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dditional Halogenated Secondary Metabolites from the Sea Hare *plysia dactylomela*

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eceived 21st November 2006, accepted in revised form 12th April 2007.

BSTRACT As part of our continuous interest in the presence of secondary metabolites in marine ɔra and fauna of Borneo, we investigated the chemical composition of sea hare (*Aplysia dactylomela*) llected from Sepanggar Island, Kota Kinabalu. Five halogenated secondary metabolites were isolated d identified as; Palisadin A (**1**, 2%), Aplysistatin (**2**, 16%), 5-acetoxypalisadin B (**3**, 2%), Palisadin B (**4**, %) and 12-hydroxypalisadin B (**5**, 2%). Similar analysis of its diet, *Laurencia snackeyi*, indicated the esence of compounds **1**, **2**, **3** and **4** only. The presence of the additional compound, 12-droxypalisadin B (**2**) is suggested to be a derivative compound due to chemical modification of ılisadin B (**4**) in the gut of the sea hare and can be explained through biogenesis. Isolated compounds so showed various levels of antimicrobial activities against environmental and clinical microbes.

BSTRAK Sehubungan dengan minat dalam kajian kandungan sebatian sekunder dari flora and una Borneo, kami telah menyelidiki kandungan kimia "sea hare" (*Aplysia dactylomela*) dari perairan ılau Sepanggar, Kota Kinabalu. Sebanyak lima sebatian sekunder berhalogen telah dipencilkan dan kenalpasti sebagai; Palisadin A (**1**, 2%), Aplysistatin (**2**, 16%), 5-acetoxypalisadin B (**3**, 2%), Palisadin (**4**, 4%) and 12-hydroxypalisadin B (**5**, 2%). Analisis kimia yang serupa telah dilakukan terhadap mpai laut (*Laurencia snackeyi*) yang merupakan makanan "sea hare" tersebut dan sebatian-sebatian (**1**),), (**3**) dan (**4**) telah dapat dikenalpasti sebagai sebatian berhalogen yang dihasilkan oleh rumpai laut sebut. Kehadiran 12-hydroxypalisadin B (**2**) dalam "sea hare" dapat dianggap sebagai sebatian terbitan, sil transformasi kimia Palisadin B (**4**) dalam perut "sea hare" dan dapat dijelaskan menerusi konsep ɔgenesis. Sebatian-sebatian yang dipencilkan juga telah menunjukkan pelbagai tahap perencatan hadap mikrob persekitaran dan klinikal.

plysia dactylomela, Halogenated Metabolites, Derivative Compound, *Laurencia snackeyi*)

INTRODUCTION

a hares (Opisthobranchia: Anaspidae) are soft-ıdied herbivorous gastropods that feed imarily on marine algae and are widely stributed throughout the tropical and btropical seas [1]. They are known for their ility to sequester secondary metabolites from etary algae. Members of the genus *Aplysia* ve narrow dietary preferences; often found to ed on a few selected algae that include *urencia* species which are known to be prolific oducers of various halogenated secondary etabolites [1, 2]. Chemistry of sea hare has en an area of intensive research for the last 30 ars, where a wide variety of halogenated and non-halogenated metabolites has been reported [3, 4, 5]. The origin of these compounds was traced to their diet. However, there were also some that were known to be derivatives of halogenated metabolites sequestered from their diet. The above observations were well documented particularly for the sea hare *Aplysia* and red algae *Laurencia* [3, 6]. Sea hare-derived natural and derivatized secondary metabolites have been known to exhibit various levels of biological activities against clinical pathogens and tumour cell lines [7, 8, 9, 10].

Information pertaining to the presence and diversity of secondary metabolites from marine invertebrates from Malaysian waters is limited.

