TESIS DOCTORAL

BIOAVAILABILITY OF CHLOROPHYLLS

Kewei CHEN

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ABBREVIATIONS

Chl: chlorophyll

OH chl *a*: 13^2-OH-chlorophyll *a*

Lactone chl *a*: 15^1-OH-lactone-chlorophyll *a*

Pheo *a*: pheophorbide *a*

OH pheo *a*: 13^2-OH- pheophorbide *a*

Lactone pheo *a*: 15^1-OH-lactone- pheophorbide *a*

Purpurin: purpurin-18 *a*

Phy *a*: pheophytin *a*

OH phy *a*: 13^2-OH- pheophytin *a*

Lactone phy *a*: 15^1-OH-lactone- pheophytin *a*

Pyro phy *a*: pyropheophytin *a*

Phytyl chlorin: phytyl chlorin e_6

Phytyl purpurin: phytyl-purpurin-18 *a*

Chl *b*: chlorophyll *b*

OH chl *b*: 13^2-OH- chlorophyll *b*

Lactone chl *b*: 15^1-OH-lactone- chlorophyll *b*

Phy *b*: pheophytin *b*

OH phy *b*: 13^2-OH- pheophytin *b*

Chl *c*: chlorophyll *c*

Pheo *c*: pheophorbide *c*

OH derivatives: 13^2-OH chlorophyll derivatives

Oxidized pheo *c*: Oxidized pheophorbide *c*

AMF: aqueous micellar fraction

pre-d: previous to *in vitro* digestion

post-d: posterior to *in vitro* digestion

AMF: aqueous micellar fraction

FN: fresh dried Nori

BN: boiled Nori

MN: microwaved Nori

FS: fresh dried Sea Lettuce

BS: boiled Sea Lettuce

MS: microwaved Sea Lettuce

FK: fresh dried Kombu

BK: boiled Kombu

MK: microwaved Kombu

HEPES: N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

BHT: butylated hydroxytoluene

BCA: bicinchoninic acid

BLT1: 2-Hexyl-1-cyclopentanone thiosemicarbazone

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl sulfoxide

PBS: phosphate-buffered saline

SR-BI: scavenger receptor class B type I

NPC1L1: Niemann Pick C1 Like 1 protein

CD36: cluster determinant 36

ABCA1: ATP-binding cassette transporter subfamily A

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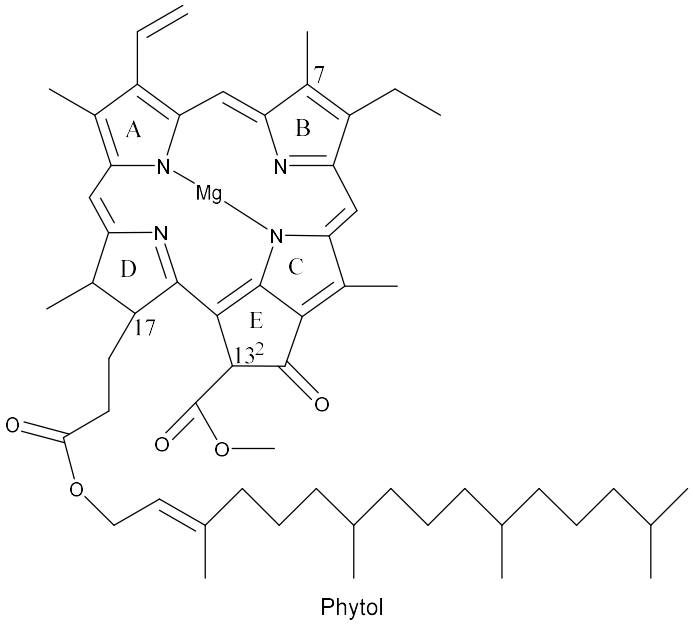
1. - INTRODUCTION

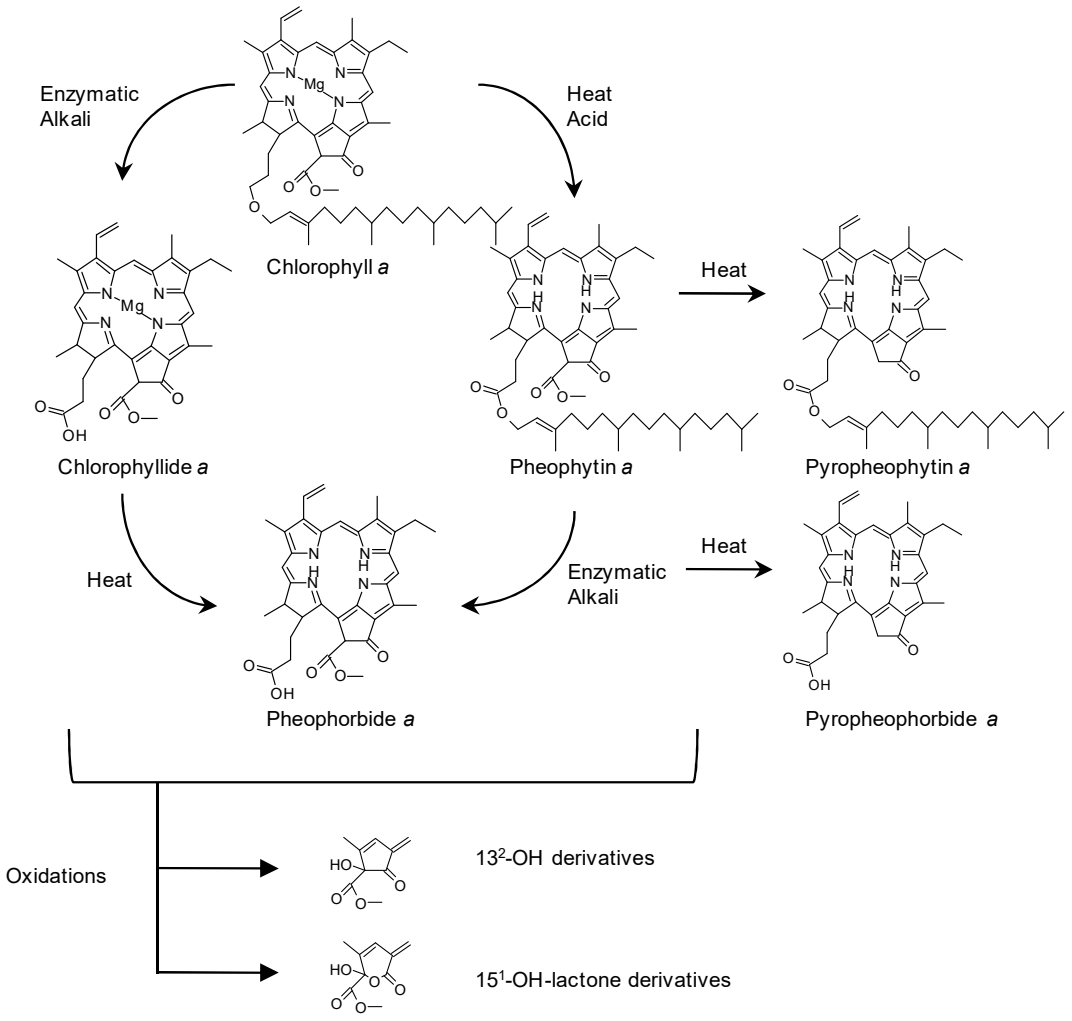
Chlorophyll pigments, the vital biomolecule for photosynthesis, are widely distributed on the earth from cyanobacteria to the chloroplasts of algae and plants where exist photosystem units. The biosynthetic machinery of plant tissues is able to metabolize more than 10^9 tons of natural chlorophylls per year (Kräutler, 2008), a figure to note the wide distribution of this family of green pigments and the significance of the intermediates and metabolites derived from that process.



Scheme 1: Chlorophyll *a* derivative.

There are many chlorophyll derivatives involved in the metabolism, storage and processing of plant materials. Take chlorophyll *a* and *b* commonly found in plant tissue for example. Native chlorophyll *a* and *b* are basically composed of porphyrin ring and phytol chain. One magnesium atom lies in the center of the porphyrin ring. De-esterification of chlorophyll *a* and *b* yields chlorophyllide *a* and *b* that can be catalyzed by chlorophyllase (Matile *et al.,* 1999) or by alkali hydrolysis (Humphrey, 1980); magnesium-dechelation yields magnesium-free compounds including pheophorbide and

pheophytin that are induced by metal-chelating substance during chlorophyll catabolism (Schelbert *et al.,* 2009), or acid or heat during food processing (Mínguez-Mosquera and Garrido-Fernández, 1989; Canjura and Schwartz, 1991; Turkmen *et al.,* 2006; Sanchez-Vega *et al.,* 2014); decarbomethoxylation at C-13^2 position yields pyro-derivatives in heated, canned, or storage food materials (Gallardo-Guerrero *et al.,* 2005; Gallardo-Guerrero *et al.,* 2008; Huang *et al.,* 2008; Loh *et al.,* 2012); finally, oxidation reactions promoted by a variety of oxidants produce 13^2-OH and 15^1-OH-lactone derivatives (Otsuki *et al.,* 1987; Yamauchi and Eguchi, 2002; Funamoto *et al.,* 2003; Roca *et al.,* 2007; Huang *et al.,* 2008; Kao *et al.,* 2011; Loh *et al.,* 2012). While related with the structure elucidation, until now, it is not available for an overall analysis of these derivatives by high resolution and mass accuracy measurements, with only chlorophyll *a* analyzed (Wei *et al.,* 2013), this suggests the need to carry out an overall investigation of structure elucidation of chlorophyll derivatives systematically, especially for 13^2-OH and 15^1-OH-lactone derivatives, since there is little information about their laboratory preparation of standard and fragmentation patterns but they are widely found in different tissues.



