

# Photochemical & Photobiological Sciences

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## PERSPECTIVE

## How green is green chemistry? Chlorophylls as a bioresource from biorefineries and their commercial potential in medicine and photovoltaics

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As the world strives to create a more sustainable environment, green chemistry has come to the fore in attempts to minimize the use of hazardous materials and shift the focus towards renewable sources. Chlorophylls, being the definitive “green” chemical are rarely used for such purposes and this article focuses on the exploitation of this natural resource, the current applications of chlorophylls and their derivatives whilst also providing a perspective on the commercial potential of large-scale isolation of these pigments from biomass for energy and medicinal applications.

### Introduction

#### I wonder why the grass is green ...

... thus begins a child's curiosity about nature in one of Jeannie KIRBY's poems.<sup>1</sup> Taking the other fundamental color in biology on board then “blood is red, and grass is green”.<sup>2</sup> Well, residing in a republic we can leave the question of blue blood to the haemocyanines of some molluscs and anthropoda. As such, the constituent pigments of red and green, heme and chlorophyll, have been termed the “*pigments of life*” by BATTERSBY<sup>3</sup> and the “*Colors of Life*” by MILGROM.<sup>4</sup> Focusing here on flora, the main color is green – indeed, the only visual evidence of life on Earth visible from outer space is the change in vegetative color. Chlorophylls are the primary photosynthetic pigments in nature and thus are central to the primary biochemical energy generation through natural solar energy conversion – photosynthesis.<sup>5</sup> Thus, from the beginning, when BERZELIUS introduced the term chlorophyll for the green of leaves, the color green has been associated with life, energy from the sun and the basics of the energy food chain.<sup>6</sup> Photosynthesis is the prime example of a ‘renewable’ energy source and photosynthesis-driven algal and plant growth provides one of the mainstays of bioresources.

Since the advent of the early industrial revolution chemistry has been based mainly on nonrenewable resources, primarily in the form of geological, organic carbon deposits. Changes in perspective, dwindling resources, and the problematic geostrategic situation in many resource areas have led to a change in emphasis on renewable resources. This paradigm shift now targets the utilization of renewable bioresources and is a cornerstone of the basic philosophy behind what is called “green chemistry”.<sup>7</sup> Here “green” implies an environmentally friendly approach but – even politically – in a sense, it goes back to the green of vegetation. A chemist can only conclude that chlorophylls in a literal sense are the ultimate green chemical compounds. They are a natural resource, function in a core role of the biosphere, and do have the potential to be used

in industrially relevant applications. At least we will attempt to take a closer look at the latter in this treatise.

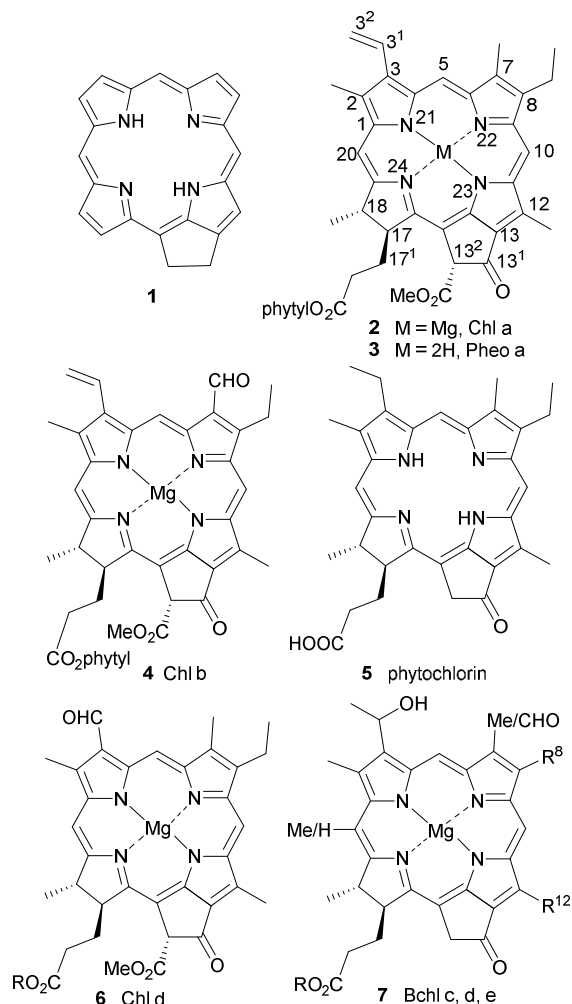
This begs the question: Is green chemistry really ‘green’? Alas, no. Even a cursory inspection reveals that all practical efforts aimed at the use of natural products and biological materials in chemistry almost solely rely on the use of non-green materials. Flipping through a current issue of the journal “Green Chemistry” reveals many intriguing articles but nothing on chlorophylls or other green dyes.

However, there is no reason to not add some color to green chemistry<sup>8</sup> and at least take a closer and serious look at chlorophylls. Thus, this essay attempts to kindle some interest in green chlorophylls. We will briefly outline the basics of their chemistry and biochemistry and then give a brief analysis of the potential of chlorophylls as a bioresource and their possible involvement in renewable energy production. It is based in part on an earlier article on this topic and also an assessment whether the situation has changed in the ten years hence.<sup>8</sup>

### Historical background

Unravelling of the structure and function took most of the past two centuries. The first landmark was the elucidation of their basic chemical structure by WILLSTÄTTER about one hundred years ago, which was honored by the Nobel Prize in Chemistry in 1915,<sup>9,10</sup> followed by more detailed chemical studies culminating in the total synthesis of chlorophyll a 50 years ago by WOODWARD.<sup>11</sup> The second half of the last century saw the bioorganic elucidation of the main steps of their biosynthesis<sup>12,13,14</sup> which was put on firmer ground in recent decades using methods from molecular biology.<sup>15,16</sup> While biophysical studies gave some understanding of the function of chlorophylls *in vivo* real breakthroughs in unravelling their molecular action were only made by X-ray crystallographic structure determination of the purple bacterial reaction center<sup>17</sup> and the bacterial light harvesting complexes.<sup>18</sup> This is now complemented by chlorophyll-protein crystal structures from Higher Plants,<sup>5,19</sup> more in depth studies on the structure, function and biosynthesis of photosynthetic pigments from

unicellular organisms and photosynthetic bacteria.<sup>20,21</sup> Lastly, the ‘enigma’ of chlorophyll breakdown was deciphered.<sup>22,23</sup>



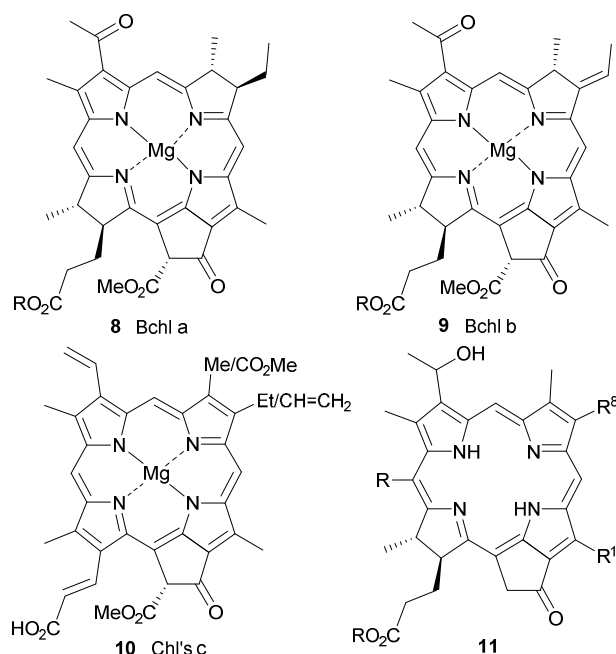
Despite an annual turnover of some 1000 million tons<sup>24</sup> and their central importance in biomass production, chlorophylls have never been used as bulk chemicals for large-scale chemical syntheses on a technical scale. Nevertheless, from the beginning chlorophylls have found widespread use – often with dubious aims (*vide infra*) – as food additives and anti-odor compounds, mostly as simple plant extracts. More recently, pheophorbide and phytychlorin derivatives have been used for the development of lead compounds for photodynamic cancer therapy and imaging<sup>25</sup> and remain under scrutiny as chromophores for solar energy conversion and hydrogen production.<sup>26</sup> Nevertheless, most of this is at a developmental stage and requires only g-scale amounts of material. The various uses will be discussed in more detail below.

## Basics of chlorophyll chemistry

### Occurrence and basic structures

Next to being the reason why grass is green and telling us the answer to the question posed in KIRBY’s poem, chlorophylls and the related bacteriochlorophylls (Bchls) are the ubiquitous pigments of photosynthetic organisms. In nature they harvest light (exciton transfer), function as the reaction center pigments

in charge separation (electron transfer),<sup>27</sup> protect the organism from light induced oxidations (photo-protection), and participate in the correct construction of the photosynthetic apparatus (regulation and protein folding). By now over a hundred chlorophyll-type pigments, *i.e.* systems with a porphyrin/phytyl skeleton **1**, are known.<sup>28</sup> Despite this multitude they share a common structure and related chemical properties. The most widely distributed pigment is chlorophyll a (Chl a) **2** which occurs in all organisms with oxygenic photosynthesis. In our green grass and Higher Plants it is accompanied in a 3:1 ratio by Chl b **4**, which carries a formyl group instead of the 7-methyl group. Characteristic for chlorophylls are the tetrapyrrole moiety with one reduced pyrrole ring and a long chain esterified terpenoid alcohol, in this case, phytyl. The green chlorophylls are magnesium chelates, however, the brown free base of Chl a, pheophytin a (Pheo a) **3**, does also occur in nature. Both chlorophylls and pheophytins can easily be isolated from plants or algae and will be our main focus here.



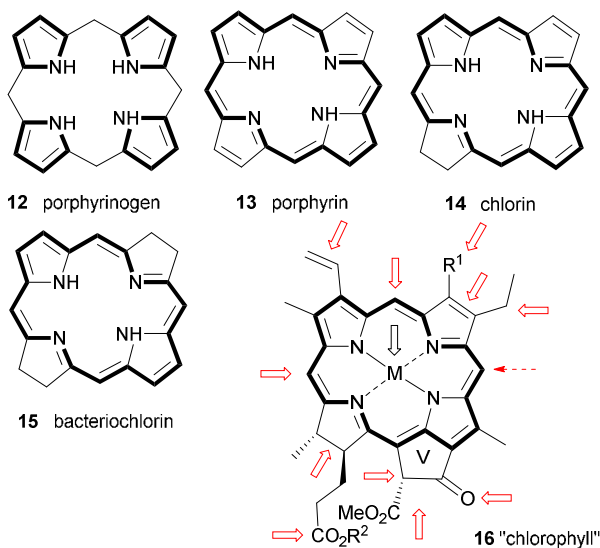
The basic skeleton of the chlorophylls a and b is that of phytychlorin **5**. Other compounds are Chl d **6** from cyanobacteria, the green bacteriochlorophylls c, d, and e **7** (which, despite the name, are chlorins, *i.e.* dihydroporphyrins). They are found in Chlorobiaceae and Chloroflexaceae and can occur as mixtures of homologs with different peripheral substituents.<sup>20,29,30</sup> The brownish-purple Bchls a **8** and b **9** are bacteriochlorophylls (7,8,17,18-tetrahydroporphyrins) and are found in photosynthetic purple bacteria (Rhodospirillales).<sup>31</sup> Other examples are Bchl g from heliobacteria and many of the (bacterio)chlorophylls occur with different esterified alcohols. To complete the confusing nomenclature, the gold-brownish chlorophylls c **10** are actually porphyrins,<sup>32</sup> while the recently identified chlorophyll f is a standard ‘chlorin-type chlorophyll’ with optimum adaptation of its absorption properties to the far red in its low-light habitat of stromatolites.<sup>33</sup>

Chemically related chlorins have also been found in many oxidoreductases, marine sponges, tunicates, and in *Bonella viridis*. Perhaps the most striking example of a non-photosynthetic use of chlorophylls is found in the deep-sea

dragon fish *Malacosteus niger*. Lurking in the deep sea, this creature reminiscent of the 'hero' in the film *Alien*<sup>34</sup> has co-opted a Bchl c/d derivative **11** and uses it as a visual pigment to see in the dark.<sup>35</sup> This and related compounds are most likely derived from bacterial or plant chlorophylls and then processed by the plant or animal.

