CHARACTERIZATION OF BROMOPEROXIDASES (BPO) INVOLVED IN THE BIOSYNTHESIS OF HALOGENATED SECONDARY METABOLITES IN RED ALGAE GENUS *LAURENCIA*

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DECLARATION

The materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

10 March 2008

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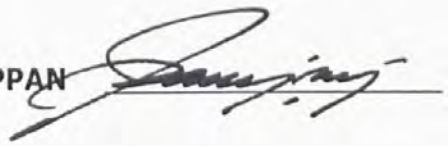

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ABSTRACT

CHARACTERIZATION OF BROMOPEROXIDASES (BPO) INVOLVED IN THE BIOSYNTHESIS OF HALOGENATED SECONDARY METABOLITES IN RED ALGAE GENUS *LAURENCIA*

This study established the presence, activity and characterization of bromoperoxidases (BPO) in two species of red algae genus *Laurencia*; *Laurencia snackyi* and *Laurencia similis*, collected from the waters of Tunku Abdul Rahman Marine Park, Kota Kinabalu, Sabah, Malaysia. The presence and utilization of BPO by both the seaweed species were apparent with the isolation of halogenated secondary metabolites; 1) *Laurencia snackyi* yielded 5-acetoxypalisadin B (**1**), Palisadin A (**2**) and Aplysistatin (**3**), and 2) *Laurencia similis* yielded 1-methyl-2,3,5,6-tetrabromoindole (**4**) and 2,3,5,6-tetrabromoindole (**5**). The specific activity of the BPO enzyme extracted from *L. snackyi* was 6.3mU/mg and ooptimum pH for enzyme activity was pH 7. Studies on the metal ions that influence the enzyme activity showed the BPO enzyme from *L. snackyi* to be vanadium-dependent. The optimum temperature for BPO from this particular species was 25°C and it was thermostable up to 70°C. Meanwhile, the specific activity of BPO from *L. simils* was 7.1mU/mg. However, the BPO for this alga species exhibits a very unusual pH characteristic, where there are two optimal pH; 5 and 7, which leads to the assumption there are two different BPOs in *L. similis*. It was found that the BPO from *L. similis* was cuprum-dependent. This is the first report of a cuprum-dependent BPO. The optimum temperature for the BPO from *L. similis* was 30°C and was also thermostable up to 70°C. The kinetic data obtained shows that both BPO enzymes from *L. snackyii* and *L. simils* are non-homogenous and the substrates inhibit the enzyme reaction at high concentrations (more than 0.6 mM MCD, 20 mM H_2O_2, 200 mM KBr for *L. snackyi* and more than 0.8 mM MCD, 5 mM H_2O_2, 200 mM KBr for *L. similis* respectively). It was found that the BPO activity of *L. similis* was approximately 2.5 times higher than the BPO activity of *L. snackyi*. Both BPOs from the *Laurencia* exhibited 1000 times lower activity as compared to other seaweeds studied to date. The fact that both species investigated in this study are prolific producers of halogenated metabolites and are tropical algae could be factors that contribute to the lowered enzyme activity. The partially purified enzymes were found to be larger than 100 kDa. Further column chromatography and SDS PAGE for both *Laurencia* algae also support the possibility of two different BPOs present in *L. similis* and only one BPO in *L. snackyi*.