Scheme 2: Main chlorophyll transformations.

Due to the ingestion of green vegetables, fruits and seaweeds, chlorophyll pigments are also involved in human diet and related food manufacturing process. It has been found that chlorophyll pigments have many beneficial effects for human health such as the antimutagenic effect (Díaz *et al.,* 2003; Simonich *et al.,* 2007), antigeno-toxic properties (Negishi *et al.,* 1997), and potent antioxidant capacity to scavenge free radicals and to prevent lipid oxidation (Lanfer-Marquez *et al.,* 2005). Despite these, researches related with the uptake of chlorophyll pigments by digestive system are scarce. After the study by Egner *et al.* (2000) that copper chlorin e_4 and copper chlorin e_4 ethyl ester were accumulated in the serum of individuals participants in a clinical trial where they were supplemented with a diet of copper chlorophyllin (mixture of modified

water-soluble chlorophyll derivatives), a few of researches were carried out for the *in vitro* digestion and following Caco-2 cell absorption of chlorophyll pigments from spinach (Ferruzzi *et al.,* 2001), peas (Gallardo-Guerrero *et al.,* 2008) or of pure chlorophyll standards (Gandul-Rojas *et al.,* 2009). It is showed that many factors including chlorophyll molecule, food matrix, processing, etc are involved and it suggests the need to investigate for a wide range of chlorophyll derivatives from natural food resources.

In this manner, edible seaweeds are considered to be ideal research materials for the investigation of chlorophyll bioavailability from natural food supplies. Bioavailability refers to the fraction of any compound ingested and made available for utilization, metabolism, and/or storage by the organism (Ferruzzi and Blakeslee, 2007). In this sense, chlorophyll pigments from edible seaweeds will be investigated from their presence in food resources to their accumulation in human intestinal cells. The selection of edible seaweeds is not only because of the increasing popularity of this seafood in western countries for the health benefits (Shahidi, 2009), but also and more importantly, due to the fact that they can provide a diverse distribution of chlorophyll derivatives such as *c* series of chlorophyll in brown algae (Fujii *et al.,* 2012), large sum of dephytylated chlorophyll (Hwang *et al.,* 2005; Ferraces-Casais *et al.,* 2012), additionally to the plant materials, besides a very different food matrix.

The general mechanism of chlorophyll absorption proposed by Ferruzzi and Blakeslee (2007) would follow routes similar to those taken by other xenobiotic compounds that require consideration of (a) efficient release of the chlorophyll from the food matrix, (b) stability to gastric and small intestinal digestive conditions, (c) solubilization of lipophilic derivatives (micellarization), (d) uptake by small intestinal absorptive epithelial cells, and (e) secretion into circulation (basal transport). Except for the report of Ferruzzi *et al.* (2002) that studied the absorption and efflux behavior of chlorophyllin on polarized Caco-2 membrane, no information is available for transportation manner of natural chlorophyll. In addition, as some transporters involved in carotenoids intestinal transportation have been described including the scavenger

receptor class B type I (SR-BI), Niemann Pick C1 Like 1 protein (NPC1L1), cluster determinant 36 (CD36) and ATP-binding cassette transporter subfamily A (ABCA1) (During *et al.,* 2005; Reboul *et al.,* 2005, 2011; Moussa *et al.,* 2008), on the contrary, nothing has been reported related with chlorophyll derivatives. These investigations will be involved in this thesis.

This thesis begins with a detailed chapter reviewing and introducing chlorophyll pigments, structures, function and localization, biosynthesis and degradation, biological actions, methods of analysis and role as additives. Due to the gap in the knowledge, 32 chlorophyll derivative standards were laboratory prepared and analyzed with high resolution time-of-flight mass spectrometry including 13^2-OH, 15^1-OH-lactone and pyro derivatives in dephytylated and phytylated of *a* and *b* series of chlorophyll, proposing new fragmentation pathways and new reaction mechanisms. Such experience allowed to characterize the five major edible seaweeds, as the information related with the chlorophyll content was almost null although highly ingested in our days. Once characterized the chlorophyll profile in fresh macro algae, the following step was to analyze the influence of different cooking methods in such phytochemicals, comparing the different food matrix effect. Following, digestive and absorbable properties of chlorophyll pigments from edible seaweeds were carried out including *in vitro* digestion, micellarization process, following cell absorption and comparison between fresh and cooked food materials. Finally, to be able to obtain information about the mechanism of chlorophyll absorption and transportation pattern it was necessary to formulate micelles with natural chlorophyll pigments to subject to highly polarized Caco-2 membrane. Finally, it was possible to propose the initial bases of the chlorophyll human intestinal membrane transportation.

2. - OBJECTIVES

Due to its essential role in the photosynthesis, chlorophylls are the most abundant pigments in nature. In spite of the omnipresence of chlorophylls from algae to higher plants, and consequently daily part of our diet, there is scarce information related with the bioavailability of chlorophylls (Ferruzzi *et al.,* 2001; Ferruzzi *et al.,* 2002; Gallardo-Guerrero *et al.,* 2008; Gandul-Rojas *et al.,* 2009). On the contrary, great advances have been made in relation with the availability of carotenoids (Garrett *et al.,* 1999; van het Hof *et al.,* 2000; Kopsell and Kopsell, 2006; Maiani *et al.,* 2009) even with few potential transporter involved (During *et al.,* 2005; Reboul *et al.,* 2005; Moussa *et al.,* 2008; Reboul *et al.,* 2011), probably due to the higher stability of these pigments. In addition, important and prominent benefits to human health have been shown for dietary chlorophylls such as antimutagenic effect (Díaz *et al.,* 2003, Simonich *et al.,* 2007), antigenotoxic properties (Negishi *et al.,* 1997), and potent antioxidant capacity to scavenge free radicals and to prevent lipid oxidation (Lanfer-Márquez *et al.,* 2005). In view of the lack of knowledge, the main aim of the present thesis is to study the bioavailability of chlorophylls from a global point of view: including an adequate analysis of chlorophyll derivatives presents in foods, effect of food cooking in chlorophyll profile, studies of *in vitro* digestion and absorption, and finally first investigations of possible transporters. Specifically, the objectives of the research are:

1- To develop a deep and **detailed characterization of all the chlorophyll derivatives potentially present in food**s. This study will allow making a proper identification of the chlorophyll derivatives in whatever matrix.

2- Once edible seaweeds are selected as reference raw material for the present doctoral thesis, and due to the gap in the scientific bibliography, one of the objectives will be **to identify the chlorophylls derivatives that characterize each group of edible macro algae**.

3- Besides fresh fruits and vegetables, our diet is composed of processed food. There are studies of the influence of different processing techniques in the chlorophyll profile of raw food material. One of the typical characteristic of macro algae is the complexity of extracellular material. Consequently, it is essential to know **the effect that different cooking methodologies can induce in the chlorophyll profile of seaweeds**.

4- Although chlorophyll derivatives are highly daily ingested, there is a strong ignorance about the behavior of these compounds during the *in vitro* digestion. Consequently, one of the main objectives of the present thesis is **to perform a complete and detailed study of the *in vitro* digestive stability and micellarization of chlorophyll derivatives present in the main edible seaweeds, including the cooking effect.** The different extracellular material of the three seaweeds will allow also evaluating the influence of the food matrix during the digestion. And the previous total characterization by HPLC-MS of the chlorophyll profile in the three seaweeds will probably permit to study the behavior of chlorophyll derivatives not analyzed before.

5- If in relation with chlorophyll performance during *in vitro* digestion the knowledge is very limited, less is known about the absorption process and nothing about transporters. In consequence, the final objective of the present work is **to gain information about the intestinal absorption of different chlorophyll derivatives to characterize the process and try to identify if any transporter could be implicated.**

3. - ABSTRACT OF THE RESULTS

The present doctoral thesis focuses on the main group of natural pigments, the chlorophylls. Although years of investigation has provided a vast information about the chemistry and biochemistry of chlorophylls, the new trends in food science give directions to human health and nutritional properties of the different phytochemicals present in our daily diet. For chlorophylls pigments such area of investigation is relatively new and consequently the reason of the present research.

The first result of the thesis is a complete review of the main characteristics of the chlorophylls pigments. This chapter includes aspects as structures, function and localization, biosynthesis and degradation, biological actions, methods of analysis and roles as food additives.

To develop the thesis it was necessary first to develop specific methods to obtain the complete set of chlorophyll standards that can be present in processed, storage or ripened foods. The 32 chlorophyll derivatives were analyzed in depth by high resolution time-of-flight mass spectrometry and studied their behavior during the MS^2 fragmentation with powerful post-processing software. It is found that, while MS^2-based reactions of phytylated chlorophyll derivatives point to fragmentations at the phytyl and propionic chains, dephytylated chlorophyll derivatives behave different as the absence of phytyl makes β-keto ester group and E ring more prone to fragmentation. The introduction of an oxygenated function at E ring enhances the progress of fragmentation reactions through the β-keto ester group, developing also exclusive product ions for 13^2-hydroxy derivatives and for 15^1-hydroxy-lactone ones. Native chlorophyllides and pheophorbides mainly exhibit product ions that involve the fragmentation of D ring, as well as additional exclusive product ions. It is noteworthy that all *b* derivatives, except 15^1-hydroxy-lactone compounds, undergo specific CO losses. It has been proposed a new reaction mechanism based on the structural configuration of *a* and *b* chlorophyll derivatives that explains the exclusive CO

fragmentation. Proposals of the key reaction mechanisms underlying the origin of new product ions have been made.

