Biologically Active Compounds Derived from Sponge (*Theonella* Sp) Associate Marine Bacteria

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ABSTRACT

The high potency of tropical marine microorganisms as a source of biologically active compounds is one of the reasons why the researchers still concern to investigate it. Indonesian sponge was known as a very potential producing active secondary metabolite. Several publications have reported that some sponge associate microorganisms contribute to producing active compounds derived from sponge. The purpose of this study was investigating the active antibacterial compounds derived from sponge (*Theonella* sp) associate microorganisms. Cultivation and characterization of the potent bacterial, extraction, purification and structural determination of antibacterial substance were conducted. Screening of antibacterial activity to the isolated bacterial strain showed that one of the strains M1Sp1.12102015.103b.3 very strongly inhibits *Vibrio eltor* and *Staphylococcus aureus*. Separation of ethyl acetate extract using open column chromatography resulted in three potent active antibacterial compounds. Identification of one of the potential active fraction using GC-MS showed that there is Pyrrolidino [1, 2-a] piperazine-3,6-dione compound. Bacterial characterization using 16S-rDNA compared with the NCBI database showed 99.87 % similarity with *Bacillus subtilis* subsp. Inaquorum.

Key Words: *marine sponge, associate microorganisms, bioactive compound.*

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INTRODUCTION

Theonella sp. sponge is known as a vey potential active compound producer such as anticancer swinholide A, anticardiovascular cyclotheonarnide A, and antiviral papuamides C & D[Error! Reference source not found.Error! Reference source not found.] [1]. The structural complexity of active compounds derived from sponge causes difficulty in synthesizing. Antimicrobial compounds of cyclic peptide theonellapeptolide[Error! Reference source not found.] [2] and cytotoxic macrolide isoswinholide B, K **Error! Reference source not found.** are the compounds derived from Theonella that is very potential, unique and complex. This study was conducted aimed at approving sponge's associate microorganisms that play an important role to construct the active compounds. A compound that was produced by *Theonella*, and also in associate microorganisms was cytotoxic bafilamycin N, O, K & M. Recently, this compound was reported to be in *Streptomyces* that was associated with *Theonella* sp.[Error! Reference source not found.]. Theopalauamide was isolated from *Theonella swinhoei* and it symbiont [Error! Reference source not found.], *Candidatus entotheonella palauensis*[Error! Reference source not found.] [6]. Polytheonamide a cytotoxic compound isolated from *Theonella swinhoei*, was synthesized by uncultivated bacteria symbiont Entotheonella[Error! Reference source not found.] [7]. Another study reported the antitumor polyketide onnamide series isolated from *Theonella swinhoei* and synthesized by associate uncultivated bacteria *Pseudomonas* sp.[Error! Reference source not found.].

Associated marine bacteria diversity is very highly dependent on environment. Tropical sponge's associated bacteria was known for the highest diversity and most potential for bioactive compounds producers. In a previous research, about 140 bacterial strains were isolated from *Theonella* sp. collected from Untung Jawa, Seribu Island.

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The most potential antibacterial strain was M1Sp1.12102015.103b.3. This strain showed strongly inhibiting *Vibrio eltor* and *Staphylococcus aureus*. Cultivation, characterization, chemical separation, purification and preliminary chemical investigation were carried out [9]. The information of active antibacterial compound derived from sponge's associate bacterial is very important to develop marine drug investigation research.

MATERIAL AND METHODS

Materials

Voucher of sponge was collected from Untung Jawa Seribu Island and directly brought to the laboratory for bacterial isolations and characterization. The media for bacterial isolation and cultivation was M1 (in 1L contained: 10 g amylum, 4 g yeast extract, 2 g of pepton and 16 g agar). Nystatine was used to suppress fungal growth. The media used for antibacterial bioassay was MHA (Muler Hinton Agar), with the bacterial tested *Bacillus subtilis*, *Vibrio eltor* and *Staphylococcus aureus*.

Extraction of potential strain was done using ethyl acetate pa. (Merck) and methanol pa (Merck). The solvents for compounds fractionation were methanol pa. (Merck) and purified distilled water.

Methods

Bacterial Isolation, cultivation and antibacterial screening test were used.

