Puupehenone, an Anticancer Produced by the Indonesian Marine Sponge *Hyrtios* sp.

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Abstract. In a continuous study on the Indonesian marine sponges, we found a *Hyrtios* sponge during our expedition in Lhok Mata Ie, an isolated place around Aceh Besar, Indonesia in 2017. The bioassay-guided separation with several steps of chromatography was applied to give pure material. Moreover, the spectroscopic analysis revealed the presence of puupehenone (1), a unique class of merosesquiterpene together with its analogs (2-3). Their structures and bioactivity against multidrug-resistant *Escherichia coli* (MDR *E. coli*) together with several cell lines are described here.

1. Introduction

The genus of *Hyrtios* (class Demospongiae, order Dictyoceratida, family Thorectidae) has proven to be an excellent source of diverse secondary metabolites with biological capacity [1]. Among the classes of secondary metabolites within this genus, there are sesquiterpenes, sesterterpenes, macrolides indole and β-carboline alkaloids [2,3]. Interestingly, the sponge *Hyrtios erectus* has been the subject of most studies for its bioactive products. Some compounds found in *H. erectus*, including indole alkaloids, β-carboline alkaloids, and sesterterpenoids, reported to have anticancer as well as antimicrobial properties [4-5].

There have been numerous discoveries of the marine sponge *H. erectus* collected from the Red Sea in Egypt, and Okinawa in Japan which contain terpenoids class of molecule while the Indonesian marine sponge *H. reticulatus* is another frequently studied which contain a potential source of novel β-carboline alkaloids [6-8]. Chemical investigations on undescribed species of the genus *Hyrtios* may provide us with a unique molecule, the puupehenone, a meroterpenoid class molecule [9].

Puupehenone (1), originally isolated by Scheuer and his co-workers from Hawaiian marine sponge *Chondrosia chucalla*, contains a quinone-methide moiety to allows the synthesis of C/D ring analog, which may occur as a result of 1,6-conjugate addition reactions by attacking C-15 of the parent compound [10]. Incredibly, this class of molecule showed significant bioactive properties. In addition to exhibiting antitumor, antiviral, antimalarial, antibiotic, immunomodulatory, and, most remarkably, antitubercular activities in vitro. Puupehenone (1) and related molecules have also attracted considerable attention in the last years [11-13].

This genus exists all over the world from the Indo-Pacific to the Red Sea. Consequently, morphology, chemical composition, and bioactivity have a strong relation. Recent studies have examined sponges from eastern Indonesia, but not those from western Indonesia. Since the marine environment in the
Indian Ocean varies from that of eastern Indonesia, the morphology and chemical composition of the species there are believed to be unique. In our chemical study of marine invertebrates from Indian Ocean littorals, we found a marine deep-sea sponge, *Hyrtios* sp. in an isolated place around Aceh Besar. Therefore, we would like to discuss about the structural elucidation on *Hyrtios* sp. which was determined by spectroscopic analysis. On the other hand, the proposed conversion of Puupehenone (1) to its derivatives together with their biological properties against multidrug-resistant *Escherichia coli* (MDR *E. coli*), HeLa and Panc-1 cell lines are described here.

2. Material and Methods

2.1. Experimental

1D and 2D NMR spectra were performed on JEOL JNM-ECZR 500 MHz spectrometer. For standards, either tetramethylsilane (TMS) at (1H, δ 0.00) and CDCl3 (13C, δ 77.1) were used and their chemical shifts are represented as ppm (δ). The HRESIMS data was recorded using a JMS-T100LP spectrometer. Jasco P-1010 polarimeter was used for measuring optical rotation. FTIR spectra were obtained on a Jasco FT/IR-6000 spectrophotometer. Shimadzu HPLC unit systems were used to perform the isolation work. In this study, Merck precoated TLC silica gel 60F254 plates and silica gel 60 were utilized for chromatography purposes.

2.2. Biomaterial

An undescribed species of the genus *Hyrtios* as VK_160511 was collected by hand using scuba at 42 m depth off Lhok Mata Ie, Aceh Besar on May 2017. The specimen was kept frozen until extraction.

2.3. Extraction and Isolation

The specimen (0.22 kg, wet) was extracted three times with acetone. The crude extract was partitioned using EtOAc and H2O to give 586.4 mg of a lipophilic extract. Furthermore, the extract was separated on a Sephadex LH-20 using a gradient system of MeOH-acetone and acetone-DCM, which was continued on a silica gel column using a stepwise gradient of n-hexane-DCM mixtures to obtain nonpolar constituents. A portion (122.6 mg) was purified on normal phase HPLC (Cosmosil 5SL-II, n-hexane-DCM, 20-1) to give compound 1 (75.4 mg, 23%). Further purification of another fraction on normal phase HPLC with n-hexane-EtOAc (500-1) gave compounds 2 (10.2 mg, 3%) and 3 (4.7 mg, 1.2%).

