CHAPTER 2
LITERATURE REVIEW

2.1 Photodynamic therapy

2.1.1 Introduction

Photodynamic therapy (PDT) is a relatively new treatment modality for premalignant and malignant cancers, as well as some malignant diseases in the eye (Dolmans *et al.*, 2003). It involves the intravenous administration or topical application of a light sensitive agent, or a photosensitiser that localises and accumulates in the cancerous tissues at higher concentration compared to its surrounding tissues. The tumour site is then irradiated at a specific wavelength and this results in the activation of the photosensitiser, causing the production of highly reactive oxygen species (ROS) known as singlet oxygen ($^1O_2$). The ROS causes necrotic or apoptotic cell death which results in irreversible photodamage to the tissues (see in Figure 2.1) (Garbo, 1996).

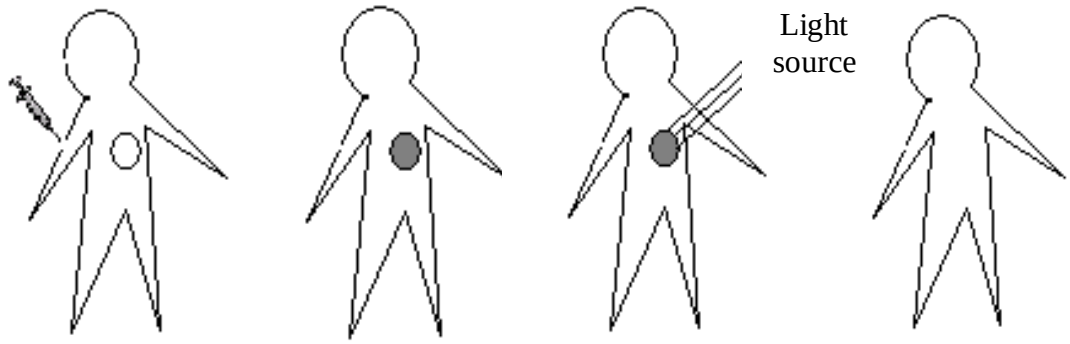


Figure 2.1: Clinical application of PDT
(Adapted from Brown *et al.*, 1999)

The selectivity of photosensitiser towards cancerous tissues is not-well studied. However, it has been hypothesised that the characteristics possessed by tumour cells, such as leaky vasculature and high lipid content, favour the binding of lipophilic photosensitisers. In addition, lower pH condition in tumour tissues and slower

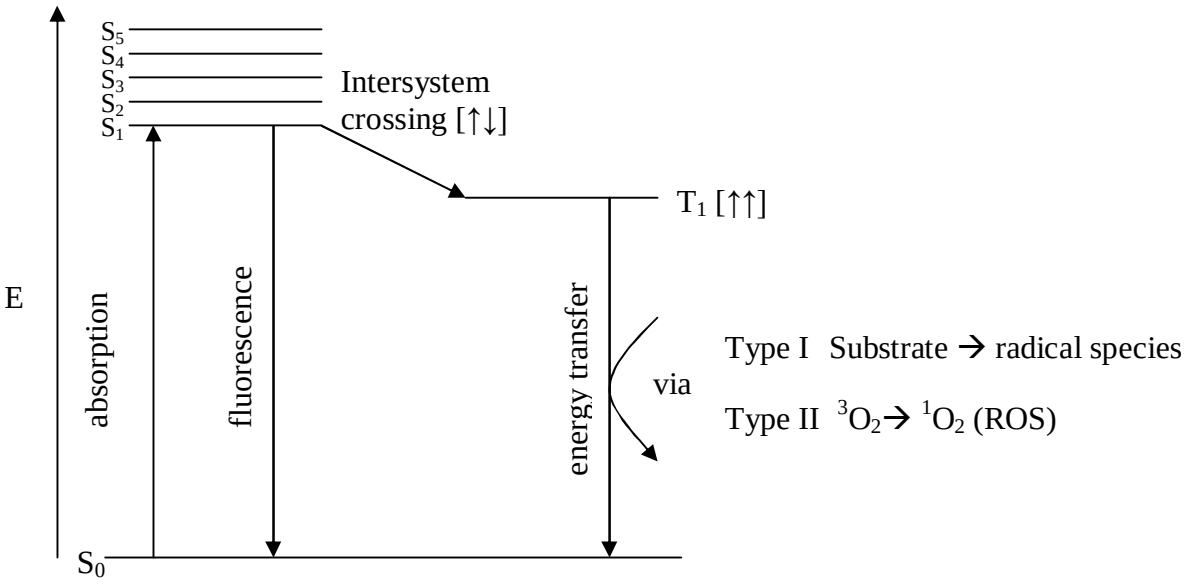
clearance rate of hydrophobic sensitisers due to poor lymphatic drainage may be the rationale behind the higher accumulation of photosensitiser in tumour cells compared to normal healthy tissues (Pervaiz, 2001).

2.1.2 Mechanism of action

The irradiation of photosensitiser excites it to a triplet state via a short-lived excited singlet state (see Figure 2.2). Some excited singlet state molecules return to its ground state and emit fluorescence light (Calzavara-Pinton *et al*., 2007) while others are converted to the excited triplet state (T_1) via intersystem crossing. The T_1- state is sufficiently long-lived enough to undergo two pathways in order to decay back to its stable ground state. Type I pathway involves reactions directly with a substrate, such as cell membrane or a molecule to form radicals via hydrogen- transfer or electron-transfer, such as superoxides, hydroperoxyls and hydroxyls. On the other hand, Type II pathway transfers the energy directly to surrounding oxygen molecules ($^3O_2$) to produce singlet oxygen ($^1O_2$) and this destroy the cells. Both reactions occur simultaneously, and the ratio between these two pathways depends highly on the type of photosensitisers, concentrations of substrates and oxygen, as well as the binding affinity of the sensitiser for the substrate (Dolmans *et al*., 2003).

Photodynamic therapy (PDT) destroys the cancerous tissues directly by the formation of ROS or other radicals. In addition, it also damages the vasculature of the tumour and its surrounding healthy vessels that supply the nutrients and oxygen to the tumour. This induces hypoxia and starvation as well as thrombus formation that results in the inhibition of tumor growth (Dolmans *et al.*, 2003*).* Photodynamic therapy (PDT) is also known to activate the patient's immune system due to the presence of inflammatory

process and vasculature damage and this is useful to eliminate any of tumour cells after treatment (Nowis *et al.*, 2005).



Where S_0, $S_1 - S_5$ are the ground singlet and excited singlet states of the photosensitiser, respectively. T_1 is the first excited triplet state of the photosensitiser. $^3O_2$ and $^1O_2$ are the ground and first excited singlet states of molecular oxygen, respectively.

Figure 2.2: Photochemistry of excited photosensitiser after irradiation
(Adapted from Calzavara-Pinton *et al.*, 2007)

2.1.3 Advantages of PDT

The selectivity of the photosensitisers towards the tumour tissues is one of the major advantages in PDT. The $^1O_2$ generated upon activation of the photosensitiser is short-lived with a half-life of < 0.04 s in biological systems; thus, the radius of the action of $^1O_2$ is < 0.02 μm (Pervaiz, 2001). This results in cell death in which the sensitiser is present upon irradiation. The selectivity of PDT is also enhanced by targeted delivery of light where only the illuminated area is destroyed as the photosensitiser is non-toxic in the absence of light.

Another advantage of PDT is its non-invasiveness as compared to other therapies such as surgeries. This is crucial particularly when the tumour removal affects the normal

functions of vital organs (Palumbo, 2007). This advantage has also widened the application of PDT in dermatology and excellent response rates have been observed in superficial skin cancer with good cosmetic results (Triesscheijn *et al.*, 2006).

Photodynamic therapy can also be repeatedly used on patients. Unlike other conventional therapies, there is no known cumulative tissue toxicity after PDT. There is also no drug resistance observed. Photodynamic therapy can be used repeatedly on the recurrent tumour sites without causing severe normal tissue damage (Triesscheijn *et al.*, 2006). This is favourable for cancer patients who have recurrence and those who are resistant towards chemotherapy.

The localisation of photosensitiser also serves as a good diagnostic tool for tumour detection. Photodynamic diagnostic (PDD) detects the difference in fluorescence emission of tissues, especially between the tumour and the normal tissues (Sieron *et al.*, 2006). This enhances the efficacy of tumour detection and enables the surgeons to remove tumour tissues more accurately. Figure 2.3 shows the clinical application of PDD.