About 1 cm^3 of sponge slice was deeply immersed in sterile seawater, and approximately 100 µL of sponge solution was transferred to micro tube containing 900 µL (serial dilutions until) 10^{-4} v/v. About 100 µL of solution was inoculated on M1-agar media plate that contained nystatin, then incubated at 28°C during 1-2 weeks. Each colony was isolated, purified and restored in freeze condition using 20 % glycerol as cryogenic agent. The single colony was cultivated on 5 mL M4 media broth during 72 hours for antibacterial screening test. After harvesting, each bacterial broth was extracted using ethyl acetate.

Approximately 100µg samples were dropped onto paper disk and laid to MHA media with pathogenic bacterial (*Staphylococcus aureus*, *Bacillus subtilis* & *Vibrio eltor*.). The antibacterial bioassay was carried out using agar disk diffusion method **Error! Reference source not found.**. After incubation during 12 hours, the zone of inhibition was determined. The most potential strain was chosen for chemical separation.

The chosen potential strain was cultivated in a semi-large scale. About 15 L M1 media broth was used for bacterial propagation. After 72 hours, the cultivar was harvested and extracted to analyze the chemicals constituents.

Extraction and separation of the potential fractions

About 15 L of bacterial broth was extracted using ethyl acetate and evaporated using rotary shaker evaporator. The extract was passed to silica gel chromatography using mobile phase hexane-ethyl acetate, gradiently (100% hexane, 80% ,70%,30%, 20%) continued to ethyl acetate-acetonitrile (100% ethyl acetate, 50, 100% acetonitrile and finally flashed with 100% methanol). All of fraction were collected and tested to determine the potent active antibacterial substance.

GC-MS Analysis

GC-MS analysis was done in Jakarta health laboratory. The instrument used was Agilent Technologies 7890, Gas Chromatograph with auto sampler and 5975 Mass Selective Detector and Chemstation data systems. The method for conditioning the GC analysis was ionization impact electron mode with an energy electron of 70 Ev. The column used was HP Ultra 2 capillary columns with a length (m) 30 x 0.25 (mm) LD x 0.25 (mm). Oven temperature was programmed as follows: initial temperature at 70°C, for 0 minutes, temperature was increased about 3oC / min until the temperature reached 150°C, then held up about 1 minute. Then temperature gradually was increased 20°C / min until the temperature reached 280°C and held up during 26 minutes. The gas carrier was helium with a flow velocity of 1 µl / minute.

Bacterial characterization

Identification of potential strain was carried out using molecular analysis based on 16S rDNA fragments in bacteria. The isolation of DNA bacterial was carried out using the PCR colony method. The cells from single colonies on solid media surface were taken using sterile toothpicks and suspended in 50 mL nuclease-free water. Cell lysis was carried out with suspended suspension for 10 seconds and incubated at 98oC for 5 minutes. Lisat is then spin down to separate the supernatant and cell debris. The supernatant was taken and used as a DNA template for PCR amplification.

Amplification of 16S rDNA fragments was done using GoTaq (Promega) with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3 ') and 1492R (5'-GGTTACCTTGTTACGACTT-3').

Purification of PCR products was carried out by PEG precipitation method [11] and continued with sequencing cycle. Sequencing cycle results were purified again with the Ethanol purification method. Nitrogen base sequences were analyzed using the automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems).

RESULT AND DISCUSSION

The screening of antibacterial properties of isolated bacteria from sponge resulted in the data of the potential strain M1Sp1.12102015.103b.3. This strain was very

active against *Staphylococcus aureus* & *Vibrio eltor*. Separation of active substances using open column chromatography generated nine fractions (Table 1). Three fractions F1, F3 and F9 were activated against *Staphylococcus aureus*.

Table 1. The fractions base to open the column separation

Fraction NO	Eluent	Yield (mg)	Diameter of inhibitions against *S aureus* (mm)
1	100 % Hexane 100%	40.7	9.0
2	Hexane-ethyl acetate (80:20)	136.6	8.4
3	Hexane –ethyl acetate(70:30)	96.0	10.5
4	Hexane-ethyl acetate (30:70)	19.2	8.3
5	Hexane-ethyle acetate(20:80)	55.6	9.8
6	Ethyl acetate 100%, ethyl acetate-acetonitrile (70:30)	143.7	9.6
7	Ethyl acetate-acetonitrile (50/50)	26	-
8	Acetonitrile 100%	85.5	8.3
9	Methanol	767.4	11. 6

Taxonomy of the potential strain and preliminary Chemical Characterization

Comparison of nucleotide sequencing base to 16Sr-RNA using Eztaxon online database www.ezbiocloud.net showed that MiSp1.12102015.103b.3 strain is a homolog with ***Bacillus subtilis*** **subsp.** ***inaquosorum*** **KCTC 13429(T).** The Accession number was AMXN01000021, similarity: 99.78%, total nucleotide compared: 1348 bp, and 3 different nucleotides.

