Phytochemistry 69 (2008) 2363–2366

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

# Streptopyrrolidine, an angiogenesis inhibitor from a marine-derived *Streptomyces* sp. KORDI-3973

Hee Jae Shin [a,*], Tae Sik Kim [a], Hyi-Seung Lee [a], Jung Youl Park [b], In-Kwon Choi [c], Ho Jeong Kwon [c]

[a] *Marine Natural Products Laboratory, Korea Ocean Research & Development Institute, Ansan P.O. Box 29, Seoul 425-600, Republic of Korea*
[b] *C & C Research Labs, 146-141, Annyung-ri, Taean-eup, Hwasung-si, Kyunggi-do 445-976, Republic of Korea*
[c] *Chemical Genomics Laboratory, Department of Biotechnology, College of Life Science & Biotechnology, Yonsei University, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-749, Republic of Korea*

ARTICLE INFO

*Article history:*
Received 4 October 2007
Received in revised form 6 May 2008
Available online 21 July 2008

*Keywords:*
*Streptomyces* sp. KORDI-3973
Angiogenesis inhibitors
Streptopyrrolidine
5-Benzyl-4-hydroxypyrrolidin-2-one
Bioactive natural products

ABSTRACT

Streptopyrrolidine, a benzyl pyrrolidine derivative, was isolated as an angiogenesis inhibitor from the fermentation broth of a marine *Streptomyces* sp. isolated from the deep sea sediment. Its structure was elucidated by extensive 2D NMR and mass spectroscopic analyses. Streptopyrrolidine exhibited significant anti-angiogenesis activity.



## 1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is crucial for the development and progression of pathophysiological changes associated with a variety of disorders, including various cancers, tumor metastases, and retinopathies (Rajesh et al., 2006). Malignant angiogenesis plays a critical role in several fatal diseases including cancer, vascular insufficiency, diabetic retinopathy, and rheumatoid arthritis by an abnormal delivering mechanism of oxygen and nutrients to cell and tissue. Because tumor angiogenesis caused by angiogenic inducers is the most critical factor in the growth of solid tumors (Bouis et al, 2006), as well as their invasion and metastasis, early control of angiogenesis is considered as a promising therapeutic strategy for the related diseases (Hanahan, 1997). Particular efforts have been placed on the discovery of small molecules that can block tumor angiogenesis (McDermott et al., 2006; Keskens and Verweij, 2006).

In our continuing efforts to search for bioactive marine natural products, we isolated an angiogenesis inhibitor from the marine bacterium, *Streptomyces* sp. KORDI-3973. In this paper, we report the isolation, physico-chemical properties, structure elucidation and anti-angiogenesis activity of streptopyrrolidine (**1**).

## 2. Results and discussion

Purification and isolation of streptopyrrolidine was guided by the anti-angiogenesis activity toward capillary tube formation of human umbilical vein endothelial cells (HUVECs). The strain KORDI-3973 was grown in 12L culture (3×) and the culture broth was separated into the mycelium and supernatant by using continuous centrifuge. The supernatant was filtered and then extracted with ethyl acetate. The EtOAc layer, which showed potent anti-angiogenesis activity, was concentrated *in vacuo* and the residual suspension was subjected to ODS open flash chromatography with a stepwise gradient mixture of MeOH/$H_2O$ as eluant. The fraction eluted with MeOH–$H_2O$ (1:1, v/v) was purified by reversed-phase HPLC (YMC ODS-A column, 10 × 250 mm; 30–65% MeOH; flow rate, 1.5 ml/min; UV detection at 210 nm) to yield a pure angiogenesis inhibitor (5.4 mg, Fig. 1). We named this compound streptopyrrolidine (**1**) because it has a pyrrolidine moiety and this study is the first report on the isolation of 1 from a natural source.

