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# Halogenated Secondary Metabolites from Sea Hare *Aplysia dactylomela*

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**ABSTRACT** Sea hare, *Aplysia dactylomela*, from North Borneo waters was investigated for its secondary metabolite content. Investigation resulted in the isolation and identification of three halogenated sesquiterpenes; Palisadin A (**1**), Aplysistatin (**2**) and 5-acetoxypalisadin B (**3**). Compounds were present in significant quantity, 5 %, 3 % and 3 % of crude extract, respectively. The same compounds were also isolated from *Laurencia snackeyi* found growing in the same location where the sea hare was collected. Further bioassay experiments showed interesting pattern in their antimicrobial activities; Palisadin A (**1**) showing > 83% inhibition against tested organisms and Aplysistatin (**2**) exhibited > 27% activity. However, 5-acetoxypalisadin B (**3**) did not show any inhibition against the tested organisms.

**ABSTRAK** *Aplysia dactylomela* dari perairan Borneo Utara diselidik untuk penentuan kandungan sebatian sekunder. Kajian ini telah membawa kepada pemencilan dan identifikasi struktur tiga sebatian sekunder berhalogen; Palisadin A (**1**), Aplysistatin (**2**) and 5-acetoxypalisadin B (**3**). Sebatian-sebatian ini wujud dalam kuantiti, 5 %, 3 % dan 3 % ekstrak kasarnya. Kesemua sebatian ini juga telah dipencil daripada alga merah *Laurencia snackeyi*, yang didapati hidup dengan liar di sekitar lokasi "sea hare" ditemui. Kajian bioesei sebatian-sebatian ini menunjukkan pola aktiviti antimikrob yang menarik; Palisadin A (**1**) mempamirkan >83% perencatan terhadap mikob yang diuji, manakala Aplystatin (**2**) pula hanya mempamirkan >27% perencatan. Walau bagaimanapun, 5-acetoxypalisadin B (**3**) tidak menunjukkan sebarang perencatan terhadap mikrob yang diuji.

(*Aplysia dactylomela*, Halogenated metabolites, *Laurencia snackeyi*, Antimicrobial activities)

## INTRODUCTION

Sea hares (Opisthobranchia: Anaspidae) are herbivorous gastropods, widely distributed throughout the tropical seas and are known to feed predominantly on marine algae and occasionally on sponges [1]. Chemical studies of this soft bodied molluscan started with the discovery of brominated sesquiterpenes, aplysin, debromoaplysin and aplysinol, from sea hare *Aplysia kurodai* Baba (1937) by Yamamura and Hirata [2]. The three brominated sesquiterpenes remained something of a curiosity until Irie demonstrated the presence of laurinterol, debromolaurinterol and related compounds among the secondary metabolites of the red alga, *Laurencia intermedia* Yamada [3, 4 and 5]. In vitro transformation of laurinterol to aplysin further suggested a model for the conversion of laurinterol from *Laurencia* sp., which was a known constituent of the sea hare's diet, into the aplysin obtained from *A. kurodai*. Sea hares are now known to sequester secondary metabolites from their diets and have proven to be a rich source of secondary metabolites, often found in higher concentration than in the source food plants [6]. Since then, more than 100 new secondary metabolites have been isolated from opisthobranch molluscs [7, 8]. The secondary metabolites that sea hares sequester from their diets are often thought to defend the sea hares against potential predators. Although there are some evidence to support this, but not without its exceptions [6, 7]. Hitherto, there has not been any published information pertaining to the chemistry of sea hares from the Malaysian

waters. Present investigation focuses on the chemistry of *Aplysia dactylomela* Rang 1828 (Gastropoda, Aplysiidae), collected from North Borneo waters and bioactive potentials of the isolated metabolites.

## MATERIALS AND METHODS

### Collection and Isolation

One sample (single animal, No.: SHK1#04) of *A. dactylomela* Rang 1828, was collected in June 2004, from the beach of Pulau Banggi, Kudat District, Sabah, Malaysia. Collected sea hare was starved for 24 hours prior to extraction; this was done to avoid isolation of compounds from plant residues in their digestive tracts.