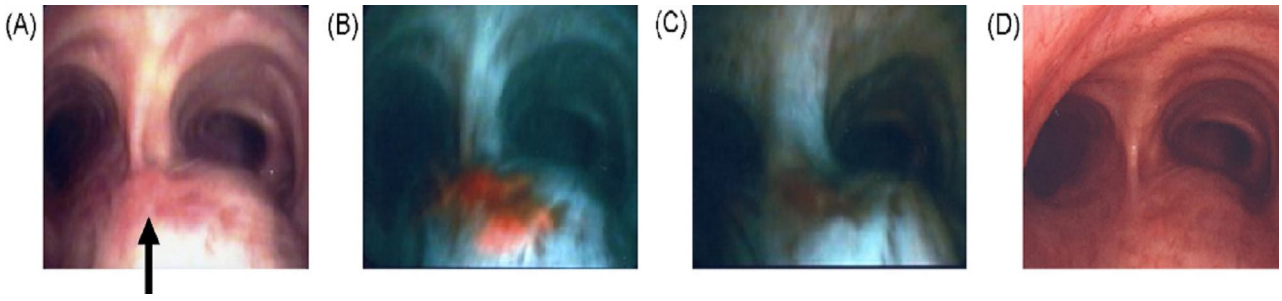


Figure 2.3: Clinical application of PDD

(A) Fiberoptic bronchoscopy revealed a superficial lesion
(B) Photodynamic diagnosis highlights the lesion using a PDT drug before PDT
(C) Loss of the red fluorescence from the tumor was confirmed by PDD immediately after PDT treatment
(D) Complete response was achieved three months after PDT

(Adapted from Usuda *et al.*, 2007)

2.1.4 Disadvantages of PDT

There are a few limitations with PDT. The major drawback of PDT is prolonged skin photosensitivity after the treatment due to the accumulation of photosensitiser in the body. For example, Photofrin®, the first generation photosensitiser, may render patients to skin photosensitivity due to its retention in the skin up to six weeks (Stenberg and Dolphin, 1998).

The incapability of light penetration into large tumours or tumours that sit deep in the body also limits the efficacy of PDT. Photosensitiser such as Photofrin® absorbs moderately at 630 nm which allows only about 1-2 mm effective penetration depth and gives only 5-10 mm therapeutic effect (Nyman and Hynninen, 2004). The invention of special light devices such as fiber optic is sometimes not sufficient to resolve the issues as some areas in the body are not accessible.

The efficacy of PDT depends fully on simultaneous exposure of the tumour to the photosensitiser and light, and their doses. The duration of photosensitiser accumulation in the body is very crucial to ensure optimum PDT response as well as good clearance from the body after the treatment to prevent skin photosensitivity. Therefore, the interval between the drug administration and light irradiation must be estimated accurately. The availability of oxygen is also essential in order to have sufficient amount for singlet oxygen production. Thus, it is recommended to perform multiple PDT sessions to allow sufficient oxygen supply at treatment site (Dolmans *et al.*, 2003).

2.2 Photosensitiser

As one of the essential components in PDT, different photosensitisers have variable efficacy on different diseases due to their photochemical and photophysical properties.

2.2.1 Characteristics of an ideal photosensitiser

Clinically, a good photosensitiser is determined by criteria such as its general toxicity, selectivity towards cancerous tissues and the ability of drug to be retained in the body prior to treatment as well as its clearance after the treatment. Several factors such as the purity, photochemical and photophysical properties are also taken into consideration in the clinical development of photosensitisers (See Table 2.1).

Table 2.1: Criteria of an ideal photosensitiser

Criteria	Characteristics
Toxicity	Should be non-toxic or minimally toxic in the absence of light
Selectivity	Should preferentially accumulate in the targeted area, such as cancerous tissues
Purity	Preferably a pure compound for easy formulation and compound identification
Photophysical properties	Should have high extinction coefficient, ε (absorption ability) at wavelength > 650 nm to allow deep penetration in the body
Photochemical properties	Should possess high triplet states yield, long triplet life-time to produce $^1O_2$ and other ROS
Ability to retain in the body and its clearance	Amphiphilic nature, where it is hydrophobic enough to bind with the membrane and retain in patients' bodies till the treatment is performed; as well as hydrophilic enough to be cleared from the body in reasonable period of time
Stability	hould be photo-stable and should not degrade easily
Production	Synthesis of the photosensitiser should be straightforward and starting materials should be readily available
Cost	Large scale production should be cost effective

(Adapted from Allison *et al.*, 2004; Maiya, 2000; Nyman and Hynninen, 2004)

In general, photosensitisers can be classified into two types based on their chemical structures, namely tetrapyrrolic and non-tetrapyrrolic. The tetrapyrrolic photosensitisers are mainly discussed in this thesis as most of the photosensitisers approved in the

market or currently in clinical trials are of tetrapyrrolic type. However, some non-tetrapyrrolic photosensitisers are also included.

2.2.2 Tetrapyrrolic photosensitiser

Tetrapyrrolic photosensitisers are classified as such that they have a core structure of cyclic tetrapyrrole (See Figure 2.4). A cyclic tetrapyrrole comprises of four pyrroles with four methine bridges connecting between the tetrapyrroles known as porphyrin. Furthermore, certain carbon positions are substituted by different substituents that distinguish the types of tetrapyrrolic derivatives. The tetrapyrrolic photosensitiser is numbered and named according to the IUPAC nomenclature in this study.

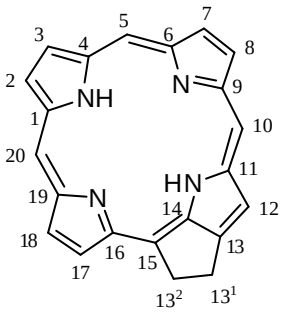


Figure 2.4: The numbering of structure of a photosensitiser according to IUPAC

As photosensitisers are pigmented compounds, they portray distinctive UV-Vis patterns based on absorption at specific wavelengths. Typically, the UV-Vis profile of a tetrapyrrolic compound shows light absorption within the 400-440 nm region , which is known as the Soret band; followed by 3-5 Q bands with one of the Q bands, namely Q_y, in the 600-700 nm region (Scheer, 2006) (See Figure 2.5). Q_y band plays a major role in PDT-related studies where its wavelength (λ) value and absorption intensity largely determines the light penetration efficacy and as a result, the effective treatment depth of the tumour in the clinical application (Nyman and Hynninen, 2004). This is due to the light with higher λ value allows deeper penetration through tissues (Sternberg and

Dolphin, 1998), thus enhancing the efficacy of reaching tumours that are seated in deeper areas or destroying tumours that are large in size.

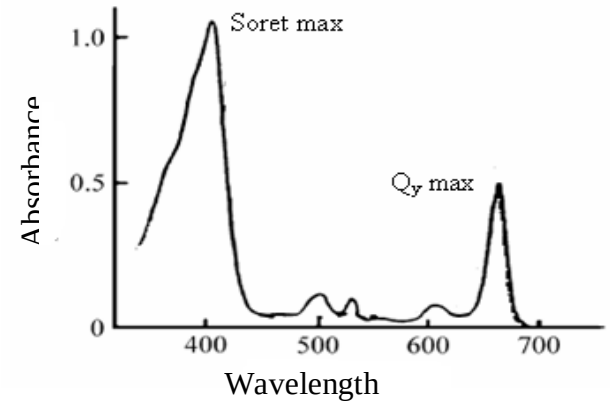


Figure 2.5: UV-Vis profile of a typical tetrapyrrolic compound
(Adapted from Nyman and Hyninnen, 2004)

However, a slight change of the compound structure affects its UV-Vis absorbance profile, thus distinguishing the compounds qualitatively. This phenomenon is observed due to the difference in the electronic transitions along the cyclic tetrapyrrole, namely porphyrin, chlorin and bacteriochlorin. Chlorin has a reduced double bond on one of the pyrrole rings in the porphyrin ring while bacteriochlorin has two reduced double bonds on opposite sides of the tetrapyrrole ring. With the reduced double bonds, Q_y max of the chlorin and bacteriochlorin are shifted to a longer region in UV absorbance as compared to porphyrin, as illustrated in Figure 2.6.