Streptopyrrolidine (**1**) was obtained as a colorless amorphous solid: $[\alpha]_D^{25}$ −12 (*c* 0.05, MeOH); IR (KBr) $\nu_{max}$ 3327, 1686, 1454, 1046 $cm^{-1}$; UV $\lambda_{max}$ (MeOH) 234 nm ($\varepsilon$ 1,300), 301 nm ($\varepsilon$ 620). The molecular formula of streptopyrrolidine was established as $C_{11}H_{13}NO_2$ by HREI-MS [*m/z* 191.0951 ($M^+$), $\Delta$ +0.5 mmu] and $^{13}C$ NMR spectroscopic analyses (Table 1). The molecular formula of **1** led to six degrees of unsaturation, three of which were due to carbon–carbon double bonds, one due to the carbonyl carbon,

* Corresponding author. Tel.: +82 31 400 6172; fax: +82 31 400 6170.
*E-mail address:* shinhj@kordi.re.kr (H.J. Shin).

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doi:10.1016/j.phytochem.2008.05.020

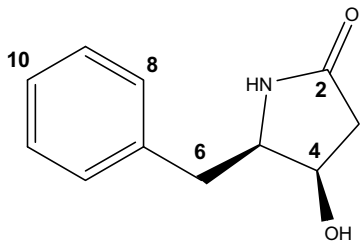


**Fig. 1.** Chemical structure of streptopyrrolidine (**1**).

**Table 1**
1H and $^{13}$C NMR spectroscopic assignments for streptopyrrolidine (**1**) in DMSO

| Position | $^{1}$H (mult, $J$ = Hz) | $^{13}$C (mult) | HMBC |
|---|---|---|---|
| NH | 7.53(1H, *s*) | – | C-3, C-4, C-5 |
| 2 | – | 174.80(*s*) | – |
| 3 | 1.96 (1H, *dd*, 16.3, 2.4)<br>2.37 (1H, *dd*, 16.3, 5.9) | 40.85 (*t*) | C-2 |
| 4 | 4.08 (1H, *m*) | 66.90 (*d*) | – |
| 5 | 3.66 (1H, *q*, 5.7) | 60.06 (*d*) | C-6, C-7a |
| 6 | 2.64 (1H, *dd*, 13.2, 6.3)<br>2.95 (1H, *dd*, 13.2, 7.8) | 34.54 (*t*) | C-4, C-5, C-7, C-8 |
| 7 | – | 138.68 (*s*) | – |
| 8 | 7.25 (1H, *d*, 6.8) | 129.23 (*d*) | C-10 |
| 9 | 7.26 (1H, *dd*, 6.8, 5.3) | 128.18 (*d*) | C-7 |
| 10 | 7.17 (1H, *t*, 5.3) | 125.97 (*d*) | C-8 |
| OH | 5.15 (1H, *br s*) | – | – |

and the rest two were ascribable to two rings. The $^{1}$H NMR spectroscopic data (Table 1) of streptopyrrolidine (**1**) indicated resonances for three aromatic protons, two methylenes, two downfield methines at $\delta$ 3.66 and $\delta$ 4.08, an amide NH at $\delta$ 7.53, and a OH at $\delta$ 5.15. The olefinic protons, two *ortho*-coupled doublets at $\delta$ 7.26 and $\delta$ 7.25 (both $J$ = 6.8 Hz) and one triplet at $\delta$ 7.17 ($J$ = 5.3 Hz), indicated the presence of a monosubstituted benzene ring. The $^{13}$C NMR spectroscopic data (Table 1) suggested that 1 contains one ketone carbon, one sp$^2$ quaternary carbon, three sp$^2$ methines, two sp$^3$ methines, and two sp$^3$ methylenes. The $^{1}$H and $^{13}$C correlations were indicated by the gHSQC spectrum. Long-range couplings from the aromatic proton at $\delta$ 7.26 (H-9) to the quaternary aromatic carbon at $\delta$ 138.68 (C-7) and from H-10 ($\delta$ 7.17) to C-8 ($\delta$ 129.23) also established the presence of a benzene ring. The $^{1}$H–$^{1}$H COSY spectrum of **1** established the partial structure from H-3 to H-6 and also showed the correlation between H-4 ($\delta$ 4.08) and the hydroxyl proton at $\delta$ 5.15 (Fig. 2). The partial structure from H-3 to H-6 and the correlation between NH and H-5 were also confirmed by analysis of the TOCSY spectrum. The presence of a 4-hydroxy-2-pyrrolidone ring was suggested by the COSY correlation peaks (H-3/H-4 and H-4/H-5) and