ABSTRAK

*Kajian ini telah mengesahkan kehadiran, aktiviti dan sifat enzim bromoperoksida (BPO) daripada dua spesis alga merah genus Laurencia iaitu Laurencia snackyi dan Laurencia similis yang dikutip dari perairan Taman Marin Tunku Abdul Rahman, Kota Kinabalu, Sabah, Malaysia. Kehadiran serta kepentingan BPO kepada kedua-dua spesis alga telah dapat disahkan dengan kewujudan metabolit sekunder berhalogen; 1) Laurencia snackyi yang mengandungi 5-acetoxypalisadin B (**1**), Palisadin A (**2**) dan Aplysistatin (**3**), manakala 2) Laurencia similis mengandungi 1-methyl-2,3,5,6-tetrabromoindole (**4**) and 2,3,5,6-tetrabromoindole (**5**). Aktiviti spesifik BPO daripada L. snackyi adalah 6.3mU/mg. Kajian yang dilakukan menunjukkan pH optimum bagi enzim BPO yang diekstrak daripada L. snackyi adalah pH 7, yang mencadangkan ia adalah BPO yang bergantung kepada vanadium. Kajian lanjutan dengan menggunakan ion-ion logam telah mengesahkan hakikat bahawa ianya bergantung kepada vanadium. Suhu optimumnya pula adalah 25.0°C dan stabil sehingga suhu 70.0°C. Bagi L. similis pula, aktiviti spesifiknya adalah 7.1mU/mg. Namun, BPO daripada alga ini menunjukkan sifat pH yang berlainan, di mana ia mempunyai dua pH optimum iaitu pH 5 dan pH 7. Ini menunjukkan kemungkinan terdapat dua jenis enzim BPO yang berlainan. Melalui kajian lanjutan mengenai ion-ion logam, didapati BPO L. similis bergantung kepada kuprum. Ini merupakan laporan pertama yang meengesahkan terdapatnya BPO yang memerlukan kuprum. Suhu optimum BPO daripada alga ini adalah 30.0°C dan ia juga stabil sehingga suhu 70.0°C. Data kinetik daripada kedua-dua BPO yang dikaji menunjukkan ia tidak homogenus dan substrat-substratnya akan mengurangkan aktiviti enzim apabila kepekatan substrat terlalu tinggi (melebihi 0.6mM MCD, 20mM H_2O_2, 200mM KBr bagi L. snackyii dan melebihi 0.8mM MCD, 5mM H_2O_2, 200mM KBr bagi L. similis). Kajian juga menunjukkan aktiviti enzim BPO daripada L. similis adalah lebih kurang 2.5 kali ganda lebih tinggi daripada L. snackyi. Enzim BPO daripada kedua-dua alga yang dikaji juga menunjukkan lebih kurang 1000 kali kurang daripada aktiviti enzim daripada alga-alga lain yang telah dikaji. Walau bagaimana pun, kedua-dua spesis alga ini merupakan penghasil metabolit berhalogen yang utama, kadar aktiviti enzim yang rendah ini mungkin disebabkan oleh faktor tersebut. Enzim BPO separa tulen menunjukkan saiz enzim BPO adalah lebih besar daripada 100 kDa. Kromatografi serta SDS PAGE juga menunjukkan kehadiran dua enzim BPO yang berbeza L. similis dan hanya satu jenis enzim BPO terkandung dalam L. snackyi.*

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF ALGAE *LAURENCIA* AND BROMOPEROXIDASE

The marine red algae from the genus *Laurencia* (division Rhodophyta, class Rhodophyceae, subclass Florideophyceae order Ceramiales, family Rhodomelaceae) was first described by Lamouroux in 1813 (Erickson, 1983). It is red in colour due to reflected red light by the phycoerythrin pigment it contains and it can live in deeper waters than other algae because of its ability to absorb blue light, which has a longer wavelength (Harper et al., 2001).

Laurencia is a very complex genus, which renders the task of identifying the various species by their morphology very difficult. Therefore, the solution to this problem may be in the chemistry of the algae itself. Chemical investigation of secondary metabolites from *Laurencia* began with the report from Obota and Fukushi in 1953 (Erickson, 1983).

Most secondary metabolites from *Laurencia* are found to be halogenated, and are believed to be synthesized and stored in cells known as 'corps en cerise' (Young, Howard & Fenical, 1980). Therefore, it indicated that only *Laurencia* species with 'corps en cerise' consist of halogenated secondary metabolites while *Laurencia* species without 'corps en cerise' will show absence of halogenated secondary metabolites (Suzuki et al., 2002). Halogenated secondary metabolites isolated from *Laurencia* are found to be mostly from the group of terpenoids and C15 acetogenins (Suzuki et al., 1996; Su, J. et al., 1995; Rovirosa et al., 1999).

To date, extensive studies on the production of halogenated secondary metabolites on the algae genus *Laurencia* have been done. These halogenated secondary metabolites are suggested to serve as a defence mechanism, due to the discovery that these metabolites have antimicrobial activities (Vairappan et al.,

2001a; Vairappan et al., 2001b; Norte, 1996; Fernandez, Souto & Norte, 1998; Nys & Steinberg, 2002; Hay, 1996; Valdebenito et at., 1982; Higgs, 1981).

The halogenated secondary metabolites produced by *Laurencia* are numerous; over 570 metabolites were produced, that are very diverse and unique in structure (Paul et al., 2001). Some of these metabolites also exhibit activities against pathogenic yeast and bacteria (Vairappan et al., 2004). This could prove to be important for various industries especially the pharmaceutical industry.

