HL
http://heteroletters.org

ISSN: 2231 – 3087(print)
ISSN: 2230 – 9632 (Online)
Coden HLEEAI
Vol. 5: (1), 2015, 21-26

ISOLATION AND CHARACTERIZATION OF THIOLUTIN FROM *STREPTOMYCES SP.* KIB0393

Yingpan Song, Jie Zhang, Gang Chen*

College of Chemistry and Chemical Engineering, Xi'an Shiyou University, Xi'an, 710065, China
E-mail: gangchen@xsyu.edu.cn

Abstract Strain Kib0393 was isolated from the soil under the herbal plant, *Panax notoginseng*, which was collected from Kunming, Yunnan, China and was identified as *Streptomyces* based on its sequence at the internal transcribed spacer (ITS) regions. From the extracts of fermentation broth, thiolutin was isolated. Its structure was elucidated by ^{1}H –NMR and the ^{13}C-NMR spectra. In addition, bioactivity research shows that thiolutin has potently antibiotic activities.

Keywords: Thiolutin, *Streptomyces*, Antimicrobial compounds, Structure elucidation

Introduction

China medicinal plants play an important role in the search for new treatments and *Panax notoginseng* is a famous traditional herb in Kunming, Yunnan, China that has been found to have hepatoprotection and antitumor activities[I]. *Streptomyces* is the largest antibiotic-producing genus, producing antibacterial, antifungal, and antiparasitic drugs, and also a wide range of other bioactive compounds, such as immunosuppressants[II]. Over two-thirds of the clinically useful antibiotics of natural origin were produced by *Streptomycetes*[III]. In this study, we report for the first time the isolation and identification of *Streptomyces sp.* Kib0393 with antibacterial activity from soil under the herb *Panax notoginseng.* We characterized the secondary metabolites of this strain and further investigated their potential antibacterial activity.

Materials and methods

Soil enrichment procedures

Two gram of rhizosphere soil was obtained from the herbal plant (*Panax notoginseng*) in Kunming,Yunnan, using previously described methods[IV]. Serial dilutions of treated samples were prepared in sterile water and inoculated onto the plates of humic acid-vitamin (HV) agar medium according to the method of Hayakawa et al[V]. Samples were also inoculated onto water yeast extract (WYE) agar[VI]. Humic acid-vitamin agar is a minimal medium containing

Na_2HPO_4 0.05%, KCl 0.17%, $MgSO_4 \cdot 7H_2O$ 0.005%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CaCO_3$ 0.002% at pH 7.2, supplemented with 0.1% (w/v) of humic acid as carbon source. Once colonies were growing on the HV and WYE agar, individual colonies were transferred onto ISP Medium 4 for further maintenance and analysis. ISP Medium 4 containing Soluble Starch 1%, K_2HPO_4 0.1%, $MgSO_4$ 0.1%, NaCl 0.1%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, $FeSO_4$ 0.0001%, $MnCl_2$ 0.0001%, $ZnSO_4$ 0.0001%, Agar 2%.

Identification of the edaphic isolate: *Stretomyces sp.* Kib0393 was grown on ISP4 agar plate for 5 days at 28°C. Genomic DNA was extracted and purified according to the general procedure. For identification and differentiation, the 16S rRNA region was amplified and sequenced. The 16S rRNA region was amplified by PCR with the universal primers PA(5'-CAGAGTTTGATCCTGGCT-3') and PB(5'-AGGAGGTGATCCAGCCGCA-3'). The PCR products were then purified with Mutifunctional Gel Extraction Kit(Bioteke) and sequenced from BeiJing Zixi Bio Tech Co., Ltd. The sequencing results were aligned with the nucleotide-nucleotide database (BLASTn) of the U.S. National Center for Biotechnology Information (NCBI) for final identification of the edaphic isolate.