### Fundamentals of chlorophyll chemistry

**Synthetic aspects.** Chlorophylls are cyclic tetrapyrroles and the heteroaromatic character of the underlying porphyrin system, the central metal, and the reactivity of the peripheral substituents govern their chemistry. At the most basic level, the degree of oxidation distinguishes the main classes of natural porphyrin derivatives. Porphyrins **13**, chlorins (7,8-dihydroporphyrins) **14**, and bacteriochlorins (7,8,17,18-tetrahydroporphyrins) **15** are exemplified in nature by heme, Chl a (**2**) and Bchl a (**8**), respectively. Typically, the higher the degree of reduction the more prone are they towards oxidation (**12**→**15**→**14**→**13**). As *N*-macroheterocycles they can accommodate almost any metal in the core; for chlorophyll, magnesium and chelates occur in nature, while the more stable copper complexes feature in industrial uses. All natural chlorophylls contain an isocyclic ring V. The aromatic character, metal-chelating macrocyclic structure, and the variability of the side chains and of the macrocycle conformation in 'chlorophyll' **16** manifest themselves in unique photophysical properties and chemical reactivities and make them the photosynthetic pigments *par excellence*.<sup>28,36</sup>



Structure **16** illustrates the various positions on the chlorophyll macrocycle through which chemical manipulations can occur. Within the aromatic pathway of the macrocycle (shown in bold) there are three free meso positions which are susceptible predominantly to electrophilic reactions, e.g. halogenations, of which the meso position closest to the reduced pyrrole ring, i.e. C-20, is most reactive. These reactions enable the generation of Bchl c and d mimics through C20 activation and further functionalizations. As the C7-C8 double bond is not part of the delocalization pathway it is susceptible to modifications such as addition/oxidation reactions, e.g., osmylation to yield *cis*-diols. Around the periphery, chlorophyll side chains can undergo standard functional group transformations, e.g., the C3 vinyl group can undergo addition

and oxidation reactions as it behaves akin to an isolated double bond under neutral and alkaline conditions, the ester groups at C17<sup>3</sup> and C13<sup>2</sup> can be hydrolyzed under acidic, alkaline or enzymatic conditions and, additionally, can be transesterified. The high reactivity of exocyclic Ring E is generally governed by enolization and follow-up chemistry of the  $\beta$ -keto ester system. The latter includes the so-called allomerization reactions, i.e. oxidative degradation reactions involving oxidations, hydroxylation, ring-opening or decarboxylations of the isocyclic pentanone ring. Synthetic advancements such as those described enable the generation of Chl analogs with tailored photophysical properties for specific applications.<sup>21,28,37,38</sup>

**Photochemistry.** Chlorophylls are macrocyclic dyes and thus depart themselves like other organic chromophores. Absorption of light will lead to the rapid formation of the lowest excited singlet state by promotion of an electron from the HOMO to the LUMO. The excited state can then either relax to the ground state *via* radiative (fluorescence) or non-radiative processes (internal conversion of vibrational relaxation). Chlorophyll fluorescence, esp. the KAUTSKY effect,<sup>39</sup> is one of the most important analytical handles in environmental photosynthesis research, including remote sensing<sup>40</sup> from outer space.<sup>41</sup> Global sun light-induced fluorescence data can be used to estimate the terrestrial gross primary production, the biggest contributor to the global carbon budget, and thus aid in carbon cycle estimates.

Another possibility is intersystem crossing to form a triplet state which again can relax either *via* radiative (phosphorescence) or non-radiative processes.<sup>42</sup> Both excited state types can take part in photochemical reactions and, in the presence of donor or acceptor units, energy transfer or electron transfer between the chromophores can compete with these processes, which would bring us into the realm of photosynthesis.<sup>5,43</sup> Formation of  $\pi$ -cation or  $\pi$ -anion radicals, that undergo further photochemical reactions, is also possible under illumination.

Within higher plants, a process known as photosensitization can also occur and this is associated with the energy transfer from photo-excited compounds, such as those described for Chl above, to generate reactive oxygen species (ROS). Two types of photosensitization can occur, namely Type 1 and Type 2. With Type 1, the activated Chl sensitizer reacts with an acceptor substrate to produce radical ions in both the sensitizer and the acceptor *via* one-electron transfers.

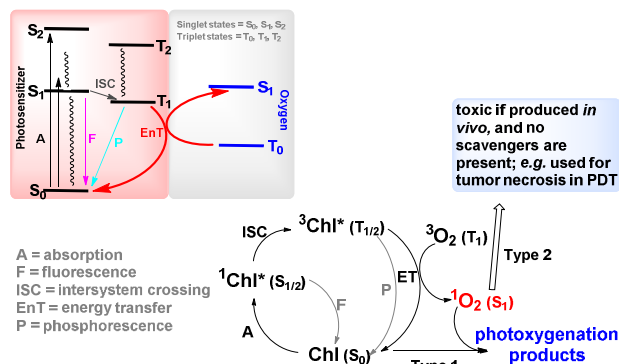


Fig. 1 Chlorophyll as a photosensitizer with modified Jablonski diagram.

These radicals can interact with oxygen to produce oxygenated products, leading to Chl destruction. In Type II, the activated sensitizer transfers its energy to ground state molecular oxygen producing the singlet oxygen excited state ( $^1\text{O}_2$ ) and regenerating the ground state Chl.  $^1\text{O}_2$  reacts with acceptor molecules to produce oxidized products and can be considered a toxic species when produced *in vivo*. Higher plants can protect themselves against photosensitization reactions which cause cell/membrane damage through scavengers of  $^1\text{O}_2$  e.g. flavonoids, and/or through compounds that quench the excited states of Chl. As Chl molecules are effective photosensitizers, this attribute makes them useful in areas such as PDT and this will be discussed in a later section (Fig. 1).<sup>44,45</sup>

Chls in natural photosynthetic systems are involved in light-harvesting (exciton transfer) as antenna pigments or charge separation (electron transfer, ET) as reaction center pigments. The antenna pigments funnel the excitation energy from the absorption of light through exciton transfer to a closely coupled pair of (B)Chl molecules in the photochemical reaction center. It is here that the ET reactions occur, whereby the excited (B)Chl transfers an electron to a nearby acceptor molecule creating a charge-separated state consisting of a reduced acceptor and oxidized Chl. After a series of ET reactions, the stored energy is stabilized in reduction equivalents and ATP.<sup>43</sup> Modelling photo-induced electron transfer (PET) artificially has received much attention and great inroads have been made towards the development of artificial photosynthetic mimics.<sup>46</sup> By in large, the majority of such systems employ porphyrin derivatives due to their ease of synthesis and handling with respect to Chl analogs. However, incorporating natural Chls or synthetic variations of such provides a more realistic representation. WASIELEWSKI and co-workers effectively presented the self-assembly of Chls as models for the natural reaction center special pair. In some instances, a single covalent linkage between two Chl molecules was incorporated to facilitate the aggregation process. Other variations include donor-bridge-acceptor systems, e.g., PPhide a- Zn(II)porphyrin dyads which show weak electronic coupling between the chromophores. Using Chl derivatives is vital for providing an accurate representation of natural processes and for mimicry within artificial constructs, especially for solar energy conversion.<sup>46</sup>

Few photochemical reactions of Chls exist, possibly due to the instability of these macrocyclic compounds outside of their natural environment upon exposure to light. The ascorbic acid induced photo-reduction of Chl a received a lot of attention due to its possible role of photo-oxidized and photo-reduced intermediates in the light conversion step in photosynthesis. Scheer *et al.* proved the structure of this photo-reduced product<sup>47</sup> which was further investigated by Smith and co-workers who demonstrated its use in the isolation of stable isobacteriochlorins from Zn(II) Chl derivatives.<sup>48</sup> A recent example of photochemical reactions with Chls involves the photo-reduced deformylation of a zinc(II) Chl d derivative, further displaying the possible utility of Chls in such photochemical reactions.<sup>49</sup>

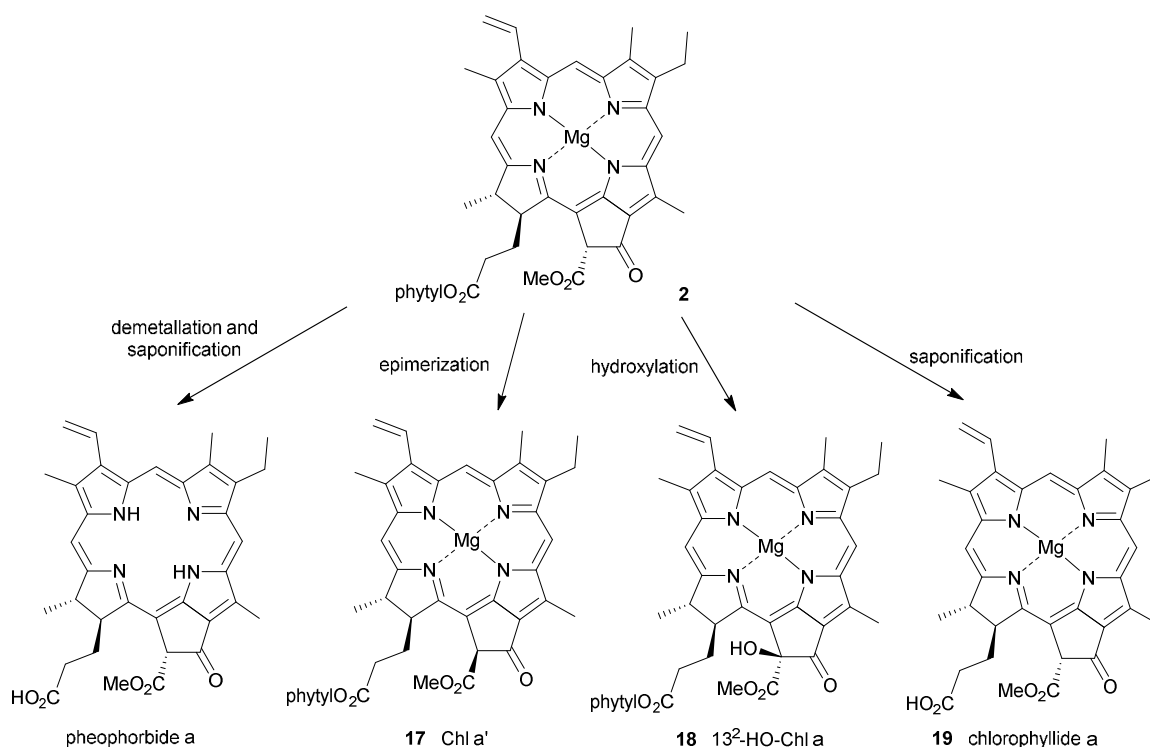
### Grass, spinach or algae? – Sourcing chlorophylls

Depending on the quantity and type of green chlorophyll that is required, numerous renewable natural sources can be used. Within higher plants and algae the pigments are bound to proteins and these complexes are held together by weak, non-covalent bonds. To liberate these pigments from the protein, the

complex must be “disassembled” in a particular solvent is required, and many different techniques have been developed for the qualitative and quantitative extraction of Chl a and b.<sup>50</sup> These methods require extraction of the pigments with an organic solvent or boiling water and, despite all efforts, no single solvent or solvent mixture has been found that will extract the pigments quantitatively and without causing degradation or chemical alteration. As the natural Chls are exceptionally labile compounds they can be susceptible to chemical alterations in the macrocycle core, at C20, and at the doubly activated 13<sup>2</sup>-position.<sup>51</sup> As a result, they are easily demetallated and/or lose the phytyl side chain to yield pheophorbides, or undergo other undesirable chemical transformations (Scheme 1).