In the pharmaceutical industry, natural products have been found to be a source of bioactive metabolites for treating diseases. Despite development of new technologies, natural products continue to be a valuable source of these bioactive metabolites. It was reported that 57% of 150 most prescribed drugs are from natural sources (Sennett, 2001). Therefore, there are high chances that halogenated secondary metabolites from the marine environment have potential as a treatment drug due to the diverse and unique structures of the metabolites from marine sources.

Based on some reports generated using some brown and red seaweeds, productions of these halogenated secondary metabolites were suggested to be catalyzed by haloperoxidases (HPO). Haloperoxidases are enzymes, which are known to catalyse the oxidation of halides using hydrogen peroxide as oxidant and therefore incorporate the halogen ions into the structure of the metabolites. Although halides are anions naturally, HPO incorporate it as cations in the metabolites. In halogenations, hypohalous acid was formed to react with a broad range of nucleophilic acceptors to perform the peroxidative synthesis of a carbon-halogen bond (Hara & Sakurai, 1998).

In 1966, L. P. Hager's group isolated the first halogenating enzyme from the fungus *Caldariomyces fumago*, which was found to be a heme-type chloroperoxidase. Hager's group also developed the monochlorodimedone assay for measuring the enzyme activity (Morris & Hager, 1966). It is also found that although chloroperoxidase is very similar to the peroxidases from the plants; horseradish and

Japanese radish in amino acid and carbohydrate contents but its catalytic activity is very different from each other (Morris & Hager, 1966).

It was found that chloroperoxidase from the fungus *Caldariomyces fumago* was able to catalyze the halogenations of many organic compounds. Its lack of substrate specificity and stereo specificity could be explained in terms of the molecular halogen or hypohalous acid addition chemistry in water, where the halogen released from the enzyme into the reaction mixture in the presence of halide anions and hydrogen peroxide (Itoh et al., 1988).

However, it has been found that chloroperoxidase was unstable due to the released active molecular halogen during reactions that denature the enzyme itself. Therefore, this enzyme could not be used as a halogenating agent in a continuous halogenation process (Itoh et al., 1988).

Another haloperoxidase that has also been discovered was bromoperoxidase. Itoh and colleagues examined the bromoperoxidase derived from the red marine algae *Corallina pilulifera* and found that the enzyme was distinguishable from the usual haloperoxidases due to its non-heme iron prosthetic group. It was also found that this particular enzyme was stable under normal reaction conditions, thus making it a suitable enzyme for the continuous halogenation processes (Itoh et al., 1988).

In nature, bromide concentration in the ocean is much lower than the concentration of chloride. However, it is found that many of the halogenated metabolites contain bromonium ion within its structure (Izumi et al., 1989) instead of chloride. Now, it is known that the enzyme bromoperoxidase (BPO), a type of haloperoxidase is responsible for the catalization that forms these unique halogenated metabolites.

Bromoperoxidase can usually be found in red algae and it catalyzes the oxidation of bromide by hydrogen peroxide, where it produces single oxygen. The process is also known as bromination from the organic substrate (Sheffield et al., 1993).

$$AH + Br^- + H_2O_2 + . \xrightarrow{BPO} A(Br) + 2H_2O$$

AH – reagent

A(Br) – metabolite that contains bromine

Figure 1.1: The reaction catalysed by bromoperoxidase in the presence of hydrogen peroxide.

So far, studies done on BPO in algae are mostly of algae in temperate climate and none were carried out on the red alga genus *Laurencia* (Itoh et al., 1988, Izumi et al., 1989, Krenn et al., 1989, Oshiro et al., 1999 & Sheffield et al., 1993). There is yet to be any reports of the presence or enzyme activities of BPO for the red algae *Laurencia* that is found abundantly in tropical waters such as Malaysia. According to Vairappan et al. (2001a), the Malaysian water contains 6 known species and several unrecorded species of *Laurencia*. The known species are *L. snackyi, L. similis, L. papilosa, L. perforata, L. majuscula,* and *L. nangii.*

Therefore, there is a need to study the bromoperoxidase of tropical algae such as the Malaysian *Laurencia* for the purpose understanding the enzyme in relation to the biosynthesis of bioactive halogenated secondary metabolites as compared to the temperate algae.