The same methodological approach has been applied for the determination of chlorophyll profile in the five major edible seaweeds. Seven new chlorophyll epimers at $C13^2$ position including chlorophyll c_1', 13^2-OH pheophorbide *a'*, pheophorbide *a'*, 13^2-OH chlorophyll *b'*, 13^2-OH pheophytin *a',* pheophytin *a',* and 15^1-OH-lactone pheophorbide *a'* were identified for the first time in edible seaweeds and they show the same fragmentation pattern as their parent chlorophyll. In addition, eight new chlorophyll derivatives including 13^2-OH chlorophyllide c_2, chlorophyll c_2, chlorophyll c_1, chlorophyll c_1', pheophorbide *d,* purpurin-18 *a*, pheophytin *d* and phytyl-purpurin-18 *a* were identified for the first time in edible macro algae and it is found that additional unsaturated position at $C17^1$-$C17^2$ impedes α-cleavage reaction in MS^2 analysis of chlorophyll *c* and the double keto rearrangement at the E ring in phytol purpurin-18 *a* and purpurin-18 *a* easily displaces the ion charge from remote positions and allows the fragmentation of the keto-lactone group. Highly surprising was the discovery of chlorophyll *d* derivatives in macro seaweeds. Chlorophyll *d* have been scarcely characterized (only in specific cyanobacteria), and pheophorbide *d* has never been reported previously. Such findings allowed by first time the complete MS^2 characterization of *d* derivatives. Chlorophyll profile in edible seaweeds differs with species. Red algae (Nori) contain mainly *a* series of chlorophyll, mainly pheophorbides and pheophytins; green algae (Sea Lettuce and Aonori), *a* and *b* series, mainly chlorophyll *a* and *b*; brown algae (Kombu and Wakame), principally *a* and *c* series, mainly pheophytins.

The study of the cooking effects on the chlorophyll profile in edible seaweeds revealed that they are associated with the processing parameters, the structure of edible seaweeds and their respective chlorophyll molecules. Pheophytinization and decarboxymethylation at $C13^2$ are favored in cooking process. Pheophytinization degree is higher for *a* series than *b* series. Oxidation reactions occur mainly for chlorophyll *a* and *b*, not for pheophytins. Due to seaweed differences in extracellular composition and

different thermal sensitivity of chlorophyll structures, generally, cooking has no effect on Nori seaweeds, and induces similar chlorophyll losses during boiling or microwaving for Kombu seaweeds. On the contrary, for green seaweeds microwaving methods is softer than boiling.

After the characterization of the chlorophyll profile of seaweeds, fresh dried and cooked, the three main macro algae were submitted to an *in vitro* digestion process, including the oral, gastric and intestinal phases to evaluate the stability of chlorophylls. In summary, three principal types of reactions were prompted: (1) oxidation reactions to produce 13^2-OH and 15^1-OH-lactone derivatives; (2) pheophytinization reaction that favors *a* series than *b* and *c* series; (3) pheophorbidation reaction that occurs obviously only when the initial chlorophyll profile for digestion is mainly composed of pheophytins. Cooking does not introduce significant modifications of chlorophyll profiles during *in vitro* digestion.

Due to their polarity, chlorophyll compounds (as many other phytochemicals) requires the incorporation in micelles previous to the absorption by the intestinal cells. Consequently, the micellarization process is an estimation of which proportion of a compound after the digestion is theoretically ready for the enterocyte in the form of micelles. The efficiency of the compound transferred from the digesta to aqueous micellar fraction (AMF) is defined as the percentage of micellarization. The research concluded that dephytylated chlorophylls are easier micellarizated than phytylated ones. The analysis of seaweeds material allowed by first time comparing three series of chlorophylls, resulting that *a* and *c* series are favored than *b* series. Finally, the detailed analysis of chlorophyll derivatives determined that oxidized chlorophyll derivatives are favored than parent chlorophylls, especially for phytylated ones. The analysis of the cooking effects on digestive and micellarization properties of chlorophyll pigments from edible seaweeds, established that cooking improves the recovery of chlorophyll pigments in edible seaweeds, especially for Sea Lettuce and Kombu, but decreases the micellarization rate of chlorophyll pigments in Nori and Kombu with that of Sea Lettuce unchanged. To obtain a general view of a combination of all the process

analyzed in the thesis (cooking, *in vitro* digestion and micellarization) on the chlorophyll profile of seaweeds, an index of processing bioaccessibility has been proposed. It showed that in the respect of chlorophyll pigments, Nori is recommended to be consumed fresh dried; while Sea Lettuce and Kombu seaweeds are better to ingest microwaved.

Followed with the physiological process, aqueous micellar fractions of chlorophyll pigments from fresh dried and cooked seaweeds were combined with DMEM and subjected to Caco-2 cell absorption. After the incubation and consequent analysis, it was observed that dephytylated chlorophylls are better absorbed than phytylated chlorophylls and that oxidation reactions are promoted during the absorption process. Generally, cooking does not affect the cell absorption of chlorophyll derivatives.

Once demonstrated that chlorophylls are absorbed by human intestinal cells, it was necessary to investigate the absorption process. It was mandatory to set up the protocol to incorporate the required concentrations of chlorophyll derivatives into the micelles. Applying the methodology, pheophorbide and pheophytin *a*-rich micelles were formulated in an absorption assay with highly polarized Caco-2 membrane cells growing on transwell plates that can present physiological intestinal environment for nutrient absorption. Pheophorbide *a* has a higher ability of incorporation into mixed micelles and higher absorption rate compared with pheophytin *a*. At 37 ^{o}C, absorption rate of pheophorbide *a* increases with increasing concentration in the mixed micelles and saturated at higher concentrations implying a facilitated transportation involved. Meanwhile the absorption rate of pheophytin *a* seems to be linearly increasing with higher concentration. At 4^{o}C, absorption rate of pheophorbide *a* and pheophytin *a* increases totally linearly, although absorption rate of both pheophorbide *a* and pheophytin *a* is obviously higher at 37^{o}C than at 4^{o}C. In conclusion, it was shown that pheophorbide *a* is actively transported into the intestinal cell while for pheophytin *a,* the most likely mechanism implied in its cell absorption process should be a passive transport.

Finally, to characterize the complete chlorophyll intestinal absorption process, other delivery protocols were investigated. These experiments permit the evaluation of transportation characteristics related with apical and basolateral surface, directionality or even efflux. Results showed that pheophorbide *a* molecule can be transported across the Caco-2 cell membrane from the basolateral side to apical side but not for the opposite direction. At difference, pheophytin *a* cannot cross the cell membrane regardless of transport direction. Hence, absorption rate of pheophytin *a* was significantly lowered by reversing the transport direction, but for pheophorbide *a*, not so significantly. In low concentrations, absorption rate of pheophorbide *a* is even faster from basolateral to apical than from apical to basolateral. Efflux experiment revealed that the majority of absorbed pheophorbide *a* and pheophytin *a* are delivered back to apical side, and others are still in the cell monolayer. Nearly none were found in basolateral side.

To conclude the thesis, taking into account the absorption process of pheophorbide *a* is mediated by a carrier, attempts to identify such transporters were made. SR-BI and NPC1L1 transporters are two of the best characterized carriers implied in the carotenoid absorption process. Subsequently, experiments of antibodies and specific inhibitor treatment against SR-BI and NPC1L1 transporters found that SR-BI, but not NPC1L1 seems to be partially involved in the intestinal absorption of pheophorbide *a* by Caco-2 cells. It is the first time that a transporter of chlorophyll derivative is identified.

4.-CHAPTER

"Chlorophylls" by Roca M, Chen K, Pérez-Gálvez A.

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5.- PAPER

"Development of an accurate and high-throughput methodology for structural comprehension of chlorophylls derivatives. (I) Phytylated derivatives". Journal of Chromatography A 2015; 1406: 99-108.

Chen K, Ríos JJ, Pérez-Gálvez A, Roca M.

6. - PAPER

"Development of an accurate and high-throughput methodology for structural comprehension of chlorophylls derivatives. (II) Dephytylated derivatives". Journal of Chromatography A 2015; 1412:90-9.

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7 SPECTROSCOPIC STUDIES OF CHLOROPHYLL DERIVATIVES IN EDIBLE SEAWEEDS

8.- *IN VITRO* DIGESTION AND MICELLARIZATION OF CHLOROPHYLL DERIVATIVES FROM DIFFERENT PREPARATIONS OF SEAWEEDS

9 CACO-2 CELL ABSORPTION OF CHLOROPHYLL DERIVATIVES

10.- DISCUSSION

Chlorophyll pigments are the most widely distributed natural pigment in the world, present in different kingdoms from protists to vegetal. In fact, our daily ingest of chlorophylls is higher than carotenoids, a class of pigments from which a lot of investigations have been done. Apart from other beneficial properties related with human health, information about their bioavailability or bioaccessibility and following transportation is limited. In this thesis, investigations were carried out associated with the structure elucidation of chlorophyll and their derivatives, characterization of the chlorophyll profile of the main edible macroalgae, bioaccessibility of chlorophyll pigments from food origin (seaweeds), cell absorption and transportation characterization of chlorophyll derivatives. In this sense, a clear picture of chlorophyll derivatives from natural occurrence to absorption and transportation by human cell membrane is obtained.