Fermentation, extraction, and isolation: The seed solution were carried out in 250mL baffle Erlenmeyer flasks. Each flask was filled with 50 mL of Tryptone Soy Broth (30g/L) and cultivated for 2 days at 28°C on a rotary shaker (250 rpm). The seed solution was cultivated in 18 L medium A at 30°C while shaking at 250 rpm for 6 days. Seventy two One-liter Baffled flasks each contained 10 ml seed solution 5.0 g glucose, 2.5g D-maltose, 1.25 g yeast extract dissolved in 250 mL of distilled water. The cultures (18 L) were centrifuged for 20 min at 6000 rpm. Then the supernatant was extracted 3 times by shaking with an equal volume of ethyl acetate. The organic phase was evaporated to dryness to yield 12.05g of ethyl acetate extract. The combined organic extracts were subjected to silica gel CC, eluting with a gradient of light petroleum-ethyl acetate (20:1) to ethyl acetate-methyl alcohol (1:1). Fractions were collected and combined by TLC examination. Fractions were purified by Sephadex LH-20 chromatography, eluting with mixtures of $CHCl_3$-MeOH (1:1). Fractions containing the desired compounds were further purified by semipreparative HPLC (HITACHI HPLC system; YMC− Triart C18 column, 250 × 10 mm; DAD detector) with a flow rate of 3 mL/min. Fractions A were isocraticly eluted by 35% MeOH for 32min,which yielded thiolutin (5.6 mg).

General: ^1H NMR (600 MHz), ^{13}C NMR (150 MHz) were recorded in DMSO using a Bruker Avance III-600 spectrometer (Bruker Corporation, Switzerland), and TMS was used as internal standard. UV spectra were recorded on Shimadzu UV-2401PC UV-VIS Recording Spectrophotometer (Shimadzu Corporation, Japan). IR spectra were measured on a Bruker Tensor 27 FTIR Spectrometer (KBr) (Bruker Corporation, Germany). ESI-MS spectra were recorded using a Waters Xevo TQ-S Ultrahigh Pressure Liquid Chromatography Triple Quadrupole Mass Spectrometer (Waters Corporation, UK). HR-ESI-MS data were obtained using an Agilent G6230 Q-TOF mass instrument (Agilent Corporation, USA). Column chromatography (CC) was performed using silica gel (Qingdao Marine Chemical Factory, China, 200–300 mesh), Sephadex LH-20 (Pharmacia Biotech Ltd, Sweden). Thin-layer chromatography (TLC) were performed using precoated silica gel GF_{254} plates (Qingdao Marine Chemical Factory). Semipreparative HPLC was performed on a Hitachi Chromaster system (Hitachi, Ltd., Japan) equipped with an YMC-Triart C_{18} column (250 mm × 10 mm

i.d., 5 μm, YMC Corporation, Japan), using a □ow rate of 3.5 mL/min at a column temperature of 25 °C, and detection was performed with a DAD detector.

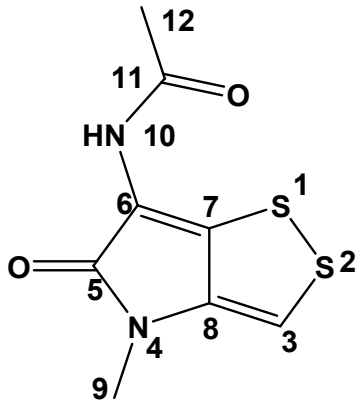

Fig. 1 Structure of thiolutin

Results and discussion

Identification of strain Kib0393

The identification of strain Kib0393 as *Streptomyces* sp. was further corroborated with analyses on its partial 16S rDNA sequence. It has been submitted to Gen Bank with an accession number KM583889. In order to establish the phylogenetic relationships between Kib0393 and other representative strains of the genus *Streptomyces*, approximately 1500-bp fragments of its 16S rDNA were PCR-amplified and 746 bp were sequenced. The BLAST analysis at the NCBI (National Center for Biotechnology Information) website revealed close matches from members of different species of the genus *Streptomyces*. Multiple alignments of the 16S rDNA gene sequence from other species of this genus and the phylogenetic tree were rebuilt with the distance data generated during alignment of these sequences. These alignments showed that strain Kib0393 exhibited a high similarity (99%) with *Streptomyces kasugaensis* **M338-M1^{T}** (Fig. 2).