This could be due to the lack of interest and awareness on the wealth of bioactive substances from marine resources. The objective of our research interest in marine natural products studies is to better understand the "chemical ecology" of marine flora and fauna of Borneo. In our first report on sea hare chemistry, we disclosed the isolation of Palisadin A (**1**), Aplysistatin (**2**) and 5-acetoxypalisadin B (**3**) from *Aplysia dactylomela* Rang 1828 collected from the northern coastal waters of Borneo [11]. Therefore, as part of our continuous study on the chemistry of sea hares, we report here, the isolation of additional halogenated secondary metabolites from *Aplysia dactylomela*, collected from western coastal waters of Borneo.

MATERIALS AND METHODS

Collection and Isolation

Two specimens (No.:SHSB1#05) of *A. dactylomela* Rang 1828, was collected in May 2005, from the vicinity of the algal bed at the coastal 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

The sea hares were starved for 24 hours and dissected for their digestive tracts. The digestive tracts (500g) were macerated and extracted in MeOH (2L) for 5 days. Extract was filtered, and the resulting methanol solution was concentrated *in vacuo* and partitioned between Et_2O and distilled H_2O in ratio of 3:1 (v/v). Et_2O solution was washed with two changes of H_2O, dried over anhydrous Na_2SO_4 and evaporated to yield 5.3g of dark yellowish paste crude extract.

Chemical profiling of the crude extract was done by spotting crude extract on SiO_2 gel F_{254} nm Thin Layer Chromatography and developed in Toluene (100%) and Hexane:EtOAc (3:1) solvent systems, and visualized by UV light (254nm) and molybdophosphoric acid. Crude extract (1600 mg) was fractioned by Si gel column chromatography (CC) with a step gradient of hexane and ethyl acetate in the ratio of 9.5:0.5, 8.0:2.0, 7.0:3.0, 6.0:4.0 and 5.0:5.0, separation procedure as reported in our previous publications [11, 12, 13]. Fractions 1, 2 and 3 were repeatedly subjected to preparative Thick Layer Chromatography (PTLC) (Merck, Kieselgel 60 F_{254S}) with $CHCl_3$ solvent system to isolate Palisadin A (**1**), Aplysistatin (**2**), 5-acetoxypalisadin B (**3**), Palisadin B (**4**) and 12-hydroxypalisadin B (**5**) (Figure 1).

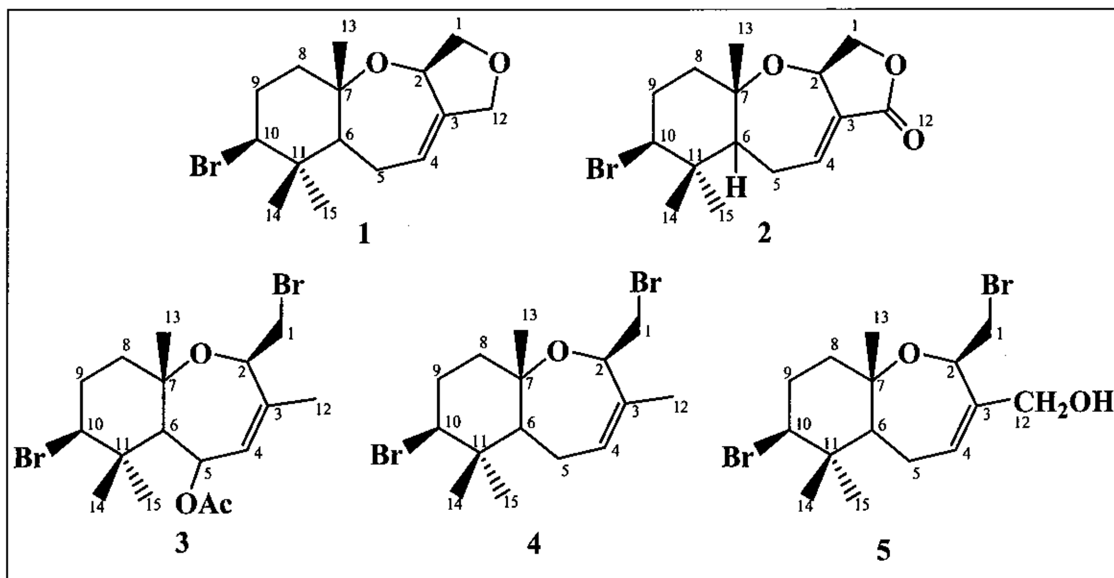

Figure 1. Halogenated metabolites isolated from *Aplysia dactylomela* collected from Sepanggar Island, Kota Kinabalu; Palisadin A (**1**), Aplysistatin (**2**), 5-acetoxypalisadin B (**3**), Palisadin B (**4**) and 12-hydroxypalisadin B (**5**).