Animal tissue (8 g) was macerated and extracted in methanol (1 L) for 5 days. Solvents were removed *in vacuo* and the extracts partitioned twice between $Et_2O/H_2O$, $Et_2O$ fraction was collected and dehydrated in $Na_2SO_4$ anhydrous for 2 hours. This was then filtered and solvents removed *in vacuo* to yield 34 mg of greenish paste, crude extract.

Chemical profiling and isolation of the halogenated secondary metabolites were carried out as reported by Vairappan [9, 10].

### *Spectroscopic Procedures*

### General Experimental Procedures

The $^1$H NMR (600 MHz), $^{13}$C NMR (150 MHz) and 2D-NMR spectra were recorded on a JEOL ECA 600 spectrometer. Melting point was measured on a Fisher Scientific micro-melting point apparatus and was uncorrected. Optical rotations were measured on a JASCO DIP-140 polarimeter, LR/HREIMS, on a JEOL JMS-A500 spectrometer. Si gel plates (Merck, Kieselgel 60 $F_{254S}$) were used for preparative TLC.

**Palisadin A** (**1**) – oil, $[\alpha]^{24}_D$ +19.0 (*c* 0.17, $CHCl_3$); $^1$H NMR ($CDCl_3$, 600 MHz) δ 0.93 (3H, s, $H_3$-14), 1.17 (3H, s, $H_3$-15), 1.27 (3H, s, $H_3$-13), 1.55 (1H, ddd, *J*= 3, 3, 13 Hz, $H_\beta$-8), 1.80 (1H, ddd, *J*=3,13,13 Hz, $H_\alpha$-8), 2.05 (1H, m, H-6), 2.26 (1H, m, H-9), 2.36 (1H, m, H-5), 3.44 (1H, dd, *J*= 8, 8 Hz, $H_\beta$-1), 3.96 (1H, dd, *J*= 5, 12 Hz, H-10), 4.07 (1H, dd, *J*= 8, 8 Hz, $H_\alpha$-1), 4.38 (1H, dd, *J*= 13, 13 Hz, H-12), 4.83 (1H, brs, H-2), 5.55 (1H, brs, H-4); $^{13}$C NMR ($CDCl_3$ 150 MHz) δ 18.68 (q, C-14), 22.60 (q, C-13), 26.96 (d, C-5), 31.50 (q, C-15), 33.36 (d, C-9), 38.18 (t, C-8), 41.65 (s, C-11), 52.46 (d, C-6), 66.98 (d, C-10), 70.74 (d, C-2), 71.73 (d, C-12), 72.68 (t, C-1), 78.65 (s, C-7), 121.83 (d, C-4), 142.52 (s, C-3); HREIMS m/z (calcd for $C_{15}H_{23}O_2Br$, ). Spectroscopy data corresponds most with data published by Paul and Fenical, [11].

**Aplysistatin** (**2**) – white crystal, $[\alpha]^{24}_D$ -29.0 (*c* 0.10, $CHCl_3$); $^1$H NMR ($CDCl_3$, 600 MHz) δ 0.97 (3H, s, $H_3$-14), 1.19 (3H, s, $H_3$-15), 1.30 (3H, s, $H_3$-13), 1.62 (1H, ddd, *J*= 3, 3, 13 Hz, $H_\alpha$-8), 1.79 (1H, ddd, *J*=3,13,13 Hz, $H_\beta$-8), 2.04 (1H, m, H-6), 2.11 (1H, m, H-9), 2.28 (1H, m, H-9), 2.55 (1H, m, H-5), 3.86 (1H, dd, *J*= 8, 8 Hz, $H_\alpha$-1), 3.93 (1H, dd, *J*= 4, 14 Hz, H-10), 4.49 (1H, dd, *J*= 8, 8 Hz, $H_\beta$-1), 5.13 (1H, brs, H-2), 6.96 (1H, brs, H-4); $^{13}$C NMR ($CDCl_3$ 150 MHz) δ 17.97 (q, C-14), 21.70 (q, C-13), 27.20 (d, C-5), 30.74 (q, C-15), 32.42 (d, C-9), 37.65 (t, C-8), 40.99 (s, C-11), 51.22 (d, C-6), 65.14 (d, C-10), 66.78 (d, C-2), 69.87 (d, C-1), 79.70 (s, C-7), 131.94 (s, C-3), 143.09 (d, C-4), 169.14 (s, C-12); HREIMS m/z (calcd for $C_{15}H_{21}O_3Br$, ). Spectroscopy data corresponds most with data published by Capon *et al.* [12] and Hoye *et al.* [13].

