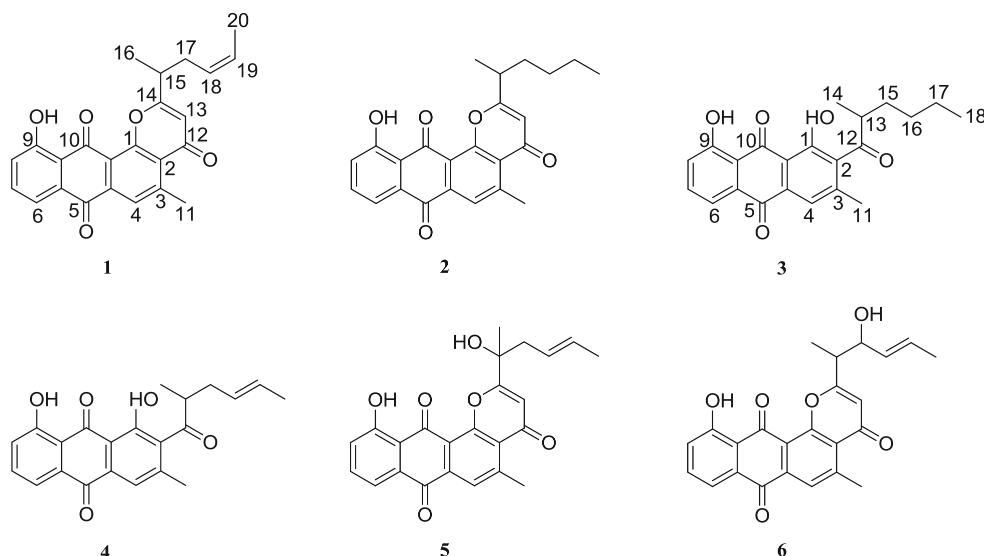

Fig. 2 Structure of isolated pluramycins (**1**–**6**)

(HMQC, COSY, and HMBC) experiments (Fig. 3 and Table S1). The *cis* geometry of the C-18, C-19 double bond could be determined by the *J* value (10.8 Hz) between H-18 and H-19.

Compound **2** was isolated as a yellow powder, and its molecular formula was found to be $C_{24}H_{22}O_5$, which possessed 14° of unsaturation, on the basis of its HR-ESI-MS (Fig. S7) (*m/z* 391.1529 $[M+H]^+$, calcd. for 391.1545). The UV spectrum of **2** showed maximal absorbance at λ_{max}(log ε) 199.0 (5.3), 206.0 (5.3), 239.0 (5.1), 267.0 (4.8), and 416.0 (4.3)nm in MeOH. Comparison of ^{1}H and ^{13}C NMR of **2** revealed a high similarity to those of **1**, except the absence of the double bond at C-18 and C-19 in **2**. As that of **1**, the ^{1}H NMR and ^{13}C NMR spectra of **2** showed a characteristic set of signals of the pluramycin class compounds, including three protons of 1,2,3-trisubstituted benzene ring H-6, H-7, and H-8 [δ_H/δ_C 7.81 (d, *J*=7.8 Hz)/119.4, 7.67 (t, *J*=7.8 Hz)/136.4, 7.36 (d, *J*=7.8 Hz)/125.4], one aromatic proton H-4 [δ_H/δ_C 8.05(s)/125.6], one olefinic H-13 [δ_H/δ_C 6.25 (s)/111.32], a hydrogen-bonded phenol proton (δ_H 12.93), and three methyl groups CH_3-11, CH_3-16, and CH_3-20 [δ_H/δ_C 3.02 (s)/24.4, 1.43 (d, *J*=6.6 Hz)/18.5, and 0.90 (t, *J*=7.2 Hz)/14.1]. Based on the above evidences together with the 2D (COSY, HMQC, and HMBC) data of **2** (Table S2 and Fig. S8–S12), the structure of **2** could be determined (Fig. 2).

Compound **3** was obtained as a yellow powder. The molecular formula was determined by HR-ESI-MS (Fig. S13) as $C_{22}H_{22}O_5$ (*m/z* 367.1568 $[M+H]^+$, calcd. for 367.1501), requiring 12° of unsaturation. The ^{1}H NMR spectrum of **3** contained resonances for two phenolic hydroxyl hydrogen [δ_H12.00 and 12.34]. Comparison of the ^{1}H and ^{13}C NMR spectra of **3** with those of **2** revealed that both shared a similar anthraquinone skeleton, and the significant differences indicated the reduction of the γ-pyrone moiety, culminating in the absence of the ethenyl group in the ^{1}H and ^{13}C NMR spectrum and the presence of an unconjugated carbonyl functionality [δ_C 209.1]. The location of the hydroxyl group [δ_H 12.34] at C-1 was corroborated by the HMBC correlation form 1-OH to C-2 [δ_C 136.4], C-10a [δ_C 114.2] and C-1 [δ_C 159.8]. All the signals of **3** were assigned unambiguously based on 2D NMR experiments (Table S3 and Fig. S14–S18) and its structure was determined as shown in Fig. 2.