These alterations/degradations can occur during the extraction process, not only promoted by chemicals which are added during the process but also as a result of the composition of the plant material. Thus it is next to impossible to extract plant materials without the concurrent formation of various Chl derivatives. Some plants may have an acidic cytoplasm resulting in demetallation and the formation of pheophytins, whilst others contain oxidative enzymes that promote oxidation reactions. During the extraction process probably the most prominent and the byproducts which are most easily formed are the allomerization products, notably epimerized **17** or hydroxylated derivatives **18** of Chl.<sup>51,52</sup> Similarly, chlorophyllase often remains active during extraction resulting in the formation of acidic chlorophyllides **19** or re-esterified derivatives thereof when alcohol is used as the extracting solvent. To circumvent chlorophyllase induced transformations, denaturation methods can be employed prior to extraction (e.g., scalding); however, this usually results in isomerization products so is usually avoided. From the reactions described above, amongst many others it is evident that great care is taken during the extraction process to minimize the formation of extraction artifacts or undesirable byproducts.

Large scale technical use of isolated Chls is hindered by the extraction process as extreme precautions are needed (inert gas, etc.) to circumvent the degradation or alteration of the labile Chls. Depending on the quantity of Chl needed, numerous “green” organisms can be used as sources, with spinach, dried alfalfa meal, and dried blue-green algae (e.g. *Spirulina*) being the organisms of choice due to their high Chl content. Various extraction methods exist and vary in terms on the solvent used and temperature to name but a few. For small scale extractions (1-5 g of plant material), the “acetone method” is commonly used, whereby the plant material is broken up *via* grinding in the presence of acetone. A typical procedure involves breaking up of the material in a blender in the presence of the extracting solvent, filtration, re-extraction of plant material, and evaporation of the combined extracts *in vacuo*. A variety of other water-miscible solvents have been utilized including DMSO, dioxane and DMF. Medium-scale extractions (up to 1 kg of plant material) are best performed using fresh spinach by first heating the material in boiling water (1–2 min), followed by filtration and extraction with methanol/petroleum ether mixtures. Typically 2 to 4 g of crude pigment extract is obtained from this method. For large-scale extractions (1–5 kg) dried alfalfa meal or air-dried blue-green algae, notably *Spirulina* are the best sources, the best results being obtained by freezing *Spirulina* with liquid nitrogen and extracting the pigments with acetone to yield about 2–5 g Chl a per kg of dried algae.



**Scheme 1.** Outline of chemical reactions of Chl a occurring during extraction.

An advantage of using *Spirulina* is that it contains only Chl *a*, which evades the *a/b* separation problem. Various chromatographic methods have been developed to separate the “a” and “b” Chls but the scale of such a separation is limited due to a high adsorbent-to-Chl ratio. Unfortunately, such a limitation prevents large-scale isolation of Chls and to overcome the use of such chromatographic techniques, chemical modifications can be exploited to transform Chls into derivatives which are easier to purify. The classic example is modification of the formyl group in pheophytin *b* with Girard’s reagent T which yields a derivative which is more straightforward to purify *via* chromatographic methods and can tolerate large quantities.<sup>53</sup>

Green biomass represents a good source of natural dyes including Chls but besides the production of “chlorophyllin” (see below) no attempts have ever been made to extract Chl or derivatives thereof on an industrial scale. A “dye fraction” is one of the components routinely obtained when green materials (grass, Lucerne, alfalfa, etc.) are processed in a green biorefinery (see below)<sup>54</sup> and thus could serve as a source of Chl derivatives. As an alternative to Chl extraction from natural sources, many efforts have been made towards the total synthesis of Chl *a* and *b*. Although it is possible to do so, the approach is a multi-step synthetic process which requires many stereochemical considerations. The overall yield from these syntheses are thus low, this making large-scale synthesis impractical. Following the first total synthesis of Chl *a* by WOODWARD and co-workers,<sup>55</sup> various efforts were made in the quest to find a more practical route for the synthesis of Chl derivatives. These included the ring closure of bilanes by MONTFORTS,<sup>56</sup> MACDONALD [2+2] type cyclizations by JACOBI<sup>57,58</sup> and the extensive work by BATTERSBY<sup>59</sup> and LINDSEY<sup>60</sup> towards substituted dipyrins and subsequent acid-catalyzed and metal-mediated cyclizations to form the chlorin

macrocycles. Although all of these efforts improved the synthesis of model compounds, large-scale production of Chls *via* such methods would not be practically feasible.

## Chlorophyll biosynthesis and degradation

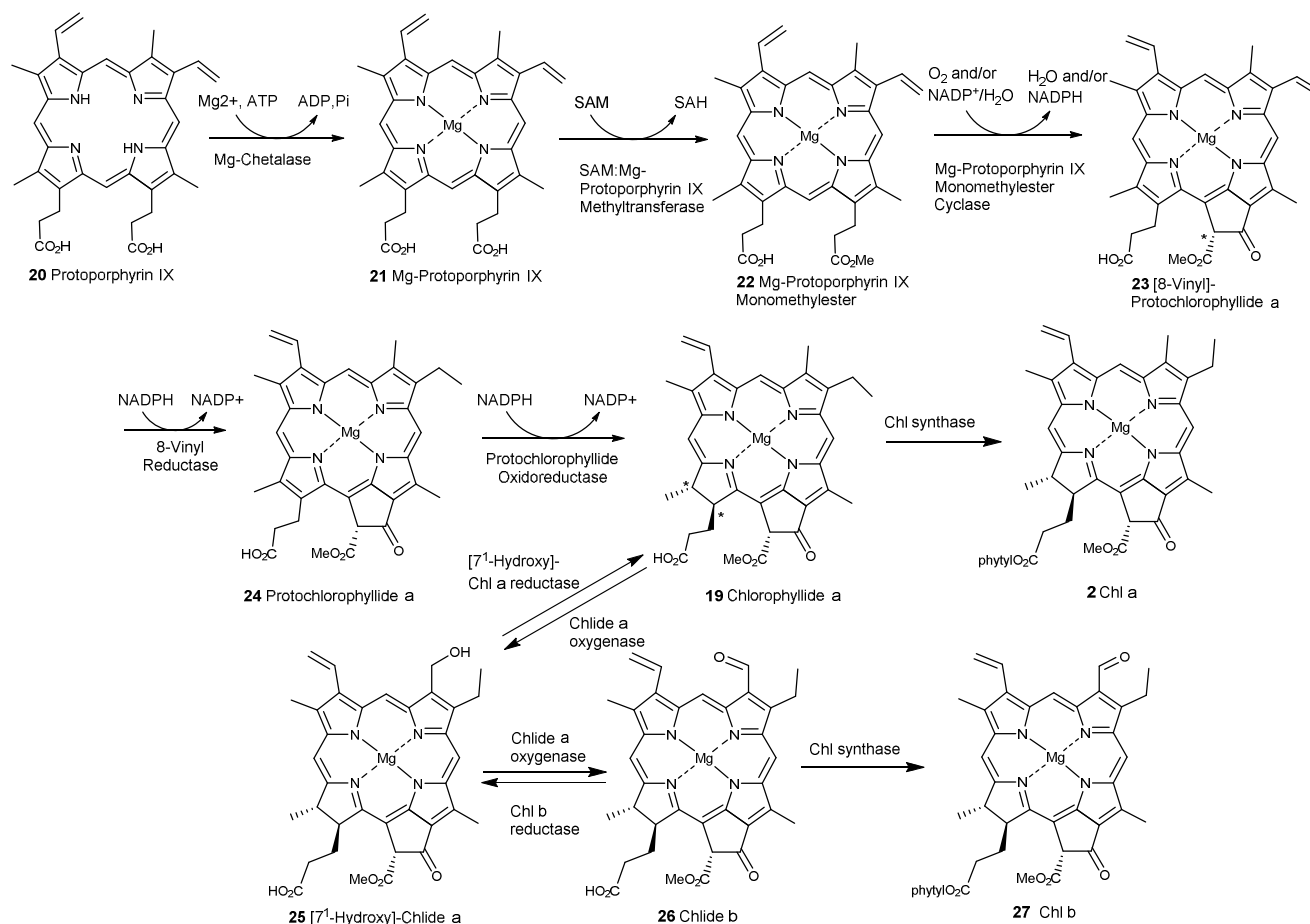
### Chlorophyll biosynthesis

If we want to take a look at the potential of chlorophylls as a bioresource or for the preparation of fine chemicals we first need to look at how nature produces and transforms these pigments. This will then allow an evaluation of what kind of compounds can be obtained from natural sources or with few, simple laboratory steps.

The first essential step for chlorophyll biosynthesis is the insertion of magnesium to protoporphyrin IX **20** by the ATP-dependent Mg-chelatase enzyme.<sup>61</sup> The resulting magnesium porphyrin **21** is now converted into the monomethyl ester **22** by a methyl transferase enzyme which first binds the porphyrin, then transfers a methyl group from *S*-adenosylmethionine (SAM) to the C13 propionic acid group. This methyl ester is an important prerequisite for the complex reaction that forms the isocyclic ring V. Firstly Mg-protoporphyrin IX monomethylester cyclase hydroxylates then oxidizes the C13<sup>1</sup> position to ketone which activates the C13<sup>2</sup> position, now connected to the C15 meso position, to yield [8-vinyl]-protochlorophyllide **a** **23**.<sup>62,63,64</sup> The proposed mechanism involves a SAM radical which abstracts a hydrogen atom from C13<sup>1</sup> and the radical generated is then converted into a putative carbocation which is subsequently hydroxylated from water. A second radical generation at C13<sup>1</sup> allows the abstraction of the proton from the hydroxy group and loss of an electron yields the keto species. Finally, a third radical formation at C13<sup>2</sup> was proposed to then react with C15.<sup>65</sup> The 3,8-divinyl compound

**23** is now reduced to the monovinyl protochlorophyllide **24**.<sup>66</sup> Alterations in this sequence, *e.g.*, first reduction of ring IV followed by reduction of the 8-vinyl group have also been reported by Tanaka's group.<sup>67</sup> The last step of modifications directly on the Chl macrocycle involves the formation of chlorophyllide **19**, *i.e.* the reduction of the porphyrin ring to a chlorin. In plants this is a light-dependent reaction catalyzed by NADPH:protochlorophyllide oxidoreductases; however, some organisms use a so-called dark variant of this process incorporating a light-independent enzyme with ATP for this reduction.<sup>68,69</sup> The light-dependent enzyme binds the substrate and co-substrate in the dark under participation of cysteine residues. Light activation then converts the enzyme-substrate complex in an active form where hydride and proton transfer

can occur, enabling the reduction. For Chl **a** the next step is the esterification of the C17 propionic acid group in **19** with phytyl pyrophosphate or geranylgeranyl pyrophosphate by the enzyme Chl synthase.<sup>70,71</sup> For Chl **b** chlorophyllide a oxygenase (a Rieske-type monooxygenase) introduces two oxygen atoms at the C7<sup>1</sup> position, the first of which can be detected *i.e.* 7<sup>1</sup>-hydroxy derivative **25**. From this derivative an intermediate geminal diol forms and spontaneously loses water in the formation of chlorophyllide **26**.<sup>72</sup> The final step utilizes the same enzyme as for Chl **a** and converts chlorophyllide **26** to Chl **b** **27**. Mutant studies indicated that this Chl **b** pathway is also reversible and allows for the conversion of Chl **b** into Chl **a** in what is known as the 'chlorophyll cycle' (Scheme 2).<sup>73</sup>



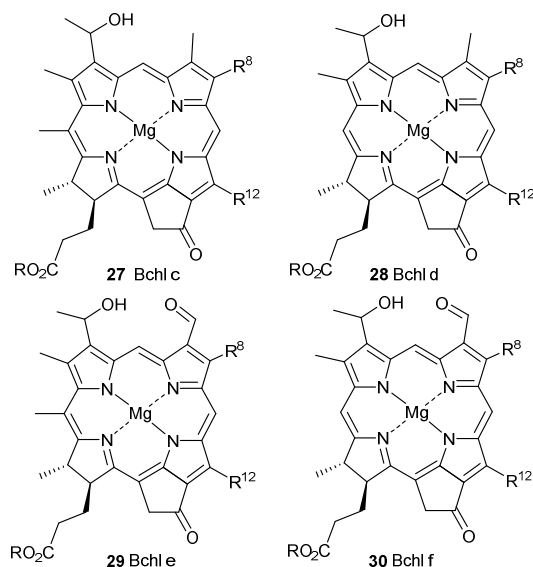
**Scheme 2.** Key steps in the biosynthesis of Chl **a** and Chl **b**.

