CHAPTER 5
DISCUSSION

5.1 The selection of seaweeds for chemical isolation

The screening of the fourteen seaweed methanolic extracts at 20 μg/mL using MTT-based bioassay has yielded four photo-cytotoxic and one cytotoxic extracts. The active extracts include the Chlorophyta (green) *Cladophora patentiramea* and *Chaetomorpha linum*; the Pheophyta (brown) *Turbinaria conoides* and *Dictyota dichotoma* as well as the Rhodophyta (red) *Gracilaria salicornia*.

However, only two species amongst the active seaweeds, namely *Turbinaria conoides* and *Cladophora patentiramea* were selected for further studies. This is because these selected seaweeds produced reasonably high yield of extract when soaked in methanol as compared to the other seaweeds (See Table 4.1). Furthermore, it was possible to obtain adequate amount of materials for extraction as the selected seaweeds were abundant at the collection site.

5.2 Compounds isolated from *Turbinaria conoides* and *Cladophora patentiramea*

A total of nine pure bioactive compounds have been isolated from both *Turbinaria conoides* (brown seaweed) and *Cladophora patentiramea* (green seaweed). These included pheophorbide-*a* methyl ester **(1)**, 13^2-hydroxypheophorbide-*a* methyl ester **(2)**, pheophorbide-*a* **(3)**, purpurin-18 methyl ester **(4)**, 13^2-methoxyl-pheophorbide-*a* methyl ester **(5)** 15^1-methoxypurpurin-7-lactone methyl diester **(6)**, 13^2-hydroxypheophorbide-*b* methyl ester **(7)** and its isomer **(8)** and 7-formyl-15^1-methoxy-7-lactone methyl diester **(9)**.

In the mean time, the photo-cytotoxic compounds in four other semi-pure fractions (Tur-10-4, Tur-17-6, Tur-me-8-3 and Cla-7-3) were most probably cyclic tetrapyrrolic derivatives based on their UV-Vis absorption profiles. The mass values obtained from these fractions suggested that these structures could be potentially novel. However, further chemical characterisation is required to confirm this.

Some compounds isolated in the study, such as compounds **1 – 4** and **7 – 8** have also been reported in other sources (Ong *et al*., 2009; Chee *et al.*, 2005; Lim *et al*., 2004; Cheng *et al*., 2001; Ocampo and Repeta, 1999). However, this is the first report on the isolation of compounds **5**, **6** and **9** from natural sources. This suggests that there is still a potential in obtaining new bioactive compounds from the seaweeds.

5.3 Chromophores of photo-cytotoxic compounds isolated

All isolated compounds in the study were of chlorophyll-*a* and –*b* types. The chlorophyll-*a* derivatives including compounds **1** – **6** were found in both green and brown seaweeds while compounds of chlorophyll-*b* type (**7** – **9**) were isolated mainly from the green seaweed, *Cladophora patentiramea*. This is in good agreement with the reported presence of chlorophyll-*b* in green seaweeds but not brown seaweeds (See Table 2.5, Chapter 2). Although the literature reported the presence of chlorophyll-*c* in brown algae (Jeffrey, 1976), the UV-Vis profiles of chlorophyll-*c* derivatives were not detected in *Turbinaria conoides* extract during the isolation process. This is most probably due to the low abundance of chlorophyll-*c* in the selected seaweed. Also, it is known that the aquatic photosynthetic pigments are largely influenced by surrounding light environment (Garrido and Zapata, 2006). Hence, distribution of chlorophyll-*c* in

brown algae may not be universal among the brown seaweeds that grow in different light conditions. This may have further caused the lack of detection of the chlorophyll-*c*-related derivatives when the starting material was inadequate (approximately 5.0 g or less per extract).

5.4 Mechanistic rationale for the compounds isolated

The identified compounds in the study are of chlorophyll degradation products. Chlorophyll breakdown is a natural process that occurs in higher plants and algae. It is known that the chlorophyll catabolism in both higher plants and algae comprise of sequential enzymatic pathways involving the removal of phytyl group, magnesium central atom and further oxidation to produce chlorophyll degradation products such as 13^2-hydroxy chlorophyll-*a*, pheophytin, pyropheophytin, pyropheophorbide, chlorophyllide and pheophorbide. In addition, the chlorophyll degradation products were also found to be further degraded into colorless metabolites, in a pathway which is better known as "oxidative chlorophyll bleaching pathway" (Hörtensteiner, 1999). Another study revealed that a monoxygenase is responsible for the oxidation of porphyrin cleavage in both higher plants and algae by tracking degradation pathway using ^{18}O-labelling experiments (Hörtensteiner, 1998). However the knowledge of actual biochemical pathways for cleavage of the porphyrin macrocycle in catabolic mechanism of algae is limited. However, this study excludes the discussion on the non-colored degradation products because photosensitisers are necessarily pigmented.