Compound 1, yellow glass; C21H28O3; [α]D20 +310 (c 1.5, CCl4); FTIR (thin film) νmax: 3384, 2956, 2889 cm⁻¹; 1H and 13C NMR see Table 1; HRESIMS m/z 329.21113 [M+H]+ (calcd for C21H29O3, 329.21116).

Compound 2, yellow glass; C22H30O3; [α]D20 +315 (c 1.6, CCl4); FTIR (thin film) νmax: 3391, 2927, 2842 cm⁻¹; 1H and 13C NMR see Table 1; HRESIMS m/z 343.22678 [M+H]+ (calcd for C22H31O3, 343.226680).

Compound 3, yellow glass; C23H34O4; [α]D20 +298 (c 1.5, CCl4); FTIR (thin film) νmax: 3402, 2985, 2872 cm⁻¹; 1H and 13C NMR see Table 1; HRESIMS m/z 375.25297 [M+H]+ (calcd for C23H35O4, 375.25295).

2.4. Bioactivity Test

A standard protocol for HeLa and Panc-1 cell lines were carried out using Dulbecco's Modified Eagle's medium (DMEM) and Roswell Park Memorial Institute medium (RPMI), respectively. Inoculated cells were incubated in 96-well plates with 100 uL of the medium under 5% CO2 at 37°C for 24 h. After incubation, 1 μL of 1-3 were added in DMSO solution, then incubated for 48 h. After the media was removed by aspiration, 100 μL of MTT solution (5 mg/mL in PBS) was added and followed by incubation for 3 h. As a final step, 100 μL of DMSO solution was added to dissolve the remaining formazan and each well was measured using a microplate reader SH-9000 at 570 nm [14].
To prepare MDR E. coli, Amp-AgNps was added to the bacterial culture at a concentration of 10^6 CFU mL\(^{-1}\) and incubated at 37°C for 24 h to determine the MIC. After 24 h, the 2 µL of treated bacterial suspension were streaked on Luria agar plates and heated to 37°C for 24 h. During incubation, fresh Luria broth was inoculated with the isolated colonies and reincubated at 37°C for 24 h. After the second cycle, bacterial cultures were exposed to the MIC concentrations for Amp-AgNps at a concentration of 10^6 CFU mL\(^{-1}\). The same process was repeated 15 times; each time, the MIC was evaluated [15].

3. Results and Discussion

Compound 1 was found to have molecular formula as C\(_{21}\)H\(_{28}\)O\(_3\) by HRESIMS data. The NMR data showed signals for four methyl singlets (\(\delta_H\) 1.22, 0.91, 0.84, 0.82; \(\delta_C\) 28.1, 15.0, 22.0, 33.7), three vinyl groups (\(\delta_H\) 6.67, 6.22, 5.85; \(\delta_C\) 140.5, 105.2, 106.1, 147.4, 129.3, 105.2), a carbonyl group (\(\delta_C\) 182.0), two sp\(^3\) methines (\(\delta_H\) 2.05, 0.95; \(\delta_C\) 54.8, 53.8), an oxygenated quaternary sp\(^3\) carbon (\(\delta_C\) 78.7), two quaternary sp\(^3\) carbons and four methylenes (Table 1). Two methyl singlets (\(\delta_H\) 0.84, 0.82, Me-18 and Me-19) showed HMBC cross-peak to \(\delta_C\) 53.8, 40.7, 33.2 (Me-18, Me-19/C-3/C-4/C-5). On the other hand, another two methyl singlets showing HMBC correlations to \(\delta_C\) 54.8, 53.8, 41.8, 40.0 (Me-20/C-1/C-5/C-9/C-10) and \(\delta_C\) 162.8, 78.7, 54.8, 39.2 (Me-21/C-7/C-8/C-9/C-13) suggested the presence of ABC heterocyclic with trans-cis fused ring (Figure 2). The remaining signals of three vinyl groups showed HMBC cross-peaks to \(\delta_C\) 129.3, 106.1 (H-11/C-12/C17), \(\delta_C\) 162.8, 182.0, 147.4, 106.1 (H-14/C-12/C-13/C-15/C-16), \(\delta_C\) 147.4, 182.0, 129.3, 162.8 (H-17/C-12/C-13/C-15) established the whole planar structure as compound 1 (Figures 1-2). After references searching, compound 1 has an identical NMR data with Puupehenone, a sesquiterpene-methylene quinone molecule [11]. Therefore, compound 1 was assigned as (5S,8S,9R,10S)-puupehenone (Figure 3).