SPECTROSCOPIC PROCEDURES

General Experimental Procedures

Spectroscopy data were measured using ^{1}H-NMR (600MHz) and ^{13}C-NMR (150MHz), JEOL ECA 600MHz; $CDCl_3$; TMS as internal standard. 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.

Palisadin A (1) – oil, $[\alpha]^{24}_{D}$ +19.0 (c 0.16, $CHCl_3$); ^{1}H-NMR and ^{13}C-NMR are as reported by Vairappan and Tan [11].

Aplysistatin (2) – white crystal, $[\alpha]^{24}_{D}$ -28.0 (c 0.14, $CHCl_3$); ^{1}H-NMR and ^{13}C-NMR are as reported by Vairappan and Tan [11].

5-acetoxypalisadin B (3) – oil, $[\alpha]^{24}_{D}$ +18.0 (c 0.15, $CHCl_3$); ^{1}H-NMR and ^{13}C-NMR are as reported by Vairappan and Tan [11].

Palisadin B (4) – oil, $[\alpha]^{24}_{D}$ +18.0 (c 0.15, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 600 MHz) δ 0.95 (3H, s, H_3-14), δ 1.15 (3H, s, H_3-15), δ 1.69 (3H, s, H_3-12), δ 1.36 (3H, s, H_3-13), δ 1.77 (2H, m, H_2-8), δ 2.05 (2H, m, H_2-5), δ 1.77 (1H, m, H-6), δ 2.25 (2H, m, H_2-9), δ 3.41 (1H, dd, J=11.0, 7.0, Hβ-1), δ 3.73 (1H, dd, J=11.0, 3.0, H_α-1), δ 3.95 (1H, dd, J=12.0, 5.0, H-10), δ 4.54 (1H, brs, H-2), δ 5.63 (1H, d, J=8.0, H-4) and ^{13}C-NMR ($CDCl_3$, 150 MHz) δ 17.9 (C-14), δ 21.0 (C-12), δ 22.0 (C-13), δ 25.9 (C-5), δ 30.7 (C-15), δ 32.9 (C-9), δ 36.2 (C-1), δ 36.7 (C-8), δ 40.8 (C-11), δ 52.8 (C-6), δ 66.3 (C-10), δ 70.7 (C-2), δ 77.5 (C-7), δ 129.4 (C-4), δ 136.1 (C-3); HREIMS M^+m/z 378/380/382 (calculated for $C_{15}H_{24}OBr_2$). Spectroscopy data corresponds with data published by Paul and Fenical [14], and Couladouros and Vidali [15].

12-hydroxypalisadin B (5) – oil, $[\alpha]^{24}_{D}$ +18.0 (c 0.15, CHCl3); ^{1}H-NMR ($CDCl_3$, 600 MHz) δ 0.93 (3H, s, H_3-14), δ 1.13 (3H, s, H_3-15), δ 1.33 (3H, s, H_3-13), δ 1.68 (1H, m, H_β-8), δ 1.86 (1H, m, H_α-8), δ 1.86 (1H, m, H-6), δ 2.27 (2H, m, H_2-5), δ 2.27 (2H, m, H_2-9), δ 3.54 (1H, dd, J=12.0, 10.0, H_β-1), δ 3.93 (1H, m, H_α-1), δ 3.93 (1H, m, H-10), δ 4.02 (1H, d, J=11.0, H_α-12), δ 4.22 (1H, d, J=11.0, H_β-12), δ 4.68 (1H, brs, H-2), δ 5.90 (1H, d, J=8.0, H-4) and ^{13}C-NMR ($CDCl_3$, 150 MHz) δ 18.0 (C-14), δ 22.0 (C-13), δ 25.9 (C-5), δ 30.7 (C-15), δ 32.9 (C-9), δ 35.8 (C-1), δ 36.7 (C-8), δ 40.8 (C-11), δ 52.4 (C-6), δ 66.1 (C-2), δ 66.1 (C-10), δ 69.6 (C-12), δ 77.6 (C-7), δ 120.7 (C-4), δ 133.2 (C-3); HREIMS M^+-Br m/z 315/317 (calculated for $C_{15}H_{24}O_2Br_2$). Spectroscopy data corresponds with data published by Paul and Fenical [14], and Couladouros and Vidali [15].