The majority of compounds isolated were methyl esters at the C-17^3 position (all compounds except compound **3**). This may have been caused by the methylation of crude extracts under acidic condition, which would have substituted the free propanoic

acid at C-17[3] with a methoxyl group contributed from methanol. The mechanism is proposed in Figure 5.1.

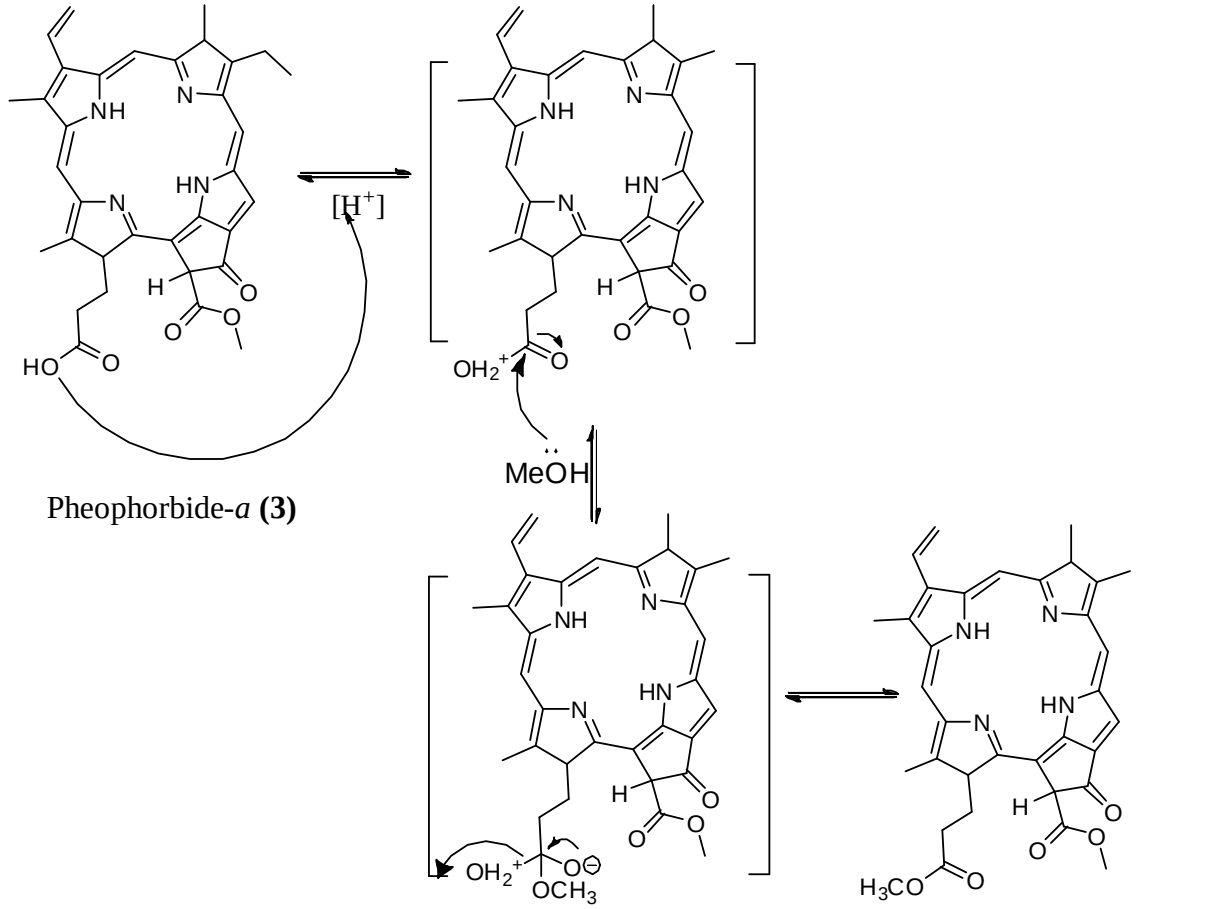

Figure 5.1:Esterification of propanoic acid at C17 to its methyl ester form

The C-13[2] position at ring E of chlorophyll derivatives is the most reactive part of the molecules. This is because the β-ketoester group in isocyclic ring E is susceptible to oxidation in alcoholic solution, a process known as Willstätter's allomerization. The formation of the isolated compounds can be explained via two main reaction pathways. Chlorophyll allomerization begins with the formation of carbanion at C-13[2] known as enolate ion **(10)**. A radical at C-13[2] **(11)** is subsequently formed and resulted in the attack of a methoxyl radical originated from methanol to produce 13[2]-methoxyl-pheophorbide-*a* **(5)** (Pathway I). Meanwhile, 13[2]-hydroxypheophorbide-*a* methyl ester