In Fig 2, the bootstrapped unrooted tree was constructed by the neighbour-joining method from the distance data generated by multiple alignment of the nucleotide sequences. The bootstrap values for major groupings of the members included in the analysis are shown on the main branches.

Structure determination of thiolutin

This antibiotic was obtained as golden yellow amorphous powders. The molecular formula of thiolutin was established as $C_8H_8N_2O_2S_2$, determined by ESIMS [found m/z 251 $[M+Na]^+$]. Spectral features common to this product included: (1) typical IR absorption bands at 3250, 1670, and 1645 cm^{-1}, accounting for two different amide groups, (2) strong UV absorptions at 243, 310 and 388 nm, (3) the appearance on the ^{1}H-NMR spectra (Fig. 3) of two singlets at 7.34 and 3.25 ppm typical for one isolated olefinic proton and one N-CH_3 group included in a amide function, respectively. A singlet at 2.02 ppm accounting for one isolated methyl (NH-CO-CH_3). The ^{1}H -NMR data and the ^{13}C -NMR data of thiolutin, are summarized in Table **1**. The ^{13}C-NMR and DEPT of this antibiotic are shown in Fig. 4 and Fig. 5 respectively: one carbonyl group (□$_C$ 166.21) , three sp^2-hybridized quaternary carbons (□$_C$

from 136.01 to 114.81), one olefinic group (δ_C 111.12), one N-CH_3 group (δ_C 27.59), one methyl (NH-CO-CH_3 δ_C 22.43). Some of these ^1H and ^{13}C NMR signals are typical of dithiolopyrrolone derivatives.