As an introductory section, a detailed chapter of chlorophyll pigments is accomplished to give information about their structures and presence in the food resources, transformations during biosynthetic and catabolic process and food processing, analysis methodology including extraction and multiple identification (UV-visible, fluorescence, mass and nuclear magnetic resonance) and their role as food coloring additives.

The lack of a complete structure elucidation of chlorophyll derivatives including 13^2-OH, 15^1-OH-lactone and pyro chlorophyll derivatives in both phytylated and dephytylated version made necessary to carry out the research of MS^n analysis of chlorophyll derivatives. Actually, in early 1990s, Schwartz research group (van Breemen *et al.,* 1991) had analyzed the main fragments ions after MS^2 analysis of the most common natural chlorophyll derivatives including chlorophyll *a* (*b*), pheophytin *a* (*b*), chlorophyllide *a* (*b*), pheophorbide *a* (*b*) and pyropheophytin *a* (*b*) with FAB mass spectrometry. While for the other 22 chlorophyll derivatives, including 13^2-OH and 15^1-OH-lactone derivatives have been frequently reported recently in natural food resources (Otsuki *et al.,* 1987; Yamauchi *et al.,* 2002; Funamoto *et al.,* 2003; Roca *et al.,* 2007; Huang *et al.,* 2008; Kao *et al.,* 2011; Loh *et al.,* 2012;) and pyro derivatives (mainly pyropheophorbide *a*) have been intensively studied in the development of photodynamic therapy (Stamati *et al.,* 2010; Zhou *et al.,* 2012), although without a proper characterization. On the contrary, there is no standard method for the laboratory

preparation of these new derivatives. In the other hand, mass techniques progress positively that allow high resolution and mass measurements available that can help to identify fragmentation ions more accurately and the powerful post-processing software allows to predict structural arrangement of new product ions not described previously. In this section, an overall method was provided for the laboratory preparations of chlorophyll standards including phytylated and dephytylated, *a* and *b* series, 13^2-OH, 15^1-OH-lactone and pyro chlorophyll derivatives. In total, 32 chlorophyll derivatives were prepared in laboratory condition. Among them, methods for 13^2-OH, 15^1-OH-lactone and pyro derivatives are newly set with different parameters (temperature, time, etc). It was found that the presence of magnesium in the central position of the cyclic tetrapyrrol structure influenced the preparation procedures of chlorophyll derivatives. Related MS^n analysis of chlorophyll derivatives, some fragmentation patterns are consistent with previous studies. As well, new fragmentation ions and patterns are found especially when additional group of 13^2-OH and 15^1-OH-lactone are introduced to the tetrapyrrol structure. By comparing fragmentation pattern of *a* and *b* series and their derivatives systematically, it was shown that in contrary to what is supposed up to now, the exclusive CO fragmentation for *b* series have the cleavage site at $C13^1$. While phytylated chlorophyll derivatives yield product ions from phytyl and propionic chains fragmentation, dephytylated chlorophyll derivatives show different reaction sites for MS^2-based reactions, as the absence of phytol makes β-keto ester group and E ring more prone to fragmentation. The introduction of functional groups at the isocyclic ring (13^2-OH, 15^1-OH-lactone and pyro rearrangement) of the chlorophyll molecule implies exclusive product ions, enhancing the progress of fragmentation reactions through the β-keto ester group. In addition, proposals of the key reaction mechanisms underlying the origin of new product ions have been made. All of these results suggest it is essential to make this thoughtful analysis of all the chlorophyll derivatives.

It is surprising that only a few of publications dealt with the bioavailability of chlorophyll compound, although they have been considered with plenty of healthy benefits and commonly taken by human beings along with green fruits and vegetables. Especially for natural chlorophyll pigments, only two reports have been found so far (Ferruzzi *et al.,* 2001; Gallardo-Guerrero *et al.,* 2008). In this manner, a comprehensive research of digestive property and following cell absorption of natural chlorophyll pigments from edible seaweeds were carried out. The reason why seaweeds were selected is due to its high content and fertile profile of chlorophyll derivatives and also

due to the emerging popularity in daily consumption for other nutrient advantages. For the enzymatic and chemical sensibility of chlorophyll compounds, effects of cooking (boiling and microwaving) were also evaluated for the bioaccessibility and cell absorption process.

Firstly, chlorophyll profiles of five edible seaweeds (Nori, Sea Lettuce, Aonori, Wakame and Kombu) with higher consumption were characterized by MS^2. A difference with microalgae, whose detailed composition is known (Garrido and Zapata, 1993; Garrido *et al.,* 2000; Zapata *et al.,* 2004), the "recalcitrant" extracellular material of macroalgae has made difficult its complete characterization, and chlorophyll composition is not an exception. Consequently, in section 7, a suitable extraction method was developed for an efficient and fast extraction of chlorophyll pigments from macro algae and notably, 8 new chlorophyll derivatives were identified from five edible seaweeds along with their MS^2 fragmentation patterns. Generally, related with chlorophyll profile, Nori, the red algae, presents around 40% of pheophorbide type and 60% of pheophytin type; Sea Lettuce and Aonori belonging to green algae, are very similar with the majority of the chlorophyll pigments being chlorophyll *a* and *b*; both Wakame and Kombu belonging to brown algae have *a* and *c* series of chlorophyll pigments. Noteworthy is the discovery by first time in brown and red seaweeds pheophorbide *d*. For a proper identification, the cyanobacteria *Acariochloris marina* was grown, as the only living organism with more than 90% of the chlorophyll profile being chlorophyll *d*. Extracted chlorophyll *d* was transformed in pheophorbide and pheophytin *d*, and used as standard to confirm the presence of such derivatives in brown and red algae. It is important to highlight that it is first time that pheophorbide *d* is described in a living organism and the complete characterization is made.

When chlorophyll pigments from edible seaweeds experience the cooking, *in vitro* digestion and sub sequential cell absorption, the chlorophyll profile is greatly modified depending on different seaweeds. Meanwhile, each step means different reactions co-concurred. Cooking implies magnesium-dechelation of chlorophyll, the yield of pyro chlorophyll derivatives, oxidation of chlorophyll *a* and *b* and total degradation of chlorophyll pigments. But depending on the different extracellular structure and the chlorophyll profile, the impact of the reactions is different. While for Nori seaweeds the cooking had no effect, for Sea Lettuce boiling and microwaving implied degradation of chlorophylls compounds at different level. At difference, Kombu cooked seaweeds also suffer net degradation of chlorophylls but at the same level for boiling and

microwaving.

The *in vitro* digestion implies magnesium-dechelation of chlorophylls, de-esterification of pheophytins, oxidation and total degradation of pigments. And finally, when the acquired AMF is subjected to cell absorption, chlorophyll pigments also experience an extra oxidation process. It can be seen that oxidation reactions occur in every step so that in the final chlorophyll profile found in Caco-2 cells, almost more than a half of chlorophyll pigments are oxidized (table 11), although this is also partly due to the oxidized pigment is better micellarizated than the parent pigment.

Edible seaweeds are good resource for the cell uptake research of chlorophyll derivatives for the reason that they not only provide different series of chlorophyll but also show a wide range of derivative distribution, from hydrophilic dephytylated derivatives (pheophorbide *a*, *c* series) to hydrophobic phytylated one (pheophytin and chlorophyll). It is the first time to study *c* series in the *in vitro* digestion and cell absorption and they are accumulated in Caco-2 cells. Comparison of different series of chlorophyll reveals that *a* and *c* series still stand out as the more bioavailable than *b* series as the ratio between *a* and *b* series is enhanced from 2.97 (table 5) in fresh Sea Lettuce to 7.07 (table 11) in Caco-2 cells and that for *a* and *c* series remains similar. This is related with the molecular structure in each series. Chlorophyll derivatives found for each series in current work are, pheophorbide *a* type, chlorophyll *a* type and pheophytin *a* type for *a* series; chlorophyll *b* type and pheophytin *b* type for *b* series; chlorophyll *c* and pheophorbide *c* type for *c* series. Both *a* and *c* series contain dephytylated chlorophyll, but do not *b* series. It has been also proved by others that dephytylated chlorophyll is easier micellarizated and absorbed than phytylated chlorophyll (Gallardo-Guerrero *et al.,* 2008; Gandul-Rojas *et al.,* 2009). This pattern is well reflected by ratio changes of dephytylated and phytylated chlorophyll from fresh dried materials to the final cell accumulation. Table 15 is a summary calculation of the ratio of dephytylated and phytylated chlorophyll in each step related with chlorophyll digestion and uptake from edible seaweeds. From this table, it can be clearly observed that dephytylated chlorophyll is much more favored than phytylated chlorophyll. The ratio enhancement of Nori and Kombu from fresh dried material to digesta is due to the de-esterification of pheophytin *a* during digestion process, while not for Sea Lettuce.

Table 15: Ratio changes of dephytylated and phytylated chlorophyll derivatives in the *in vitro* digestion and cell absorption process of fresh dried seaweeds.

	Ratios of dephytylated and phytylated chlorophyll derivatives			
Seaweeds	Fresh dried material	Digesta	Aquatic micellar fractions	Accumulated in cells
Nori	0,63±0,11	1,11±0,10	3,15±0,41	10,06±1,71
Sea Lettuce	0,08±0,00	0,07±0,01	0,39±0,04	0,99±0,28
Kombu	0,28±0,00	0,66±0,23	1,17±0,09	1,62±0,13

The fresh dried material (Nori, Sea Lettuce and Kombu) were subjected to an *in vitro* digestion to form digesta, then aquatic micellar fraction was obtained by ultracentrifuge, diluted, and mixed with DMEM for the cell absorption. Finally, chlorophyll pigments were found to be accumulated in cells.