**5-Acetoxypalisadin B** (**3**) – oil, $[\alpha]^{24}_D$ +19.0 (*c* 0.17, $CHCl_3$); $^1$H NMR ($CDCl_3$, 600 MHz) δ 1.03 (3H, s, $H_3$-14), 1.22 (3H, s, $H_3$-15), 1.61 (1H, m, $H_\alpha$-8), 1.65 (3H, s, $H_3$-13), 1.75 (1H, m, H-6), 1.77 (3H, s, $H_3$-12), 1.86 (1H, m, $H_\beta$-8), 2.10 (3H, s, $H_3$-16), 2.18 (1H, m, $H_\alpha$-9), 2.30 (1H, m, $H_\beta$-9), 3.43 (1H, dd, *J*= 8, 11 Hz, $H_\alpha$-1), 3.72 (1H, dd, *J*= 3, 11 Hz, $H_\beta$-1), 3.88 (1H, dd, *J*= 4, 13 Hz, H-10), 4.45 (1H, d, *J*= 9 Hz, H-2), 5.71 (1H, d, *J*= 6 Hz, H-4), 5.80 (1H, d, *J*= 8 Hz, H-5); $^{13}$C NMR ($CDCl_3$ 150 MHz) δ 18.75 (q, C-14), 21.25 (q, C-12), 21.55 (q, C-16), 25.29 (q, C-13), 30.88 (q, C-15), 32.81 (d, C-9), 34.83 (t, C-1), 39.37 (t, C-8), 41.38 (s, C-11), 53.82 (d, C-6), 66.12 (d, C-10), 69.74 (d, C-5), 70.15 (d, C-2), 126.98 (d, C-4), 142.25 (s, C-3), 171.14 (s, C-17); HREIMS m/z (calcd for $C_{17}H_{26}O_3Br_2$, ). Spectroscopy data corresponds most with data published by Paul and Fenical, [11].

### Biological Tests

The antimicrobial bioassays for the isolated halogenated compounds were carried out using seven species of pathogenic yeast, six species of environmental bacteria and five species of terrestrial fungi. Assays were performed as previously described by Vairappan [9, 10].

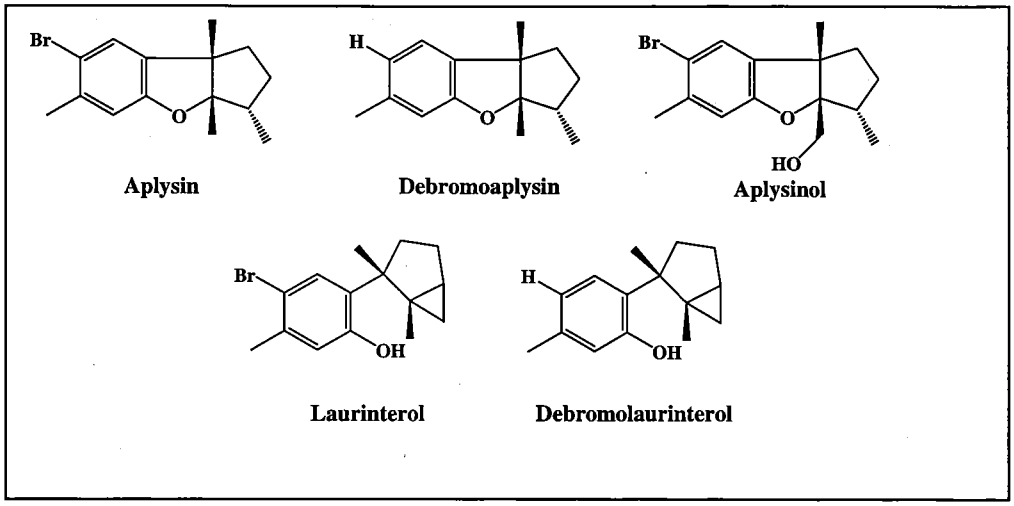


**Figure 1.** Halogenated metabolites first reported to be associated with *Aplysia-Laurencia* investigation; aplysin, debromoaplysin, aplysinol, laurinterol and debromolaurinterol