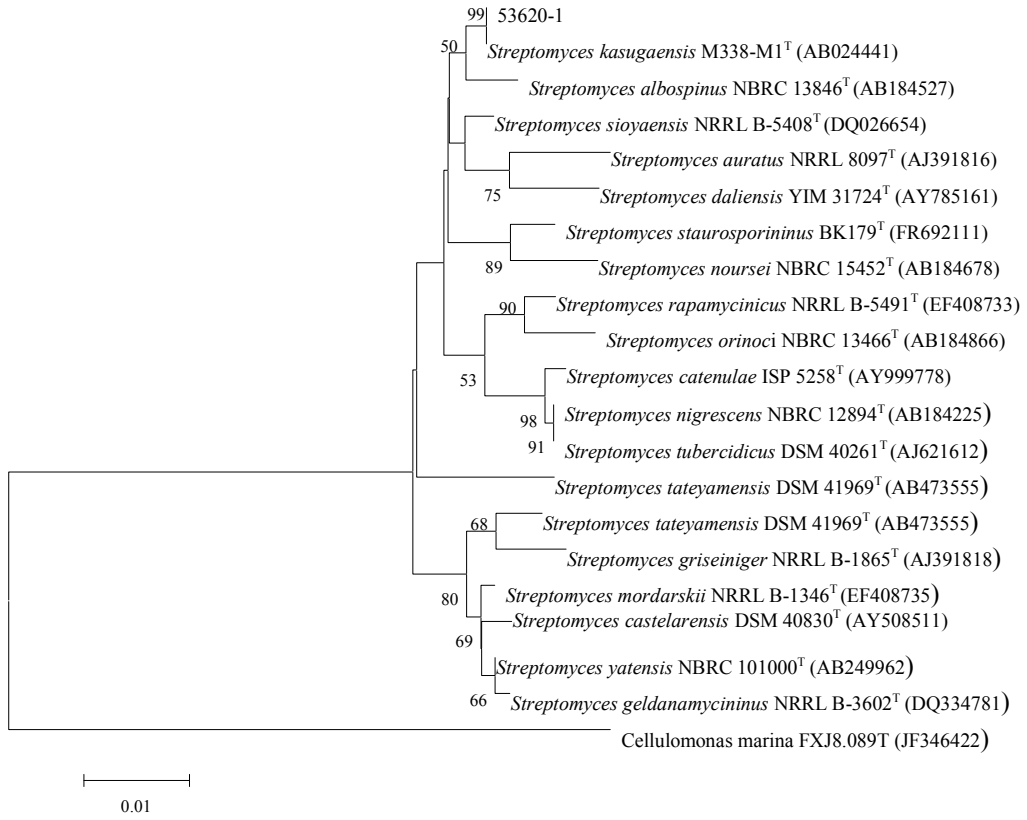

Fig. 2 Phylogenetic relationship of Streptomyces sp. Kib03931 with 20 species of the family Streptomycetaceae based on partial 16S rDNA sequences.

Table **1**. ^1H -NMR data and the ^{13}C-NMR data of thiolutin (DMSO-d_6 δ[ppm]).

Position	^1H NMR (600 MHz)	^{13}C NMR (150 MHz)
3	7.34	111.12
5	—	166.21
6	—	114.81
7	—	136.01
8	—	132.45
9	3.25	27.59
10	10.01	—
11	—	168.90
12	2.02	22.43