Cooking effect is evaluated for determine whether it improves the bioaccessibility and cell uptake of chlorophyll pigments and it has been found no influence when the micellar chlorophyll pigments are supplied to cell absorption. But it does affect the *in vitro* digestion and following micellar fractions. Generally, cooking can enhance the recovery rate of chlorophyll derivatives indicating more pigments were ready for the micellarization process. Nevertheless, cooking decreases the micellarization rate for Nori and Kombu, not for Sea Lettuce. Consistently, micellarization process of hydrophilic pheophorbide is depressed by cooking. In this sense, the processing bioaccessibility which combines cooking effects on the digestive materials, recovery rate and micellarization process together gives specific information about the amounts of chlorophyll that are ready for the enterocytes absorption, and results show the effect of cooking needs to be treated differently with different seaweeds.

According to the present investigations and previous study, it has been known that chlorophyll pigments can be absorbed by human cell lines (Ferruzzi *et al.,* 2001; Gallardo-Guerrero *et al.,* 2008; Gandul-Rojas *et al.,* 2009), while none was known related their absorption process, circulation and potential involved transporter, etc. Due to this, Caco-2 cells were grown on transwell plates to differentiate into polarized absorptive cell monolayers for the membrane transportation study of chlorophyll pigments. When Caco-2 membrane were subjected with mixed micelles of pheophorbide *a* or pheophytin *a*, it is showed that pheophorbide *a* involves a facilitated absorption in the tested ranges while for pheophytin *a*, totally passive absorption. The additional phytol chain affects molecular properties such as polarity, molecular size and solubility in micelles, thus resulted in different behavior of pheophorbide *a* and

pheophytin *a* not only in the micellarization process but also cell absorption. Their differences are also reflected in the directionality experiment. It is surprisingly found that pheophorbide *a* is effectively transported from basolateral side to the apical side compared with the opposite, especially in lower concentrations. For pheophytin *a*, the absorption rate from basolateral side to apical is much lower than that from apical side to basolateral. The efflux experiments showed that after 24 h, over a half of the absorbed pheophorbide *a* and pheophytin *a* are secreted to the apical side with only a very small amount of pheophorbide *a* found in the basolateral side, and more will be effluxed to apical with incubation time prolonged. With the available data and references, it is very difficult to speculate the potential transportation mechanisms for pheophorbide *a* and pheophytin *a*. Only references from related phytochemicals such as carotenoids and lipid vitamins give information about transporter involved (Reboul and Borel, 2011). Thus antibody of SR-BI and its inhibitor plus antibody of NPC1L1were tested with the absorption experiment of pheophorbide *a* due to the facilitated transportation found in the present work. Finally, it seems SR-BI is involved in the apical uptake of pheophorbide *a* absorption by Caco-2 membrane.

11. - CONCLUSSIONS

1. The central coordination of the macrocycle with magnesium (chlorophylls) or with hydrogen atoms (pheophytins) affects the preparation of 13^2-OH, 15^1-OH-lactone and pyro chlorophyll derivatives
2. In phytylated chlorophylls, the fragmentations mainly correspond to the phytyl chain and progressive breakdowns from the propionic chain at C17 (excluding the pyro-derivatives).While for dephytylated chlorophylls, the absence of phytol makes β-ketoester group and E ring more prone to fragmentation.
3. The introduction of functional groups at the isocyclic ring (13^2-OH, 15^1-OH-lactone and pyro rearrangement) of the chlorophyll molecule enhances the progress of fragmentation reactions through the β-keto ester group, developing exclusive product ions..
4. Contrary to the present thoughts, the cleavage site for the exclusive CO fragmentation in *b* series is at $C13^1$.
5. Chlorophyll epimers at $C13^2$ position show the same fragmentation pattern as their parent chlorophyll.
6. Eight new chlorophyll derivatives have been identified by first time in the five major seaweeds, and a new chlorophyll pigment, pheophorbide *d,* have been elucidated structurally and identified in living organisms.
7. The analysis of the fragments ions after MS^2 analysis by first time of chlorophyll *c* derivatives has demonstrated that unsaturations in the propionic unit at C17 reduce the McLafferty rearrangements and in consequence impedes the fragmentation at this position.
8. The analysis by first time of the fragmentation products of different purpurin-18 *a* derivatives has shown that the existence of alternative reactions affecting the V ring indicates that the double keto rearrangement at the E ring easily displaces the ion charge from remote positions and allows the fragmentation of the keto-lactone group.
9. The main parameter that determines the cooking effects on the chlorophyll profile in edible seaweeds is associated with the structure of edible seaweeds. Generally, cooking has no effect on Nori seaweeds, induces similar chlorophyll losses during boiling or microwaving for Kombu seaweeds and on the contrary, for green

seaweeds microwaving methods is softer than boiling.

10. The main reactions that cooking induces in the chlorophyll profile of seaweeds are: pheophytinization (higher for *a* series than *b* series), decarboxymethylation at $C13^2$ and oxidation reactions (mainly for chlorophyll *a* and *b*, but not for pheophytins).
11. During *in vitro* digestion, three types of reactions were prompted: (1) oxidation reactions to produce 13^2-OH and 15^1-OH-lactone derivatives; (2) pheophytinization reaction that favors *a* series than *b* and *c* series; (3) pheophorbidation reaction that occurs obviously only when the initial chlorophyll profile for digestion is mainly composed of pheophytins. Cooking does not introduce significant modifications into *in vitro* digestion.
12. The recovery during *in vitro* digestion and the micellarization of the chlorophyll pigments are directly affected by the extracellular matrix of the different seaweeds.
13. During micellarization process, dephytylated chlorophylls are easier micellarizated than phytylated ones; *a* and *c* series are favored than *b* series and oxidized chlorophyll derivatives are highly favored than parent chlorophyll, probably by their higher polarity.
14. Cooking improves the recovery of chlorophyll pigments in edible seaweeds, especially for Sea Lettuce and Kombu, but decreases the micellarization rate of chlorophyll pigments in Nori and Kombu with that of Sea Lettuce unchanged. Taking into account all the process studied, which is represented by the processing bioaccessibility index, in the respect of chlorophyll pigments, Nori is recommended to be consumed fresh dried; while both Sea Lettuce and Kombu, microwaved.
15. The absorption process supposes that pheophorbides are better transported than pheophytins, due to its high polarity, and an increase in the oxidation reactions occurs for chlorophyll derivatives. Generally, cooking does not affect the cell absorption of chlorophyll derivatives from edible seaweeds.
16. Pheophorbide *a* is absorbed in Caco-2 cells by a facilitated transportation, while pheophytin *a* is transported by a passive diffusion.
17. In the assay of transportation directionality, pheophytin *a* is inefficiently absorbed from basolateral side to apical side compared with from apical side to basolateral side. While for pheophorbide *a*, it can be delivered efficiently from basolateral side to apical side especially in low concentrations. Meanwhile, pheophorbide *a* can be transported across the membrane efficiently only from basolateral side to apical side.

18. In the efflux experiment, pheophorbide *a* and pheophytin *a* show similar pattern that the majority are effluxed to apical side with traces of pheophorbide *a* and none of pheophytin *a* found in the basolateral side.
19. It seems SR-BI is involved in the apical absorption of pheophorbide *a.*

12. - ANNEXES

Annex 1. - Retention time, elemental composition and exact mass of all the chlorophyll derivatives analyzed in the thesis.

Annex 2. - Chlorophyll structures of all derivatives analyzed in the thesis

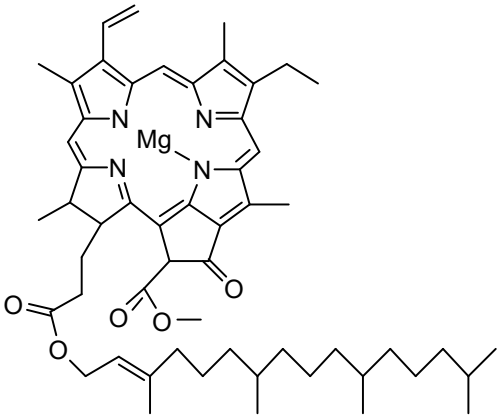
Annex 1. - Retention time, elemental composition and exact mass of all the chlorophyll derivatives analyzed in the thesis.