Theoretically one could imagine using alterations in the biosynthesis for a production or accumulation of biosynthetic intermediates or derivatives. Well known examples for this are the porphyrias, where heme biosynthesis disorders result in the formation of 'atypical' porphyrins and the accumulation in the body of humans. The resulting concentration of free base porphyrins, which are photochemically active, can then lead to increased photosensitivity and severe diseases.<sup>74</sup> Historically this was used, for example, by Hans FISCHER in the 1920s to identify novel porphyrins from the blood and urine of his coworker Matthias PETRY, who suffered from congenital erythropoietic porphyria and ultimately succumbed to the disease.<sup>75</sup> Plants are even more susceptible to photooxidations and the one confirmed case of a plant 'porphyria' (somewhat

sensationally called "vampire plants" in a review.<sup>76</sup>) involved a mutation for uroporphyrinogen decarboxylase and resulted in necrotic spots on the leaves of maize accumulation of uroporphyrin.<sup>77</sup>

All curiosities aside, biological methods can be used for practical chemical purposes. For example, the Bchls **c**, **d**, and **e** from green sulfur bacteria (**27**, **28**, and **29**) comprise a rather complex group of chlorin-type pigments with variations in the C7, C8, C12 and C20 substituents (see also 7). Based on chemical analogies between Bchl **c** and Bchl **d** a bacteriochlorophyll **f** **30** (*e.g.*, a 20-demethyl-Bchl **e**) had been proposed to exist but never been isolated from natural sources.<sup>78</sup> However, a laboratory mutant of a bacterium which normally produces Bchl **e** with inactivation of the

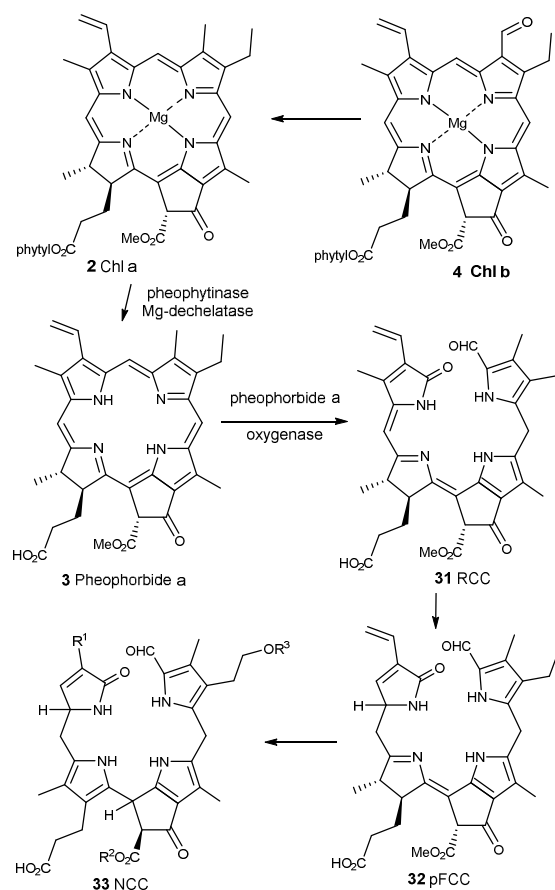
bacteriochlorophyll C20 methyltransferase resulted in a species which produced Bchl f.<sup>79,80</sup>



### Biological chlorophyll degradation – Deciphering the enigma

**Plant senescence.** Next to the green color of grass, the most visible effect of chlorophyll biochemistry is its ‘disappearance’ in autumn. The green color is then replaced by the yellow, red, brown and purple autumn colors the result of the dominance of carotenoid and anthocyanine dyes in the plants after plant senescence.<sup>24</sup> Chlorophyll biosynthesis is required for plant growth and thus has a direct economic production impact in agriculture and forestry. Chlorophyll catabolism has a more indirect economic impact through fall foliage tourism, notably in the New England states of the USA and parts of Canada.<sup>81</sup> During the low light conditions of winter times the photosynthetic machinery is downsized and this releases chlorophylls and carotenoids, which normally also act as photo-protective agents for the plants. As outlined below ‘free’ chlorophylls are potent photosensitizers and can generate reactive oxygen species which would damage the plants. Thus, prevention of photo-damage, rather than recycling of nutrients, is the primary rational behind Chl degradation.<sup>82</sup> While phytol can be stored in gerontoplasts after transesterification to phytol acetate, recent studies also indicate a degree of reutilization of Chlide and phytol in the cyanobacterium *Synechocystis*.<sup>83,84</sup>

In terms of chlorophyll biochemistry the unravelling of the ‘enigma’<sup>24</sup> of chlorophyll degradation remained unsolved until the early 1990s. Up to then the only known biological degradation reactions were similar to those occurring during chlorophyll extraction. Thus, demetallation, dephytylation and reactions of the isocyclic ring V but any steps after, especially the fate of the porphyrin ring system, remained hidden in the secret code of nature. Unlike deciphering the German enigma machine during WW II, it did not take a collaboration of Polish and British cryptographers and the massive efforts of Bletchley Park<sup>85</sup> to decipher this biological enigma. Work by the groups of KRÄUTLER, MATILE, and GOSSAUER showed that the central step is a ring-opening reaction at the 5-position.<sup>22,23,83,86</sup> However, unravelling the whole pathway of chlorophyll catabolism by KRÄUTLER required almost as painstaking an effort as that of Alan TURING and co in generating ‘Ultra’.<sup>23,85</sup>



**Scheme 3.** Key steps in the natural degradation of chlorophylls in Higher Plants.

Scheme 3 illustrates the key steps in our current understanding of chlorophyll breakdown. Cleavage of the chlorophylls at C5 is different from the natural breakdown of heme, which is oxidatively ring-opened with loss of the meso carbon atom and initial formation of a verdoheme.<sup>87</sup> Chl b can only be degraded when it is transformed into Chl a *via* a chlorophyll b reductase enzyme. The crucial steps during Chl a degradation begin with the removal of the phytol chain by pheophytinase enzyme and the removal of Mg(II) *via* a Mg-dechelataase enzyme to yield pheophorbide a. Following this, an oxidative ring opening at C5 occurs, *via* the incorporation of molecular oxygen to form a non-green product, referred to as the red chlorophyll catabolite (RCC) **31**, a red tetrapyrrolic compound. This reaction is catalyzed by the ferredoxin-dependent Rieske-type monooxygenase, pheophorbide a oxygenase (PaO). RCC is bound strongly to the enzyme, inhibiting its activity and the bound RCC is reduced directly by RCC reductase to yield the free primary fluorescent catabolite (pFCC) **32**. This key step in the Chl degradation is dependent on the cooperation of the membrane bound PaO and the stroma located RCC-reductase. pFCC undergoes other modifications at various positions, giving rise to several fluorescent products, collectively referred to as FCCs (fluorescent chlorophyll catabolites). FCC derivatives do not accumulate during Chl breakdown as they are exported from the chloroplast into the cytosol where they are isomerized to non-fluorescent chlorophyll catabolites (NCCs) **33** in a slightly acidic medium; along with some changes in the side chains aimed at increasing the hydrophilicity of the breakdown products.

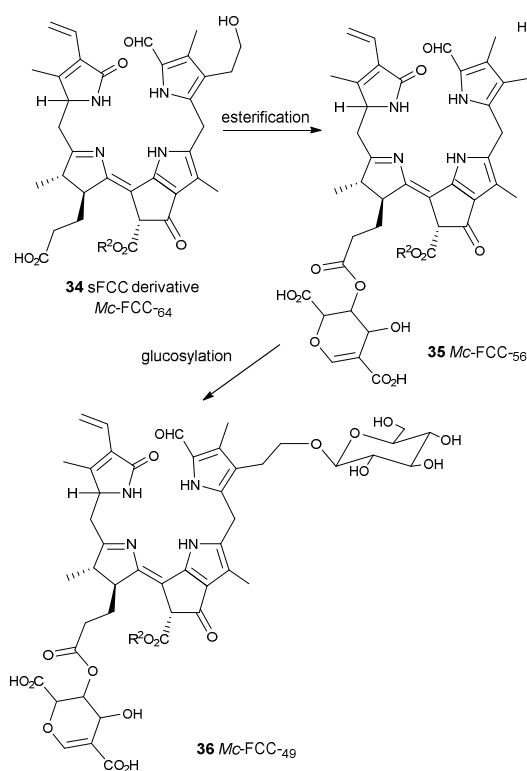


**Banana ripening.** A more direct example for the economic ‘consequences’ of chlorophyll catabolism relates to our supermarket behavior. When shopping for bananas we will go for the nice yellow ones, while the green ones and definitely the ones with black splotches will be non-sellers. No surprise at this juncture, yes, this also has something to do with chlorophylls and resulted from KRAÜTLER’s work on chlorophyll degradation, too. Summarized in a recent publication, aptly in the journal *Gerontology*,<sup>88</sup> the ageing and ripening of bananas and other fruits involves chemistry similar to that just described (Scheme 4). The first step will appear logical from the section on chlorophyll senescence.<sup>87,88</sup> The green bananas break down chlorophyll during ripening, albeit under formation of different catabolites.

In contrast to other fruits, whereby chlorophyll breakdown typically gives rise to colorless, non-fluorescent tetrapyrroles (termed NCCs), in banana ripening, chlorophylls degrade to yield a variety of unique chemically stabilized fluorescent catabolites (FCCs **34**, **35**, and **36**) which accumulate in the peels of yellow bananas, causing them to glow blue under UV light. The intensity of this luminescence (emission wavelength at 447 nm) is strongest for fresh ripe, yellow bananas and decreases during the further aging of the fruit. The most abundant FCC in yellow banana peels is Mc-FCC-<sub>56</sub> **35** (Scheme 4), whereby the free carboxylic acid group of FCC is esterified with a daucyl group. This group somewhat ‘stabilizes’ the catabolite from acid-catalyzed conversion to NCC and provides an explanation as to why these derivatives accumulate in yellow banana peels. These FCCs can be referred to as persistent FCCs and provide an intricate feature of chlorophyll breakdown.<sup>89</sup>

In the course of the aging process of bananas, there is a short period whereby dark spots appear on the peel and these typically suggest deterioration post-harvesting and coincide with the ‘fading’ of the luminescence of the peel. In conjunction with these spots, “blue halos” or intense, blue luminescent rings develop around these senescence-associated dark spots (not to be confused with dark spots connected with physical damage or ‘bruising’). These halos consist of FCCs, as determined *via in vivo* fluorescence analysis, and the main contributor to this phenomenon is the polar catabolite Mc-FCC-<sub>49</sub> **36** (Scheme 4). This represents another persistent FCC and is generated from Mc-FCC-<sub>56</sub> **35** through enzyme catalyzed hypermodification. Dark-senescent associated spots are thought to occur due to spontaneous oxidative processes and are a sign of ‘over-ripeness’. It is obvious that these natural FCCs can provide a useful non-invasive tool for the monitoring of ripening in bananas, and also for the study of cellular processes preceding cell death in plants.