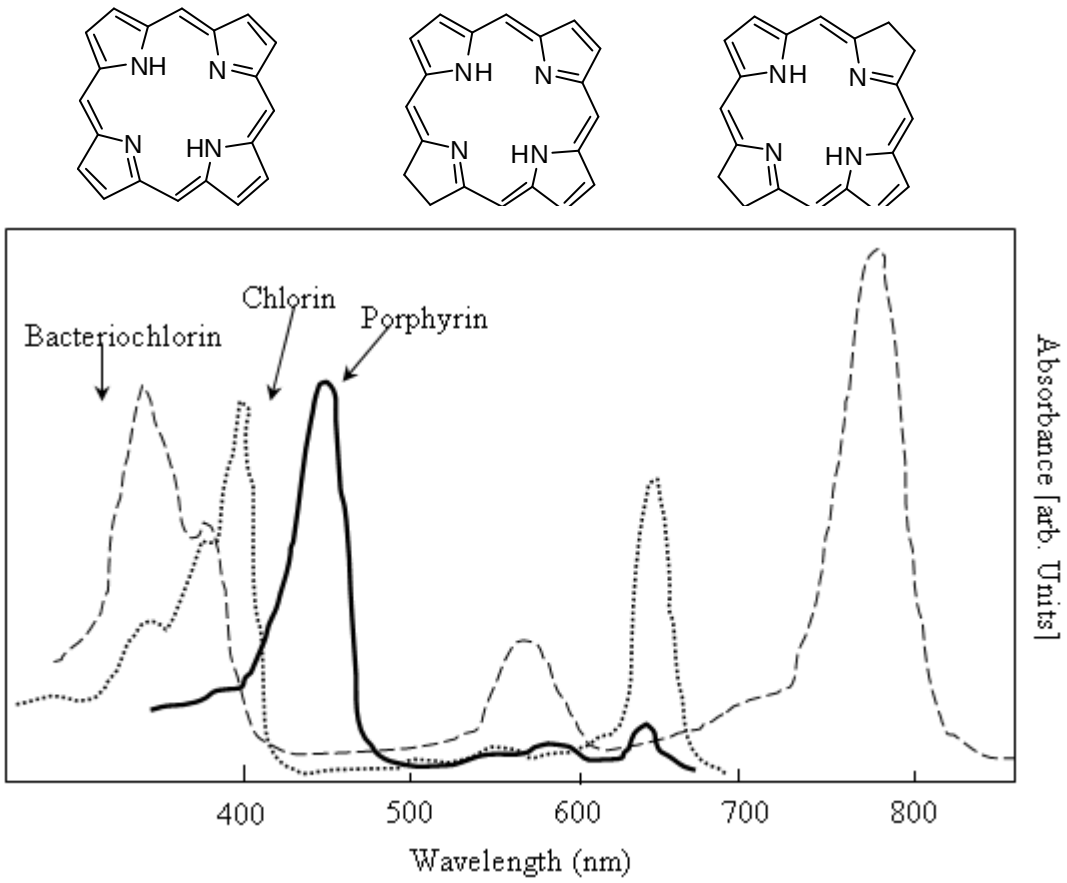


Figure 2.6: Structures of different cyclic tetrapyrroles and their UV-Vis patterns (Adapted from Kobayashi *et al.,* 2006)

The presence of side chains with different functional groups (electron-releasing or -withdrawing) can also influence the shifts of Soret and Q bands in the UV-Vis spectroscopy (Kobayashi *et al.,* 2006). For example, Both the compounds 13^2-hydroxypheophorbide-*a* methyl ester **(2)** and 13^2-hydroxypheophorbide-*b* methyl ester **(7** and **8)** possess the core structure of the chlorin type (See Figure 4.9 and Figure 4.32). However, the presence of an aldehyde group at C-7 (ring B) of the chlorin ring in 13^2-hydroxypheophorbide-*b* methyl ester alters the pattern of Soret band as well as shifts the Soret max to approximately 434 nm. Consequently, chlorophyll-*a* derivatives have a Soret max at 390-410 nm and a Q_y max at 660-670 nm while chlorophyll-*b* derivatives have a Soret max that is shifted to 430-440 nm and the Q_y max at 650-660 nm. It is also noted that Q_y band of 13^2-hydroxypheophorbide-*a* methyl ester exhibits a higher Q_y

max (λ_{max} = 662 nm) with stronger intensity than of 13^2-hydroxypheophorbide-*b* methyl ester (λ_{max} = 654 nm). This results in the difference of the Soret/Q_y band ratio amongst the chlorophylls which is mainly due to different attached substituent to the macrocycle. In another example, chlorophyll-*d* has a much lower Soret/Q_y band ratio (ca.0.85) as compared to chlorophyll-*a* (ca. 1.3) because of the presence of an aldehyde group on ring A of the macrocycle (Kobayashi *et al.*, 2006), as shown in Table 2.2 and Figure 2.7.

Table 2.2: Molar extinction coefficient (ε) of chlorophyll-*a* and –*d* in diethyl ether at room temperature

Compounds	ε (10^3 M^{-1} cm^1) at Soret band	ε (10^3 M^{-1} cm^1) at Q_y band	Soret/Q_y band ratio (ca.)
Chlorophyll *a*	115	89.8	1.3
Chlorophyll *d*	87.6	98.9	0.85

(Adapted from Kobayashi *et al.*, 2006)

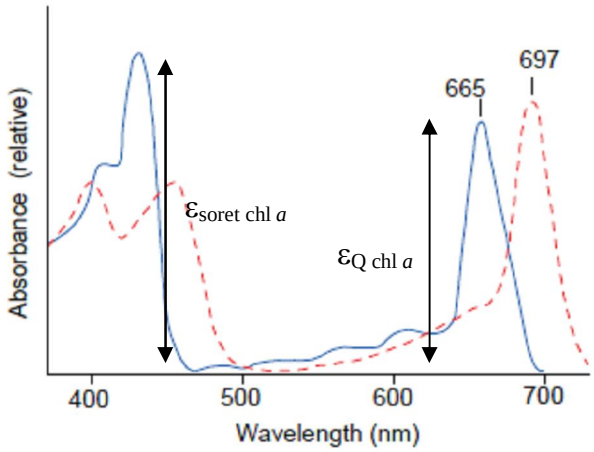


Figure 2.7: Comparison of UV-Vis profiles of chlorophyll-*a* (blue) and chlorophyll-*d* (red) with molar extinction coefficient, ε of chlorophyll-*a*
(Adapted from Larkum and Hühl, 2005)

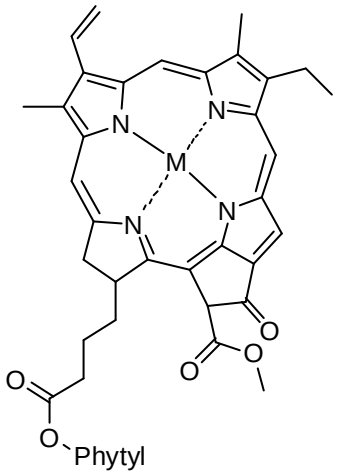
In addition, the Soret band region also differentiates the metallated-chlorophyll type compounds from the demetallated ones. The removal of magnesium reduces the symmetry of macrocycle (Kobayashi *et al.*, 2006) and shifts the absorbance up to 30 nm in the Soret band region. For example, the demetallated chlorophyll-*a*, pheophytin-*a* has

a lower Soret band absorbance (410 nm) as compared to chlorophyll-*a* (430 nm), as described in Table 2.3 and Figure 2.8.

Table 2.3: Maxima absorbance of metallated and demetallated chlorophyll derivatives in diethyl ether

Types of Compound	Maxima Absorbance (nm)
Chlorophyll-*a*	*430*, 661
Pheophytin-*a*	*410*, 505, 667

(Adapted from Almela *et al.*, 2000)



Chlorophyll-*a* : M = Mg
Pheophytin-*a* : M = 2H

Figure 2.8: Structures of chlorophyll-*a* and pheophytin-*a*

2.2.2.1 Tetrapyrrolic photosensitisers with porphyrin core structure

2.2.2.1.1 Haematoporphyrin derivative (HpD)

Haematoporphyrin derivative (HpD) is the first photosensitiser approved in the market. It was first discovered by Meyer-Betz's self-experiment in 1913 where the area that was exposed to the light showed photosensitivity. It was found out by Schwartz later in early 1950s that it was an oligometric mixture of haematoporphyrin (Hp) that caused the skin photosensitivity. The oligometric mixture, known as HpD, was obtained in a reaction of Hp treated with sulphuric acid in acetic acid, followed by strong alkali treatment (See Figure 2.9).