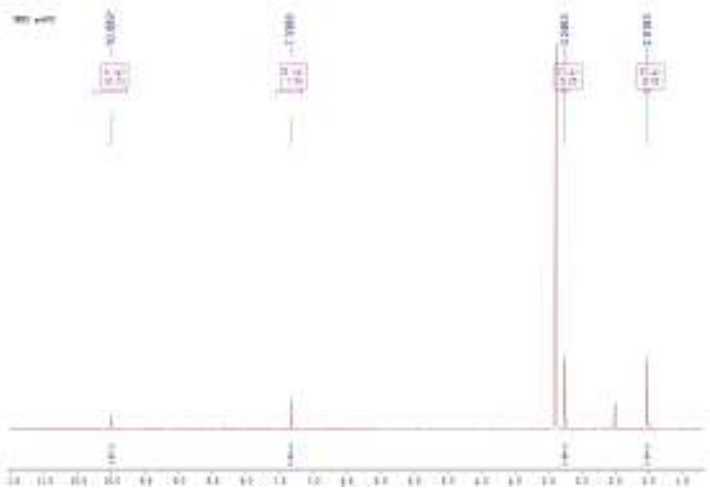

Fig. 3 ^{1}H-NMR of thiolutin

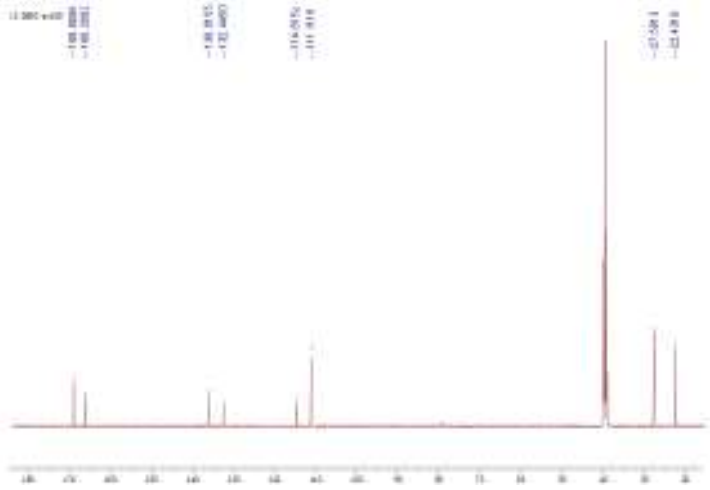

Fig. 4 ^{13}C-NMR of thiolutin

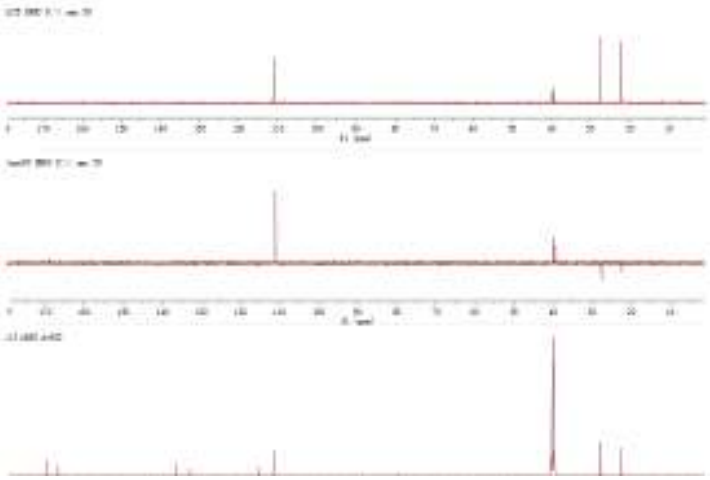

Fig. 5 DEPT of thiolutin

Antibacterial activity

Thiolutin appears to possess activity against eukaryotic cells because it has antifungal activity and is reported to be moderately toxic in mice[VII]. It was shown to potently inhibit endothelial cell adhesion with an IC_{50}<1 μM and to inhibit S180 tumor-induced angiogenesis in mice[VIII]. On the basis of the staphylococcal cross-resistance patterns thiolutin is worthy of further study since it overcames rifampin-resistant genotypes. It could have an application in the treatment of infections caused by rifampin-resistant and sensitive staphylococci[IX].

Acknowledgments

This work was financially supported by the grants from National Science Foundation of China (21376189), Scientific and Technological Plan Projects of Shaanxi Province (2014TG-09) and Scientific Research Program Funded by Shaanxi Provincial Education Department (2013JK0647).

References

I. F.D. Chen, M.C. Wu, H.E. Wang, J.J. Hwang, C.Y. Hong, Y.H. Huang, S.H. Yen, Y.H. Ou, Sensitization of a tumor, butnotnormal tissue, to the cytotoxic effect of ionizing radiation using Panax notoginseng extract, Am J Chin Med 29, 517 (2001).

II. M.G. Watve, R. Tickoo, M.M. Jog, B.D. Bhole, How many antibiotics are produced by the genus Streptomyces?, Arch. Microbiol 176, 386 (2001) .

III. T. Kieser, M.J. Bibb, M.J. Buttner, K.F. Chater, D.A. Hopwood, Practical Streptomyces Genetics (2nd ed.). Norwich, England: John Innes Foundation. (2000).

IV. A. Gonzalez, Investigation of the distribution, diversity, and dynamics of mesophilic nd thermophilic actinomycetes that produce hydrolytic enzymes and antifungal metabolites (Ph.D. thesis). University of Idaho, Moscow, ID (2004).

V. M. Hayakawa, Y. Yoshida, Y. Iimura, Selective isolation of bioactive soil actinomycetes belonging to the Streptomyces violaceusniger phenotypic cluster. J Appl Microbiol 96, 973 (2004).

VI. R.M. Atlas Handbook of microbiological media. CRC Press, Boca Raton, FL (1993)

VII. H. Seneca, J. H. Kane, and J. Rockenbach, Bactericidal, protozoicidal and fungicidal properties of thiolutin. Antibiot. Chemother. **2,** 357 (1952)

VIII. K. Minamiguchi, H. Kumagai, T. Masuda, M. Kawada, M. Ishizuka, T. Takeuchi, Thiolutin, an inhibitor of HUVEC adhesion to vitronectin, reduces paxillin in HUVECs and suppresses tumor cell-induced angiogenesis. Int J Cancer 93, 307 (2001).

IX. A. O'neill, B. Oliva, C. Storey, A. Hoyle, C. Fishwick, and I. Chopra, RNA Polymerase Inhibitors with Activity against Rifampin-Resistant Mutants of Staphylococcus aureus. American Society for Microbiology 44, 3163 (2000).

Received on December 23, 2014.