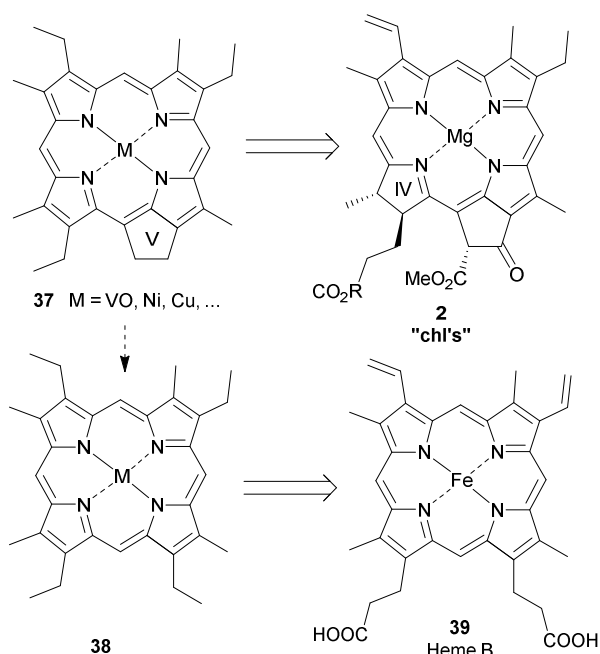
The massive consumption worldwide of bananas is largely due to their cheap price. Although other factors influence this cost which will not be discussed, it can be in part attributed to the fact that only one banana variety is produced on a worldwide scale, the Cavendish. This enables multinational companies to ripen the fruit in bulk at the same time in an almost uniform fashion. In spite of this, the UN agricultural organization estimates that 30-40 % of bananas are discarded based solely on their appearance. It is thus critical to control the level of “artificial ripening” and the use of UV lamps for the detection of FCCs and hence ripening could have a major economic impact, helping with prolonging the narrow shelf-life window for bananas.<sup>90</sup>



**Scheme 4.** Chlorophyll catabolites during banana ripening.

Overall, Chl breakdown can be seen as a process for the recycling of nutrients and the dismantling of Chl/protein complexes to release free Chl molecules for their degradation to occur. The Chl phototoxic agent is broken down which enables this “detoxification” process, i.e. Chl catabolism, vital for plant protection, senescence and fruit ripening. Other ripening fruits such as the yellow pear and apple varieties also contain NCCs which are made more polar through the glycosylation of the hydroxyl ethyl side chain (of type **33**). This suggests common biochemical pathways exist in multiple fruits for Chl breakdown.<sup>91</sup>

**Geological chlorophyll degradation.** What happens if there is more time than for a quick banana run to the supermarket? Contrary to the biological chlorophyll breakdown the basic principles behind the geological breakdown have been known for 80 years.<sup>92</sup> Up to a few percent of all annual primary organic production is accumulated in sediments and under appropriate conditions such as low thermal or oxidative stress, significant amounts of organic compounds will remain identifiable in sediments as fossil molecules and this includes porphyrins and related compounds.<sup>93</sup> Such diagenic compounds can serve as geochemical biomarkers as long as they retain enough structural resemblance to biochemical compounds. Indeed, porphyrins are found in many deposits and their concentration in sediments can vary from 1 to 3,000 ppm with lower concentrations in coal than in crude oil. They have been termed geoporphyrins, petroporphyrins, or sedimentary porphyrins,<sup>94</sup> for an excellent and still fairly up-to-date review see Callot.<sup>95</sup>



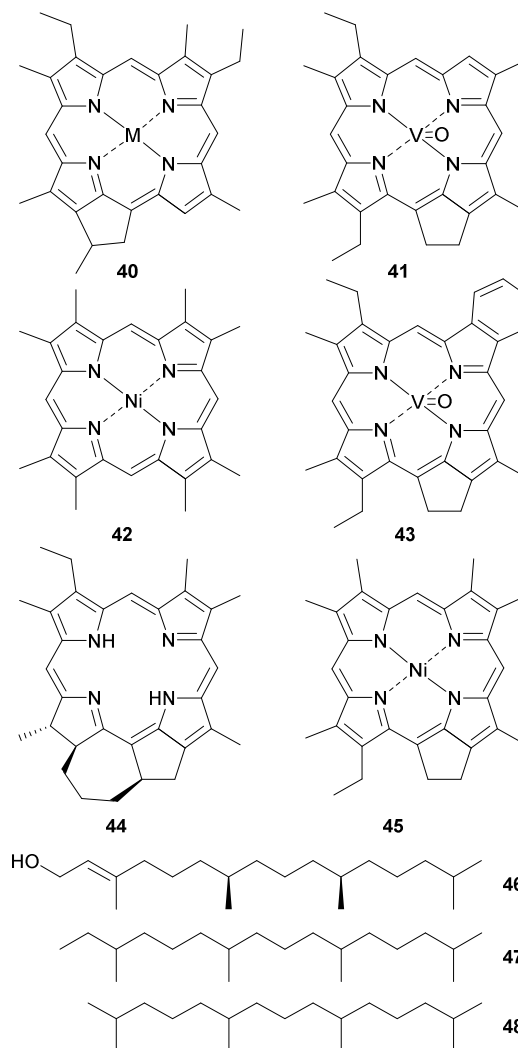
**Scheme 5.** TREIBS's hypothesis for the formation of geoporphyrins from chlorophyll.

With the large biomass accumulated in geological deposits tetrapyrroles have been isolated from almost all sediments with organic matter and are found at geological ages ranging from the Precambrian to recent. A scheme for their diagenetic formation was first proposed by TREIBS based on the close structural resemblance between geoporphyrins and biological pigments, when he suggested that they are geoporphyrins are derived from natural chlorophylls and porphyrins (Scheme 5).<sup>91</sup> This has been more than vindicated and over a 100 petroporphyrins have been identified. Indeed, TREIBS's discovery of porphyrins in crude oil also spawned the beginning of a new scientific discipline – organic geochemistry.<sup>96</sup>

The best known examples of geoporphyrins are metal complexes of desoxyphyloerythroporphyrin **37** (a derivative of phytoporphyrin in modern nomenclature), which still contains the five ring systems as found in Chl a. Cleavage of ring V in **37** could explain the occurrence of etioporphyrins such as **38**; alternatively, these could be formed from heme-type pigments (**39**). Nevertheless, 'green flora' has more biomass than 'red fauna' and CALLOT estimated the relative contributions of organic matter to sediments as 100,000 : 1, respectively.<sup>95</sup>

Based on macrocycle structure comparisons petroporphyrins are derived from almost all different types of chlorophylls. For example, **40** appears to be derived from Chl c, while **41** stems from Chl b. Geoporphyrin **42** could be derived from any cyclic tetrapyrrole precursor, while others such as **43** cannot easily be assigned to a (known) biological porphyrin. The constitution of these compounds has been confirmed in many cases by total synthesis and a simple chemical rationale can be given for their mechanistic formation. Clearly, extreme diagenetic conditions are involved, which include geological timescale, heat, pressure, catalysts, and more. The chemical reactions of this geological porphyrin laboratory are simple ones. They include loss of magnesium, remetallation with V=O, Ni or other metals, vinyl and keto group reduction, cleavage of the methyl ester, decarboxylation,

and aromatization of ring IV.<sup>95</sup> Complete oxidation to the porphyrin level does not always occur. Petrochlorins such as phytochlorin **5** and the butanochlorin **44** have been isolated. Likewise, the nonmacrocylic parts of chlorophylls, *i.e.* derivatives of the esterified isoprenoid alcohols at C17<sup>3</sup> are found in carbon sediments. Examples are fossils of phytol **46**, *e.g.*, phytane **47** and pristane **48**.<sup>97</sup>



Next to presenting an interesting object for scholarly research on their formation, petroporphyrins offer a practical entry into oil analysis and identification. Different deposits have dissimilar porphyrin compositions, both with regard to type and relative content of pigments. This is the result of the paleoenvironment and the composition of petroporphyrins is a fingerprint for a given source.<sup>98,99,100</sup> In practical terms this is more difficult than it sounds, as porphyrins might not be released from certain fractions, might not solubilize under the analytical conditions used, require prior desulfurization, or be degraded further. Thus modern methods focus more on mass spectrometric analyses of bulk samples than HPLC analysis of soluble fractions.<sup>101,102</sup> Analytical studies offer the possibility of using geoporphyrin fingerprinting to track the origin of crude oil samples.<sup>103,104</sup> Together with other biomarker analyses this offers a potential use in the context of oil spill analysis with regard to environmental impact and legal liability or as a

control and enforcement tool in geopolitics during oil embargoes.

In contrast to the biological chlorophylls the geological petroporphyrins are exceptionally stable compounds. However, besides their importance in palaeobiology, geochemistry and as an analytical tool, they are of no industrial importance as a chemical sources of porphyrins. However, even biological samples containing chlorophyll can last a long time under the right conditions. For example, the Tyrolean iceman “Ötzi”, the 5300 year old natural mummy from the Chalcolithic found in the Ötztal Alps, carried maple leaves as an isolation material for storing hot embers in a birch-bark container, with still intact chlorophyll after five millennia.<sup>105,106</sup>

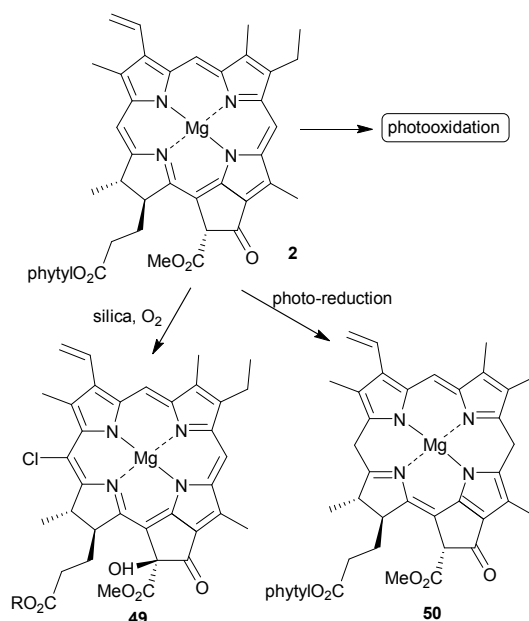
Chlorophyll can even turn into ‘stone’. The mineral abelsonite was shown to consist of crystalline (deoxophylloerythroetioporphyrinato)nickel(II) **45** and is clearly derived from chlorophyll. First found in the Green River Formation in Utah,<sup>107</sup> it is a secondary mineral and its structure was confirmed by total synthesis.<sup>108,109</sup> Contrary to common belief, porphyrins can form relatively large crystal lattices. For example, 5,10,15,20-tetraphenylporphyrin crystals can easily be grown up to several cm in size, while stable porphyrin dication salts have been grown up to > 1×1×1 cm in size for neutron studies diffraction.<sup>110</sup>

**Chemical chlorophyll degradation.** We all know how quickly green plant material turns non-green in isolation, which should not to be confused with dark-induced senescence, *e.g.*, covered lawn areas. Clearly chlorophylls are chemically not very stable compounds. This is due to a) their photochemistry (*vide supra*) and b) phytochlorins contain many reactive positions where degradation reactions may be initiated. Roughly speaking these are side chain transformations, alterations at and cleavage of ring V, and reactions involved the tetrapyrrole ring system. All three can occur during isolation and purification of chlorophylls and must be considered when choosing a specific isolation protocol from biomass. A classic example are the allomerization reactions mentioned above; 13<sup>2</sup>-hydroxylation is a common problem when using silica (esp. TLC plates) for purification.<sup>111,112</sup> Unwanted macrocycle modifications can occur as well. In chlorophylls the C20 position is highly reactive towards electrophilic substitution and meso chlorination from chloride salts has been observed during chromatography (*e.g.*, **49**, Scheme 6).<sup>113</sup>

Chlorophylls are also prone to oxidative chlorin → porphyrin conversions. Hydroporphyrins with hydrogen atoms at the sp<sup>3</sup>-hybridized centers of the reduced pyrroles ring(s) can be oxidized to the respective porphyrin by various oxidants including oxygen.<sup>114,115</sup> The opposite, *i.e.* macrocycle reductions, can occur as well, albeit only under more controlled conditions. A typical example is the photochemical KRASNOVSKII reduction to yield **50**.<sup>116,117</sup> A detailed analysis of ‘chlorophyll chemistry’ will be given in a forthcoming monograph; for now the reader is referred to the classic reviews.<sup>28,36,37,114</sup>

The fading of color in chlorophyll samples is mainly due to photooxygenation.<sup>118</sup> As outlined in the section on photochemistry (Figure 1), chlorophylls are potent photosensitizers and will produce singlet oxygen in the presence of air or triplet oxygen.<sup>119,120</sup> This can result in self-destruction of chlorophylls *via* photosensitized autoxidation. The underlying chemistry is highly complex and varies from pigment to pigment and comprehensive mechanistic studies are still lacking.<sup>121,122</sup> These reactions are more pronounced in

solution.<sup>123</sup> In our hands the easiest way to store chlorophyll samples is in freeze-dried form at –80 °C. Photooxidation does contribute to side reactions in food stuff.<sup>124</sup> While undesirable in the context of product stability, the underlying principle offers the best prospects for practical applications of chlorophyll derivatives. This relates to the specific use of Chls as photosensitizers in synthetic (photo)chemistry and as drug candidates in medicinal chemistry (photodynamic therapy,<sup>25</sup> see below).