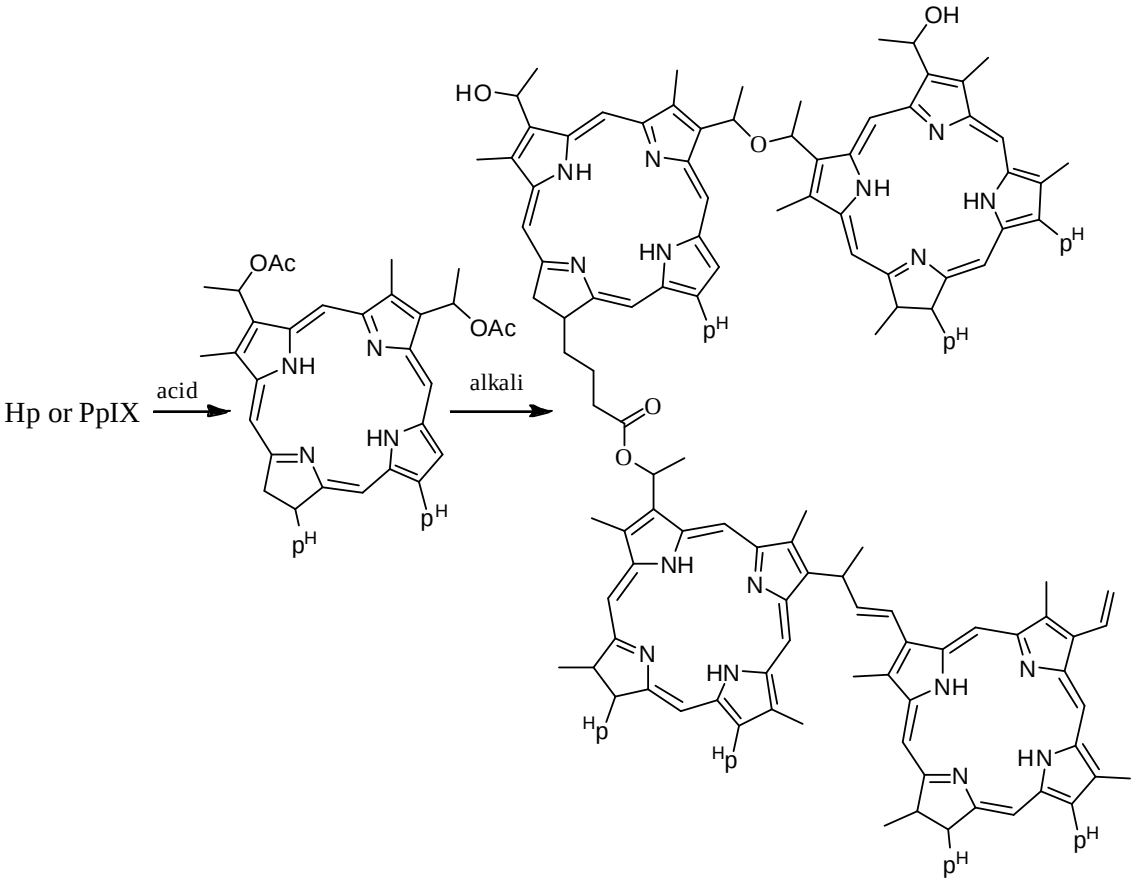


Figure 2.9: Formation of oligometric HpD from haematoporphyrin
(Adapted from Sternberg and Dolphin, 1998)

The commercialised HpD known as "Fraction D" was separated partially by HPLC. The ratio of monomers, dimers and oligomers in Fraction D is estimated to be 14:19:67 as compared to HpD with a ratio of 22:23:55. The largest oligomer found in Protofrin

comprises nine porphyrin units. In HpD, oligomers with 2-10 porphyrin units are linked by ether, ester or carbon-carbon bridges and the average molecular weight of HpD is about the weight of 4 porphyrin units (Nyman and Hynninen, 2004).

HpD was widely studied in various clinical applications since early 1970s and has received great responses due to its low dark toxicity and selective accumulation in tumor sites (Sternberg and Dolphin, 1998). The oligometric material was later developed and commercialised with the brand Photofrin® and has been approved in Europe and North America for various cancers as shown in Table 2.4.

Despite having favourable clinical development, there is concern about the complexity of mixture that causes difficulties in reproducibility in synthesis process and also ambiguity about the actual active component of the mixture. Other disadvantages include prolonged skin photosensitivity, low fluorescent quantum yield and low rate of reactive oxygen species generation (Palumbo, 2007), along with limited light penetration, as the activation wavelength is only about 630nm (Stemberg and Dolphin, 1998).

Table 2.4: Regulatory approval of commercial haematoporphyrin derivative, Photofrin®

Country	Approved Indications
Canada	Prophylactic treatment of recurrent papillary bladder cancer(1993) Palliative treatment of completely and partially obstructive esophageal cancers (1995); and lung cancer(1999)
Denmark	Treatment of lung and esophageal cancer (1999)
France	Treatment of recurrent lung and esophageal cancer (1996)
Finland	Treatment of lung and esophageal cancer (1999)
Germany	Treatment of early stage lung cancer (1997)
Iceland	Treatment of lung and esophageal cancer (1999)
Italy	Reduction of endobronchial obstruction or endobronchial mucosal lesions caused by non-small cell lung cancer or by metastases of other tumor cells to the lung; palliative treatment of malignant dysphagia caused by esophageal carcinoma (2000)
Japan	Treatment of early stage lung cancer (1994), gastric and cervical cancers including cervical dysplasia and inoperable superficial esophageal and gastric cancers (1996)
The Netherlands	Treatment of superficial lung cancers, and palliative treatment of obstructive lung and esophageal cancers (1994)
United Kingdom	Treatment of lung and esophageal cancer (1998)
United States	Palliative treatment of totally or partially obstructing esophageal cancers that are unsuitable for Nd:YAG therapy (1995), curative treatment of microinvasive endobronchial non-small cell lung cancer (NSCLC) in patients, who are not indicated for surgery and radiotherapy (1998), and palliative treatment of late NSCLC (1998), treatment of pre-cancerous lesions in Barrett's Esophagus (2003)

(Adapted from Rogers, 2005)

2.2.2.1.2 Phthalocyanines

A porphyrin ring expanded with four benzene rings substituted at β-pyrrolic positions and nitrogens substituted at the methine-bridges, namely phthalocyanine (Figure 2.10) improves the absorption of wavelength to 10 000 $M^{-1}cm^{-1}$ (HpD = ~10^3 $M^{-1}cm^{-1}$) as well as moves the λ_{max} to 680 nm (Allen *et al.*, 2001). In addition, phthalocyanine has rapid clearance from the PDT-treated patient's body which minimizes the skin photosensitivity issue that is observed when patients are treated using HpD. The central metal atom attached to phthalocyanines also helps in improving the photophysical and photochemical properties. Phthalocyanines attached with diamagnetic metals including

Zn^{2+}, Al^{3+} and Ga^{3+} have been observed to have increased the triplet-state yield which increase the singlet oxygen generation upon activation by light (Allen *et al.*, 2001).

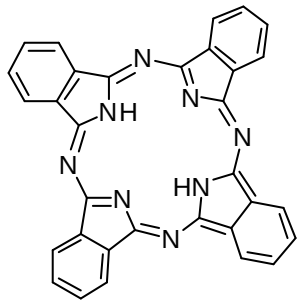


Figure 2.10: Structure of phthalocyanine

The drawback observed for phthalocyanines is the high hydrophobicity of the structure. Thus polar substituents are often attached to overcome the solubility problems. However, the polar substituents cause aggregation which affects the photo-killing activity of photosensitisers. Hence, compatible drug delivery system is required to sustain the drug efficacy in clinics such as liposomes (Allen *et al.*, 2001).

2.2.2.1.3 Lutetium texaphyrin (Lu-Tex)

Lutetium texaphyrin (Lu-Tex) is a modified porphyrin. It is tripyrrolic, pentaaza-expanded with 22π-electron system rather than 18π-electron system in porphyrin (Mody and Sessler, 2001), as shown in Figure 2.11. Unlike many other photosensitisers, Lu-Tex is water soluble due to its polyether and hydroxyl functional group. It also absorbs broadly at 474 nm and near infra-red region at 732 nm with absorption strengths of 125,900 $M^{-1}cm^{-1}$ and 42,000 $M^{-1}cm^{-1}$ respectively. Its amphiphilic and broad fluorescence emission characteristics make it not only as a good biolocalisation agent (Blumenkranz *et al.*, 2000), but also as a good photosensitiser as it generates high triplet state yield as well as high production of singlet oxygen (up to 70%) (Mody and Sessler, 2001).