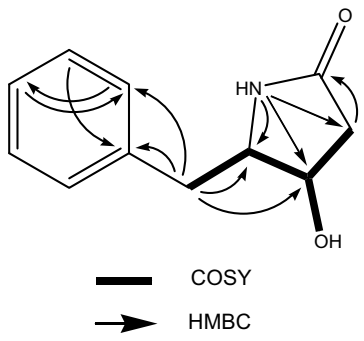


**Fig. 2.** Key HMBC and COSY correlations of streptopyrrolidine (**1**).

the HMBC correlations from NH to C-3, C-4, and C-5. The HMBC correlations from H-6A ($\delta$ 2.64) and H-6B ($\delta$ 2.95) to C-4, C-5, C-7, and C-8 established the link between the benzyl group and 4-hydroxy-2-pyrrolidone. This linkage was also supported by the ROESY correlation between H-5 ($\delta$ 3.66) and H-8 ($\delta$ 7.25). Thus, the gross structure of streptopyrrolidine (**1**) was elucidated to be 5-benzyl-4-hydroxypyrrolidin-2-one (Fig. 1). The relative stereochemistry of streptopyrrolidine (**1**) was successfully assigned by analyses of the ROESY spectrum. Strong NOE correlations observed between H-5 ($\delta$ 3.66) and H-4 ($\delta$ 4.08) and NH ($\delta$ 7.53) suggested that these protons are on the same face of the ring. The absolute configuration of streptopyrroldine remains unknown and we are consequently very interested in elucidating this matter.

The 4-hydroxy-2-pyrrolidinone ring system in streptopyrrolidine is present in many biologically active compounds (e.g., nootropic drug oxiracetam) and it could act as a versatile intermediate for the syntheses of a wide variety of $\gamma$-amino acids (GABA), substituted 2-pyrrolidinones (e.g., cynometrine and cynodine) as well as pyrrolidines (Huang et al., 1999; Park et al., 2003). Streptopyrrolidine (**1**) was reported as a synthetic intermediate (Kondekar et al, 2004; Poncet et al., 1990). However, this study is the first report of its isolation and complete structural assignment from a natural source. Additionally, the bioactivities of streptopyrrolidine (**1**) have also never been reported. In our HUVECs based capillary tube formation assay (Jeon et al., 2005), streptopyrrolidine (**1**) significantly blocked the capillary tube formation of the cells at the same potency as a known angiogenesis inhibitor, SU11248 (Blansfield et al., 2008). In the absence of vascular endothelial growth factor (VEGF), cultured HUVECs on the Matrigel formed an incomplete and narrow tube-like capillary sprout (Fig. 3a) but the obvious endothelial cell sprouting was stimulated by the treatment of VEGF resulting in an extensive network of thick tubes as shown in Fig. 3b. Treatment of HUVECs with either streptopyrrolidine (**1**) or SU11248 resulted in a dramatic inhibition of tube formation (Fig. 3c and d). Inhibitory effects of streptopyrrolidine (**1**) on tube formation were shown as either narrow or broken tube networks on the Matrigel (indicated by arrow). In addition, streptopyrrolidine (**1**) exhibited anti-angiogenesis activity without showing cytotoxicity against HUVECs at the concentration of 100 μg/ml.