	Compound	t_R (min)	Molecular formula	$[M+H]^+$ (*m/z*) MW. calc.
	15^1-Hydroxy-lactone chlorophyllide *b*	2.40	$C_{35}H_{32}MgN_4O_8$	661.2143
	13^2-Hydroxy-chlorophyllide *b*	3.10	$C_{35}H_{32}MgN_4O_7$	645.2194
	15^1-Hydroxy-lactone-chlorophyllide *a*	4.00	$C_{35}H_{34}MgN_4O_7$	647.2351
	Pyro-chlorophyllide *b*	4.10	$C_{33}H_{30}MgN_4O_4$	571.2190
	15^1-Hydroxy-lactone pheophorbide *b*	4.30	$C_{35}H_{34}N_4O_8$	639.2449
1	13^2-Hydroxy-chlorophyllide *a*	4.77	$C_{35}H_{34}MgN_4O_6$	631.2402
2	13^2-Hydroxy-chlorophyll c_2	5.00	$C_{35}H_{28}MgN_4O_6$	625.1932
3	Chlorophyll c_2	5.10	$C_{35}H_{28}MgN_4O_5$	609.1982
4	Chlorophyllide *a*	5.18	$C_{35}H_{34}MgN_4O_5$	615.2452
	13^2-Hydroxy- pheophorbide *b*	5.20	$C_{35}H_{34}N_4O_7$	623.2500
	Chlorophyllide *b*	5.30	$C_{35}H_{32}MgN_4O_6$	629.2245
5	15^1-Hydroxy-lactone pheophorbide *a*	5.55	$C_{35}H_{36}N_4O_7$	625.2657
6	Chlorophyll c_1	6.00	$C_{35}H_{30}MgN_4O_5$	611.2139
6a	Chlorophyll c_1'	6.20	$C_{35}H_{30}MgN_4O_5$	611.2139
5a	15^1-Hydroxy-lactone pheophorbide *a*'	6.30	$C_{35}H_{36}N_4O_7$	625.2657
	Pheophorbide *b*	6.50	$C_{35}H_{34}N_4O_6$	607.2557
7	Pheophorbide *d*	6.50	$C_{34}H_{34}N_4O_6$	595.2551
	Pyro-chlorophyllide *a*	6.80	$C_{33}H_{32}MgN_4O_3$	557.2398
8	13^2-Hydroxy-pheophorbide *a*	6.83	$C_{35}H_{36}N_4O_6$	609.2708
	Pyro- pheophorbide *b*	7.70	$C_{33}H_{32}N_4O_4$	549.2496
8a	13^2-Hydroxy-pheophorbide *a*'	7.71	$C_{35}H_{36}N_4O_6$	609.2708
9	Pheophorbide *a*	7.90	$C_{35}H_{36}N_4O_5$	593.2759
9a	Pheophorbide *a*'	8.70	$C_{35}H_{36}N_4O_5$	593.2759
10	Pyro-pheophorbide *a*	9.34	$C_{33}H_{34}N_4O_3$	535.2704
11	Purpurin-18 *a*	9.70	$C_{33}H_{32}N_4O_5$	565.2446

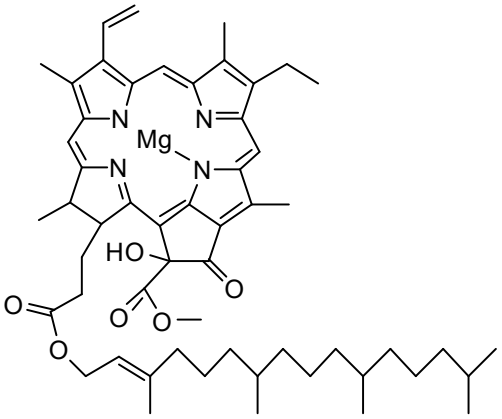
	Compound	t_R (min)	Molecular formula	$[M+H]^+$ (*m/z*) MW. calc.
12	15^1-Hydroxy-lactone chlorophyll *b*	15.1/13.9	$C_{55}H_{70}MgN_4O_8$	939.5117
13	13^2-Hydroxy-chlorophyll *b*	16.3/15.6	$C_{55}H_{70}MgN_4O_7$	923.5168
13a	13^2-Hydroxy-chlorophyll *b'*	16.70	$C_{55}H_{70}MgN_4O_7$	923.5168
14	15^1-Hydroxy-lactone chlorophyll *a*	17.7/16.5	$C_{55}H_{72}MgN_4O_7$	925.5324
15	Chlorophyll *b*	17.8/17.1	$C_{55}H_{70}MgN_4O_6$	907.5219
15a	Chlorophyll *b'*	18.50	$C_{55}H_{70}MgN_4O_6$	907.5219
16	13^2-Hydroxy-chlorophyll *a*	18.6/18.0	$C_{55}H_{72}MgN_4O_6$	909.5375
	Pyro-chlorophyll *b*	18.6	$C_{53}H_{68}MgN_4O_4$	849.5164
	Pyro-chlorophyll *a*	21.00	$C_{53}H_{70}MgN_4O_3$	835.5371
17	Chlorophyll *a*	19.4/21.0	$C_{55}H_{72}MgN_4O_5$	893.5426
17a	Chlorophyll *a'*	20.20	$C_{55}H_{72}MgN_4O_5$	893.5426
	13^2-Hydroxy-pheophytin *b*	19.20	$C_{55}H_{72}N_4O_7$	901.5474
	15^1-Hydroxy-lactone pheophytin *b*	19.60	$C_{55}H_{72}N_4O_8$	917.5423
18	15^1-Hydroxy-lactone-pheophytin *a*	21.3/24.2	$C_{55}H_{74}N_4O_7$	903.5630
19	13^2-Hydroxy-pheophytin *a*	23.2/26.5	$C_{55}H_{74}N_4O_6$	887.5681
19a	13^2-Hydroxy-pheophytin *a'*	23.80	$C_{55}H_{74}N_4O_6$	887.5681
21	Pheophytin *a*	25.2/27.5	$C_{55}H_{74}N_4O_5$	871.5732
21a	Pheophytin *a'*	25.70	$C_{55}H_{74}N_4O_5$	871.5732
	Pheophytin *b*	25.70	$C_{55}H_{72}N_4O_6$	885.5525
	Pyro-pheophytin *b*	28.40	$C_{53}H_{70}N_4O_4$	827.5470
20	Pheophytin *d*	24.40	$C_{54}H_{72}N_4O_6$	873.5525
22	Phytyl-purpurin-18 *a*	27.60	$C_{53}H_{70}N_4O_5$	843.5419
23	Pyro-pheophytin *a*	27.8/31.5	$C_{53}H_{72}N_4O_3$	813.5677