Antibacterial Bioassay

Isolated compounds were tested for their biological activities against six strains of environmental bacteria and seven strains of human pathogenic bacteria. Details of the test organisms are given in Table 1. Potency of these compounds was compared against a range of commercially available antibiotics; Novobiocin (NB30), Minocycline (MI30), Vancomycine (VA30), Cefazoline (CZ30) and Kanamycine (K30). Concentrations of tested compounds and antibiotics were standardized at 30 μgdisc^{-1} as recommended by the CLSI (Clinical and Laboratory Standard Institute), formerly known as NCCLS. Antimicrobial bioassays were performed as previously described by Vairappan [11, 12, 13].

Table 1. Antimicrobial activities of halogenated metabolites as compared to commercial antibiotics.

	COMPOUNDS TESTED									
TESTED MICROBES	1	2	3	4	5	K30	CZ30	MI30	NB30	VA30
PATHOGENIC YEAST										
Candida albicans (ATCC)	8	10	-		14	-	-	13	-	-
Candida albicans (51)	9	18	-	-	16	-	-	20	-	-

Table 1. Antimicrobial activities of halogenated metabolites as compared to commercial antibiotics (continued)

	COMPOUNDS TESTED									
TESTED MICROBES	**1**	**2**	**3**	**4**	**5**	**K30**	**CZ30**	**MI30**	**NB30**	**VA30**
Candida albicans (G588)	-	18	-	-	-	-	-	-	-	-
Candida albicans (G670)	9	16	-	-	12	-	-	19	-	-
Candida albicans (U1515)	10	10	-	-	-	-	-	18	-	-
Candida albicans (U1580)	8	8	-	-	10	-	-	14	-	-
Cryptococcus neoformans	-	10	-	-	-	17	26	44	38	22
ENVIRONMENTAL BACTERIA										
Clostridium cellobioparum	8	8	-	-	10	20	30	36	20	24
Clostridium sordelli	8	10	-	-	12	16	24	18	26	14
Clostridium novyi	8	8	-	-	10	16	-	24	26	20
Proteus vulgaris	10	8	-	-	12	-	50	20	60	-
Vibrio alginolyticus	8	10	-	-	10	14	18	22	26	18
Vibrio parahaemolyticus	8	8	-	-	12	20	14	32	24	20
PATHOGENIC BACTERIA										
Enterococcus faecalis	12	10	-	-	18	14	16	-	-	18
Escherichia coli	10	8	-	-	16	12	12	18	20	14
Salmonella typii	8	8	-	-	16	16	20	10	-	20
Staphylococcus aureus	8	10	-	-	12	16	26	28	-	24
Vibrio cholerae	-	-	-	-	-	16	10	36	22	18

Inhibition Zone Diameter: mm, - : No Inhibition. Compound concentration: 30 μgdisc^{-1} (CLSI levels)

