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Secondary metabolite produced from marine bacterium *Streptomyces* sp. strain ND7c

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ABSTRACT

Actinomycetes are filamentous gram-positive bacteria that can be found abundantly in both terrestrial and marine environment. These bacteria are known as producers of many bioactive compounds through the production of secondary metabolites for their survival and adaptation in nature and have been widely used today as therapeutic agents. Marine actinomycetes have been the focus of research over the past decade for new drugs discovery due to its unique adaptation in the harsh sea environment. It is believed that marine actinomycetes could produce compounds that are rare and unique compared to the terrestrial actinomycetes. Marine sponges often harbor dense and diverse microbial communities including actinobacteria. One secondary metabolite, thymine was isolated and identified from marine Streptomyces sp. strain ND7c. Its structure was determined by spectroscopic analysis including MS, 1D and 2D NMR, as well as by comparison with reported data.

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1 INTRODUCTION

Marine bacteria are considered to play a central role as symbionts of most marine invertebrates and also represent one of the most novel biomedical resources remaining to be explored (Fenical, 1993). Marine microorganisms have been the important study in recent years because of production of novel metabolites which represent various biological properties such as antiviral, antitumor or antimicrobial activities. These secondary metabolites serve as model systems in discovery of new drugs (Bernan *et al.*, 1997; Fenical, 1997). The studies of the secondary substances produced by marine micro-organisms have obtained many significant achievements in the world (Radjasa and Sabdono, 2003). Among the secondary metabolites from marine microorganisms, there are many compounds having interesting biological activities that should be useful to development for their pharmaceutical uses. Metabolites from microorganisms are a rapidly growing field, due, at least in part, to the suspicion that a number of metabolites obtained from algae and invertebrates may be produced by associated microorganisms (Debbab *et al.*, 2010). Meanwhile, the search of bioactive secondary metabolites from marine microorganisms is not widely explored in Vietnam

(Minh *et al*, 2012; Do *et al*., 2012). Although marine actinomycetes have a lot of potential compared to terrestrial actinomycetes, the major challenge in exploiting their potential. In the course of screening program, the Ethyl Acetate extract of a *Streptomyces* sp. from marine sponge of Ha Tien Sea, Kien Giang province, Vietnam exhibited an inhibition activity against *Salmonella typhimurium, Escherichia coli, Bacillus cereus* and *Candida albicans*. In this paper, the report of the isolation and structural elucidation of secondary metabolites from the cultures broth of *Streptomyces* sp. is the difficulty in isolating these microbes from its environment (Murphy *et al.*, 2009).

2 MATERIALS AND METHODS

2.1 Actinomycete material

The marine sponge was collected in Ha Tien Bay, Kien Giang province in April 2016. The sponge sample (1 g) was added to the 10 mL of sterile sea water in a conical flask. The flask was agitated for about one hour. The marine sediment was filtered and the filtrate was serially diluted to obtain 10^{-1} to 10^{-7} dilutions using the sterilized sea water. An aliquot of 100 µL of each dilution was spread on the media. Different media like Starch Casein Agar (SCA) was used for isolation of actinomycetes. The media containing 50% of sterile sea water were supplemented with rifampicin (5 µg/mL) and nystatin (25 µg/mL) (Himedia Mumbai) to inhibit bacterial and fungal contamination, respectively. The petriplates were incubated up to 3 weeks at 28°C. The isolated discrete colonies were observed and used for identification.

The obtained strain *Streptomyces* sp. was identified using 16S rRNA gene sequencing method. The universal primers including forward primer, 5'-AGA GTT TGA-TCA TGG CTC A-3', and reverse primer, 5'- AAG GAG GTG ATC CAG CC- 3', were used for amplifying nearly full length of 16S rRNA gene sequence (about 1500 bp.). The obtained sequence was analyzed by comparing with bacterial 16S rRNA sequences in GenBank by BLAST N, which showed 99% similarity with *Streptomyces* sp. 2011 (GenBank Accession No. JF751041.1).

2.2 General Experimental Procedures

2.2.1 Fermentation, extraction and isolation

Streptomyces sp. ND7c strain was cultured in 250 ml flasks at 30°C for 24 hours with shaking at 150 rpm. Fermentation was carried out in 100 L fermenter with 50 L medium 2216 and 10% bacterial inoculum at 30°C for 52 hours. Neutral pH was maintained by NaOH or HCl 1N. Natural products were extracted by liquid-liquid extraction with solvents as ethyl acetate, dichloromethane.

Compounds isolation was performed by column chromatography (CC), using ~~a~~ silica gel (Kiesel gel 60, 70-230 mesh and 230-400 mesh, Merck, Germany). Acetone, chloroform and *n*-hexane were used as eluent. Thin-layer chromatography (TLC) used pre-coated silica gel 60 F254 alumium sheet (Merck, Germany).