Annex 2. - Chlorophyll structures of all derivatives analyzed in the thesis

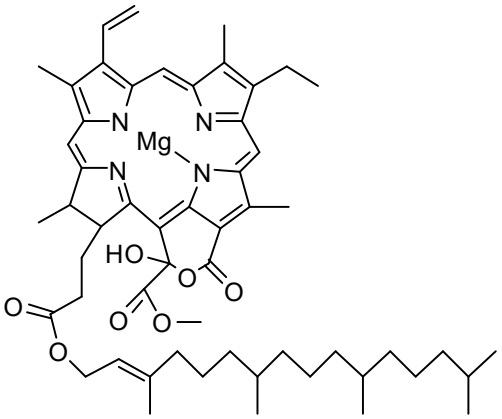
Chlorophyll *a* derivatives



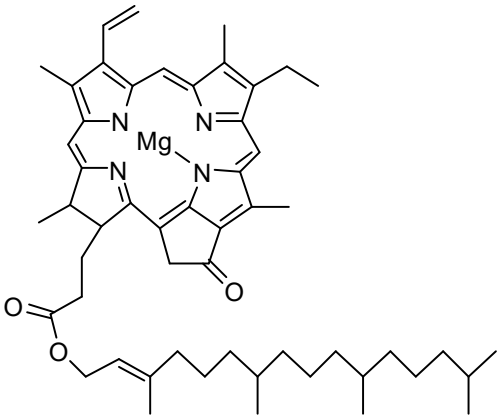
Chlorophyll *a*



13^2 OH-chlorophyll *a*



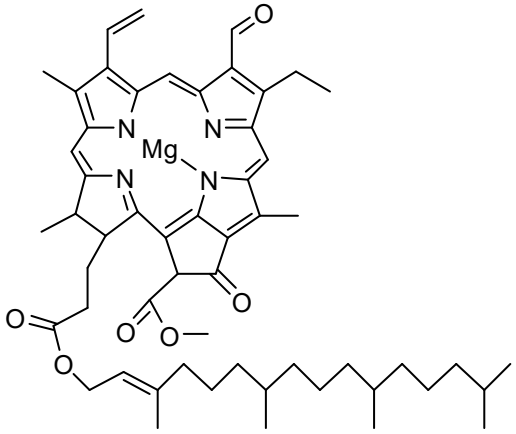
15^1 OH-lactone-chlorophyll *a*



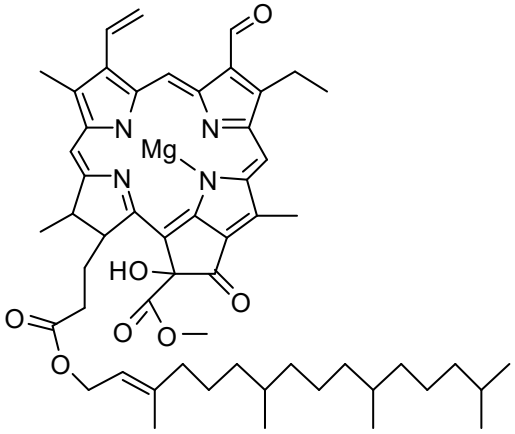
Pyro-chlorophyll *a*

Annex 2.1

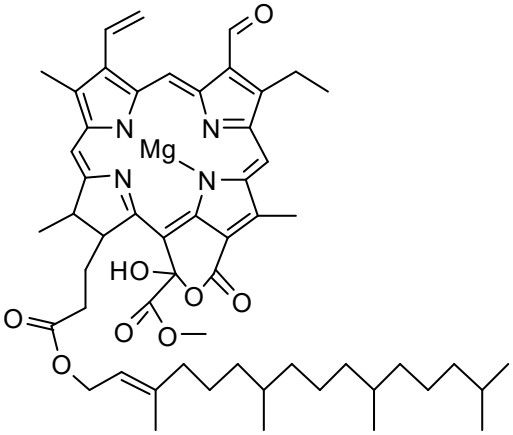
Chlorophyll *b* derivatives



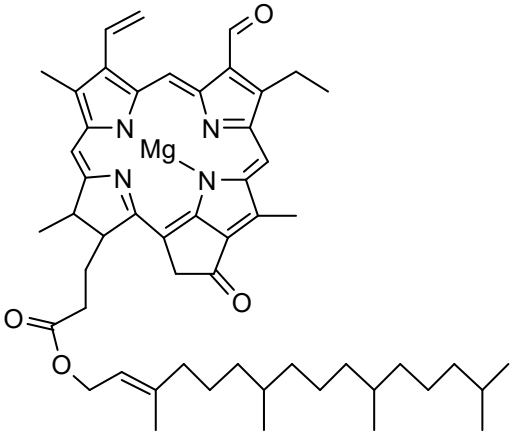
Chlorophyll *b*



13^2 OH-chlorophyll *b*



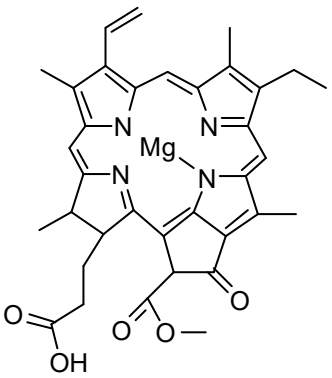
15^1 OH-lactone-chlorophyll *b*



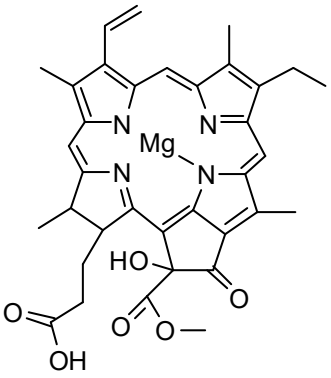
Pyro-chlorophyll *b*

Annex 2.2

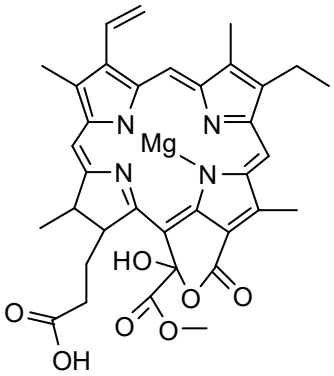
Chlorophyllide *a* derivatives



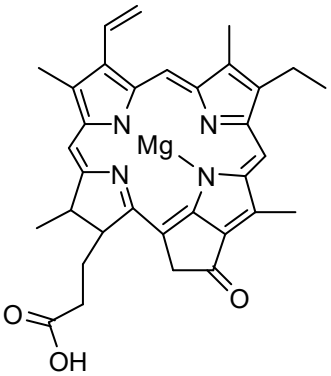
Chlorophyllide *a*



13^2 OH-chlorophyll a



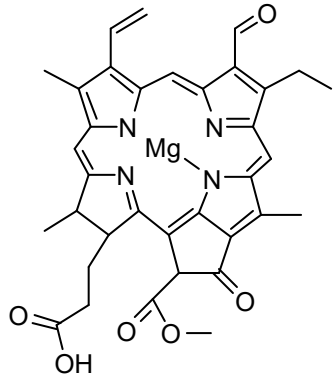
15^1 OH-lactone-chlorophyll *a*



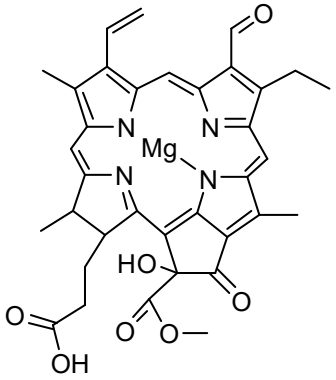
Pyro-chlorophyll a

Annex 2.3

Chlorophyllide *b* derivatives



Chlorophyllide *b*



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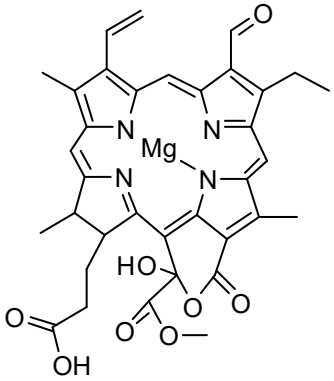

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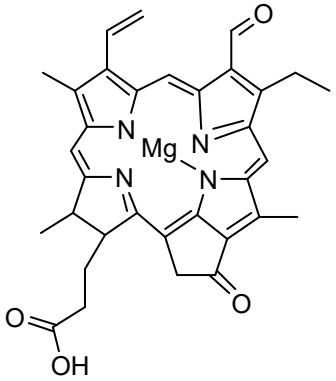
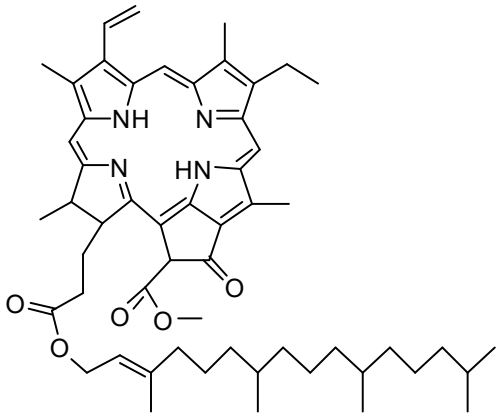


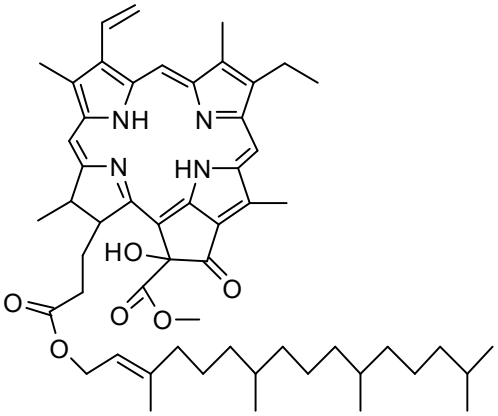
Pyro-chlorophyllide *b*

Annex 2.4

Pheophytin *a* derivatives



Pheophytin *a*



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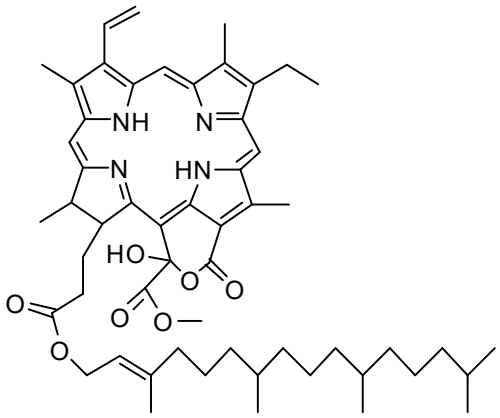

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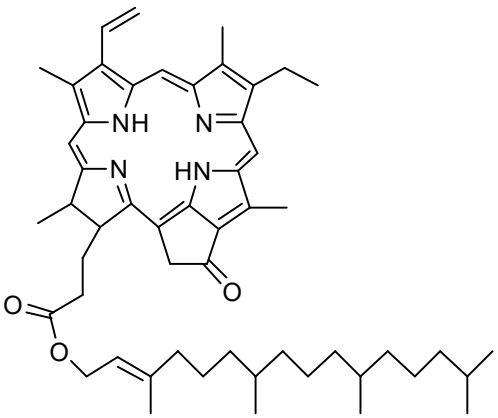
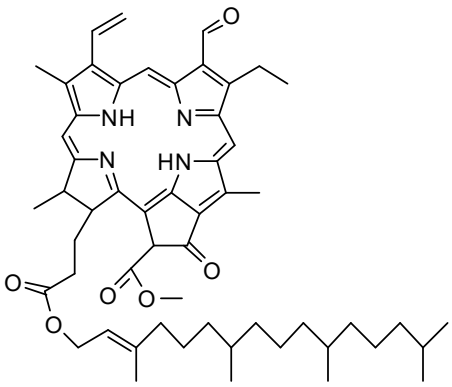


Pyro-pheophytin *a*

Annex 2.5

Pheophytin *b* derivatives



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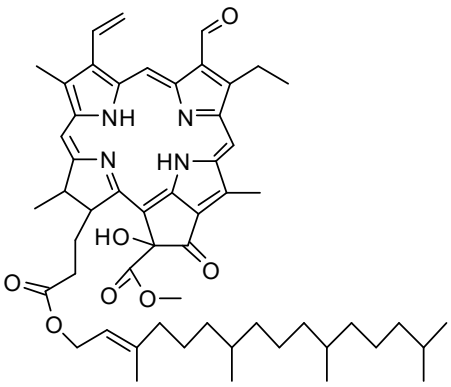

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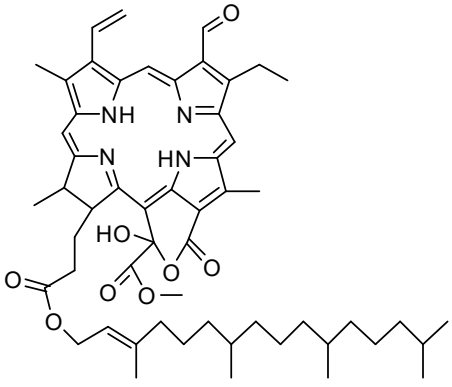