*Concluding remarks:* Streptopyrrolidine (**1**) is expected to be a unique small molecule bioprobe for studying angiogenesis. It is

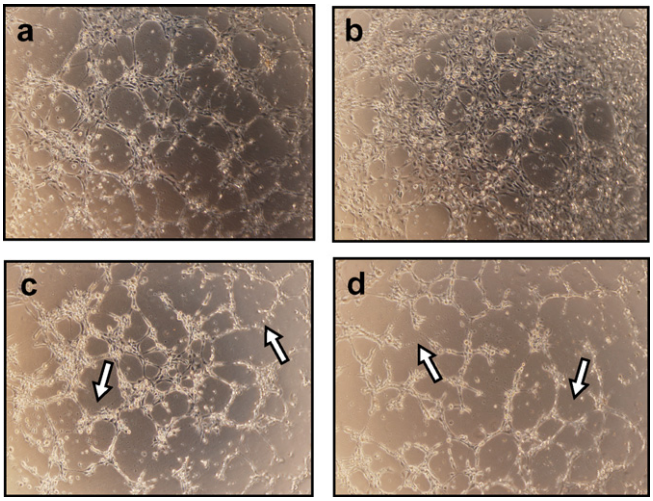


**Fig. 3.** Effect of streptopyrrolidine on tube forming ability of HUVECs: (a) Serum free; (b) VEGF alone; (c) VEGF + streptopyrrolidine (10 μM); (d) VEGF + SU11248 (10 μM). Each value represents mean ± SE from three independent experiments. Arrows indicate either narrow or broken tube formed by VEGF-stimulated HUVECs after drug treatment.

unclear, however, why deep sea bacteria produce this angiogenesis inhibitor and what its actual role in the natural environments. Some microbial natural products, such as fumagillin from the fungus *Aspergillus fumigatus*, exhibit anti-infective and anti-angiogenic activities. It has been shown that this class of anti-angiogenic agent exhibits its activity by inhibition of the biochemical activity of target protein in endothelial cells, and which resulted in suppression of cell proliferation. Accordingly, the microorganisms either in their territorial or marine environments may produce this class of natural product to either protect themselves or survive from other eukaryotic organisms.

The unique chemical structure distinct from known angiogenesis small molecule inhibitors and anti-angiogenic activity at non-toxic threshold dose of streptopyrrolidine will serve the basis for the development of new anti-angiogenic agents. Detailed studies on the anti-angiogenic activity of streptopyrrolidine as well as the identification of cellular target of the compound will help to decipher the anti-angiogenic mechanism of the compound.

## 3. Experimental

### 3.1. General procedures

IR spectra were recorded on a Mattson Galaxy FT-IR spectrophotometer, whereas optical rotation measurements were acquired using a JASCO digital polarimeter with a 5 cm cell at 25 °C. UV spectra were measured in methanol using a Milton-Roy spectrophotometer, whereas NMR spectra were recorded on a Varian Unity 500 NMR spectrometer in $CD_3OD$-$d_4$ at 300 K. $^1H$ and $^{13}C$ NMR spectra were measured at 500 and 125 MHz, respectively. The resonances of residual $CD_3OD$-$d_4$ at $\delta_H$ 3.30 and $\delta_C$ 49.0 were used as internal references for $^1H$ and $^{13}C$ NMR spectra, respectively. Mass spectra, including high resolution mass measurements, were measured by using a JEOL JMS-SX 102A mass spectrometer provided by the Korea Basic Science Institute, Seoul, Korea. All solvents used were either spectral grade or were distilled from glass prior to use.

### 3.2. Isolation and taxonomy of the strain KORDI-3973

The marine bacterium, *Streptomyces* sp. KORDI-3973, was isolated from a deep-sea sediment sample collected at Ayu Trough, after applying dry heat to the sediment. One gram of sediment was incubated for 50 min at 60 °C and resuspended in 20 ml of autoclaved seawater. After filtration and dilution with autoclaved, 0.1 ml aliquots were spread onto modified Bennett's agar (Atlas, 1993) plates. The plates were incubated for 14 days at 30 °C, and the resulting colonies were transferred and maintained on the modified Bennett's agar. The strain KORDI-3973 is a Gram-positive actinomycete which formed well-developed substrate mycelium and aerial mycelium. Good growth was observed on ISP-2, ISP-4, and modified Bennett's agar. The best medium for the culture of this strain was modified Bennett's agar, on which it grew abundantly. Substrate and aerial mycelium color were medium-dependant. This strain formed light gray or pale yellow mycelium on ISP-2, ISP-4, and modified Bennett's agar. The strain was identified as *Streptomyces* sp. on the basis of 16S rDNA sequence analysis. Furthermore, a BLAST search of 16S rDNA sequence available in the DDBJ/EMBL/GenBank database showed the highest similarity of 99% with *Streptomyces* cf. *griseus* (AY207610). The GenBank accession number of the 16S rDNA sequence of the strain KORDI-3973 is EU689096. The strain is currently deposited in the Korean Culture Center of Microorganisms (KCCM), with the name of *Streptomyces* sp. KORDI-3973 under the accession number of KFCC11406P.

