

Diet-Derived Halogenated Metabolite from the Sea Hare *Aplysia parvula*

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ABSTRACT As part of our continuous interest in the diversity of halogenated metabolites in the red algae genus *Laurencia* and their grazers, the sea hare genus *Aplysia* in Sabah coastal waters, we report the chemical composition of *Aplysia parvula* collected from Sepanggar Island, Kota Kinabalu. Chemical analysis resulted in the isolation and identification of three sesquiterpenes; palisadin A (**1**), iso-obtusol (**2**) and elatol (**3**). Compounds were present as 13.9%, 6.7%, and 8.5% of crude extract, respectively. A similar analysis of its diet from the same location, *Laurencia snackeyi* (Weber-van Bosse) Masuda and *Laurencia majuscula* (Harvey) Lucas, showed the existence of palisadin A, palisadin B, aplysinatin and 5-acetoxypalisadin B in *Laurencia snackeyi*, while *Laurencia majuscula* contained elatol and isoobtusol. Hence, it is suggestive that *A. parvula* is capable of selective sequestering of compounds derived from its diet. Based on the chemicals sequestered, it is confirmed that *A. parvula* is an oligophagous feeder. Its ability to selectively sequester palisadin A and not the other syndreans, reflects on the complexity of its digestive glands. All three compounds also showed potent antimicrobial, antifeedant and cytotoxic activities.

ABSTRAK Sehubungan dengan minat kami dalam penyelidikan sebatian berhalogen 'seahare' dari perairan Sabah, kami ingin melaporkan komposisi kimia sebatian berhalogen dari *Aplysia parvula* dari Pulau Sepanggar, Kota Kinabalu. Hasil kajian telah membawa kepada pemencilan dan identifikasi tiga sesquiterpene; Palisadin A (**1**), iso-obtusol (**2**) and elatol (**3**). Sebatian-sebatian ini wujud dalam kuantiti yang signifikan 13.9%, 6.7%, and 8.5% dalam ekstrak kasarnya 'sea hare' yang dikaji. Analisis kimia sedemikian keatas dietnya, *Laurencia snackeyi* (Weber-van Bosse) Masuda and *Laurencia majuscula* (Harvey) Lucas, telah menunjukkan kehadiran palisadin A, palisadin B, aplysinatin and 5-acetoxypalisadin B dalam *Laurencia snackeyi*, manakala *Laurencia majuscula* mengandungi elatol and isoobtusol. Maka, dapat disyorkan bahawa *A. parvula* dapat melakukan menepuan sebatian kimia daripada dietnya secara selektif. Berdasarkan bahan kimia yang ditepukan, dapat dikenalpasti bahawa *A. parvula* merupakan pemakan oligofagus. Kebolehan untuk melakukan penemuan selektif terhadap palisadin A dan bukan 'syndrean' yang lain dapat membayangkan betapa kompleksnya kelenjar penghadamannya. Semua ketiga-tiga sebatian ini telah menunjukkan aktiviti antimikrob, 'antifeedant' dan sititoksik.

(Keywords: *Aplysia parvula*, *Laurencia majuscula*, *Laurencia snackeyi*, chemical ecology, halogenated metabolite.)

INTRODUCTION

Sea hares (Opisthobranchia: Anaspidea) are herbivorous gastropods with a soft body which feed predominantly on marine algae and can be found abundantly throughout the tropical/subtropical seas [1]. They generally consume seaweeds that are chemically rich in secondary metabolites and concentrate these metabolites in their digestive glands [2]. Sea hares from the genus *Aplysia* are known to feed on red algae including *Laurencia* that are known to be prolific producers of halogenated secondary metabolites [3, 4, and 5]. Seaweed-derived halogenated metabolites are either accumulated as they are, selectively sequestered or subjected to chemical modifications in their digestive system [6].

As a result sea hares accumulate a variety of metabolites and some are known to exhibit various types of biological activities [7, 8, 9, and 10]. Based on the existing reports, sequestered secondary metabolites are suggested to act as defensive chemicals for sea hares [11]. The ability of their digestive glands to facilitate chemical modifications makes these gastropods an attractive research subject for natural products chemists [5].

Hitherto, very few findings on the chemistry and the interrelationship between *Laurencia* and sea hares from Malaysian waters have been published. In our previous work, we reported the isolation of five sesquiterpenes from *Aplysia dactylomela* Rang collected from the Western and Northern coast of

Sabah [11]. Hence, as part of our continuous effort to study the chemical ecology of sea hares we report herein the halogenated metabolites of *Aplysia parvula* Guilding Morch from the Western coast of Sabah waters.

MATERIALS AND METHODS

Sample Collection

Specimens of *A. parvula* were collected in May 2007 from seaweed beds from the waters of Sepanggar Island, Kota Kinabalu, Sabah, Malaysia. (06°03'350"N, 116°04'140"E). Latitude and longitude were recorded using GPS 12XL (GARMIN Olathe, KS, USA).