1.2 OBJECTIVES

a. Extraction, isolation and characterization of secondary halogenated metabolites present in *Laurencia snackyi* and *Laurencia similis*.

b. Extraction and determination of the enzyme activity from the extracted crude enzyme.

c. Enzyme optimization (enzyme concentration, pH, temperature and metal co-factor). Optimum condition will be determined by altering each factor that could influence the enzyme activity. The presence of metal co-factor will be determined by the addition of vanadium, cobalt, cuprum, ferum and zinc.

d. Determination of the enzyme kinetics and molecular size of partially purified BPO.

CHAPTER 2

LITERATURE REVIEW

2.1 STRUCTURAL DIVERSITY OF HALOGENATED METABOLITES FROM *LAURENCIA*

Numerous studies had been done on algae from the genus *Laurencia* in the field of natural products. It is known that *Laurencia* produce structurally unique halogenated secondary metabolites (Vairappan & Tan, 2005), predominantly in two major biosynthetic groups, which are terpenoids that consist of sesquiterpenes, diterpenes and triterpenes; and C_{15}-acetogenins (Rovirosa et al., 1999). Meanwhile, bromoindoles are classified as miscellaneous secondary metabolites (Erickson, 1983).

2.1.1 Sesquiterpenes

The red algae genus *Laurencia* is known to produce principally bromine-containing sesquiterpenes (Fernandez et., al, 2005) and the secondary metabolites from this group can be further divided into 26 different structural classes with at least 16 of which are novel and only found in *Laurencia* (Hay, 1996). Some of the sesquiterpenes that were found include (16*R*, 9*R*, 10*S*)-10-bromo-9-hydroxy-chamigra-2,7(14)-diene (**a**) (*Z*)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**b**) and (*E*)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**c**) from Okinawan *Laurencia* species (Vairappan, et al., 2001b), 2,10-dibromo-3-chloro-α-chamigrene **(d)** and laurinterol **(e)** from various Japanese *Laurencia* species (Suzuki et al., 2005) and Aldingenin A **(f)** from *Laurencia aldingensis* (Carvalho et al, 2003).

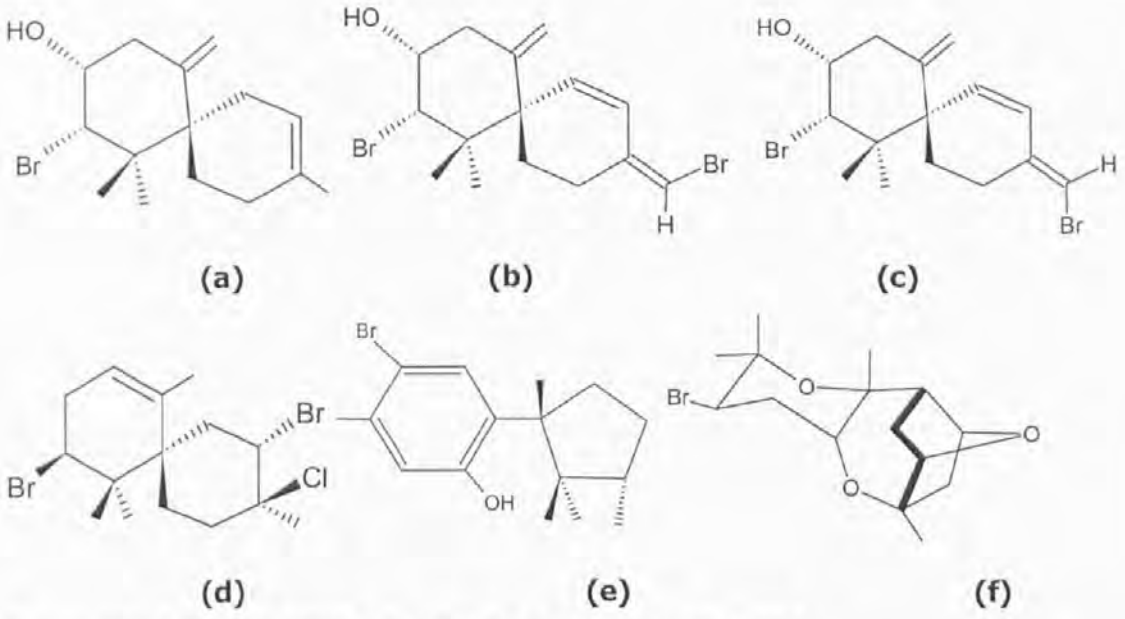


Figure 2.1: Sesquiterpenes from various species of *Laurencia*
Source: Vairappan, et al. (2001b), Suzuki et al. (2005) and Carvalho et al. (2003).