RESULT AND DISCUSSION

Digestive glands of two *A. dactylomela* specimens were macerated and extracted in MeOH for five days. Resulting MeOH extract was concentrated *in vacuo* and partitioned twice between Et_2O and H_2O. The resulting Et_2O fraction was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to yield 5.3 g of crude extract. Chemical profiling of the crude extract was done by spotting the crude extract on SiO_2 gel Thin Layer Chromatography, developed in Toluene and Hexane:EtOAc (3:1) solvent systems, and visualized by UV light (254 nm)

and molybdophosphoric acid. Purification of the crude extract was done in two stages; 1) SiO2 gel column chromatography, and 2) Preparative Thin Layer Chromatography (PTLC). First, a total of 1600 mg crude extract was separated via SiO_2 gel column chromatography with a step gradient of Hexane:EtOAc, gradient ratio of 9.5:0.5, 8.0:2.0, 7.0:3.0, 6.0:4.0 and 5.0:5.0. Fractions were subjected to analytical Thin Layer Chromatography analysis and the presence of halogenated metabolites was detected in fractions 1, 2 and 3. These fractions were then subjected to PTLC in $CHCl_3$. Total of five halogenated secondary metabolites were isolated and identified using spectroscopy data to be Palisadin A (**1**, 2%), Aplysistatin (**2**, 16%), 5-acetoxypalisadin B (**3**, 2%), Palisadin B (**4**, 4%) and 12-hydroxypalisadin B (**5**, 2%) (Figure 1) [11, 14, 15]. Similar extraction, separation and identification of halogenated metabolites of *Laurencia snackeyi* collected from the same location revealed the presence of only Palisadin A (**1**, 0.5%), Aplysistatin (**2**, 4.8%), 5-acetoxypalisadin B (**3**, 0.9%) and Palisadin B (**4**, 2.5%). Their relative concentration in the sea hare's guts suggested accumulation or concentration effect as a consequence of continues grazing.

Out of the five halogenated metabolites isolated from *A. dactylomela*, four were also found to be present in *Laurencia snackeyi* (Weber-van Bosse) Masuda that grows in the same location where the sea hares were collected (unpublished data). During specimen collection, the two specimens of *A. dactylomela* used in this investigation were seen grazing among clumps of *L. snackeyi* (Figure 2), and the juveniles of this species were found attached on the thalli of this particular *Laurencia* as reported in our earlier findings [11]. Thus, based on our observation and available data, it is strongly suggested that the *A. dactylomela* lives and feeds on its host plant (*L. snackeyi*) and sequesters the algae's metabolites in its body. Similar phenomenon has also been reported for members of *Aplysiidae*, where the sequestered metabolites are often thought to protect the sea hares against potential predators [16, 17, 18].

The presence of the additional compound, 12-hydroxypalisadin B (**5**) in *A. dactylomela* is suggested to be a derivative metabolite and could be a result of chemical transformation that occurred in the gut of the sea hare, probably facilitated by pH and digestive enzymes. The existence of this compound could be easily explained based on the biogenesis of Palisadin B (**4**) (Figure 3).

Based on Figure 3, metabolites isolated from sea hare and *L. snackeyi* are proposed to be derived from snyderane's pathway where snyderols are formed from acyclic precursor nerodiol by bromonium-ion-induced carbocyclization [19]. Chemical processes such as bromination, dehydration and hydrolysis take place to produce compounds with snydrane skeletons. Isomeration converts snydrane to Palisadin B (**4**) which acts as a precursor. In addition, Palisadin B undergoes acyclic oxidation to produce 12-hydroxypalisadin B (**5**) and 5-acetoxypalisadin B (**3**). Further, debromonization of 12-hydroxypalisadin B (**5**) facilitates in the production of Palisadin A (**1**). It is also reasonable to propose and rationalize the absence of 12-hydroxypalisadin B (**5**) in the seaweed due to swift transformation of Palisadin B (**4**) to Palisadin A (**1**) in the seaweed. In *L. snackeyi*, this process is believed to be facilitated by bromoperoxidases (BPO) enzymes, hence explaining the narrow preference in compound type. But, in sea hare's digestive glands, the absence of BPO and mere chemical reaction mediated changes could explain the formation and retention of 12-hydroxypalisadin B (**5**).