### 3.3. Cultivation of the producing strain

Strain KORDI-3973 was cultured in twenty 2 l Fernbach flasks containing 600 ml of modified Bennett's medium consisting of 0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1% dextrose, 100% sea water, buffered with 10 ml of 1.0 M Tris buffer. Fermentation was carried out at 30 °C for 7 days on a rotary shaker set at 200 rpm.

### 3.4. Extraction and isolation of streptopyrrolidine (**1**)

The culture broth of *Streptomyces* sp. KORDI-3973 was separated into the mycelium and supernatant by using continuous centrifuge. The supernatant was then filtered (0.2 μm pore-size membrane filter) to obtain a cell-free supernatant, followed by extraction with EtOAc. The EtOAc layer, which showed potent anti-angiogenesis activity, was concentrated to dryness using a rotary evaporator under vacuum at 40 °C. The residual suspension (1.47 g) was subjected to ODS open flash chromatography with a stepwise gradient mixture of MeOH/$H_2O$ as eluant. The fraction (550 mg) eluted with MeOH–$H_2O$ (1:1, v/v) was purified by reversed-phase HPLC (YMC ODS-A column, 10 × 250 mm; 30–65% MeOH in $H_2O$; flow rate, 1.5 ml/min; UV detection at 210 nm) to yield a pure angiogenesis inhibitor, streptopyrrolidine (1, 5.4 mg).

### 3.5. Streptopyrrolidine (**1**)

Colorless amorphous solid; $[\alpha]_D^{25}$ −12 (*c* 0.05, MeOH); IR (KBr) $\nu_{max}$ 3327, 1686, 1454, 1046 $cm^{-1}$; UV $\lambda_{max}$ (MeOH) 234 nm ($\varepsilon$ 1300), 301 nm ($\varepsilon$ 620); $C_{11}H_{13}NO_2$ by HREI-MS [*m/z* 191.0951 ($M^+$), $\Delta$ +0.5 mmu]; For $^1H$ and $^{13}C$ NMR spectroscopic data, see Table 1.

### 3.6. Tube formation assay

Capillary tube formation of endothelial cells *in vitro* was assessed as described previously (Jeon et al., 2005). Briefly, the HUVECs ($1 \times 10^5$ cells) were inoculated on the surface of the Matrigel, and either streptopyrrolidine (**1**) or SU11248 was treated for 6–18 h in the presence or absence of VEGF (vascular endothelial growth factor). The morphological changes of the cells and tubes formed were observed under a microscope and photographed at 100 × magnification using JVC digital camera (Victor, Yokohama, Japan). Tube formation was quantified by counting the number of connected cells in randomly selected fields at 100 × magnification and dividing that number by the total number of cells in the same field.

## Acknowledgment

The authors express gratitude to S.-H.Yang and K.K. Kwon, Korea Ocean Research & Development Institute, for the identification of the microbial strain. Thanks are also extended to Y.H. Kim, Korea Basic Science Institute, Daejeon, Korea, for providing mass spectroscopic data. This research was supported in part by the Ministry of Maritime Affairs and Fisheries, Korea (MarineBio 21 program), Korea Ocean Research and Development Institute (Grant PE98230 to S.K.K. and PE98210 to H.S.P), the National R&D Program for Cancer Control, Ministry of Health and Welfare (0620360-1), and the Brain Korea 21 Project (to H.J.K.).

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