Scheme 6. Selected chlorophyll ‘degradation’ reactions.

## Green biorefineries

### Green biorefinery concept

Chemistry, life, and sustainable economic growth are dependent on molecular and industrial raw materials. Only two sources exist for the latter: geological deposits and biomass. Supplies of the former are limited and its use is neither sustainable nor environmentally friendly. Akin to the use of petroleum, which is utilized for its various uses in fractionation refineries, the industrial use of biomass requires dedicated concepts and factories. These biorefineries are based on a combination of technologies for the collection, fractionation, transformation and use of biogenic raw materials.

Depending on the starting material many different biorefinery concepts are possible. In practical terms, three main ones are currently under development: a) Whole crop biorefineries are based on cereals or maize; b) lignocellulose feedstock biorefineries using “dry” cellulose containing biomass or wastes; c) the green biorefinery, with its use of “wet” biomasses such as grass, lucerne, alfalfa, clover, etc.<sup>125</sup>

Chlorophyll can only be obtained from ‘green’ biorefineries, but it is not the only, nor the main target fraction of these. Biorefineries are multi-product systems which offer an entry into several different chemical classes, depending on the underlying plant physiology and thus the exact ‘flow-chart’ of their product fractionation can differ significantly. In terms of fractionation the green biomass is first separated into a fiber-rich press cake and a green juice (Fig. 2). The press cake

contains cellulose, starch and other organic compounds and is the primary source for bulk raw materials or biomass energy generation (syngas and synfuels). The juice contains free amino acids, proteins, organic acids and other compounds, minerals and dyes. At present it is mainly used for the generation of lactic acid (derivatives), ethanol, amino acids and proteins and as a feeder for biotechnology or biogas.

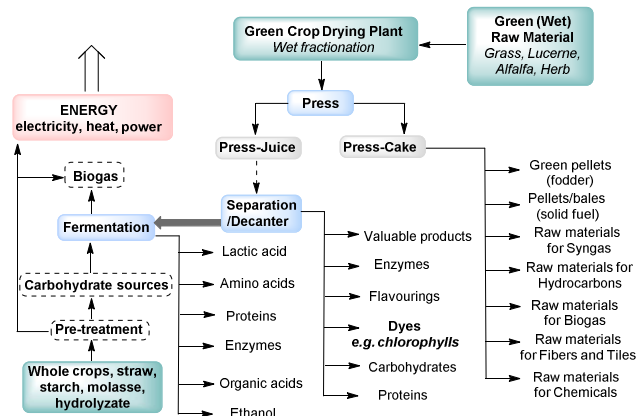


Fig. 2 Concept of a green biorefinery as proposed by Kamm and Kamm.<sup>125,126</sup>

### Chlorophylls and green biorefineries

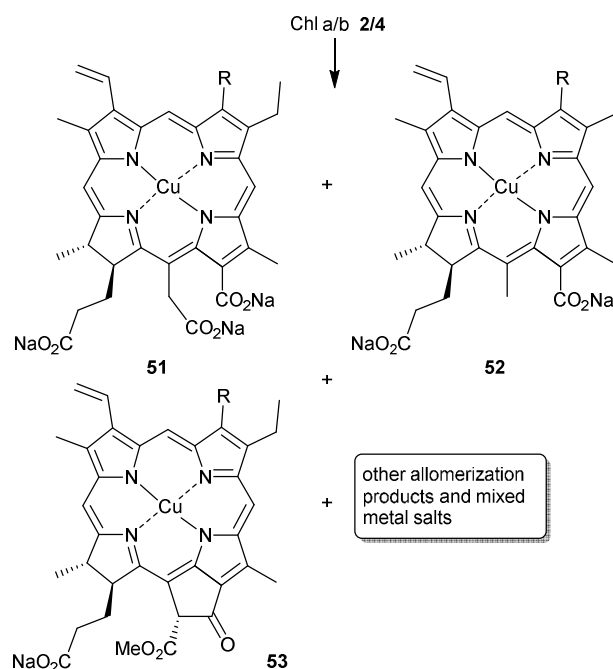
At present no practical use is made of the chlorophylls from biorefineries. Industrial uses of green plant pigments are solely based on the dedicated extraction of chlorophyll from bioresources without use of the remaining plant material. On the other hand, biorefineries utilize most materials *except* for chlorophylls, despite listing chlorophylls and pigments as one of the target fractions.<sup>127</sup> In part this is the result of persisting misconceptions in biorefinery research. Often, a green “pigment/dye” fraction is thought to imply that the content can be used as an industrial pigment without taking into account the heterogeneity of the relevant biomass fraction and the chemical instability of the natural tetrapyrrole pigments. Likewise, advances in biomimetic photosynthesis using *artificial* systems is often used as an argument for the use of *natural* biorenewable systems.<sup>128</sup> However, it must be admitted that the current state of the art of biorefineries requires a focus on one dedicated class of compounds of potential use (next to energy), at least in economic terms.

For a synthetic organic chemist a biorefinery has several limitations that currently negate any use of the “green fraction”. Any mass-produced large-scale crop that can be used as raw material will contain Chl a and Chl b, compelling a chromatographic or chemical separation of the two pigment series if pure starting materials are required. Additionally, the green pigment fraction contains many other pigments that cannot be removed by a simple filtration or extraction step. Again, the necessary chromatography means additional costs and environmental impact due to solvent use. The green fraction is also one of the smallest in terms of mass but most complex in composition. Typically, the chlorophyll content in green feed stocks and blue-green algae is 0.2 and 2–3 %, respectively. Thus, unlike the situation for sugars, the use of chlorophylls from biorefineries as bulk chemicals is economically not feasible. At this time biorefineries allow no use of the green fraction on an industrial scale. However, this is partially a result of the absence of any related studies in this

area, the lack of appropriate extraction methods to yield pigments in high purity, non-existing industrial chlorophyll chemistry, and the lack of large scale practical uses for chlorophyll-derived pigments.

### Commercial use of chlorophyll(in)

Chlorophyll derivatives have been used in daily life for a long time. In Europe its main use has been as a green pigment in the nutrition industry, in fact two of the well-known and often mysterious “E” ingredients in food stuff are chlorophyll related: Food additive E140 is chlorophyll and E141 is chlorophyllin. Typical uses are in beverages, pasta, cakes, sweets, ice-cream, and more. Historically it has also been used in candles and as a lipophilic oil bleaching additive; to neutralize the yellow color of oils in food stuff or giving them a greener hue.<sup>129,130</sup>



Scheme 7. Preparation of “chlorophyllin”.

While often labelled as ‘chlorophyll’ most of these materials do not contain the chemical compound chlorophyll (a/b) but rather derivatives which are called chlorophyllin (and metal complexes thereof).<sup>9,131</sup> This is related to the low stability of Chls as outlined above. “Chlorophyllin” is an inhomogeneous, water-soluble material. Its preparation involved the saponification of the phytol side chain with sodium hydroxide and exchange of the central magnesium atom for copper or other metals. Hence, one can find names such as ‘copper-chlorophyllin’ or ‘zinc-chlorophyllin’. The harsh reaction conditions (and the use of the natural Chl a/b mix) results in the formation of a mixture of chemical compounds (Scheme 7). Constituent components are derivatives 3<sup>1</sup>,3<sup>2</sup>-didehydrorhodochlorin (e.g., the chlorin e<sub>6</sub> **51** and e<sub>4</sub> **52** derivatives in the old nomenclature), pheophorbide salts (such as **53**) and the typical allomerization products.<sup>132,133</sup> In contrast to chlorophyll chlorophyllin is a stable pigment with intense light green to dark blue-green color. Related formulations and trade names are sodium zinc chlorophyllin, chlorophyll paste, oil-soluble chlorophyll, and sodium magnesium chlorophyllin.

The preparation of chlorophyllin also releases significant amounts of phytol **46**, a colorless oil by-product.<sup>134</sup> Historically, the ‘non-green’ part of chlorophyll compound has found uses in the synthesis of vitamin K1 (phylloquinone)<sup>135,136</sup> and as the lipophilic part of vitamin E.<sup>137</sup> Today, it appears to be only used in small quantities in the fragrance industry.<sup>138</sup>

Practical chlorophyll use saw a first boom in the 1940ties. Commercial products included bathroom tissue, diapers, chewing gum, bed sheets, shoe liners, toothpaste,<sup>139</sup> and other daily products, all containing “chlorophyll”.<sup>140</sup> The main selling point was as an anti-odorant and in dental hygiene.<sup>130</sup> To date, chlorophyll preparations are still available as OTC medicine to reduce fecal odor due to incontinence or to reduce odor from a colostomy or ileostomy. Other applications involved use in wound healing, germ killing, and the treatment of infections and inflammations (use of bandages, antiseptic ointments, and surgical dressings). While many studies on apparent medical benefits were published during this early period many of the putative effects remain unconfirmed or have not been substantiated. In line with the opening line of this article any visitor to Eire could do a simple ‘experiment’. Picturesque sheep dot the Irish landscape and they spend their whole life doing one thing: eating grass, aka, chlorophyll. Those who venture close to the animals may test the validity of the anti-odor effects of chlorophyll at their own peril.

Chlorophyll use became more fashionable again in the 1980ies when consumer demand for natural pigments in food and other products increased and when ‘phytochemicals’ became a catch word and its natural connotation was misinterpreted to mean safer and ‘healthier’. Note, legally, dyes derived directly from biomass may not be termed a “natural color”, although they may not require certification by the FDA. Present day use of chlorophyllin is still mainly as a food coloring agent in Europe and in Asia. In the USA the FDA exempts chlorophyllin from certification and allows its use as a food additive in citrus-based beverages for <0.2 % of dry weight and in dentifrices which are drugs at <0.1 % of weight. The only FDA certified green food color (Green No. 3, Fast Green) is a triphenylmethane dye.

The current annual chlorophyllin production is difficult to assess. While estimated to be several thousand tons worldwide, the industrial production of “chlorophyll” is spread over many countries and mostly involves smaller production facilities. Most of the main distributors on the internet indicate an individual monthly production of one to five tons although the actual content of the chemical ‘chlorophyllin’ in the sold ‘chlorophylls’ varies widely.

The advent of the internet with its direct marketing possibilities has spawned a surge in other alleged applications. Even a quick and by no means exhaustive internet search reveals claims for: wound healing, treatment of ulcers, treatment of acute and chronic hepatitis, treatment and prevention of cancer, recovery of liver function as an anti-septic, anti-odorant in the digestive system, treatment of iron deficiency anemia, and many more. These claims have resulted in a growing market for chlorophyll formulations as dietary supplements. With more and more information only downloaded by the general public from the internet and taken at face value, the situation is reminiscent of the snake oil times. Every conceivable health benefit is claimed for this “whole food” and “wonder tonic” and we have reached a point where an unbiased, critical and scientific analysis of these claims must be made. While it is unlikely that such dietary supplements are harmful they certainly do not help the wallet of the believing

customers. For now a few excerpts from web pages may serve to illustrate the state of affairs: “Chlorophyll is one of the greatest food substances for cleansing the bowel and other elimination systems, such as the liver and the blood. – Chlorophyll is effective against anemia and stimulates the production of red blood cells in the body. It also helps carry oxygen around the body and to the brain. – Brain Food – Energise for Life – chlorophyll should eventually be recognized as the major cancer preventative that it is – ...it essentially has the same effects in the body as iron, so it builds our own blood naturally. – mega-chlorophyll – “...liquid chlorophyll is .., an ad-hoc replacement for red blood cells, and carrying oxygen directly to your body’s most vital tissues.” As dubious as most of these claims are, it does not mean that there are no potential medical benefits. There is growing evidence for a possible medicinal use of chlorophylls, which will be detailed below.