All the five halogenated metabolites were tested for their antimicrobial potentials; Table 1 shows the compounds activities against selected range pathogenic yeast, environmental and pathogenic bacteria. Potent antimicrobial activities were shown by Palisadin A (**1**), Aplysistatin (**2**) and 12-hydroxypalisadin B (**5**). Their relative potentials were compared to five types of commercially available antibiotics at concentrations suggested by CLSI (Clinical and Laboratory Standard Institute) (Table 1).

Figure 2. Sea hares grazing on red algae, *Laurencia snackeyi* in seaweed meadow at Sepanggar Bay, Kota Kinabalu, Sabah.

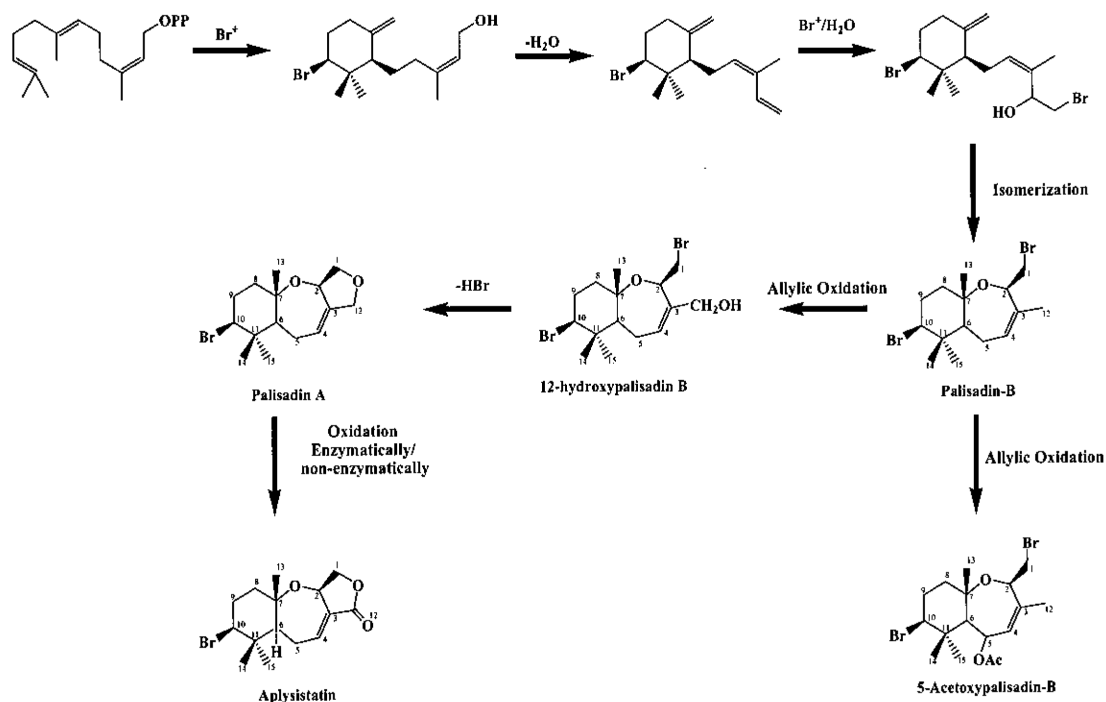

Figure 3. Biogenesis pathway for halogenated metabolites with syndrean skeleton.

CONCLUSION

Present investigation and previous studies by other groups have shown that sea hares of the genus *Aplysia* have special preference for red algae *Laurencia* as their diet. In Sabah waters, *A. dactylomela* seem to selectively prefer *L. snackeyi* as its diet although other species of *Laurencia* are found growing in the same seaweed meadow. Apart from their ability to accumulate and concentrate halogenated metabolites sequestered from the algae, this study has shown that sea hares also facilitate the derivatization of sequestered compounds in their digestive glands, producing a wider variety of structurally interesting halogenated metabolites. However, the practical use of these compounds for the sea hare still remains unclear due to difficulty in establishing reliable ecologically relevant bioassay. Therefore, it is only suggestive that these compounds could have a role in their defence to evade predators or bacterial infection.

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