Phytochemistry Letters 3 (2010) 194–197

Contents lists available at ScienceDirect

Phytochemistry Letters

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

An angiogenesis inhibitor isolated from a marine-derived actinomycete, *Nocardiopsis* sp. 03N67

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ARTICLE INFO

Article history:
Received 9 April 2010
Received in revised form 14 July 2010
Accepted 18 July 2010
Available online 30 July 2010

Keywords:
Marine actinomycetes
Angiogenesis inhibitors
HUVECs
Diketopiperazine
Cyclo-(L-Pro-L-Met)

ABSTRACT

Cyclo-(L-Pro-L-Met) was isolated from the fermentation broth of a marine-derived actinomycete *Nocardiopsis* sp. 03N67 by chromatographic analysis and showed anti-angiogenesis activity against human umbilical vein endothelial cells (HUVECs). The structure and absolute stereochemistry of this compound were determined based on extensive spectroscopic data analysis, and Marfey's method, respectively.

1. Introduction

Angiogenesis is an essential step in tumor cells proliferation, development, and metastasis (Risau, 1997). It is generally accepted that there are two stages of tumor progression regarding its vasculature (Folkman, 1995; Reynolds et al., 1992). In initial vascular stage of tumor growth (tumor mass < 0.5 mm), it receives nutrition and oxygen from blood by diffusion. When tumor mass grows larger than 0.5 mm, nutrition through diffusion is not sufficient for further growth (vascular stage) and therefore, the formation of new blood vessels (angiogenesis) is necessary to draw nutrition directly from the systemic circulation (Folkman, 1990; Gimbrone et al., 1972). The tumor remains in a dormant state until it can stimulate blood vessel growth from nearby pre-existing capillaries (Folkman, 1972; Horak et al., 1992; Weidner et al., 1993). Inhibiting angiogenesis has been considered as an important anticancer strategy to suppress tumor growth and metastasis.

As marine environmental conditions are extremely different from terrestrial ones, so that marine microorganisms have different characteristics from those of terrestrial counterparts and, therefore, might produce different types of bioactive compounds in challenging living conditions (Lam, 2006). As a part of our on going search for bioactive secondary metabolites, we isolated a rare bioactive diketopiperazine, *cyclo*-(L-Pro-L-Met), from a marine actinomycete *Nocardiopsis* sp. 03N67. In this paper, we report the isolation, absolute stereochemistry and anti-angiogenesis activity of *cyclo*-(L-Pro-L-Met, **1**, Fig. 1).

2. Results and discussion

Compound **1** was readily identified as *cyclo*-(L-Pro-L-Met) by comparison of its NMR (Table 1) and MS data with those reported (Jayatilake et al., 1996). The molecular formula of compound **1** was assigned as $C_{10}H_{16}N_2O_2S$ based on its ^1H and ^{13}C NMR and MS data. This compound was first reported from an Antarctic sponge-associated bacterium, *Pseudomonas aeruginosa.* Marfey's method was applied to assign the absolute configuration of the Pro and Met residues resulting from acid hydrolysis of compound **1**. HPLC analysis of the 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) derivatives of the acid hydrolysate of compound **1** gave the almost same -retention times as those prepared from samples of authentic L-Pro and L-Met, suggesting that both amino acids have L-configuration. The specific rotation value of $[\alpha]_D$-82.2° reported for this known compound (Jayatilake et al., 1996) and a similar specific rotation value of $[\alpha]_D$-80.0 observed for compound **1** suggested the same configuration for both isolates. To the best of our knowledge, we first isolated compound **1** from an Arctic seaweed-associated actinomycete *Nocardiopsis* sp. 03N67. Additionally, the bioactivities of compound **1** have never been reported. Recent reports have shown that

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doi:10.1016/j.phytol.2010.07.005