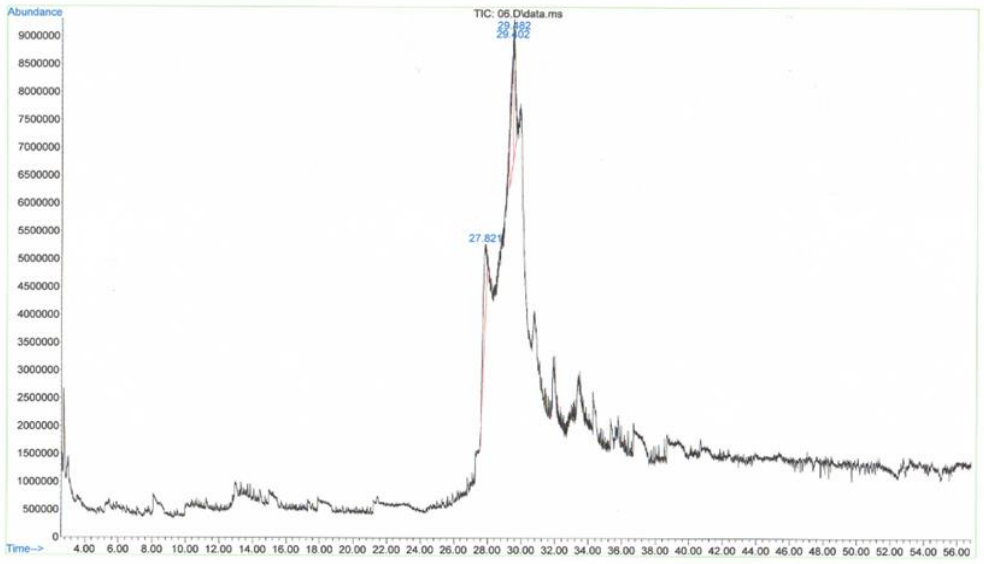

Figure 1. GC-MS chromatogram of fraction 9

The chemical structural analysis using GC-MS analysis of fraction 9 showed that the peak with retention time of 27.824 minutes had a molecular weight of 154.1 g/mol and had 97% similarity in fragmentation with the pyrrolidino [1,2-a] piperazine-3,6-dione as compound 1. Compound 1 was used as an analgesic, especially for antiepileptics and neurodegenerative (US patent no. US6306060). Compound 2 contained in fraction 6 was 3-benzylhexahydropyrrolo [1,2-A]pyrazine-1,4-dione at 31,368 minute MW : 244.1 g/ mol. The previous scientists reported that this compound was isolated from *Streptomyces* sp VITOK9 collected from the salts spring habitat **Error! Reference source not found.** and *Pseudonocardia endophytica* VUK-10 collected from mangrove ecosystem. The biological activity of this compound was reported as antifungal, moderate cytotoxic against normal cell-line, and showed hemolytic **Error! Reference source not found.**, antibacterial, and anticancer activity **Error! Reference source not found.**. The structure of both of potential compounds is described in Figure 2.

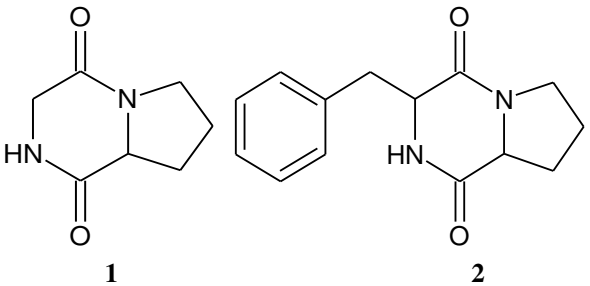

Figure 2. The active compound 1: [1, 2-a] piperazine-3,6-dione, compound 2 : 3-benzylhexahydropyrrolo[1,2-A]pyrazine-1,4-dione

CONCLUSION

Sponge's associated bacteria was a very potent producing antibacterial compound. This study concering the sponge Theonella associated bacteria showed that one of bacterial strain confirmed to *Bacillus subtilis subsp. inaquosorum* a very potential producing active compound. The preliminary study of chemicals constituent using GC-Mass showed that there are potential active compounds of pyrrolidino [1,2-a] piperazine-3,6-dione and 3-benzylhexahydropyrrolo [1,2-A]pyrazine-1,4-dione.