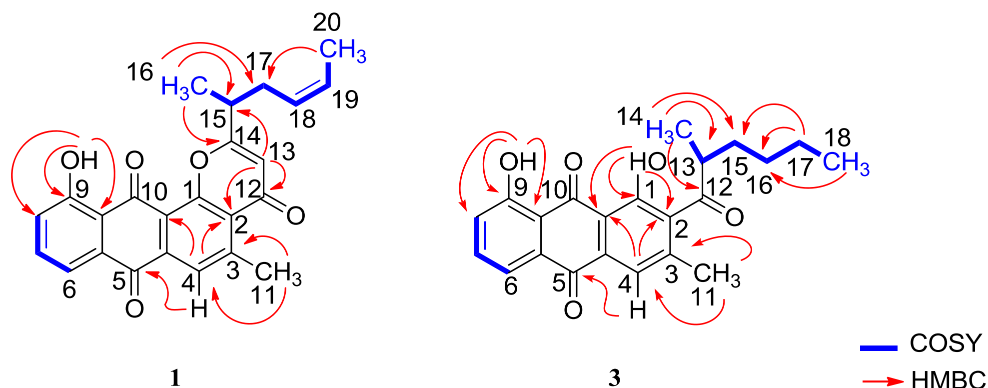

Fig. 3 Observed COSY and key HMBC correlations of **1** and **3**

Table 2 Antimicrobial activities of compounds 1–6

Organism (strain)	Minimum Inhibitory Concentration (μg/mL)				
	1	2	3	4	Control
Bacillus Calmette-Guérin (Pasteur 1173P2, BCG)	100	>100	6.25	25	0.05[a]
Staphylococcus aureus (ATCC 6538)	50	50	>100	25	1[b]
Methicillin-resistant *S. aureus* Clinical strain of Chaoyang Hospital	50	50	>100	25	1[b]
Bacillus subtilis (ATCC 6633)	100	>100	>100	50	0.5[b]
Pseudomonas aeruginosa (PAO1)	>100	>100	>100	>100	1[c]

[a] Isoniazid
[b] Vancomycin
[c] Ciprofloxacin

Compound **4** was obtained as a yellow powder, and the HR-ESI-MS data (Fig. S19) of **4** suggested the molecular formula $C_{22}H_{20}O_5$ (*m/z* 365.1440 $[M+H]^+$, calcd. for 365.1344). In the ^1H NMR spectrum signals of **4** were very similar to those of **3** except for the presence of double bond at C-16 and C-17 in **4** [δ_H/δ_C 5.40 (dt, *J*=17.6, 6.6 Hz)/127.5, 5.55 (dq, *J*=17.6, 6.6 Hz)/126.4]. The *trans* geometry of the C-18, C-19 double bond could be determined by the *J* value (17.6 Hz) between H-16 and H-17. Thus, the structure of **4** could be determined, and all the NMR signals were assigned unambiguously based on 2D NMR experiments (Table S4, Fig. S20–S24, and Fig. 2).

All of the isolated compounds have been evaluated for their biological activities against *M. bovis* BCG, *S. aureus*, MRSA, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (Table 2). Compounds **3** and **4** showed significant activity against BCG with the MIC values of 6.25 and 25 μg/mL, respectively. Compound **1** showed weak activity with an MIC value of 100 μg/mL, whereas **2**, **5**, and **6** were inactive against BCG. These results indicated that the absence of the γ-pyrone is probably essential for the BCG inhibitory activity. On the other hand, compounds **1** and **4** exhibited anti-Gram positive bacteria activity including anti-BS, anti-SA, and anti-MRSA. However, compounds **2**, **3**, **5**, and **6** were unable to inhibit these activities up to 100 μg/mL, suggesting that hexenyl side chain at C-13 or C-15 is important for these microbe inhibitory activities.

Time-kill experiments of the new pluramycin-type compounds against BCG were performed (Fig. 4). Antimicrobials were considered bactericidal at the lowest concentration that reduced the original inoculum by ≥3 log_{10} RFU/mL (99.9 %) at each of the time periods and bacteriostatic if the inoculum was reduced by 0 to 3 log_{10} RFU/mL (Tatiana et al. 2005). As shown in Fig. 4, maximum killing for heraclemycin C was observed at concentrations of 8× the MIC, with a 1.2-log drop in the numbers of RFU/mL