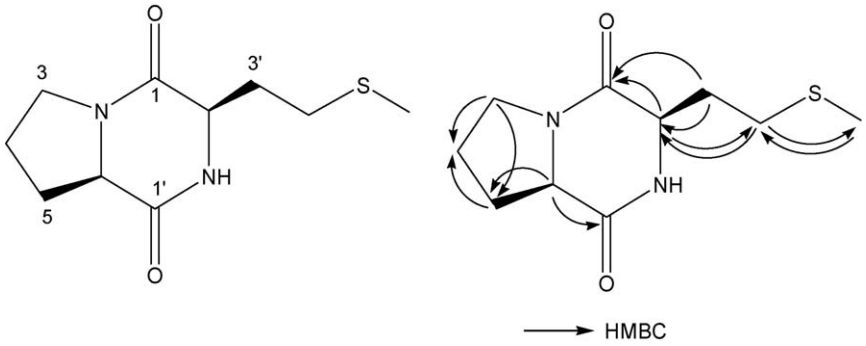

Fig. 1. Structure and key HMBC correlations of *cyclo*-(L-Pro-L-Met).

several diketopiperazines inhibit angiogenesis, thereby suppressing tumor growth (Brooks et al., 2004; Maloney et al., 2009). To explore the anti-angiogenic activity of compound **1**, we investigated the effect of compound **1** on angiogenic phenotypes of endothelial cells. In our HUVECs based capillary tube formation and invasion assays, compound **1** significantly blocked the capillary tube formation induced by tumor necrosis factor alpha (TNF-α) in a dose dependent manner (Fig. 2). In the absence of TNF-α, cultured HUVECs sprouted incompletely on the Matrigel to form narrow tube-like capillary whereas endothelial cell sprouting was stimulated by TNF-α resulting in extensive network like thick capillary tube formation. In case of invasion assay, migration/invasion of HUVECs was reduced with increasing concentration of compound **1** induced by TNF-α. Compound **1** showed concentration dependent angiogenesis inhibition in the both assays. Trypan blue staining was performed in parallel with these angiogenesis assays and cytotoxicity was not observed at concentrations used in this study.

Diketopiperazines possess diverse biological activities such as antitumor (Nicholson et al., 2006; van der Merwe et al., 2008), antifungal (Houston et al., 2004), antibacterial (Fdhila et al., 2003), and antihyperglycemic (Song et al., 2003) activities. Due to their chiral, rigid, and functionalized structures, they bind to a large variety of receptors with high affinity, giving a broad range of biological activities (Martins and Carvalho, 2007). Therefore, diketopiperazines are attractive structures for the discovery of new lead compounds for the rational development of new therapeutic agents.

In conclusion, *cyclo*-(L-Pro-L-Met) and the like diketopiperazines are the smallest cyclic peptides, commonly biosynthesized from amino acids by different organisms, including mammals, and are considered to be secondary functional metabolites or side products of terminal peptide cleavage and mainly act as cell signaling molecules. The bioactivities of *cyclo*-(L-Pro-L-Met) have never been known. Here, we identified *cyclo*-(L-Pro-L-Met) as a new anti-angiogenic agent. *Cyclo*-(L-Pro-L-Met) inhibited TNF-α-induced tube formation and invasion at 10 μM, a concentration at which no cytotoxicity was observed. However, it is unclear why

Table 1
NMR data of *cyclo*-(L-Pro-L-Met) in CD_3OD.

Position	^1H (mult., J = Hz)	^{13}C	COSY	HMBC
Pro				
1		172.7		
2	4.24, t (1H, 7.8)	60.4	H-3	C-1, C-3
3	2.31, m (2H)	29.4	H-2, H-4	C-4, C-5
4	2.02, m (2H)	23.6	H-3, H-5	C-5
5	3.52, m (2H)	46.5	H-4	C-4
Met				
1′		168.0		
2′	4.27, t (1H, 5.3)	55.4	H-3′	C-1′, C-4′
3′	2.20, m (1H)	30.3	H-2′, H-4′	C-1, C-2′
	2.07, m (1H)			
4′	2.61, m (2H)	30.5	H-3′	C-2′, S-CH_3
S-CH_3	2.10, s (3H)	15.2		C-4′

