Pyrimidines from the Philippine Marine Sponge *Aaptos suberitoides*

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Abstract—The pyrimidines thymine and uracil were isolated from the ethyl acetate extract of the marine sponge *Aaptos suberitoides* collected in Kalangahan, Luga-it, Misamis Oriental, Philippines. The extract showed to be toxic against brine shrimp nauplii at LC_{50} of 954.99 ppm. It also displayed significant activities towards *Staphylococcus aureus* and *Escherichia coli*. The elucidation of the structures of thymine and uracil were aided by spectroscopic techniques such as Fourier Transform - Infrared Spectroscopy (FT-IR), Ultraviolet Spectroscopy (UV), ESITOF Mass Spectroscopy (ESITOF-MS), 1-D (^{1}H, ^{13}C) and 2-D (COSY, HMQC, HMBC) Nuclear Magnetic Resonance (NMR).

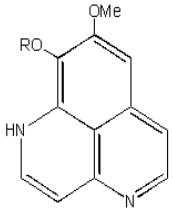
Index Terms— Characterization, Crude extract, Secondary metabolites, Spectroscopy

1 INTRODUCTION

MARINE organisms evolved differently from terrestrial organisms. Because of greater competition of existence in their habitat, marine organisms secrete antibacterial proteins and bioactive secondary metabolites for bacterial defense as part of their metabolism that help them survive [1]. Marine sponges, being sessile, are one of the marine organisms that attract the interest of many natural product chemists because of its unique characteristics in adapting to the harsh and diverse marine environment. The Philippines, as an archipelago, offers vast potentials from its marine resources, sponges in particular, waiting to be utilized as source of essential bioactive substances with pronounced pharmacological potentials. The challenge now lies in the effective exploration of the rich chemical diversity offered by marine life.

This research study involved isolation and elucidation of one metabolite from the marine sponge, *Aaptos suberitoides* (Brønsted, 1934). This marine sponge is a yellow-brown sponge classified under Demospongiae, order Hadromerida, family Suberitidae and genus Aaptos. The distribution of these sponges span within the region of Malaysia, Indonesia, Palau, Vietnam and have fairly widespread in South East Asian wasters. Report of its abundance also includes the Red Sea, Egypt [2]. The most promising compounds isolated from these sponge species are the marine alkaloids aaptamines and demethlyaaptamine (**1**). These compounds have been reported to exhibit a high potent α-adrenoreceptor-blocking activity on vascular smooth muscle and have been shown to be cytotoxic toward murine leukemia cell P388 and other human tumor cell lines [3][4][5].

In 2007, De Voogd, N.J. did an assessment of the possible mariculture potential of selected sponges possessing pharmaceutically promising compounds in the area of Spermonde Archipelago, Indonesia. A. suberitoides was one of the sponges that showed mariculture prospects [6].



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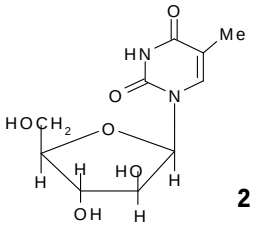
Aaptamine : R = Me
Demethylaaptamine: R = H

Pyrimidines, as well as purines, are vital components of all living cells and involved in several biological processes interacting with the synthesis and function of nucleic acids. The most promising cell growth-inhibiting molecules ever isolated from the marine world were the unusual pyrimidine nucleosides spongothymidine (**2**) and spongouridine (**3**) from the Caribbean sponge Cryptotethia crypta. Few examples of the compounds synthesized from these nucleosides are the anti-viral Ara-A (active against Herpes virus), anti-tumour Ara-C (effective in acute lymphoid leukemea) and Ara-T which inhibits the replication of some DNA viruses. [7][8].



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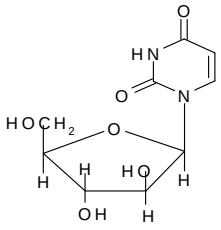

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2 EXPERIMENTAL SECTION

Fresh sample of the marine sponge *A. suberitoides* was collected at the approximated location of 8°20'45.51" N and 124°15'23.43"E by hand scuba at a depth of 5 – 10 meters by local divers on January 13, 2008 at Kalangahan, Lugait, Misamis Oriental, Mindanao, Philippines. The identification of the sponge was done by Dr. Rob W.M. Van Soest, the Head

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In the laboratory, 600 g of the wet sponge sample was washed with sterile sea water, macerated / cut into small pieces and steeped in methanol for three days inside the refrigerator. The solution was then filtered and the pooled MeOH extracts was concentrated *in vacuo* at room temperature to give the aqueous MeOH extract (96.0 g). The resulting aqueous methanol extract was then sequentially partitioned with hexane and ethyl acetate. Upon concentration in vacuo, the hexane (**ASH**, 2.21 g, wet weight), ethyl acetate (**ASEA**, 3.49 g, wet weight) and methanol (**ASM**, 5.33 g, wet weight) extracts were obtained. All three extracts were subjected to toxicity and antimicrobial tests.