Chemical Analysis

Sea hares were starved for 24 hours prior to extraction; this was done to avoid isolation of compounds from algal residues (diet) in their digestive tract. Digestive tract of four specimens of *A. parvula* weighing at 25 g in total was extracted in MeOH for three days. The MeOH extract was concentrated *in vacuo* and partitioned twice between Et₂O and distilled H₂O. The resulting Et₂O fraction was dried over Na₂SO₄ anhydrous and concentrated *in vacuo* to yield 28 mg of crude extract. Chemical profiling and isolation of the halogenated metabolites were performed on a High Performance Liquid Chromatography (HPLC) system consisting of a Shimadzu SLC-10A Controller, SPD-20A UV-Vis Detector, and a Ross plotter. Chromatography analysis was performed on a 10 x 250 mm Phenomenex Luna Phenyl Hexyl reversed phase column with acetonitrile-water (70:30, v/v) as mobile phase. The HPLC operating parameters were as follows: injection volume, 5 mL (Analytical) and 100 mL (Semi-preparative); column flow rate, 2.0 mL/min; chromatographic run time, 50 min; UV spectra recording, 220 nm. The three compounds were present as major peaks in HPLC profile and it corresponded to SiO₂ F_{254nm} Thin Layer Chromatography developed in hexane: EtOAc (3:1), sprayed with 5% molybdophosphoric acid in ethanol. Compound identification was carried out based on spectroscopic data.

Spectroscopic Procedure

Spectroscopy data were measured using ¹H-NMR (600 MHz) and ¹³C-NMR (150 MHz), JEOL ECA 600 MHz; CDCl₃; with TMS as internal standard. The melting point was measured on a micro-melting point apparatus (Fisher Scientific) and was uncorrected. Optical rotations were measured on a JASCO DIP-140 polarimeter and LR/HREIMS, on a JEOL JMS-A500 spectrometer.

Bioassay

Cytotoxic ~ P388 murine leukemia cells (JCRB 17) were cultured in RPMI 1640 medium (Nissui Pharm. Co., Tokyo) supplemented with 100 µg/mL of kanamycin (Nacalai Tesque Inc., Kyoto), 10% of fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), and 10 µM/mL of 2-hydroxyethyl disulfide (Nacalai Tesque Inc. Kyoto) at 37°C under an atmosphere of 5% CO₂. To each well of 96-well microplate which contained 100 µL of tumor cell suspension of 1x10⁴ cells/mL, 100 µL of test solution (sample dissolved in RPMI 1640 medium) was added and the plates were incubated for 96 h. After addition of 50 µL of 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT) saline solution (1 mg/mL) to each well the plates were incubated for 3 h under the same conditions. The mixtures were centrifuged and the supernatants were removed. The precipitates obtained were dissolved in DMSO, and absorbance at 550 nm was measured with a dual wavelength flying spot scanning densitometer.

RESULTS AND DISCUSSION

Chemical profiling of the extract was performed *via* reversed phase HPLC as described in the earlier section. Figure 1 shows the presence of three major peaks at 13.50 min, 16.20 min, and 16.65 min. All three peaks were collected and spotted on SiO₂ gel Thin Layer Chromatography, developed in toluene and hexane: EtOAc (3:1) solvent systems, visualized by UV light (254 nm) and molybdophosphoric acid. Upon repeated purification steps, these compounds were isolated in pure form and subjected to spectroscopic data measurements. Physical properties and detailed ¹H-NMR, ¹³C-NMR and 2D-NMR data lead to the identification of these three compounds (Figure 2) and these are given below:

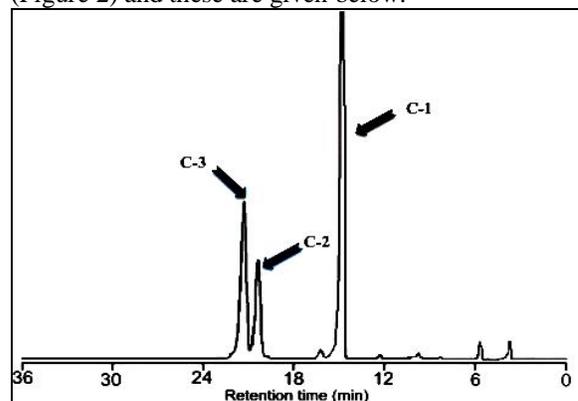


Figure 1. Relative retention time of compounds 1, 2 and 3 in their crude methanol extract of *Aplysia parvula* (C-1: 13.50 min, C-2: 16.20 min, and C-3: 16.65 min).