## Potential use of chlorophyll derivatives

### ‘Green’ chlorophyll chemistry

Finally, let’s take a look at the potential for practical and commercial applications of chlorophylls. With the present state-of-the-art of industrial use the inherent lability of the natural chlorophylls precludes any direct commercial use. Exceptions are dried algae or plant materials or freeze-dried extracts thereof, mostly in the context of chlorophyllin applications. But, such use does not relate with a green biorefinery approach<sup>141</sup> and even if a suitable application would be identified, regulatory problems might arise with respect to GMP standards, at least in the medical area. The development of practical uses of the chlorophyll pigments mandates a simple and facile extraction process and simple chemical transformations that yield chemically defined materials of high purity and stability. These requirements are counteracted by the need to produce chemicals that are still reactive enough, *i.e.* with functional groups, which permit transformation into looked-for and marketable target compounds.

Many different extraction methods have been developed for small-scale and laboratory production. Mostly these differ in the choice of plant or algal material or practical experiences of the individual groups and it should be possible to adapt these to technical-scale production.<sup>142</sup>

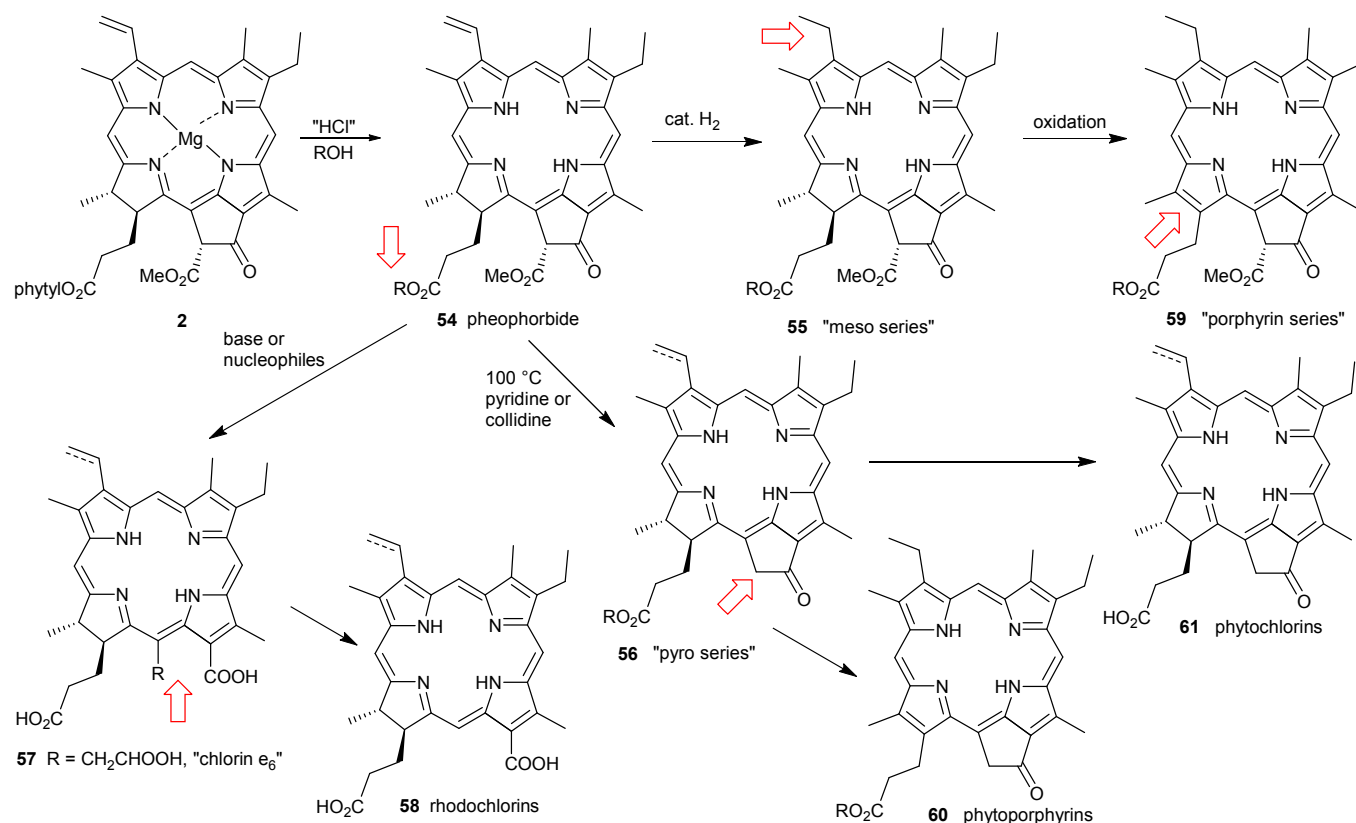
But what kind of compounds can be obtained from a chlorophyll fraction? If we leave aside the chlorophyll a/b separation issue, several possibilities exist to utilize chlorophyll derivatives as starting materials for other fine chemicals. For reasons of stability any suitable use will involve an initial conversion of the chlorophylls to the pheophorbides, *i.e.* demetallation and hydrolysis of the 17<sup>2</sup>-ester (Scheme 8). Preferably, this reaction is performed in the presence of an alcohol to yield a pheophorbide ester **54**, which can be handled in organic solvents. Note, that one of the advantages of chlorophyllin is its excellent water solubility (~200 g.L<sup>-1</sup>). However, anything akin to a synthetic chemistry of chlorophyllin is nonexistent. Many researchers prefer to hydrogenate the 3-vinyl group in pheophorbides to an ethyl residue **55**. Called “meso” compounds in the older literature, this prevents the formation of product mixtures under reducing conditions. The stability of the chlorophylls can also be enhanced by removing the double activated C13<sup>2</sup> position and thus preventing the formation of allomerization products. The simplest way is to remove the 13<sup>2</sup> ester group *via* demethoxycarbonylation to yield the “pyro” compound **56**.<sup>53</sup>

Alternatively, ring V can be cleaved with various nucleophiles to yield chlorin  $e_6$  **57** and/or various other derivatives of rhodochlorin **58**. Further stabilization can be achieved through oxidation of the chlorins to the respective porphyrins (**59,60**), which is possible at any stage. Historically, rhodochlorin and phytychlorin derivatives served as very important intermediates in chlorophyll chemistry, during studies on their constitution<sup>9,143</sup> and total synthesis.<sup>114,144</sup> Today interest has waned in these and PDT-related studies and investigations of the bacteriochlorophylls c, d, e, and f remain the only active areas of synthetic chlorophyll chemistry.<sup>25,37,145,146</sup>

Any putative use of chlorophylls as fine chemicals will have these or closely related compounds as its starting point. In fact, the preparation of chlorophyllin uses similar principles: exchange of magnesium to copper to increase stability and ester saponification to yield a water soluble compound. Nevertheless, due to the drastic reaction conditions allomerization occurs to varying degrees and often the Chl a/b mixture is used. Thus, as described, chlorophyllin is not really a chemically defined pure compound. Chlorophyll chemistry in general is well developed, however requires significant laboratory skills. Continuing interest in hydroporphyrins for applications in PDT (see next section) and their photobiological relevance have led to a

portfolio of reactions which can be performed on a multigram scale. However, most of these reactions are not "green chemistry" compliant reactions in terms of solvents, reagents and atom economy.

Compounds such as those illustrated in Scheme 7 are accessible from crude Chl a in a few steps with standard chemicals. In parallel porphyrin chemists are continuously adding new reactions to this area, especially using organometallic reagents.<sup>147</sup> Areas of contemporary interest are chlorin-based electron transfer compounds<sup>148,149</sup> and photosynthesis mimics,<sup>150</sup> drug development,<sup>25,145</sup> and the bacteriochlorophylls from the green sulfur bacteria.<sup>149</sup> Surprisingly, as the most prominent photoactive pigments in nature and with their proven photosensitizing activity, there is almost no use in synthetic photochemical transformations. Attempts are however under way to utilize them for the coupled productions of basic chemicals in conjunction with CO<sub>2</sub>-fixing systems.<sup>151</sup> Any use of biorefineries for chlorophyll production does not require any specific developments in synthetic Chl chemistry; this is well in hand. Rather, the biotechnological side of resource maximization and the technical problems of the extraction processes need to be resolved.



**Scheme 8.** Selected chlorophyll transformations and key intermediates in chlorophyll chemistry.

### Chlorophylls in medicinal chemistry

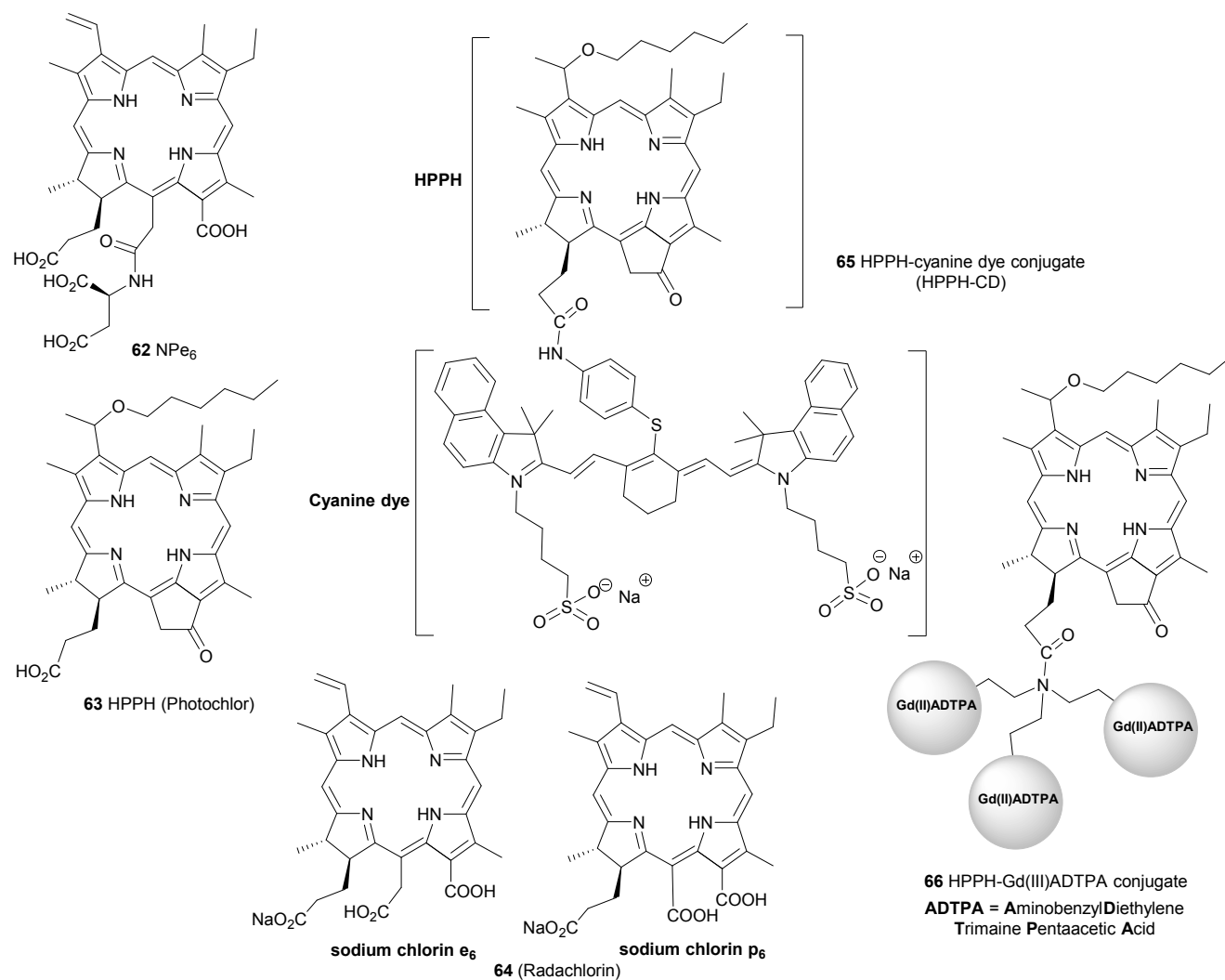
**Photodynamic therapy and theranostics.** Photodynamic therapy (PDT) presents the one clearly established medicinal application of chlorophyll derivatives to date.<sup>25,152</sup> Adopting semi-synthetic methodologies for the chemical transformations of Chl a described above is advantageous as these tend to have lower production costs in comparison to fully synthetic

analogues. Additionally, the likelihood of biocompatibility is enhanced as being related to natural systems they should in theory demonstrate good membrane passage and improved clearance *in vivo*. PDT relies on the selective accumulation of a photosensitizer in target tissue where it can be activated with light to produce reactive oxygen species, primarily the toxic singlet oxygen resulting in *e.g.* tumor necrosis as outlined in Figure 1. Although developed in the early seventies, this

concept is only now making its potential felt in oncology, as antiviral and antibacterial therapeutic options, and in the treatment of diseases like age-related macular generation and psoriasis.<sup>153-157</sup>