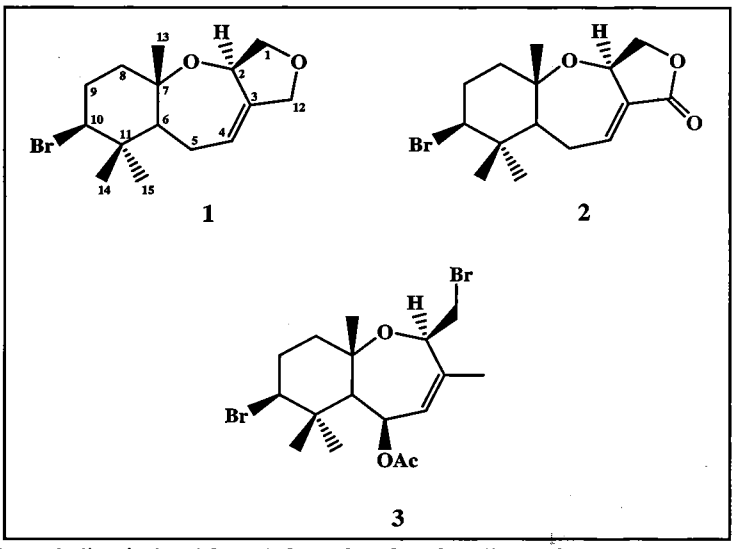


**Figure 2.** Halogenated metabolites isolated from *Aplysia dactylomela* collected from North Borneo waters; Palisadin A (**1**), Aplysistatin (**2**), and 5-acetoxypalisadin B (**3**).

## RESULTS AND DISCUSSION

One relatively small specimen of *A. dactylomela* was collected, macerated and extracted in methanol for five days. The methanol extract was concentrated in *vacuo* and partitioned between $Et_2O/H_2O$, $Et_2O$ fraction dried over $Na_2SO_4$ anhydrous and the resulting $Et_2O$ fraction concentrated *in vacuo* to give crude extract (34 mg). Crude extract was examined by $SiO_2$ gel Thin Layer Chromatography (TLC) in Toluene and Hexane:EtOAc (3:1) solvent system, visualized by UV light (254 nm) and molybdophosphoric acid. Presence of three bright blue spots were visualized at $R_f$ 0.20 (Compound **1**), 0.10 (Compound **2**) and 0.25 (Compound **3**), in toluene solvent system. Due to the limited amount of crude extract, separation was performed *via* Preparative Thin Layer Chromatography (PTLC) in toluene and hexane:ethyl acetate (3:1) solvent systems, repeatedly. Total of 3 halogenated secondary metabolites were isolated and identified *via* spectral data to be Palisadin A (**1**), Aplysistatin (**2**) and 5-acetoxypalisadin B (**3**) (Figure 2) [11, 12 and 13].

All the three halogenated metabolites isolated from *A. dactylomela* are known to be present in *Laurencia snackeyi* (Weber-van Bosse) Masuda that grows at the location where this sea hare was collected [14]. In the field, tiny sea hare juveniles of *A. dactylomela* were observed attached to branches of *L. snackeyi*, where their cryptic coloration made them very well camouflaged. Therefore, based on observation and available data (unpublished), it is suggested that *A. dactylomela* grazes on *L. snackeyi* and sequesters the plant's secondary halogenated metabolites in its body. Similar phenomenon has been reported for *A. dactylomela* that grazes on other *Laurencia* species in temperate waters [15, 16, 17, 18 and 19]. Two of these metabolites, Palisadin A (**1**) and aplysistatin (**2**) were previously isolated from *A. angasi* (*A. dactylomela*) that grazes on *Laurencia filiformis* (C. Agardh) Montagne and was reported by Pettit *et al.* [20].