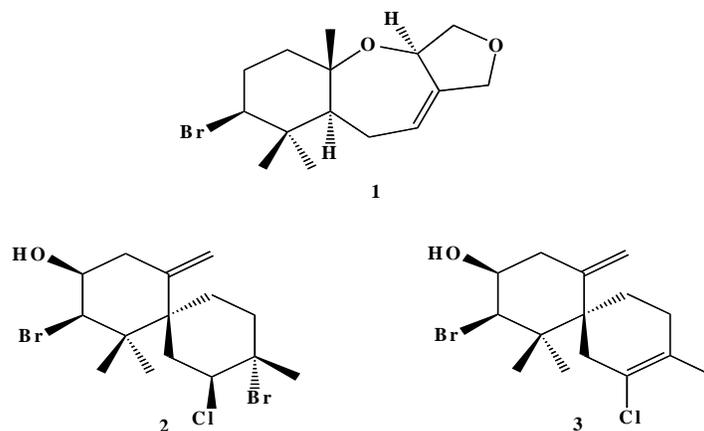


Figure 2. Chemical structure of palisadin A (**1**), elatol (**2**), and iso-obtusol (**3**) isolated from crude methanol extract of *Aplysia parvula*.

Compound 1 – oil, $[\alpha]_D^{24} +19.0^\circ$ (*c* 0.16, CHCl_3); $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ are as reported by Vairappan et al. (2007) [10] and Paul and Fenical (1980) [12]. Compound **1** was identified as palisadin A and was present as 13.9% of its crude extract.

Compound 2 – solid, $[\alpha]_D^{24} +24.6^\circ$ (*c* 0.50, CHCl_3); $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ are as reported by Wessels et al. (2000) and Vairappan (2003) [8,10]. Compound was identified as iso-obtusol and was present as 6.7% of its crude extract.

Compound 3 – oil, $[\alpha]_D^{24} +75.3^\circ$ (*c* 0.40, CHCl_3); $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ are as reported by Wessels et al (2000) and Vairappan (2003) [8,10]. Compound **3** was identified as elatol and was present as 8.5% of its crude extract.

Similar chemical analysis carried out on its natural seaweed diet such as *Laurencia snackeyi* and *L. majuscula* revealed that palisadin A was found in *Laurencia snackeyi* (Weber-van Bosse) Masuda, together with palisadin B, aplystatin and 5-acetoxypalisadin B (Figure 3). In *L. snackeyi*, aplystatin was present as major metabolite followed by 5-acetoxypalisadin B, palisadin A and palisadin B, as 14.6%, 8.5%, 6.2% and 5.1% of its crude seaweed extract, respectively [11]. While elatol and iso-obtusol were found in *Laurencia majuscula* (Harvey) Lucas that was found growing in the same location from where the sea hares were collected (Figure 4). In *L. majuscula*, isoobtusol and elatol were present as 8.6% and 6.4% respectively. During our field investigation, *A. parvula* was seen attached to the thalli of these seaweeds. This is the first reported sighting and isolation of metabolites from *A. parvula* from Malaysian waters. Chemical analysis of *A. parvula* from other regions such as Japan and

Australian showed the presence of diet derived metabolites such as costatone, isolaurenisol acetate and allolaurinterol acetate, but these compounds were not detected in the HPLC analysis of our specimens (Figure 5) [1,9,13].

In our previous investigation, we reported *Aplysia dactylomela* that feeds only on *L. snackeyi* although there were other *Laurencia* species growing in the vicinity [11]. Therefore it was established that *Aplysia dactylomela* is a specialist feeder. On the contrary, *A. parvula* seems to be feeding on both *Laurencia* species based on field observation and chemical analysis. This indicates that it is an oligophagous feeder and it corresponds with other studies on *A. parvula* carried out in Japan and Australia [1, 13]. It is believed that the sea hare sequesters and harbors these metabolites in its digestive tract to be utilized as defense mechanism [3, 14, and 15]. The presence of only palisadin A derived from *L. snackeyi*, as compared to *A. dactylomela* that contained the other three metabolites and few modified ones is another interesting observation. Selective retention of palisadin A in such a high concentration should have a meaning, perhaps as an important defense compound for this organism. In nature, palisadin A is an unstable compound and could easily be converted to aplystatin. Hence, its ability to exist as a sole syndrean metabolite in *A. parvula* reflects on the unique pH and biochemical properties of this animal's digestive glands.

All three halogenated metabolites isolated from *A. parvula* were tested for their antimicrobial potential against marine environmental bacteria, human pathogenic bacteria and pathogenic yeast.