2.2.2 Structural elucidation and identification

High resolution ESI mass spectra were measured a FT-ICR MS VARIAN 910 spectrometer. NMR spectra were recorded on a Brucker AM500 FT-NMR spectrometer with TMS as internal standard from Institute of Chemistry, Vietnam Academy of Science and Technology.

2.3 Isolation and purification

The obtained culture broth (50 L) was extracted with ethyl acetate (25 L × 3 times). The combined organic solutions were then decanted, filtered and concentrated under reduced pressure to yield 5.2 g of crude extract which was chromatographed on a silica gel column using a gradient of 1-100% acetone in *n*-hexane to afford six fractions F1-6.

The fourth fraction F4 (620 mg) was continued to subject to CC with chloroform as eluent to give 4 subfractions F41-44. The subfraction F42 (43 mg) was re-crystallized in *n*-hexane to obtain pure compound DIEP4 (9 mg).

2.4 Spectral data of isolated compound

ESI-MS (m/z): 125.04 $[M-H]^-$. ^{1}H-NMR (DMSO-d_6, 500 MHz, δ_H ppm): 1.72 (3H, *d*, 2.0, H-7), 7.23 (1H, *s*, H-6), 10.54 (1H, *s*, H-1), 10.96 (1H, *s*, H-3). ^{13}C-NMR (DMSO-d_6, 125 MHz, δ_C ppm): 11.7 (C-7), 107.6 (C-5), 137.6 (C-6), 151.4 (C-2), 164.9 (C-4).

3 RESULTS AND DISCUSSION

The compound DIEP4 obtained as white needle crystals, mp. 316–317°C, had good solubility in $CHCl_3$. The molecular formula of it was speculated to be $C_5H_6N_2O_2$ (calc. for 126.04, 4 degrees of unsaturated) on the basic of the ESI-MS (by the quasi-ion *m/z* 125.04 $[M-H]^-$) and 1D-NMR data.

The ^{1}H-NMR spectrum of DIEP4 (DMSO-d_6, 500 MHz) gave typical signals of a double-bond methine group at δ_H 7.23 (1H, *s*), one methyl group at δ_H 1.72 (3H, *d*, 2.0), two –NH groups at δ_H 10.54 and 10.96 in sequence.

Its ^{13}C-NMR spectrum (DMSO-d_6, 125 MHz) combined with DEPT spectrum of it showed five carbon signals contained: two carbonyl groups at δ_C

151.4 and 164.9, one double-bond quaternary carbon at δ_C 107.6, one double-bond methine carbon at δ_C 137.6, and one methyl group at δ_C 11.7.

Based on mentioned MS and NMR spectral data, the structure of DIEP4 was determined as a heterocyclic compound, a form of two nitrogen atoms contained aromatic ring. Besides, by the comparation of ^{1}H-NMR data with those given in the literature [Quyen *et al*., 2015] (Table 1), DIEP4 was identified to be thymine (Fig. 1). Furthermore, the HSQC (Heteronuclear Single Quantum Coherance) and HMBC (Heteronuclear Multiple Bond Correlation) between protons and carbons in the NMR spectral of DIEP4 (Table 1) agreed with the structure of thymine.

Thymine also identified from extracted from *Xestospongia testudinaria* collected from Vietnam sea (Cuong *et al*., 2007).

Table 1: NMR spectral data of DIEP4 and thymine

No.	DIEP4 (*)				Thymine (**) [Quyen]
	δ_H, J (Hz)	**δ_C**	**DEPT**	**HMBC (^{1}H→^{13}C)**	**δ_H, J (Hz)**
1	10.54 (1H; *s*)				
2		151.4	>C<		
3	10.96 (1H; *s*)				
4		164.9	>C<		
5		107.6	>C<		
6	7.23 (1H; *s*)	137.6	=CH	2, 4, 7	7.06 (1H; *s*)
7	1.72 (3H; *d*; 2,0)	11.7	-CH_3	4, 5, 6	1.86 (3H; *s*)

Note: (*) Recorded in DMSO-d_6, 500/125 MHz; (**) recorded in CD_3OD, 500/125 MHz

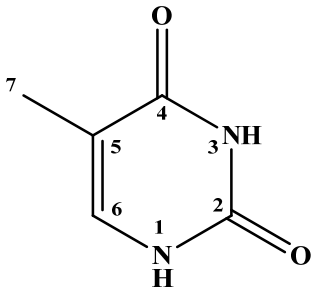

Fig. 1: The chemical structure of thymine

4 CONCLUSION

From the ethyl acetate extract of *Streptomyces* sp. ND7c strain, by using chromatography and modern spectral methods, the first time a secondary metabolite was isolated and identified as thymine. Thymine was a bioactive compound as previous of Zing *et al*. (2011) when they isolated and identified thymine from *Penicillium* sp. P-1, A fungal endophyte in *Huperzia serrata*.

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