(2) is thought to be produced via two pathways, namely via direct nucleophilic attack of hydroxyl radicals (Pathway I) or via homolytic cleavage of C-13^2- hydrogen peroxide **(13)** (Pathway II). It is proposed that pathway II predominates when the reaction is carried out under extreme anhydrous condition. The formation of derivatives with anhydride ring E (in **6** and **14)** is also described with a similar reaction pathway. The carbon $C13^2$ radical **(11)** undergoes homolytic cleavage of hydrogen peroxide induced by the attack of methoxyl radical to C-13^1 to form an intermediate **(15)** (Pathway III). Further nucleophilic attack at the carbonyl group at C-15^1 produced the hydroxyl- **(14)** or methoxyl **(6)** anhydride ring, as shown in Figure 5.2 (Hynninen and Hyvarinen, 2002).

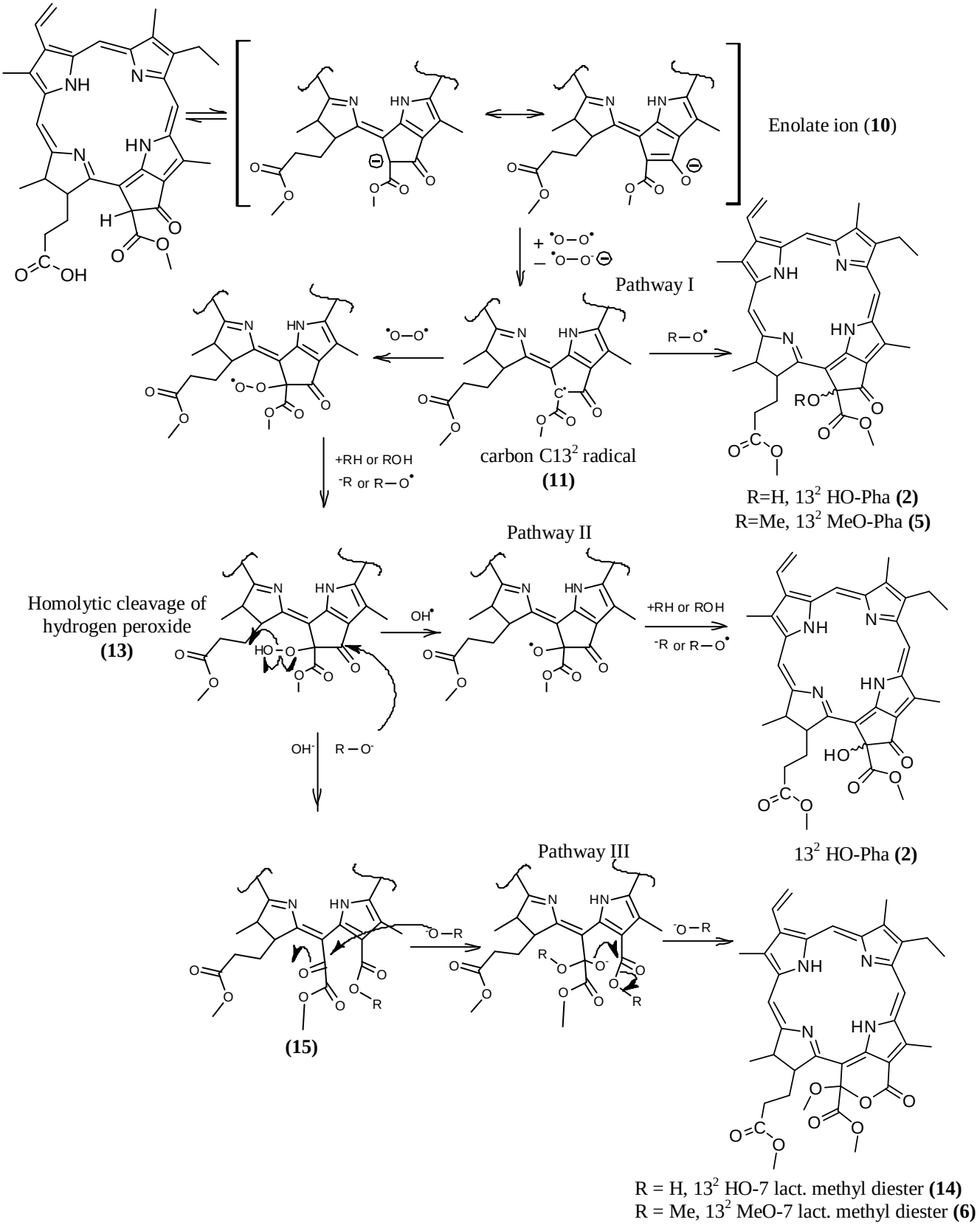

Figure 5.2: Mechanisms of chlorophyll allomerization proposed by Hyninnen and Hyvarinen (Adapted from Hynninen and Hyvarinen, 2002)

5.5 Appraisal of the strategies employed in the study

5.5.1 Methylation of crude extracts

The first attempt of crude extracts isolation yielded bioactive compounds in very low amounts. Also, the compounds that were isolated were in the form of free propanoic acids as well as methyl esters at the C-17[3] position. Therefore, in an attempt to reduce the complexity of the crude extract during scale-up fractionationation, methylation of the crude extract under acidic condition was performed in order methylate all free acids to their methyl ester forms. It was observed that the pre-treatment of the crude extract indeed increased the yields of the same compounds isolated from the methylated extracts as compared to those from their respective untreated extracts. For example, the yield of pheophorbide-*a*-methyl ester from the methylated *Turbinaria conoides* extract was higher than its non-treated extract (yield: 0.69% vs 0.08%)

5.5.2 Bioassay-guided isolation

In this study, the selected methanolic extracts caused photo-cytotoxicity against > 50% of HL60 cells at 20 μg/mL upon light activation while minimal cytotoxicity is observed in the absence of light. Subsequent chromatographic isolation and purification of the fractions selected on the basis of their photo-cytotoxicity have led to the yield of pure compounds that exhibited photo-killing of > 93% of HL60 cells at 5 and 10 μg/mL concentrations, hence demonstrated that the bioassay-guided isolation was an efficient strategy in identifying potentially bioactive compounds.

5.6 Areas for further studies