2.1.2 Diterpenes

Diterpenes from *Laurencia* are derived from bromonium-ion induced cyclization products. There are four diterpene skeletons that are derived from *Laurencia*. These diterpene skeletons are Obtusanes, Irieanes, Labdanes and Neoconcinnanes (Erickson, 1983). Some diterpenes found in *Laurencia* are (3R,5S,6S,9S,10S)-3-bromo-6,12-diacetoxy-13-hydroxylabd-8(19),14-diene **(g)** (Suzuki et al., 2005), (-)-Paniculatol (3R,6S,8S,13R) **(h)** (Briand et al., 1997) and Laukarlaol **(i)** (Su et al., 1995).

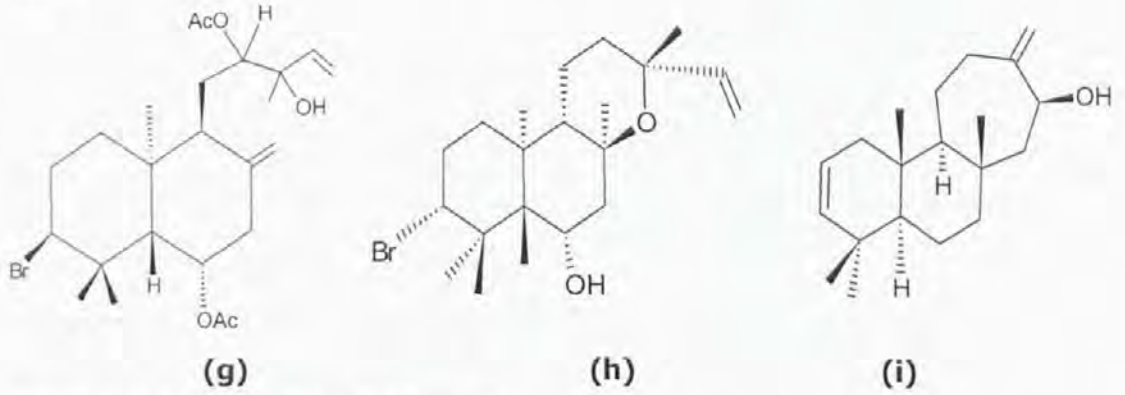
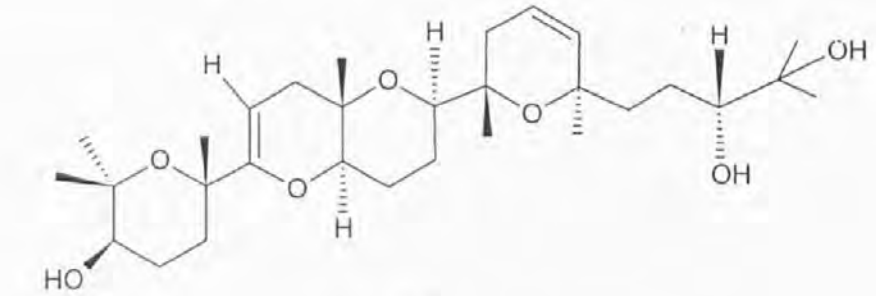


Figure 2.2: Diterpenes from *Laurencia*.
Source: Suzuki et al. (2005), Briand et al. (1997) and Su et al. (1995).

2.1.3 Triterpenes

Tripterpenes are found mainly in *Laurencia* and also sponges from *Axinellidae* family and are reported to have polyether moiety (Souto et al., 2002). Examples of triterpenes found in *Laurencia* are such as martiriol **(j)**, Pseudodehydrothyrsiferol **(k)** and dioxepandehydrothyrsiferol **(l)** (Manriquez et al., 2001).