Initially, it was assumed that aplysistatin (**2**) was an oxidation product of palisadin A (**1**), but was later isolated from *L. filiformis*. Our finding, where *A. dactylomela* accumulates diet-derived halogenated secondary metabolites, is of no surprise since sea hares are well known for their ability to sequester such metabolites from their dietary algae. Similar cases involving *A. dactylomela* was also reported by McPhail and co-workers when they reported nidificene and prepacifenol epoxide (metabolites of *Laurencia*) from sea hares collected from South Africa [1]. Besides, metabolites of *Laurencia poitei* Lamouroux (dactylene, dactyloxene-A, dactyloxene-B and dactyloxene-C) were also found to be present in *A. dactylomela* [21].

**Table 1.** Antimicrobial activity of halogenated metabolites isolated from *Aplysia dactylomela.*

| | Compounds Tested | | |
|---|---|---|---|
| **Tested Microbes** | **Compound 1** | **Compound 2** | **Compound 3** |
| **PATHOGENIC YEAST** | | | |
| *Candida albicans* (ATCC) | + | + | - |
| *Candida albicans* (G561) | + | + | - |
| *Candida albicans* (G588) | - | - | - |
| *Candida albicans* (G670) | + | - | - |
| *Candida albicans* (U1515) | + | - | - |
| *Candida albicans* (U1580) | + | + | - |
| *Cryptococcus neoformans* | - | - | - |
| **ENVIRONMENTAL BACTERIA** | | | |
| *Clostridium cellobioparum* | + | - | - |
| *Clostridium sordelli* | + | - | - |
| *Clostridium novyi* | + | - | - |
| *Proteus vulgaris* | + | + | - |
| *Vibrio alginolyticus* | + | + | - |
| *Vibrio parahaemolyticus* | + | - | - |
| **TERRESTRIAL FUNGI** | | | |
| *Mucor sp.* | +++ | - | - |
| *Aspergillus niger* | ++ | - | - |
| *Aspergillus terreus* | + | - | - |
| *Penicillium sp.* | + | - | - |
| *Monilia sp.* | - | - | - |

Inhibition zone diameter; +++: 19-24 mm, ++: 12-18 mm, +: 7-12 mm, - : no inhibition.
Compound concentration: 30 $\mu$gdisc$^{-1}$ (NCCLS levels)

Apart from that, detailed spectroscopy analysis of the isolated compounds also revealed significant differences in chemical shifts of some protons and carbons of these compounds as compared to the ones reported by Paul & Fenical [11], Capon *et al.* [12] and Hoye *et al.* [13]. However, independent spectral analysis resulted in the similar molecular structures. Detailed spectroscopic data are shown in the Materials and Methods section. In depth comparison and discussion of chemical shift values of our data with that of published data will not be done in this paper. However, the differences observed between these data could be due to the usage of much advanced NMR instrumentation as compared to the time of earlier findings were reported.

Isolated metabolites were tested for their antimicrobial potentials; Table 1 shows the 3 compounds' activities against a wide range of pathogenic and terrestrial microorganisms. Potent antimicrobial activities were exhibited by Palisadin A (**1**) and Aplysistatin (**2**). Aplysistatin (**2**) has been well documented as highly cytotoxic metabolites and was initially reported to be isolated from an Australian sea hare *Aplysia angasi*, then from *L. filiformis* [20]. It was reported potent against murine lymphocytic leukemia P-388 and growth of the new P-388 in vitro cell line. Our on going study has also shown that Palisadin A (**1**) and Aplysistatin (**2**) has strong tendency to be toxic against post-larval sea bass fish fries (on going study). These compounds may play a significant role in the sea hare either as antimicrobial agent or substance to evade predators. However, tangible proof is often a problem due to the difficulty in establishing ecologically relevant bioassay. Even if it is possible, results are often subjected to various unavoidable assumptions. Hence, at this point it is only suggestive that the isolated compounds might play these roles in this animal.

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