![Figure 1. Puupehenone (1) and its analogs (2 and 3).](image-url)

Compound 2, C\(_{22}\)H\(_{30}\)O\(_3\), was analyzed by HRESIMS data which was accounted to have a methoxy group (\(\delta_H\) 3.81; \(\delta_C\) 56.8). The remaining four methyl singlets and vinyl groups indicated the same skeleton as compound 1 (Table 1). However, HMBC cross-peaks between two vinyl groups (\(\delta_H\) 6.56, 6.46; \(\delta_C\) 109.1, 103.7, H-14, H-17) suggested the whole planar structure containing aromatic moiety (Figure 2). As conclusion, compound 2 was assigned as (5S,8S,10S)-16-methoxypuupehenol (Figure 1).

Compound 3 was found as C\(_{22}\)H\(_{30}\)O\(_4\), which confirmed the presence of two methoxy groups (\(\delta_H\) 3.83, 3.44; \(\delta_C\) 56.2, 56.5). The presence of aromatic signals (\(\delta_H\) 6.35, 6.75; \(\delta_C\) 103.5, 119.9, 141.2, 146.5, 148.6, 114.5) confirmed the whole planar structure as derivative of compound 2. Therefore, compound 3 was assigned as (5S,8S,9S,10S,11S)-11-16-dimethoxypuupehenol (Figure 1).
Interestingly, as we found unique merosesquiterpenoids, Puupehenone (1) together with its derivatives (2 and 3), it also revealed their proposed chemical conversion on 1 to form 2 and 3. The presence of resonance on oxygen atom in carbonyl may generate isomerization to form aromatic moiety. Moreover, the rearrangement of a vinyl group at C-11 together with solvolysis reaction by methanol will introduce compound 2. On the other hand, a water molecule was attached on C-11 to form alcohol moiety due to the cleavage of a vinyl group. The presence of methanol also has an opportunity to give solvolysis product as compound 3 (Figure 4). The presence of methoxy groups on compounds 2 and 3 suggested they are likely to be artifacts products.
Compounds 1-3 showed moderate cytotoxicity with MIC values of 28.2, 27.5, and 29.4 μg/mL, respectively, against MDR *E. coli*. On the other hand, the strongest activity was shown against HeLa and Panc-1 cell lines with IC₅₀ value of 0.6, 0.8, 1.1 μg/mL and 1.3, 1.1, 1.7 μg/mL, respectively.

Over millions of years, bacteria evolved sophisticated resistance mechanisms to avoid destroying themselves with antimicrobial molecules. Many bacterial cells can employ multiple mechanisms of resistance on their own to resist an antibiotic's effects, as bacterial classes of resistance are usually achieved by a number of biochemical pathways [16]. In the case of bioactivity on Puupehenones (1-3) against MDR *E. coli*, the study showed that genes that encode fluoroquinolone (FQ) targets, the enzymes DNA gyrase, and topoisomerase IV expel the drug from the cell have been mutated in *E. coli*. Moreover, the protein Qnr protected the FQ target site by means of self-mechanistic mechanisms. Consequently, Puupehenones (1-3) were not effective for antibacterial MDR *E. coli*.

Quinone-containing natural products are highly reactive chemical compounds that are found in both marine and plant organisms [11,17]. Since Puupehenone (1) has a quinone moiety, it generates a more powerful anticancer than its derivatives (2-3). In eukaryotic cells, reactive oxygen species (ROS) are produced by metabolism in mitochondria and other organs that consume large amounts of oxygen. The most reactive ROS contain unpaired electrons (free radicals), including O₂⁻ and •OH. Despite not being a free radical, hydrogen peroxide (H₂O₂) has the ability to diffuse through cell membranes. Also, it is
capable of being converted into OH by the Fenton reaction. Moreover, the low concentrations of ROS are necessary for certain functions in the cell to take place normally. However, cancer cells are believed to have a higher concentration of ROS than normal cells thus requiring more ATP to leads to a major formation of superoxide radical anions through the electron transport chain [18]. As a result of this imbalance, Puupehenon (1) may play a role as chemotherapy can be used to target cancer cells to release more radical species which can make the situation more critical, resulting in their death.

4. Conclusion

The discovery of Indonesian marine deep-sea sponge *Hyrtios* sp. in an isolated place around Aceh Besar. Chemical investigation revealed the presence of unique merosesquiterpenoids, Puupehenone (1) together with the analogs (2 and 3). Biological activity on them indicated the stronger activity against HeLa and Panc-1 cell lines with IC₅₀ value of 0.6 μg/mL and 1.3 μg/mL, respectively, than MDR *E. coli* as moderate cytotoxicity with minimum inhibitory concentration (MIC) values of 28.2 μg/mL. These results also give us information on the availability of unique metabolites together with potential biological activity around Aceh Besar.

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