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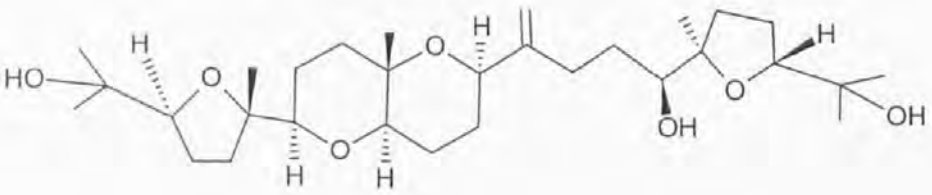

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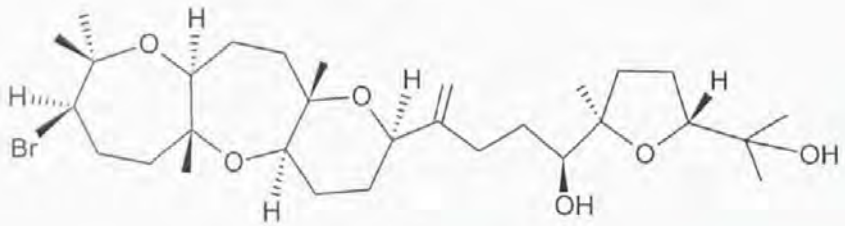


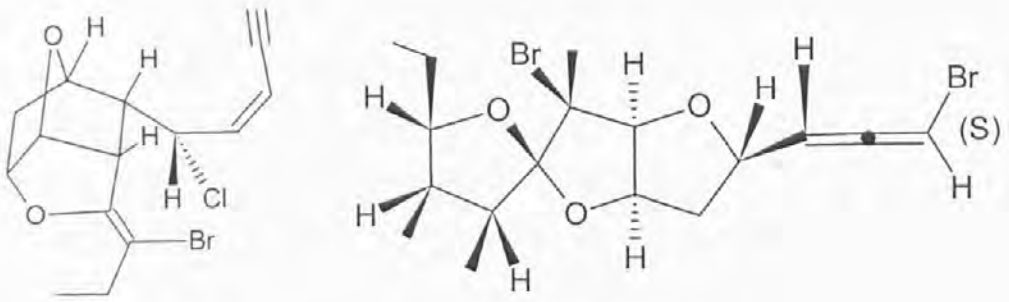
(l)

Figure 2.3: Triterpenes from *Laurencia*
Source: Manriquez et al. (2001).

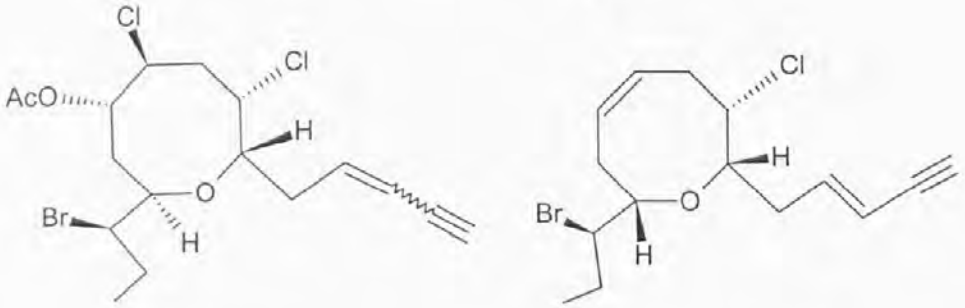
2.1.4 C_{15}-acetogenins

Non-terpenoid C_{15}-acetogenins from *Laurencia* arise from the fatty acid metabolism and they are classified as acyclic, heterocyclic or carbocyclic (Erickson, 1983).

C_{15}-acetogenins that have been found include (12E)-lembyne-A **(m)** (Vairappan, et al., 2001b), chinzallene **(n)** (Suzuki et al., 2005), 13-epilaurencienyne (3Z) **(o)** and 13-epipinnatifidenyne (3E) **(p)** (Iliopoulou et al., 2002).



(m) **(n)**

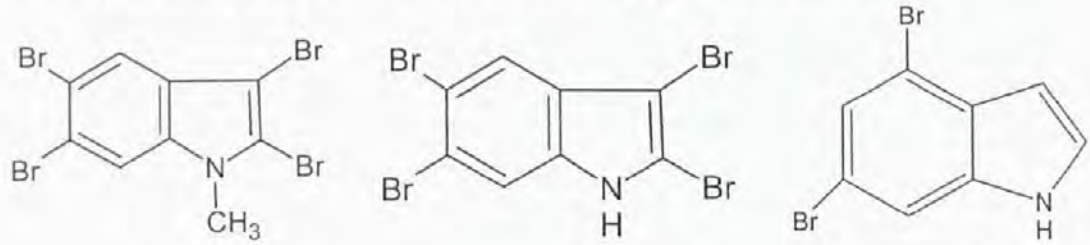


(o) **(p)**

Figure 2.4: C_{15}-acetogenins from *Laurencia*
Source : Vairappan, et al. (2001b), Suzuki et al. (2005) and Iliopoulou et al. (2002).