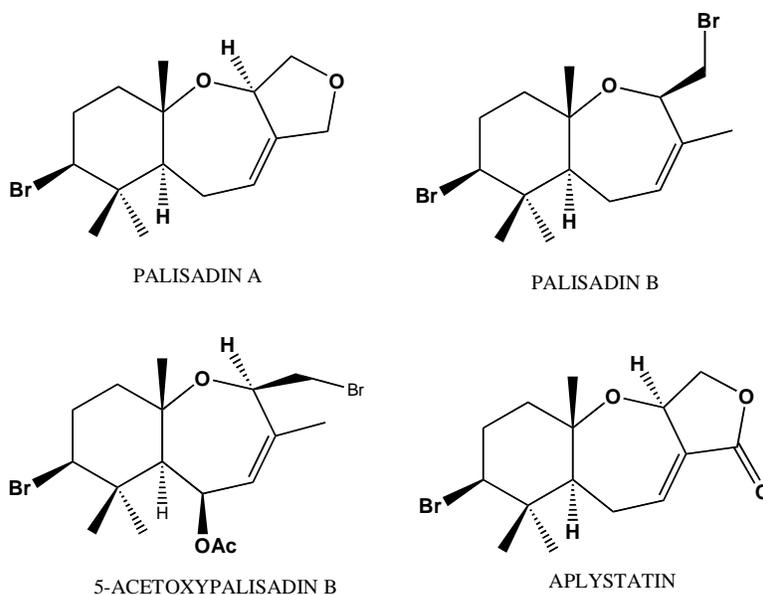


Figure 3. Chemical structure of palisadin A, palisadin B, 5-acetoxypalisadin B and aplystatin isolated from crude methanol extract of *Laurencia snackeyi*.

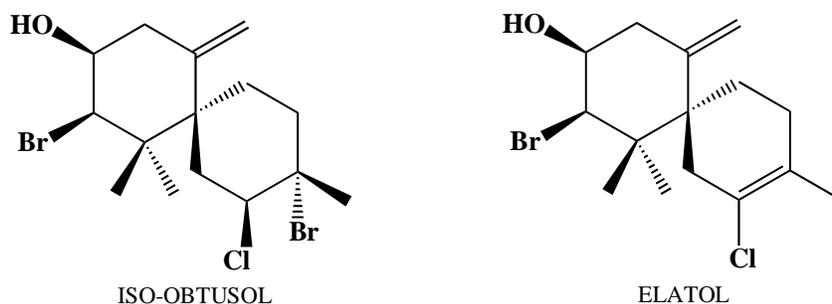


Figure 4. Chemical structure of elatol and isoobtusol isolated from crude methanol extract of *Laurencia majuscula*.

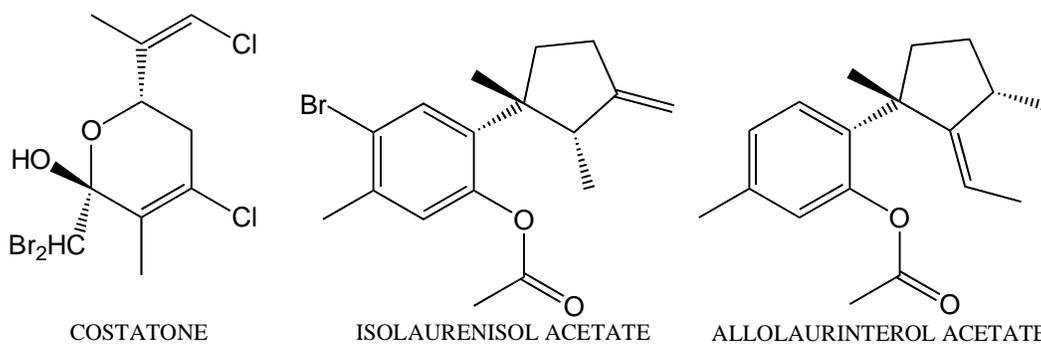


Figure 5. Chemical structure of costatone, isolaurenisol acetate and allolaurinterol acetate isolated from crude methanol extract of Australian *Aplysia parvula*.

Results are as reported by Vairappan (2003) and Vairappan et al. (2007) [10, 11]. Additional cytotoxic test against P-388 cell lines showed apoptosis of the cell lines in 24 hours at concentration as low as 0.05 mg/ml, 0.025 mg/ml and 0.025 mg/ml for palisadin A, iso-obtusol and elatol, respectively.

CONCLUSIONS

Based on our ongoing investigation and previous reports by various researchers, it can be suggested that sea hare from the genus *Aplysia* have preference for red algae genus *Laurencia* as its diet, although

they are also seen grazing on blue green algae. In Sabah waters, *A. parvula* has shown to prefer the red algae *L. snackeyi* and *L. majuscula*. In addition to their ability to sequester and harbor defensive metabolites in its digestive tract, this investigation has also disclosed that *A. parvula* is an oligophagous feeder on the red algal genus *Laurencia*. The biological activities exhibited by these compounds are very broad and could very well assist this vulnerable soft-bodied organism to defend itself against disease, biofoulers and microbes.

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