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Conflict of interest

No conflict of interest was reported.

REFERENCES

[1] Sipkema D, Franssen MC, Osinga R, Tramper J, Wijffels RH. Marine sponges as pharmacy. Marine biotechnology. 2005 Jun 1;7(3):142. https://doi.org/10.1007/s10126-004-0405-5.

[2] Tsuda M, Shimbo K, Kubota T, Mikami Y, Kobayashi JI. Two theonellapeptolide congeners from marine sponge Theonella sp. Tetrahedron. 1999 Aug 20;55(34):10305-14.

[3] Sinisi A, Calcinai B, Cerrano C, Dien HA, Zampella A, D'Amore C, Renga B, Fiorucci S, Taglialatela-Scafati O. Isoswinholide B and swinholide K, potently cytotoxic dimeric macrolides from Theonella swinhoei. Bioorganic & medicinal chemistry. 2013 Sep 1;21(17):5332-8. http://dx.doi.org/10.1016/j.bmc.2013.06.015

[4] Chen YH, Yang JC, Lu MC, Weng CF, Su YD, Kuo J, Wu YC, Sung PJ. Bafilomycins N and O, novel cytotoxic bafilomycin analogues produced by Streptomyces sp. GIC10-1 isolated from marine sponge Theonella sp. Tetrahedron. 2017 Aug 24;73(34):5170-5.

[5] Yang F, Li YY, Tang J, Sun F, Lin HW. New 4-methylidene sterols from the marine sponge Theonella swinhoei. Fitoterapia. 2018 Jun 1;127:279-85. https://doi.org/10.1016/j.fitote.2018.03.006.(http://www.sciencedirect.com/science/article/pii/S0367326X18302405)

[6] Schmidt EW, Obraztsova AY, Davidson SK, Faulkner DJ, Haygood MG. Identification of the antifungal peptide-containing symbiont of the marine sponge Theonella swinhoei as a novel δ-proteobacterium,"Candidatus Entotheonella palauensis". Marine Biology. 2000 Jul 1;136(6):969-77.

[7] Freeman MF, Vagstad AL, Piel J. Polytheonamide biosynthesis showcasing the metabolic potential of sponge-associated uncultivated 'Entotheonella'bacteria. Current opinion in chemical biology. 2016 Apr 1;31:8-14. http://dx.doi.org/10.1016/j.cbpa.2015.11.0021367-5931/.

[8] Piel J, Hui D, Wen G, Butzke D, Platzer M, Fusetani N, Matsunaga S. Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge Theonella swinhoei. Proceedings of the National Academy of Sciences. 2004 Nov 16;101(46):16222-7.

[9] Sinisi A, Calcinai B, Cerrano C, Dien HA, Zampella A, D'Amore C, Renga B, Fiorucci S, Taglialatela-Scafati O. Isoswinholide B and swinholide K, potently cytotoxic dimeric macrolides from Theonella swinhoei. Bioorganic & medicinal chemistry. 2013 Sep 1;21(17):5332-8. http://dx.doi.org/10.1016/j.bmc.2013.06.015.

[10] Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. Journal of pharmaceutical analysis. 2016 Apr 1;6(2):71-9. http://dx.doi.org/10.1016/J.Jphar.2015.11.005

[11] Hiraishi A, Kamagata Y, Nakamura K. Polymerase chain reaction amplification and restriction fragment length polymorphism analysis of 16S rRNA genes from methanogens. Journal of fermentation and bioengineering. 1995 Jan 1;79(6):523-9.

[12] Sanjenbam, P., Kannabiran, K. Bioactivity of Pyrrolo[1,2-a] pyrazine-1,4-dione-hexahydro-3-phenyll ethyl-Extracted from Streptomyces VITOK-9from the salt spring habitate of Mampur, India. Asian Journal of Pharmaceutics. 2016; 10(4): 265-269. DOI : http://dx.doi.org/10.22377/ajp.vio.04.865

[13] Mangamuri UK, Muvva V, Poda S, Chitturi B, Yenamandra V. Bioactive natural products from Pseudonocardia endophytica VUK-10. Journal of Genetic Engineering and Biotechnology. 2016 Dec 1;14(2):261-7. http://dx.doi.org/10.1016/j.geb.2016.10.002.