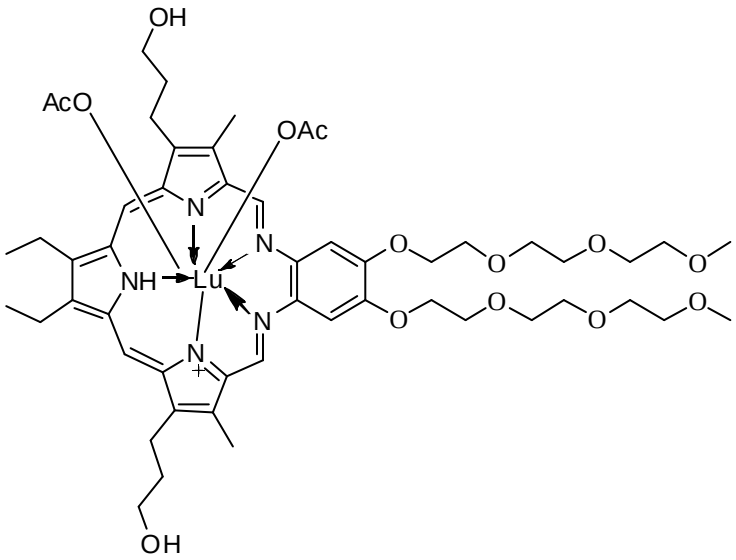
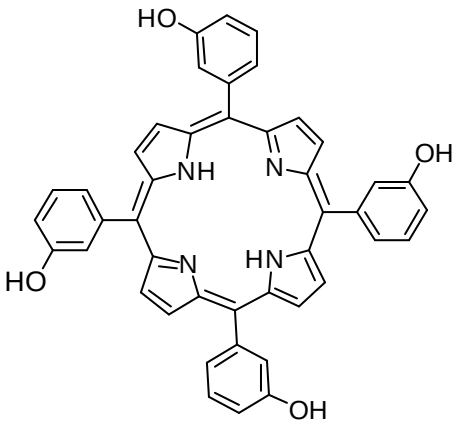


Figure 2.11: Structure of lutetium texaphyrin (Lu-Tex)

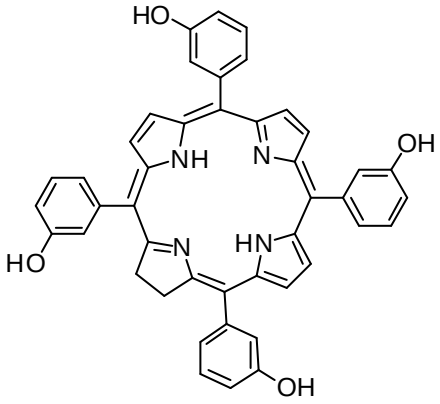
2.2.2.2 Tetrapyrrolic photosensitisers with chlorin and bacteriochlorin core structures

2.2.2.2.1 5,10,15,20-tetra(3-hydroxyphenyl)porphyrin (m-THPP) and its chlorin derivative (m-THPC)

The substitution of the meso positions of a porphyrin with substituents such as 3-hydroxyphenol, as illustrated in Figure 2.12 shifts the λ_{max} value to 650 nm and increase the drug efficacy of the drug to 25-30 times as compared to HpD. The structure has also been reduced to a chlorin form (m-THPC) and this further exhibits excellent photophysical properties including higher potency as compared to m-THPP (Nyman and Hynninen, 2004). The chlorin derivative also demonstrates a high tumour to normal tissues selectivity and relatively shorter skin photosensitivity as compared to Photofrin® (Berenbaum *et al.*, 1993). With its excellent photophysical properties and pharmacokinetic profile, mTHPC has been commercialised as Foscan® and is extensively utilised especially in head and neck cancers in European Union countries, Norway and Iceland.



5,10,15,20-tetra(3-hydroxyphenyl) porphyrin (mTHPP)



5,10,15,20-tetra(3-hydroxyphenyl) chlorin (mTHPC)

Figure 2.12: Structures of 5,10,15,20-tetra(3-hydroxyphenyl)porphyrin and its chlorin derivative

However, a few drawbacks were also observed in Foscan®. Patients may experience significant pain as the drug is administered during the treatment. Clinicians would also have to take into account the optical properties of tumour in order to prevent excessive light penetration which may cause other complications such as vascular damage, especially when the tumour is present in vital parts of the body such as head and neck. Another significant disadvantage is that Foscan® is active even under room light. Hence, treatment has to be conducted in a dark room and patients will have to avoid exposure of light up to one week after treatment (Allison *et al.*, 2004).

2.2.2.2.2 Benzoporphyrin derivatives

Benzoporphyrin derivatives are synthesised with structure of a chlorin ring attached with a benzene ring. Amongst the benzoporphyrin derivatives, benzoporphyrin derivative monoacid ring A (BPD-MA) (Figure 2.13) has been subjected to Phase II clinical trial due to its excellent photophysical properties, including strong absorption of 33 000 $M^{-1}cm^{-1}$ at λ_{max} of approximately 688 nm as well as relatively high triplet and singlet-state yield (Aveline *et al.*, 1993). The presence of benzene ring at the chlorin

increases the lipophilicity of the compound which enhances the intracellular uptake and localisation in the membrane layer of the cells (Chowdhary *et al.*, 2003). It is also rapidly cleared as compared to many other photosensitisers and this minimises the risk of systemic photosensitivity (Schmidt-Erfurth and Hasan, 2000). To date, BPD-MA is marketed as a liposomal benzoporphyrin derivative with the brand Visudyne® that is used in various ocular-related treatments (Prescription Indication of Visudyne, Novartis).

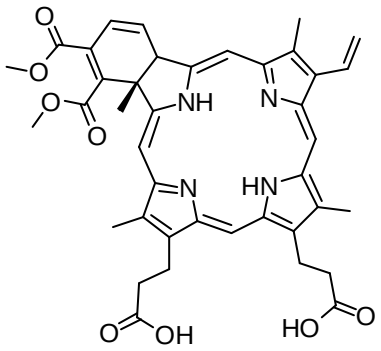
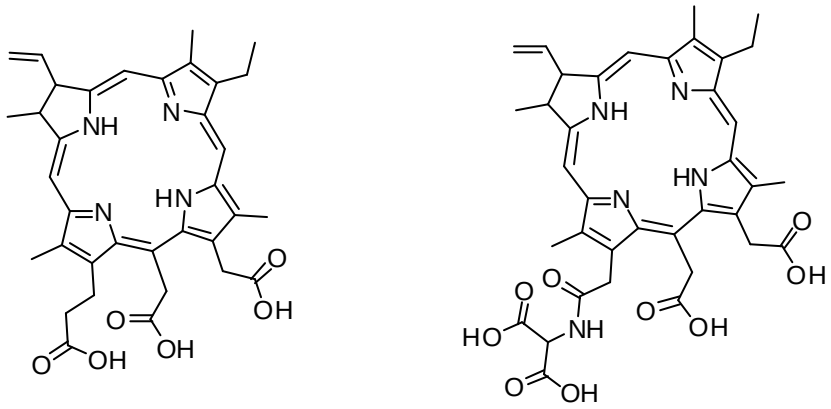


Figure 2.13: Structure of benzoporphyrin derivative monoacid ring A

2.2.2.2.3 Mono-L-aspartyl-chlorin e6 (NPe6)

Mono-L-aspartyl-chlorin e6 (NPe6) is also a chlorin-based photosensitiser with an L-aspartic acid attached to the propionic acid of chlorin e6 (ce6) through an amide bond, forming a structure as shown in Figure 2.14. Mono-L-aspartyl-chlorin e6 (NPe6) is more hydrophilic as compared to ce6 due to the presence of an extra free carboxylic acid. Furthermore, NPe6 is found to have slight increase of singlet oxygen yield than ce6 (Nyman and Hynninen, 2004) with absorption of 40000 $M^{-1}cm^{-1}$ (Palumbo, 2006) at λ_{max} = 664 nm (Kondo *et al.*, 2007). It has also rapid clearance time as compared to HpD and this reduces the prolonged skin photosensitivity after PDT (Nyman and Hynninen, 2004).