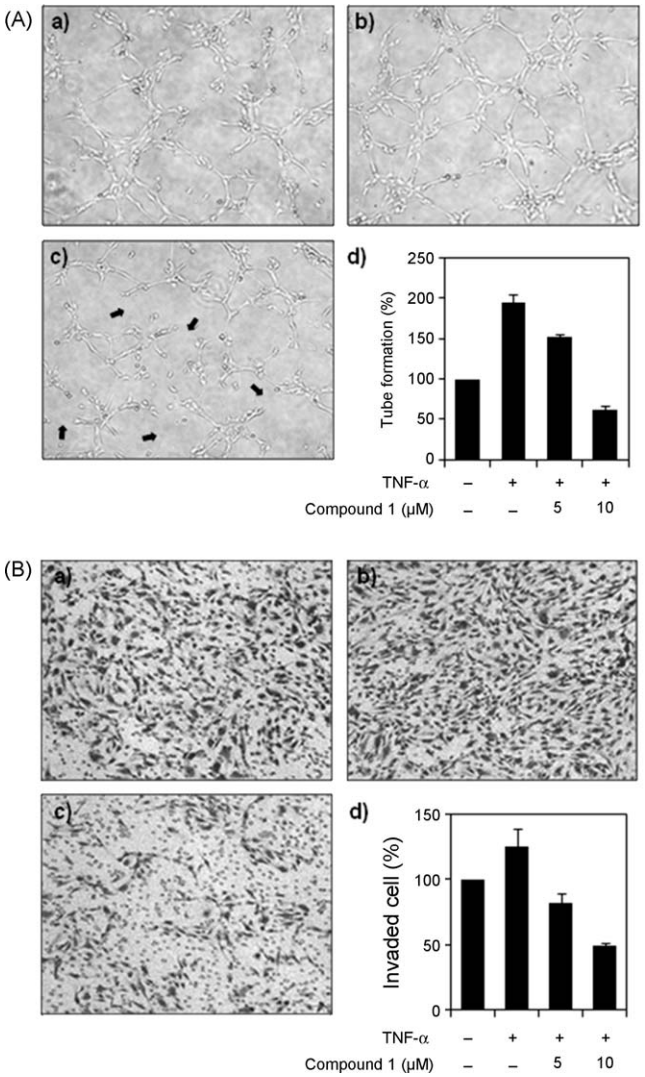

Fig. 2. Inhibition of angiogenic activities by *cyclo*-(L-Pro-L-Met) *in vitro*. (A) Effect of *cyclo*-(L-Pro-L-Met) on TNF-α-induced capillary tube formation in HUVECs: (a) non-treated cells. (b) TNF-α (50 ng/mL) alone. (c) TNF-α and *cyclo*-(L-Pro-L-Met) (10 μM). (d) Quantitative analysis of tube formation. Arrows indicate narrow or broken tubes formed by TNF-α-induced HUVECs after *cyclo*-(L-Pro-L-Met) treatment. (B) Effect of *cyclo*-(L-Pro-L-Met) on TNF-α-induced invasion of HUVECs: (a) non-treated cells. (b) TNF-α (50 ng/mL) alone. (c) TNF-α and *cyclo*-(L-Pro-L-Met) (10 μM). (d) Quantitative analysis of invaded cells. Each value represents mean ± SE from three independent experiments. $^*p < 0.01$ versus TNF-α control.

this strain produces *cyclo*-(L-Pro-L-Met) which shows anti-angiogenesis activity against HUVECs. The reason behind this may be the deterrence of predation from eukaryotic organisms. Anti-angiogenesis activity of *cyclo*-(L-Pro-L-Met) is an encouraging bioprobe to develop new anticancer therapeutics from such type of small molecules in near future.