Toxicity test made use of the Brine Shrimp Lethality Test (BSLT). It involves the mortality of cultured brine shrimp, *Artemia salina* Leach, towards the extract under study. The measure of toxicity of the extract is determined by the lethal concentration for 50% mortality, as acute LC_{50} (after 6 h) or chronic LC_{50} (after 24 h). Reed-Muench method is the technique use to obtain such measurements, where 95% confidence limit can also be calculated [9]. Antimicrobial activities of the extracts were indicated using gram-positive bacteria *S. aureus*, gram-negative bacteria *E. coli*, and mold species *A. flavus* employing the Paper Disc Diffusion method.

The structures of the metabolites were determined by spectroscopic techniques such as Ultraviolet/Visible Absorption Spectroscopy (UV – VIS), Infrared Radiation (IR), Mass Spectrometry (MS), one-dimensional and two-dimensional Nuclear Magnetic Resonance (NMR). The NMR, IR and MS measurements were conducted in Japan through Dr. Shinji Ohta of Nagahama Institute of Bio-Science and Technology.

Base on its weight and assay results, the EtOAc extract (**ASEA**, 325.3 mg) was subjected to Gravity Column Chromatography (GCC) using silica gel (Scharlau 70-230 mesh ASTM) packed into a 30 x 2.5 cm column eluted with EtOAc in hexane (40-100% with 10% increment) and MeOH in EtOAc (0-100% with 10% increment). This process yielded 9 major fractions among which the second fraction, **ASEA2** was purified further on a modified glass column of 20 x 1 cm, with silica gel and eluted by EtOAc in hexane (0-100%) and MeOH in EtOAc (50-50% and 100-0%) to give **ASEA2.8**, a tlc and HPLC-pure isolate.

The structure elucidation of the metabolites in **ASEA2.8** was done primarily by Mass Spectrometry (MS), one-dimensional (^{1}H-NMR, ^{13}C-NMR) and two-dimensional (H-H COSY, HMBC, HMQC) Nuclear Magnetic resonance (NMR). MS was done using Electronspray Ionization Time of Flight Mass Spectrometry (ESITOFMS). ^{1}H, H-H COSY, HMBC and HMQC spectra were taken at 400 MHz frequency while ^{13}C spectra was recorded at 100 MHz, recorded on a JEOL AL-400 NMR instrument. The reference solvent used was CD_3OD with chemical shift given on δ (ppm) scale (^{1}H 3.30, ^{13}C 49.00). The establishment of the fragments was also aided by its absorption of the ultra violet (UV) using Shimadzu 160A UV-Vis Spectrophotometer, and infrared (IR) radiations using Horiba FT-IR 720 Spectrometer (900-4000 cm^{-1} wavelength range and 92.50-100.8 transmittance range).

3 RESULTS AND DISCUSSION

3.1 Bioactivity of ASEA

The Brine Shrimp Lethality Test (BSLT) showed that the LC_{50} of **ASEA** (the extract from where the pyrimidines were isolated) is 954.99 ppm against *Artemia salina* nauplii (Table 1). It also exhibit significant activity against S. aureus and E. coli in the Paper Disc Diffusion Method (Table 2).

3.2 Characterization of ASEA2.8

Isolate **ASEA2.8** was obtained as a white amorphous solid. Its FT-IR spectrum revealed C=O and C=C stretching vibrations at 1670.05 cm^{-1} and 1712.48 cm^{-1}. N-H and C-H stretching bands were evident at 3444.24 cm^{-1}, 3328.53 cm^{-1} and 2923.56 cm^{-1}. The range of bands at 1600 cm^{-1} – 1000 cm^{-1} displayed the presence of C-N and C-C stretching. The UV spectrum showed two absorption maxima at 211.0 nm and 261.0 nm suggesting the presence of C=C and C=O. Mass

TABLE 1
ESTIMATED LC_{50} AND SE LC_{50} OF THE CRUDE EXTRACTS AGAINST THE BRINE SHRIMP A. SALINA

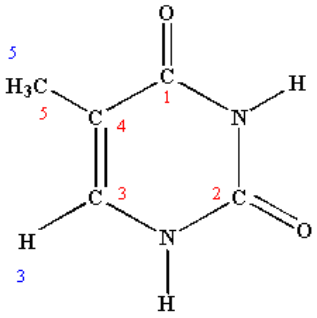
Extract	***Hexane***	***Ethyl Acetate***	***Methanol***
LC_{50} , ppm	421.70	954.99	578.10
Standard Error (SE LC_{50})	1.13	1.09	1.13
95% Confidence range of the (LC_{50})	419.44 – 423.96	952.81 - 957.17	575.84 – 580.36