2.1.5 Bromoindoles

Bromoindoles from *Laurencia* are classified as miscellaneous metabolites (Erickson, 1983). This is due to relatively less bromoindoles that were isolated from *Laurencia* as compared to the other groups of metabolites. Bromoindoles isolated from *Laurencia* are 1-methyl-2,3,5,6-tetrabromoindole **(q)**, 2,3,5,6-tetrabromoindole **(r)** (Vairappan et al., 2004) and 4,6-dibromoindole **(s)** (Erickson, 1983).



(q) **(r)** **(s)**

Figure 2.5: indoles from *Laurencia*
Source: Vairappan et al. (2004) and Erickson (1983).

2.2 BIOACTIVITY OF THE HALOGENATED METABOLITES FROM *LAURENCIA*

The halogenated secondary metabolites from *Laurencia* are found to be bioactive against microbes and grazers (Vairappan & Tan, 2005). Studies done by Vairappan and colleagues (2001b) and Vairappan (2003) in demonstrating the bioactivity of the metabolites from *Laurencia* showed that the metabolites extracted were bioactive although the algae were collected from different climate regions. (The metabolites tested were (12E)-lembyne-A **(A)** from *L. mariannensis*; 2-bromo-3-chloro-5-acetoxy-chamigra-7(14),9-dien-8-one **(B)**, (6R,9R,10S)-10-bromo-9-hydroxy-chamigra-2,7(14)-diene **(C)**, (Z)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene **(D)** and (E)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene **(E)** from *L. majuscula*; laurinterol **(F)** and isolaurinterol **(G)** from *L. nidifica*. – in table 2.1; elatol **(H)**, iso-obtusol **(I)** from *L. majuscula* – in table 2.2).

Table 2.1: Antibacterial activity of the halogenated compounds against marine bacteria isolated from algal habitats in the Japanese coastal waters.

Test bacteria	Compounds[a]			
	A	**C**	**F**	**G**
Alcaligenes aquamarinus	++	++	-	-
Alteromonas sp.	-	-	++	+++
Azomonas agilis	++	++	+++	+++
Azobacter beijerinckii	-	++	++	-
Erwinia amylovora	++	++	+++	+++
Escherichia coli	++	++	++	+++
Halobacterium sp.	-	-	-	-
Halococcus sp.	-	-	-	-

Source: Vairappan et al. (2001b).

[a] Inhibition zone diameter; +++: 19-24mm, ++: 12-18mm, +: 7-12mm, -: no inhibition. Concentration: 90µg/disc.

Table 2.2: Comparative antibacterial activity of elatol **(H)**, iso-obtusol **(I)** and six types of commercially available antibiotics against tested strains of human pathogenic bacteria.

	Compounds tested							
	Elatol **(H)**	Iso-obtusol **(I)**	AMC	MOX	CEC	CRO	K	NET
Streptococcus hemolyticus	+	-	+++	+++	+++	+++	+++	-
C. freundii	-	+	+	++	+++	+++	+++	-
E. coli	-	++	+++	++	+++	++	-	-
K. pneumoniae	+++	+++	+	+++	++	+++	+++	+
Pseudomonas sp.	++	-	-	-	-	+	-	++
Salmonella sp.	++++	+++	+++	+++	+++	+++	+++	-
S. aureus	+	-	-	++	++	++	-	++
S. epidermis	+++	-	+++	+++	+++	++	+	-

Source: Vairappan (2003).
Inhibition zone diameter; ++++: 25-30mm, +++: 19-24mm, ++: 12-18mm, +: 7-12mm, -: no inhibition. Compound concentration: 30µg.

These halogenated secondary metabolites are bioactive due to the unique presence of the bromonium ions in their structure; therefore the enzyme responsible for the biosynthesis of these halogenated metabolites is a highly interesting subject to be studied.

2.3 BROMOPEROXIDASE

Red algae were known to contain many halogenated metabolites, therefore the study of the enzyme directly responsible for its production have long been studied.

In the study of *Corallina pilulifera* (Itoh et al, 1988), bromoperoxidase was found to catalyze a range of organic compounds that generally consist of cyclic β-diketones, substituted phenols, substituted alkenes and nitrogen-containing or sulphur-containing heterocycles. Bromoperoxidase from *C. pilulifera* had also been identified to contain a specific active site, which could explain the different substrate specificity and reaction rates in table 2.3.

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