Pheophorbide-related derivatives, namely compounds **1 – 5** and **7 – 8** were well-studied for their *in vitro* and *in vivo* photo-cytotoxicity in various cancers (Chee *et* al, 2005; Choi *et al.,* 2004; Lee *et al.*, 2004; Lim *et al*., 2004; Hajri *et al*., 2002; Di Stefano *et al.*, 2001). However, there is limited literature for compounds with the structures such as compounds **6** and **9**. Therefore, it is worth carrying out further evaluation on the biological activities of the compounds against different cancer cell lines as well as establishing more information on the photophysical and photochemical properties of these compounds.

The isolation work in this study has yielded only known compounds rather than new structures. This may be because the bioactive compounds that are structurally new existed only in low amounts and they were masked by the known active components which were in high abundance. This problem may be overcome by first enriching the extracts with the low abundance compounds by removal of the known and abundant structures such as the chlorophylls, prior to the first MTT screening. This way, the probability of getting positive hits due to the presence of tetrapyrrolic derivatives may be reduced.

Dereplication is one strategy that may be employed to help in the selection of bioactive extracts most likely to contain novel structures for further isolation and purification. The components present in the extracts can be analysed via HPLC-MS. By comparing the HPLC-MS data with that of known compounds in terms of characteristics such as the UV-vis profiles, mass values or HPLC retention time, we can rule out those bioactive

extracts that contain major known compounds, subsequently avoid repetition in isolating known compounds.

The effort of seeking novel PDT compounds from seaweeds should be continued as the chemical diversity in marine ecosystem is usually different from those in the terrestrial systems and the former has not been extensively studied. Moreover, the type of marine samples for further studies should include microalgae which have not been investigated in the current study. There is a potential in getting compounds that are potentially new in the microalgae as the chemical defence system in microalgae may be different from seaweeds due to their morphology.

The likelihood of obtaining novel chromophores may be enhanced via proper selection of algae species and their ecosystem origins. For example, seaweeds that grow in extreme environments such as deep seas and mangrove areas may produce different secondary metabolites than those of at the seashores (Cragg *et al.*, 2009).