Chlorin e6 Mono-L-Aspartyl-chlorin e6

Figure 2.14: Structures of chlorin e6 and mono-L-aspartyl-chlorin e6

2.2.2.2.4 Pheophorbide-*a*

Pheophorbide-*a* is one of the degradation products from chlorophyll-*a* (See Figure 2.15) which has a chlorin skeleton. Chlorophyll-based photosensitisers have slightly different chemical structure from other tetrapyrrolic photosensitisers discussed previously. They have β-ketoester isocyclic ring E attached to ring C of the core structure, where it acts as the main reactive site in derivatisation for various chlorophyll-based compounds. The formation of pheophorbide-*a*' involves the cleavage of a magnesium central atom and a phytyl group at $C17^3$ position from chlorophyll-*a* to form a metal-free chlorin with a free propionic acid at $C17^3$. Its free carboxylic acid group increases its hydrophilicity as compared to chlorophyll-*a* as well as allows better solubility in physiological liquid and absorption into the body (Nyman and Hynninen, 2004). The degradation shifts the λ_{max} to 668 nm (chlorophyll-*a*, λ_{max} = 660 nm) as well as causes a slight increase of triplet state yield and singlet oxygen yield as compared to chlorophyll-*a*. In other words, pheophorbide- *a* has favourable photophysical properties which make it a better photosensitiser as compared to other naturally occurring chlorophylls degradation products such as pheophytin and chlorophyllide.

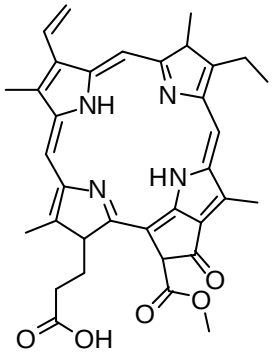


Figure 2.15: Structure of pheophorbide-*a*

2.2.2.2.5 Palladium-bacteriopheophorbide-*a*

Bacteriopheophorbide-*a* (See Figure 2.16) is a degradation product from natural occurring bacteriochlorophyll-*a*. Bacteriochlorophyll-*a* has a bacteriochlorin skeleton with strong absorption coefficient of 4-10 x 10^4 $M^{-1}cm^{-1}$ at much higher wavelength region (> 700 nm). In addition, high singlet oxygen yield is another superior feature found in bacteriochlorophyll (Brandis *et al.,* 2006). However, the compound is easily oxidised to its porphyrin form and therefore requires stabilisation via certain procedures, including metallation, exocylic ring fusion or attachment of possible substituents to the tetrapyrrole ring (Nyman and Hynninen, 2004).

Palladium-bacteriopheophorbide-*a* has been recently developed as one of the promising PDT drugs due to its superior photophysical properties (Garbo *et al.,* 2004). The attachment of palladium as a central metal atom in the structure has improved the triplet oxygen yield of the compound to almost 99% and the compound is rapidly cleared after PDT. Palladium-bacteriopheophorbide-*a* is marketed as Tookad® with λ_{max}=762 nm and currently in clinical trials for prostate cancer (Weersink *et al.,* 2005).

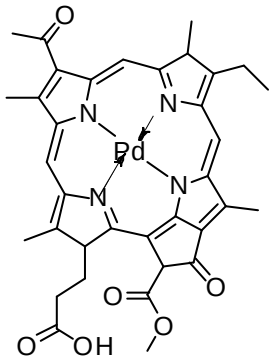
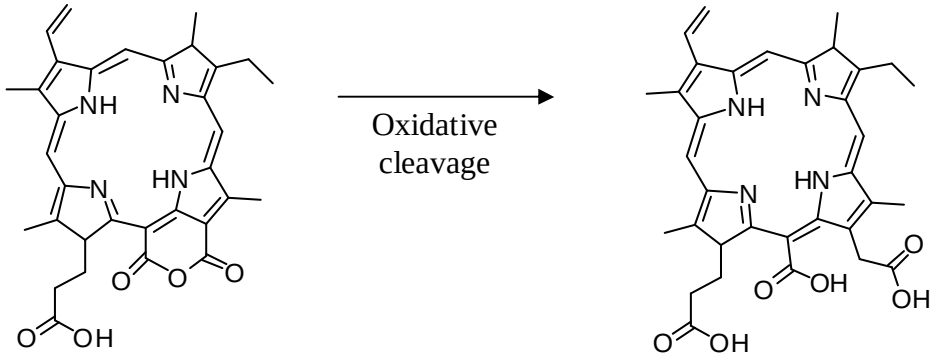


Figure 2.16: Structure of palladium-bacteriopheophorbide-*a*

2.2.2.2.6 Purpurin-18

Purpurin-18 is another chlorophyll degradation product with an anhydride ring instead of a β-ketoester isocyclic ring E attached to ring C of a chlorin structure (Garbo, 1996), as shown in Figure 2.17. The attachment of the anhydride ring to chlorin has shifted the λ_{max} to 698 nm with the absorption strength of 45 000 $M^{-1}cm^{-1}$. Purpurin-18 is unstable when it is administrated into body as the anhydride ring is easily cleaved via nucleophilic reactions. However, the ring opening of the anhydride ring produces another photo-active agent known as chlorin p6 thus this does not reduce the photo-killing efficacy of the compound (Nyman and Hynninen, 2004).



Purpurin-18 Chlorin p6

Figure 2.17: Structures of Purpurin-18 and its oxidation product after oxidative cleavage in body

2.2.3 Non-tetrapyrrolic photosensitisers

2.2.3.1 Hypericin

Hypericin was first isolated from St. John's Worts (*Hypericum perforatum)* by Brockmann and co-workers in 1942 together with another analog known as pseudohypericin. It has a different core structure from the typical tetrapyrrolic photosensitisers where it comprises eight aromatic rings with strong acidic properties due to the presence of phenolic rings (See Figure 2.18). Formation of stable hypericin anions due to the preferential loss of proton of hydroxyl group at C3 position results in the occurrence of hypericin as a potassium salt in nature (Vollmer and Rosenson, 2004). It is also amphiphilic for having both hydrophobic aromatic ring and hydrophilic residues in the structure. It exhibits the strongest absorption at 590 nm with high singlet oxygen quantum yield and minimal dark toxicity properties making it a very promising photosensitiser (Agostinis *et al.*, 2002).

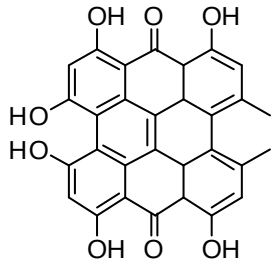
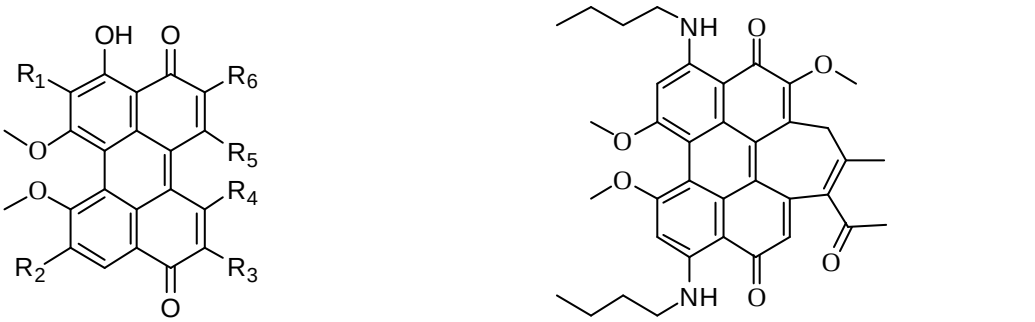


Figure 2.18: Structure of hypericin

2.2.3.2 Hypocrellin

Hypocrellins are the lipophilic peryloquinone derivatives originated from a parasitic fungus of *Sinarundinaria* plant species named *Hypocrella bambuase* (See Figure 2.19). It has favourable light absorption in the red region (λ_{max}=658nm), high singlet oxygen generation yield, rapid clearance from the body as well as feasibility of site-specific modification. Therefore, various approaches have been taken to optimise the photosensitising properties of the compound (He *et al.*, 2000; Estey *et al.*, 1996). This

includes nucleophilic and alkylamino substitution at the phenolic groups at C4 and C9 positions. For example, 4,9-bis(butylamino)-derivative of hypocrellin B (See Figure 2.19) has increased molar extinction coefficient and decreased dark toxicity compared to its parent compound (Wainwright, 1996).