3. Experimental section

3.1. General experimental procedures

Specific rotations were measured on a JASCO digital polarimeter (DIP-1000) with a 0.1 dm cell at 23 °C. ESI-MS experiments were performed on a Thermo Finnigan mass spectrometer. 1D and 2D NMR spectra were obtained on a Varian Unity 500 spectrometer (^{1}H at 500 MHz, 20 °C and ^{13}C at 125 MHz, 24 °C). The resonances of residual CD_3OD-d_4 at δ_H 3.31 and δ_C 49.15 were used as internal references for ^{1}H and ^{13}C NMR spectra, respectively. HPLC was performed using a PrimeLine Binary pump with a Shodex detector (RI-101). IR spectra were recorded on a JASCO FT/IR-4100. UV spectra were measured in methanol using a Shimadzu spectrophotometer (UV-1650PC). All solvents used were either spectral grade or were distilled prior to use.

3.2. Isolation and taxonomy of strain 03N67

The strain 03N67 was isolated from a seaweed (*Undaria pinnatifida*) sample collected from the Arctic expedition in 2003 by serial dilution technique. In brief, one gram of the seaweed sample was blended, diluted in sterilized sea water (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) in aseptic conditions and 100 μL from each dilution were spread on modified Bennett's agar medium (0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1% dextrose, 100% natural sea water, 1.8% agar and pH 7.2 before sterilization). The plates were incubated for 14 days at 30 °C, and the resulting colony of strain 03N67 was isolated and maintained on the modified Bennett's agar. The strain 03N67, which formed well-developed white substrate mycelium and aerial mycelium on modified Bennett's agar medium. The strain was identified as *Nocardiopsis* sp. on the basis of 16S rDNA sequence analysis. The strain is currently deposited in the Microbial Culture Collection, KORDI, with the accession number *Nocardiopsis* sp. 03N67 under the curatorship of H.J.S.

3.3. Seed and mass cultures of strain 03N67

The composition and pH of the seed medium were same as modified Bennett's medium. The medium was dispensed at 50 mL in 250 mL conical flask. A single colony from the agar plate was inoculated into the flask and incubated at 30 °C at 120 rpm for 3 days. An aliquot (0.2%, v/v) from the seed culture was inoculated into a 20 L fermenter containing 17 L of culture medium. The composition of the mass culture medium was same to that of the seed culture medium. The production culture was carried out at 30 °C at 120 rpm for 7 days and then harvested.

3.4. Extraction and isolation

The fermentation broth (17 L) was centrifuged at 60,000 rpm by using continuous centrifuge, and the supernatant was extracted with EtOAc (17 L × 2). The EtOAc layer was concentrated to dryness using a rotary evaporator under reduced pressure at 40 °C. The residual suspension (1.2 g) was subjected to ODS open chromatography with a stepwise gradient mixture of MeOH and H_2O as eluant. The fraction eluted with MeOH: H_2O (2:3, v/v) was further fractionated by medium pressure liquid chromatography (MPLC) and fraction 5 (33.1 mg) containing the compound *cyclo*-(L-Pro-L-Met) was purified by reversed-phase HPLC (YMC ODS-A column, 250 × 10 mm i.d.: 40% MeOH; flow rate: 1.5 mL/min; detector: RI, t_R: 26 min) to yield pure compound **1** (3.6 mg).

3.5. Absolute configuration of compound 1

Marfey's method was performed to determine the absolute configuration of methionine and proline moiety in compound **1** as described previously (Marfey, 1984). In short, 100 μg of compound **1** was taken in 500 μL glass vial and hydrolyzed by heating at 110 °C for 12 h with 200 μL 6 N HCl. Upon removal of excess HCl under reduced pressure, 0.1% solution (100 μL) of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) in acetone, followed by 1.0N $NaHCO_3$ (20 μL) was added to derivatise the hydrolysate and heated at 40 °C for 1 h, cooled to room temperature, and then neutralized with 1N HCl (20 μL). In a similar fashion, standard L-Pro, D-Pro, L-Met, and D-Met were derivatized separately with Marfey's reagent. The derivatives of the acid hydrolysate and the standard amino acids were subjected to RP-HPLC analysis (YMC ODS-A column, 50 mm × 2.0 mm i.d.) at 25 °C using the following gradient program: solvent A, MeOH with 0.1% TFA; solvent B, CH_3CN with 0.1% TFA; linear gradient, 10–40% of A for 40 min with UV detection at 340 nm; flow rate: 0.2 mL/min. The retention times for the FDAA derivatives of L-Pro, D-Pro, L-Met, and D-Met were 22.30, 24.87 30.41, and 37.10 min, respectively, whereas those for the FDAA derivatives of Pro and Met in the hydrolysate of compound **1** were 22.80 and 31.13 min, respectively.