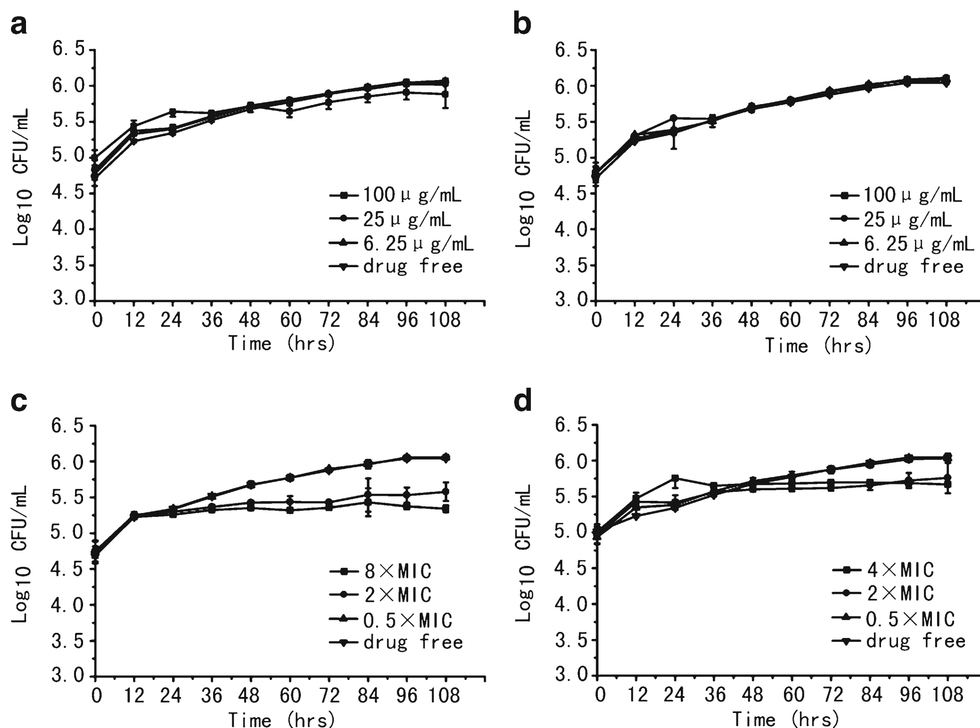

Fig. 4 Killing activities of compounds **1**–**4** against *M. bovis* BCG. Compounds were added to cultures at time zero, and samples were processed as described in "Materials and Methods". Bacterial strains: Bacillus Calmette-Guérin Pasteur 1173P2. **a** Heraclemycin A (**1**), (**b**) heraclemycin B (**2**), (**c**) heraclemycin C (**3**), (**d**) heraclemycin D (**4**)

occurring by 108 h after the addition of the compound, consistent with a bacteriostatic effect (Fig. 4c). Just slightly less than 1.2-log drop in RFU/mL was also observed for heraclemycin D at 2× and 4× the MIC (Fig. 4d). However, even in the presence of 100 μg/mL heraclemycins A and B, there were no obvious differences between compounds treatment and control, suggesting an inactive effect on mycobacteria (Fig. 4a, b).

Discussion

Natural products have been a rich source of lead molecules in drug discovery since they possess high chemical diversity and many unique biological activities (Kellenberger et al. 2011). Around 50 % of all small molecules approved drugs up to 2007 were either directly derived from naturally occurring compounds or were inspired by a natural product (Li and Vederas 2009). Natural products derived from microbes and medicinal plants have played a critical role in TB drug discovery.

Previous studies on the bioactive substances from endophytes indicated several compounds with antibacterial (Castillo et al. 2003; Miller et al. 1998), antivirus (Guo et al. 2000), anticancer (Strobel et al. 1996), antioxidant (Strobel et al. 2002), insecticidal (Daisy et al. 2002), and antidiabetic (Zhang et al. 1999). In our present investigation, we identified the first example of pluramycin analogues produced by TCM endophytic microbes and the first BCG-selective pluramycin which indicates a potentially unique mode of action. Previously reported pluramycin antibiotics containing the 4H-anthra [1,2-b] pyran-4,7,12-trione nucleus to which amino sugars such as angolosamine and vancosamine are typically attached by *C*-glycosidic linkage at C-6 and C-8 (Schumacher et al. 1995) were found to have versatile and strong antimicrobial and anticancer activities (Brockmann 1968; Ogawara et al. 1966). Compared to other natural sources, endophytes are a poorly investigated group of microorganisms that represent 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 (Strobel et al. 2004). Our investigations confirm that screening on endophytes offers a valuable approach towards the discovery new anti-TB natural products. In addition, our study extends knowledge of the secondary metabolism of an endophyte associated with TCM *H. souliei* (Y3111). Bioassay-guided fractionation yieded the structurally diverse heraclemycins A–D, and in particular heraclemycin C, which showed strong anti-BCG activity with a MIC value of 6.25 μg/mL.

Therefore, we have demonstrated that screening for antitubercular small molecules from endophytic microbes associated with TCMs can facilitate the discovery of new natural products and potentially new and novel molecular scaffolds for drug discovery.

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