Hypocrellin 4,9-bis(butylamino)-derivative of hypocrellin B

Figure 2.19: Structures of hypocrellin and its derivative

2.2.4 Photosensitisers from marine algae

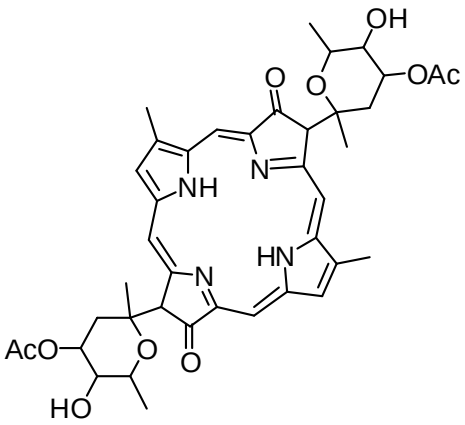


Figure 2.20: Structure of tolyporphin

Two of the compounds which have potential use as photosensitisers of marine algae origin are tolyporphyrin and phycocyanin. Tolyporphyin (TP) was first isolated from the blue green alga *Tolypothrix nodosa* in 1992. It has a chlorin core structure with different side residues as compared to chlorophyll *a*. The residues include a methyl group at C3, two carbonyl groups at C7 and C17 respectively as well as the lack of an isocylic ring E fused to the chlorin (See Figure 2.20). In addition, it exhibits a unique

characteristic that is not observed in other photosensitisers with the presence of two C-glycosyl rings attaching at C8 and C18 of the chlorin ring (Prinsep *et al.*, 1992). The substitution of the side residues increases the hydrophilicity of the compound as compared to HpD. The unique substitution of tolyporphin may have also contributed to the difference in the subcellular localisation and biodistribution properties in comparison with other examined photosensitisers thus significantly increase its drug efficacy as a photosensitiser *in vivo*. It has been observed to selectively bind to endoplastic reticulum membranes and has a higher concentration ratio of tumor to blood ($[TP]_{tumor}/[TP]_{blood}$ = ~100) than n-(4-butanol) pheophorbamide-*a* (Ph4-OH) (~3) and H-hexyl ether of 6-methylpyropheophorbide-*a* (MPPH) (~1.5) (Morlière *et al.*, 1998).

Photodynamic efficacy of phycocyanin extracted from *Spirulina platensis* was also evaluated in the treatment of human arterioesclerofic plaque (Pádula *et al.*, 1996) and bovine coronary artery endothelium (Morcos *et al.*, 1991). Phycocyanin (See Figure 2.21) was demonstrated to kill gram-positive bacteria *Staphylococcus epidermidis* but not gram-negative bacteria *E. coli* strains via singlet oxygen generation (type II pathway) upon light activation. Further investigation proposed that the insensitivity against gram-negative bacteria was due to the incapability of phycocyanin binding to the outer membrane of the bacteria that prohibit the penetration of hydrophobic or high molecular weight compounds into the cells. The studies also suggested that phycocyanin induces DNA photo-oxidation upon light activation (Pádula *et al.*, 1996).

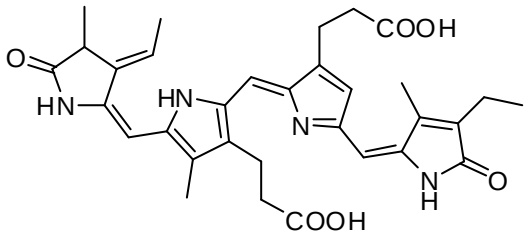


Figure 2.21: Structure of phycocyanin

Other phycobiliproteins, including R-phycoerythrin, C-phycocyanin and allophycophyanin have also displayed better photophysical properties than HpD, such as high molar extinction coefficient and reduced skin photosensitivity. However, more studies have to be carried out to validate the potential issues in human immune response towards the proteins (He *et al*., 1997).

2.3 Algae

Algae are defined as an oxygen producing photosynthetic organism that lacks sterile tissue around its sexual reproductive structure(s) (Andersen, 1992). They exist in habitats of various temperature and geological conditions from sea and freshwater to hot desert sand and on hot springs, as well as on snow and ice. Algae are present in various shapes and forms, ranging from small, single-celled organisms such as phytoplankton, to complex multicellular organisms, such as giant kelps. Single-celled algae are often called microalgae while multicellular algae are commonly known as macroalgae, or seaweeds in marine.

Seaweeds, which are also known as benthic marine algae, are mainly located in aquatic environment. They can be found in the coastal region between high tide to low tide and in the sub-tidal region up to a depth where 0.01 % photosynthetic light is available. Seaweeds are not similar to the higher plants. They have whole plant body that is called a thallus, which comprise the holdfast for anchorage, stipe that absorbs nutrients from the surrounding water as well as functioning as a support for the blade that is involved in photosynthesis to produce their own food (See Figure 2.22). These parts of the seaweeds do not have the same functions as the roots, stem or leaves of higher plants have although their morphological structures may be plant-like (Dhargalkar and Kavlekar, 2004).

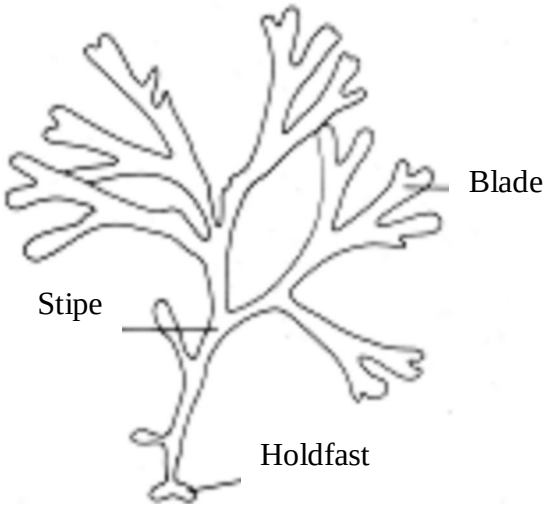


Figure 2.22: Morphology of seaweed
(Adapted from Dhargalkar and Kavlekar, 2004)

2.3.1 Classification of algae

The taxonomic study of algae has been very difficult due to their complex evolutionary mechanisms as well as their diverse physical structures. Algae are classified into Domain Eukarya according to the Three Domain System, which includes the multicellular phyla such as Pheophyta, Rhodophyta and Chlorophyta. Other microalgae such as the diatoms, dinoflagellates and golden algae also belong to this domain. Meanwhile, the cyanobacteria which are commonly known as blue-green algae are categorized in the Domain Bacteria due to similarities to bacteria, especially in the absence of a true nucleus (Oswald *et al.*, 2007).

Generally, the algae are differentiated according to the types of pigments present (See Table 2.5). Meanwhile, other characteristics such as chloroplast structure, the chemistry of cell walls, number and position of flagellae, and the form of food reserves in cells are also taken into consideration for classification.

Table 2.5: Classes of seaweeds

Class of Seaweeds	Types of photosynthetic pigments	Types of reproduction	Uses	Examples
Chlorophyta (green)	chlorophyll-*a, b*	asexual or sexual reproduction, some undergo alternation of generations	Food consumption	*Chlamydomas*, *Caulerpa* and *Spirulina*
Rhodophyta (red)	chlorophyll-*a, d,* phycobiliproteins such as phycocyanin, allophycocyanin and phycoerythrin	Asexual (in form of fragmentation) or sexually (spore formation)	Phycocolloid production	*Eucheuma, Gracilaria* and *Kappaphycus*
Pheophyta (brown)	chlorophyll-*a, c,* fucoxanthin	Sexual with alternation of generation between two multicellular stage	Alginate production Food consumption	*Fucus, Sargassum* and *Turbinaria* *Laminaria* (Kombu) and *Undaria* (wakame)

(Adapted from Dhargalkar and Kavlekar, 2004; Zemke-White and Ohno, 1999, Clark, 1999)

2.3.2 Biodiversity of marine algae in Malaysia

Being one of the twelve mega-biodiversity countries (Paine, 1997), Malaysia is blessed with abundant species of marine algae around the coastal zones. The compilation of biodiversity information of marine algae in Malaysia has largely been contributed by Phang *et al.* with the earliest publication dated in the year 1991. There are a total of Malaysian marine algae with 373 specific and intraspecific taxa of Malaysian marine algae including 17 taxa of Cyanophyta, 102 Chlorophyta, 182 Rhodophyta and 72 Phaeophyta (Phang, 2006).

2.3.3 Uses of seaweeds

Seaweeds, especially the red and brown ones, are the main source of phycocolloids including agar, carrageenan and alginate (See Table 2.5). Seaweed species such as

Eucheuma and *Gracilaria* are widely cultivated in the coastal zones of Sabah to supply the raw materials in the industrial scales (Phang, 2006).

Seaweeds have also been one of the feedstuff ingredients for farm animals, poultry and aquaculture. It is known that the feed has improved the fertility of the farm animals as well as providing sufficient nutrients to promote better quality of fish and prawn culture due to its enriched nutrients (Dhargalkar and Pereira, 2005).