TABLE 2
ANTIMICROBIAL SCREENING RESULTS OF THE CRUDE EXTRACTS

Extract	***Mean Diameter Zone of Inhibition (mm)***		
	Test Organism		
	S. aureus	E. coli	A. flavus
Hexane (ASH)	8 ± 1	-	-
Ethyl Acetate (ASEA)	20 ± 1	18 ± 1	8 ± 1
Methanol (ASM)	8 ± 1	10 ± 1	-

S. aureus = Gram +; E. coli = Gram - ; A. flavus = Fungi
Disc diameter = 6 mm

spectral analysis substantiated with NMR data indicate that **ASEA2.8** contains, as major components, a mixture of thymine (**4**) and uracil (**5**) with molecular formula $C_5H_6N_2O_2$ and $C_4H_4N_2O_2$, respectively. The spectrum obtained by ESITOF Mass Spectroscopy displayed the thymine M^+ at *m/z* 126.9041.



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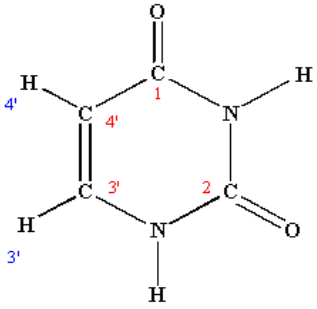

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Inspection of the ^{1}H-NMR spectrum revealed the presence of the downfield vinylic protons at δ 7.38 ppm (d, J = 8 Hz) and δ 7.21 ppm (s) corresponding to H-3' and H-3 of uracil and thymine, respectively. These vinylic protons were shifted downfield due to the effect of the –NH- proton neighbors. The doublet signal at δ 5.60 ppm (J = 8 Hz) denotes the proton H-4' in uracil. The methyl group in thymine is indicated by the broad singlet at δ 1.84 ppm.

The ^{13}C-NMR spectrum displayed the signals for the carbonyl carbons at δ 167.13 ppm (C-1) and δ 153.46 ppm (C-2). The two C=O carbons of thymine and uracil exhibited the same chemical shifts as the two compounds are similar except for the methyl group in thymine. The signals at δ 143.36 ppm, δ 138.91 ppm, δ 110.26 ppm and δ 101.57 ppm correspond to the vinylic carbons C-3', C-3, C-4 and C-4', respectively. The methyl carbon signal was indicated at δ 12.17 ppm. The ^{1}H and ^{13}C-NMR data for isolate **ASEA2.8** are summarized in the table 3.

TABLE 3
^{1}H AND ^{13}C-NMR DATA OF THYMINE AND URACIL IN ASEA2.8

Position Number	δ ^{1}H [a], ppm (multiplicity, J in Hz)	δ ^{13}C [b], ppm
1		167.13 (s)
2		153.46 (s)
3	7.21 (s)	138.91 (d)
3'	7.38 (d, 8 Hz)	143.36 (d)
4		110.26 (s)
4'	5.60 (d, 8 Hz)	101.57 (d)
5	1.84 (bs)	12.17 (q)

a Data measured in CD3OD at 400 MHz.
b Data measured in CD3OD at 100 MHz.

Data from the H-H COSY, HMBC and HMQC spectra also aided the aided elucidation of the pyrimidines. The HMQC spectrum enabled the assignment of vinylic and methyl protons to their corresponding carbons. The COSY spectrum illustrated the coupling of the vinylic protons (H-3' to H-4') in uracil and the coupling of H-3 to H-5 in thymine.
The HMBC spectrum enabled the placement of the carbonyl carbons. The signal for H-3′ (δ 7.21 ppm) showed correlation to both the carbon signals at δ 167.13 ppm and 153.46 ppm, which are both 3 bonds away from it. However, the methyl protons at δ 1.84 ppm was correlated only with the carbonyl signal at δ 167.13 ppm thus confirming the assignment of such signal to C-1.

Further confirmation of the structures of the pyridines in **ASEA2.8** was provided by the good agreement of their NMR data with those in literature [10][11].

4 CONCLUSION

Spectroscopic analyses of the amorphous solid isolate from the ethyl acetate extract, **ASEA2.8**, of the marine sponge *A. suberitoides* revealed the presence of a mixture of the pyrimidines thymine and uracil.

5 RECOMMENDATIONS

It is recommended that greater volume of raw sponge material should be utilized at the first methanolic extraction to acquire a substantial amount of EtOAc extractFurthermore, it is proposed that other fractions from EtOAc and methanol should be purified further, especially those of considerable quantity. The biological potential of the pyrimidines may go beyond toxicity and antibiotic activity. Therefore, it is also suggested that other assay methods should be employed on the **ASEA** extract.

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