3.6. Characteristics of compound 1

White amorphous solid; $[\alpha]_D$-80.0 (c 0.05, MeOH); ^{1}H, ^{13}C and 2D NMR data in CD_3OD given in Table 1. ESI-MS: at *m*/*z* 251 $(M + Na)^+$ and at *m*/*z* 227 $(M - H)^-$, calcd. 228 for $C_{10}H_{16}N_2O_2S$; λ_{max} (ε) 210 (11255); IR (neat) ν_{max} at 3709 and 1705 cm^{-1}.

3.7. Tube formation assay

Matrigel (10 mg/mL) was added to a 48-well plate and allowed to polymerize for 1 h at 37 °C. HUVECs (6×10^4 cells) were seeded onto the surface of the Matrigel in a 48-well plate. Compound **1** was then added for 6–18 h at 37 °C in the presence or absence of TNF-α. The morphological changes of the cells and tubular structure formed were observed under a microscope (IX71, Olympus) and photographed at 100× magnification with a camera (DP70, Olympus).). 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.

3.8. Invasion assay

The invasiveness of HUVECs was examined *in vitro* using a Transwell chamber system with polycarbonate filter inserts with 8.0-μm-sized pores. Briefly, the lower side of the filter was coated with gelatin (10 μL, 1 mg/mL), and the upper side was coated with Matrigel (10 μL, 3 mg/mL). HUVECs (7×10^4 cells) were placed in the upper part of the filter, and compound **1** was added to the lower parts in the presence of TNF-α (50 ng/mL). Then the chamber was incubated at 37 °C for 18 h. The cells were fixed with 70% MeOH and stained with hematoxylin and eosin. The cell invasiveness was measured by counting the whole-cell numbers on the lower side of the filter using a microscope at 100× magnification. Cells were also photographed at 100× magnification with a camera.

3.9. Cell proliferation assay

HUVECs (7–11 passages) were grown in EGM-2 medium supplemented with 10% FBS. HT1080 (fibrosarcoma, human) cells were grown in MEM containing 10% FBS and 1% antibiotics. HeLa (cervical carcinoma, human), HepG2 (hepatocellular carcinoma, human), and MCF-7 (breast carcinoma, human) cells were maintained in DMEM with 10% FBS and 1% antibiotics. NF-kB-bla HEK293T CellSensor™ cells were grown in DMEM containing 10% FBS, 1 mM sodium pyruvate, 100 U/mL penicillin, 0.1 mM non-essential amino acids, 100 μg/mL streptomycin, and 5 μg/mL blasticidin. All cell lines were maintained at 37 °C in a humidified 5% CO_2 incubator. The cell proliferation assay was conducted using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, and cytotoxicity was analyzed using Trypan blue exclusion (Jung et al., 2007).

3.10. Statistical analysis

Results are expressed as the mean ± standard error (SE). Student's *t*-test was used to determine statistical significance between control and test groups. A *p*-value of <0.05 was considered statistically significant.

Acknowledgements

The authors express gratitude to Y.H. Kim, Korea Basic Science Institute, Daejeon, Korea, for providing mass data. This research was supported in part by the Ministry of Land, Transport and Maritime Affairs, Korea, Korea Ocean Research and Development Institute (Grants PE98472 to D.H.K. and PE98494 to H.S.P.) and the Translational Research Center for Protein Function Control (NRF 2009-0083522 to H.J.K.).

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