Seaweed biomass is also a great source of renewable energy. The anaerobic conversion of seaweed biomass via consumption of solar energy and carbon from ambient CO_2 supplies biofuel (Klass, 2004) that provides clean fuel without contributing lead to pollution. The use of seaweed biomass for energy has been extensively studied in power generation and biofuel supply (Ross *et al.*, 2008).

Seaweeds are found with numerous growth promoting hormones like auxin and cytokinins as well as other minerals and micronutrients which make a very good fertilizer. In addition, the seaweed-based fertilizer is biodegradable and non-polluting that it is very environmental friendly to be used. To date, it has been marketed as liquid rich seaweed fertilizer (LSF) and was found to show improvement in germination, seedling vigour, fruit settling as well as increase the weight of fruits in certain crops (Edward *et al.*, 2003).

2.3.4 Anti-cancer compounds from marine algae

The collection of organisms from the ocean has been providing the natural product chemists with a largely untapped resource that offers a range of unique structures (Fenical, 1997) including terpenes, acetogenins, alkaloids, polyphenolics, polyketides,

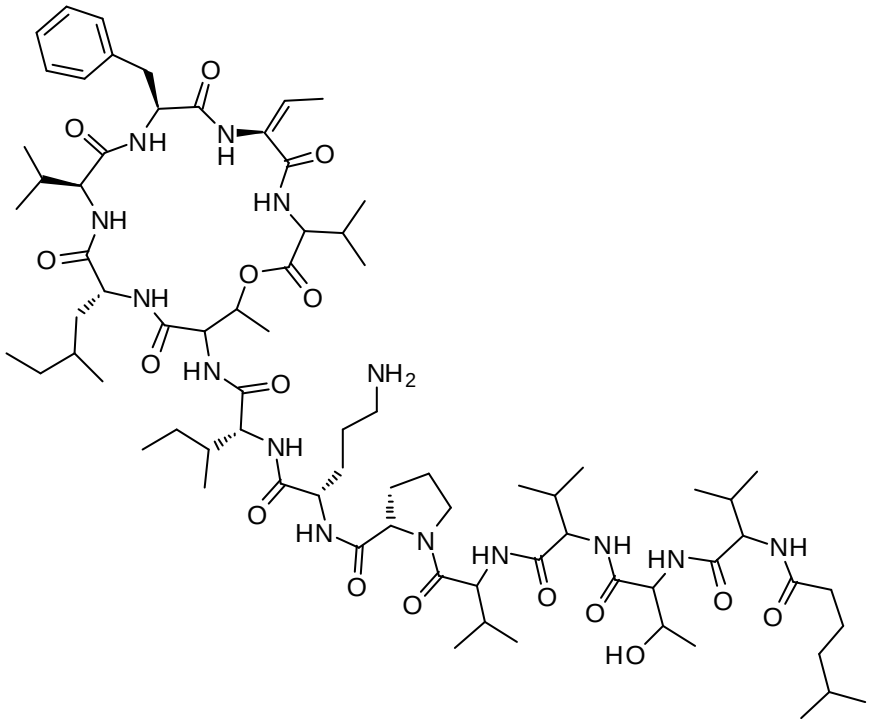
steroids, peptides, shikimic acid derivatives, sugars, and a variety of mixed biogenesis metabolites (Mayer and Gustafsonb, 2006; Hay and Fenical, 1996; Simmons *et al*., 2005). Some of these compounds differ fundamentally from terrestrial secondary metabolites in that they are halogenated and often possess chemical structures that are unprecedented among terrestrial organisms (Hay and Fenical, 1996).

To date, marine algae contribute to 25% of the marine natural product present today (Kijjoa and Sawangwong, 2004) and several candidates have exhibited remarkable anticancer property. Kahalalide F ((a), see Figure 2.23) is a cyclic depsipeptide isolated from green alga *Bryopsis sp.* as well as from mollusk *Elysia rufescens.* It is one of the marine metabolites that showed excellent cytotoxicity against various solid tumour cell lines at micromolar concentrations (Hamann and Scheuer, 1993). It has been brought forward to phase I clinical investigation in advanced androgen refractory prostate cancer (Rademaker-Lakhai *et al.*, 2005) as well as phase II clinical trials in advanced malignant melanoma (Martín-Algarra *et al.*, 2009) and advanced hepatocellular carcinoma (Jimeno *et al.*, 2004).

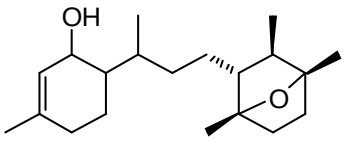
In addition, two terpene compounds isolated from a tropical marine red alga genus known as *Laurencia*, namely laurenditerpenol ((b), see Figure 2.23) and dehydrothyrsiferol ((c), see Figure 2.23), have also been found to show potential as anticancer agents. Laurenditerpenol from *Laurencia intricata* has demonstrated an inhibitory effect towards transcription factor hypoxia-inducible factor-1 HIF-1, which plays a major role in promoting the growth of cancer cells under hypoxic conditions (Mohammed *et al.*, 2004). Meanwhile, dehydrothyrsiferol, a triterpenoid from red alga *Laurencia viridis spec. nov.* has demonstrated *in vitro* cytotoxicity against a number of cancer cells (Fernández *et al.*, 1998). Further investigation of dehydrothyrsiferol's

cytotoxic effect also demonstrated a higher apoptosis rate in human estrogen receptor (ER^-) breast cancer cell lines (Pec e*t al.,* 2003).

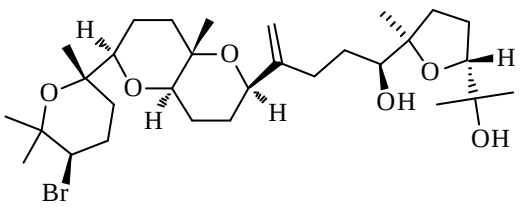
GA3P polysaccharide is a D-galactan sulphate associated with L-(+)-lactic acid isolated from the marine microalga Gymnodinium sp. It induced apoptosis on human myeloid leukemia K562 cells (Sogawa *et al.,* 2000) and was later found to inhibit topoisomerases I and II effectively apart from showing moderate *in vitro* cytotoxicity against a panel of cancer cell lines (Umemura *et al.,* 2003). Figure 2.23 showed the diversity of anticancer metabolites that was discussed above.



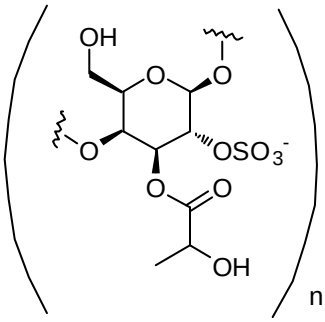
(a) Kahalalide F



(b) Laurenditerpenol



(c) Dehydrothrysiferol



(d) Monomer unit of GA3P polysaccharide

Figure 2.23: Anti-cancer compounds derived from marine algae
(Adapted from Mayer and Gustafson, 2006)

2.4 Sourcing photosensitisers from marine algae

The chlorophylls have been abundantly isolated from the nature sources for the past few decades. They are used as starting materials for further synthetic modification of the tetrapyrrole structures to improve its PDT efficacy. This reduces the cost and time spent as compared to total synthesis of a similar chemical structure from simple binding blocks (Nyman and Hynninen, 2004).

However, it is known that the major disadvantage of tetrapyrrolic photosensitisers is the photo-instability of the compounds which cause photo-degradation, or better known as photo-bleaching. (Ferreira *et al.*, 2008). Furthermore, the self-aggregation property of tetrapyrrolic photosensitisers also affects the triplet state lifetime and consequently reduces the singlet oxygen quantum yield *in vivo* (MacDonald and Dougherty, 2001). In addition, tetrapyrrolic compounds have lower intensity of light absorption in near infra-red as compared to its UV-visible region which causes generalized photosensitivity under light exposure (Nyman and Hynninen, 2004). Therefore, it is worth sourcing for other potential structures that could overcome the current limitations observed.

Marine algae are enriched with a variety of photosynthetic pigments, which is one of the essential prerequisites for a photosensitiser. It is also noted that different types of algae comprise of different photosynthetic pigments due to their habitats and many of them are not commonly seen in terrestrial organisms. Therefore, it may be a good chance of obtaining derivatives of tetrapyrrolic photosensitisers that may not have been observed in those of terrestrial origin, for example: tolyporphin, which have been discussed earlier (See Section 2.3). More importantly, marine organisms can provide an opportunity for seeking new chromophores that may be a better drug leads for further

optimization to overcome the drawbacks observed with the tetrapyrrolic photosensitisers.