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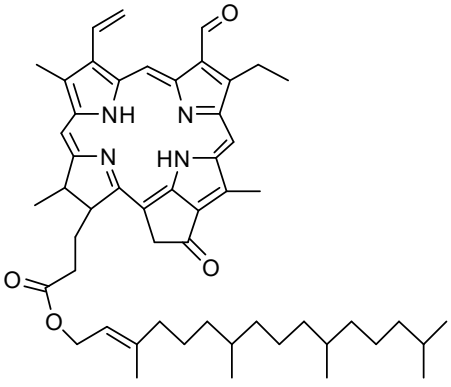
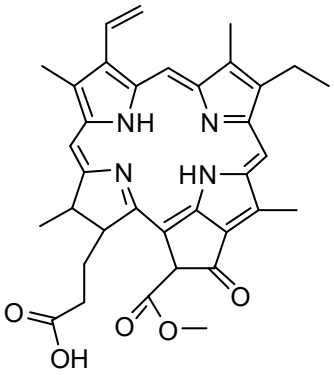


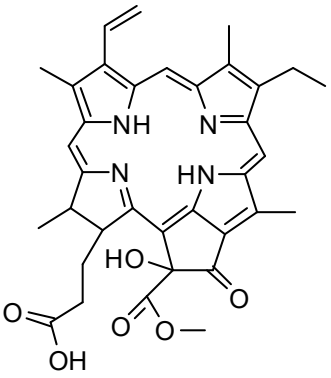
Pyro-pheophytin *b*

Annex 2.6

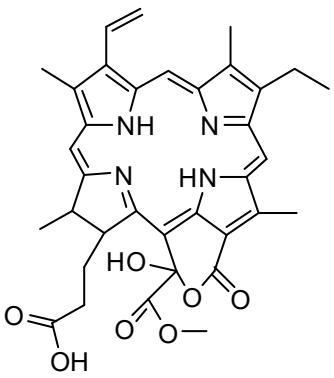
Pheophorbide *a* derivatives



Pheophorbide *a*



13^2 OH-chlorophyll pheophorbide *a*



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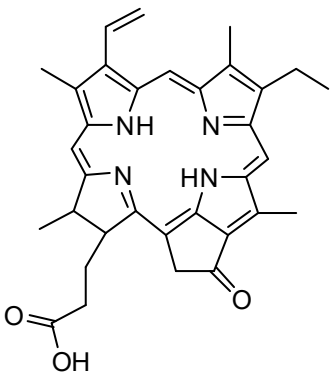
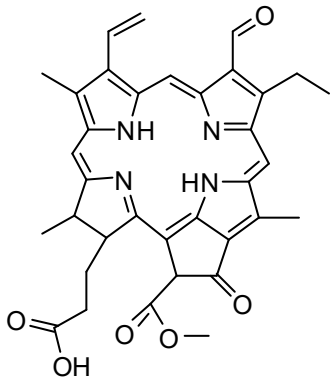


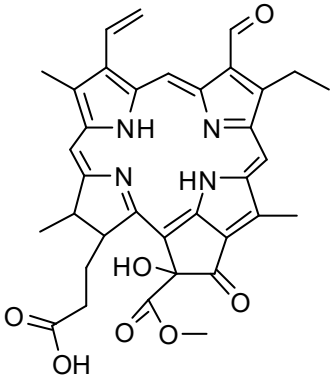
Pyro-pheophorbide *a*

Annex 2.7

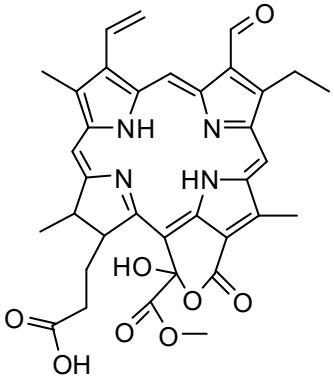
Pheophorbide *b* derivatives



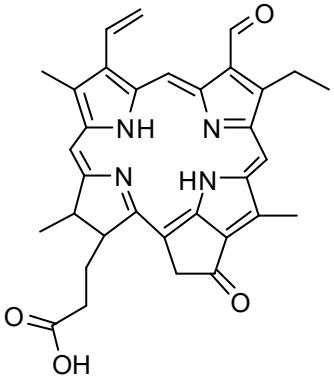
Pheophorbide *b*



13^2 OH-chlorophyll pheophorbide *b*



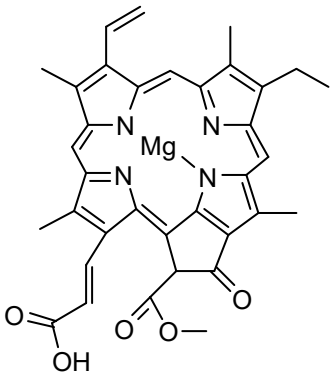
15^1 OH-lactone-pheophorbide *b*



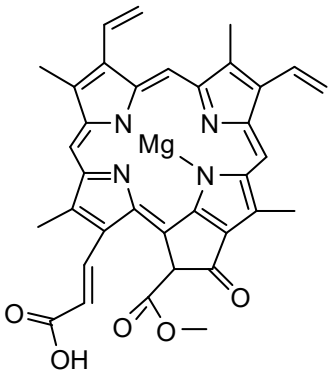
Pyro-pheophorbide *b*

Annex 2.8

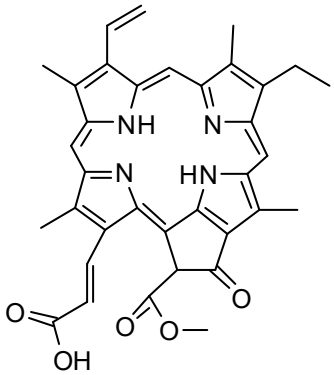
Serie *c derivatives*



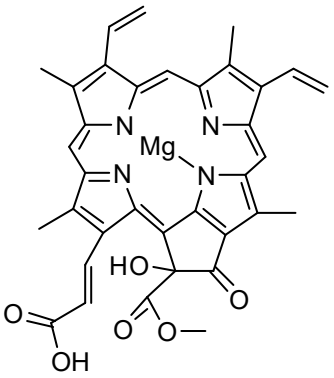
Chlorophyll c_1



Chlorophyll c_2



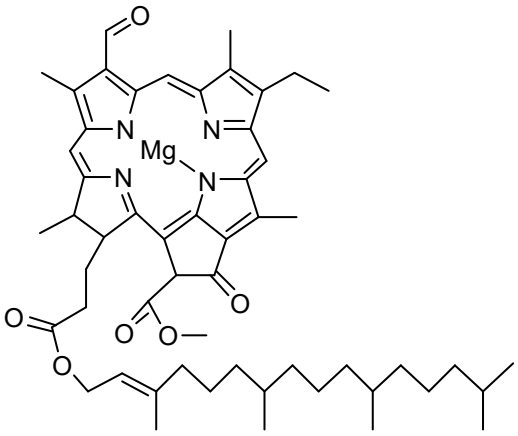
Pheophorbide c_1



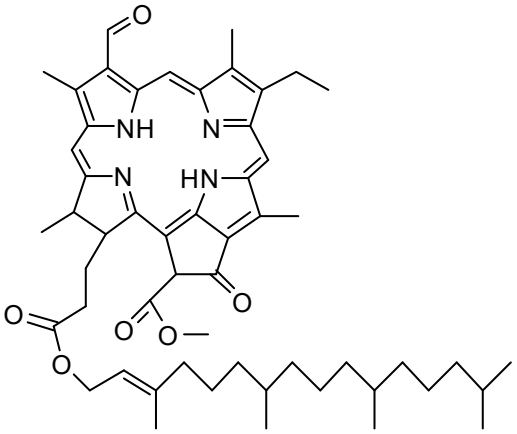
13^2 OH-chlorophyll c_2

Annex 2.9

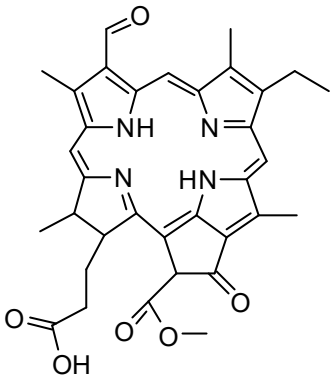
Serie *d* derivatives



Chlorophyll *d*



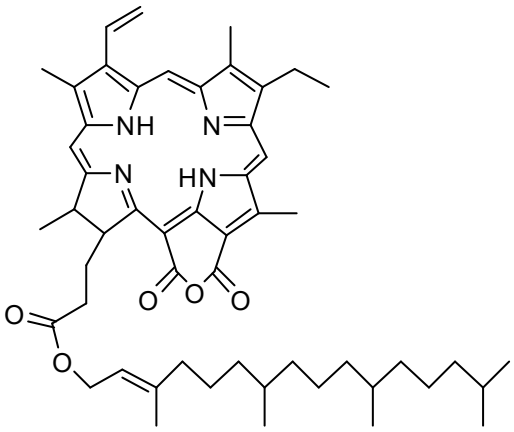
Pheophytin *d*



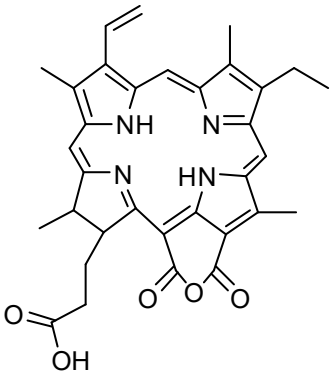
Pheophorbide *d*

Annex 2.10

Purpurin derivatives



Phytyl-purpurin-18 *a*



Purpurin-18 *a*

Annex 2.11

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