In order to be an efficient photosensitizer, a number of requirements should be fulfilled including: good singlet oxygen generation; high absorption coefficients in the 700-800 nm region; no dark toxicity and selective accumulation in the tumor/lesion; high stability.<sup>155</sup> Although several porphyrin-based compounds have been approved for medicinal applications and others are in Phase-2 trials, their widespread applicability is hindered by common drawbacks such as poor selectivity and poor clearance which leads to prolonged photosensitization. Additionally, these derivatives absorb weakly in the therapeutic window (650-800 nm) enabling only

shallow tumors to be treated.<sup>157</sup> As light penetration through biological tissue is wavelength dependent it is advantageous to use photosensitizers that can be activated at longer wavelengths. In many respects, natural Chls fulfill many of the requirements for a good PS and are devoid of the drawbacks described above with porphyrin derivatives. For these reasons, chlorophyll derivatives have emerged as attractive photosensitizers, many semi-synthetic derivatives of which are currently under active investigation and show great promise.<sup>25</sup> In spite of superior photophysical properties, natural Chls are chemically unstable outside of their natural environment when exposed to light but efforts have been made to generate synthetic mimics of these compounds with optimum properties.



**Figure 3** Selected chlorophyll derivatives currently in use or in advanced clinical trials for PDT and HPPH-conjugates as imaging agents.

Many PS drugs which are currently approved or which are in clinical trials are based on the structures of natural Chls and Behls. Of these NPe<sub>6</sub> **62** (also known as mono-L-aspartyl chlorin) is approved from lung cancer treatment in Japan. It is a hydrophilic PS due to the presence of an aspartyl residue and is generated through the synthesis of pheophorbide a from Chl a. Radachlorin® **64** is also a Chl a derivative generated through semi-synthetic methodologies. It is a mixture of three chlorins:

sodium chlorin e<sub>6</sub>, sodium chlorin p<sub>6</sub> and a third unknown derivative. It is currently in clinical trials in Russia for several cancer indications. The most promising Chl derivatives for PDT stem from the “pyro series”, and selected examples are shown in Figure 3.

HPPH or Photochlor® **63** is derived from pyropheophorbide a. It exhibits no long term phototoxicity and is in advance clinical trials for numerous cancer indications. To

advance the applicability of HPPH, polyacrylamide nanoparticles as a carrier system incorporating the PS were analyzed. An enhanced localization of the PS in the tumor and similar PDT efficacy and phototoxicity compared to the free drug was observed *in vivo*.<sup>158</sup> Derivatives similar to HPPH also have the potential to be used as two-photon absorption photosensitizers, although this is still at the early stage of PS development.<sup>145,159</sup> Chl analogs have shown promise as antimicrobial PDT agents. Chlorin *e*<sub>6</sub> and its hydrogenated derivative have demonstrated complete inhibition of Gram positive microorganisms and, whilst only displaying low inhibition of Gram negative microorganisms, Chl PS have the potential to be optimized for such purposes, *e.g.*, *via* the use of cationic derivatives which may assist with uptake.<sup>160</sup>

In addition to PDT, efforts have been made to generate so-called “multi-modality” drugs both for tumor diagnosis and treatment or “theranostics. Leading the field in this area are HPPH conjugates bearing a) fluorophores for NIR fluorescence imaging **65** b) contrast agents such as Gd(II) derivatives for MRI, with Gd(II) as the paramagnetic ion **66**. Here *in vivo* results demonstrate the ability of this conjugate to perform akin to Magnavit (a FDA approved Gd(III) contrast agent) at 10 times lower dosage. Additionally, radiolabelled HPPH conjugates have the ability to be used for nuclear imaging. Such advances demonstrate the applicability of semi-synthetic Chl analogues in medicine and hence the need for larger scale production.<sup>145</sup>

**Back to the future.** Some of the dubious health claims for chlorophylls notwithstanding there is potential for Chl use in medicinal chemistry outside of PDT. For example, sodium metallochlorophyllins appear to interfere with hepatic microsomal drug-metabolizing enzymes through reduction of heme and cytochrome P450 levels.<sup>161</sup> Antimutagenic effects, both *in vitro* and in animal models, have been proven, notably against benzopyrenes.<sup>162</sup> Likewise, there are indications for an anti-carcinogenic role.<sup>163,164</sup> For example, an animal study showed inhibition of dioxin absorption and increased fecal excretion of dioxin.<sup>165</sup> Chemopreventive uses have also been tested in Humans and indicate the possibility to lower the bioavailability of aflatoxin-type food toxins.<sup>166</sup> An increasing number of publications, albeit sometimes conflicting, indicates an anti-clastogenic activity of chlorophyllins, a DNA protective and gene regulatory role, and anti-oxidant activity.<sup>167</sup> At the very least, these results indicate the need for further research and offer the promise of some future Chl applications. Note, that some of the current trends in photodynamic therapy are disinfection and dental hygiene.<sup>168</sup> A reinvestigation of the putative uses of chlorophylls in the 1940ties in the context of

the ongoing revival of anti-microbial therapy<sup>169</sup> appears to be worthwhile.

### Chlorophylls in biohydrogen production and solar energy conversion

The use of chlorophyll derivatives in technical applications is still in the early developmental stage. Topics of current interest are both solar energy conversion and photo-induced biohydrogen production.

**Photo-induced hydrogen generation.** A promising approach to biohydrogen production derives from renewable biomass resources with large carbohydrate composition incorporated into so-called “biofuel cells” or BFCs. Many different methodologies exist to exploit biomass hydrogen production including pyrolysis, gasification along with biological processes such as biophotolysis and fermentation. Advancements in this field have led to the developments of photoelectrochemical fuel cells and also to that of hybrid photoelectrochemical biofuel cells which is an amalgamation of a BFC and a dye-sensitized solar cell (DSSC, see section below). In both systems, a photosensitizer is used to harness visible light energy and, through a series of redox reactions with other components of the cell, results in hydrogen gas generation (*i.e.* photo-induced hydrogen generation).<sup>170-172</sup> Although an area that is extensively studied, chlorophylls as the photosensitizing component have received little attention.

A representative investigation on the biohydrogen evolution uses Zn(II)-Chl a. This is prepared from Chl a derived from *Spirulina* and shows superior photostability and photosensitizing activity compared to the natural magnesium complex.<sup>173-175</sup> The use of chlorophyll derivatives is based on their absorption in the visible region. Besides the photosensitizer the photo-induced hydrogen generation system also requires an electron donor (for example, NADH regenerated by GDH), an electron relay ( $MV^+ / MV^{2+}$ ) and a catalyst (for example, colloidal platinum). A schematic representation of this type of system is shown in Fig. 4.<sup>176,177</sup> More recently, chlorophyll chlorin *e*<sub>6</sub> derivative (copper chlorophyll trisodium salt) has shown promise in a hybrid cell as it is more stable than natural Chls.<sup>175</sup> Chls represent an attractive PS source due to their low toxicity, abundant availability and low costs. Using semi-synthetic methodologies more tailored Chl derivatives could be utilized for this purpose and would open an avenue to more economical dyes for biohydrogen fuel cells.

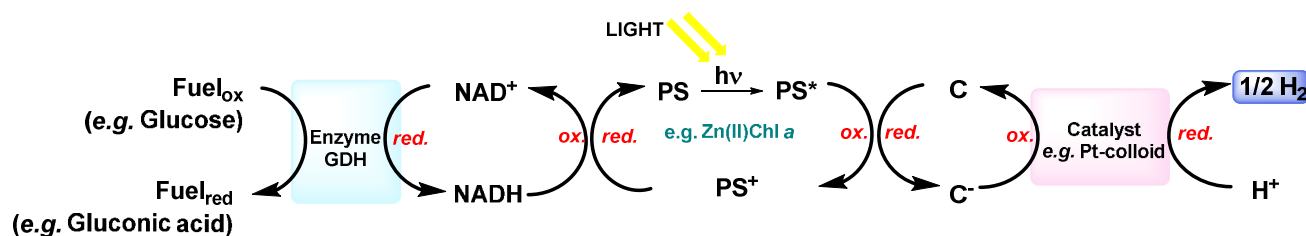


Fig. 4 Schematic representation of a visible light-induced hydrogen production system from biomass. GDH = glucose dehydrogenase; ox = oxidation; red = reduced; NADH = nicotinamide adenine dinucleotide (reduced form); NAD<sup>+</sup> = nicotinamide adenine dinucleotide (oxidized form); PS = photosensitizer; C = catalyst.



**Photovoltaic systems with chlorophylls.** Due to their central role in photosynthesis the use of chlorophylls in photovoltaic systems might be obvious. However, the low (photo)stability prompted most researchers in this area to use more stable synthetic (metallo)porphyrins and phthalocyanines. Nevertheless, chlorophyll derivatives have helped to advance the field and deserve a 'second look'. Historically, one of the first systems studied by GRÄTZEL in his quest for the most efficient dye-sensitized solar cell (DSSC)<sup>178</sup> utilized Chl a, along with other chlorophyll derivatives such as chlorin *e*<sub>6</sub> (a constituent of chlorophyllin **51**) for the photosensitization of nanocrystalline TiO<sub>2</sub> films in 1993 and represented the first trial of chlorophylls in a DSSC.<sup>179,180</sup> In these devices the green pigment absorbs visible light and is photoexcited to an excited singlet state. In the next step its emission is quenched by TiO<sub>2</sub> with effective electron injection from <sup>1</sup>(Chl)\* into the TiO<sub>2</sub> conduction band (Fig 5). The ultimate aim of DSSC development is to produce stable solid state photovoltaic cells of low cost with good solar energy conversion efficiency. In this regard, Chls are an obvious choice due to their panchromatic light absorption and abundant supply.

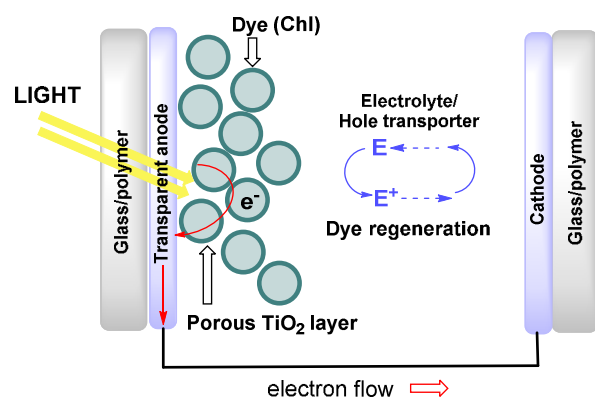


Fig. 5 Schematic representation illustrating how a DSSC works.

Currently the best performing dyes in DSSCs are synthetic, *e.g.*, organometallic compounds such as ruthenium(II) polypyridyl complexes<sup>179</sup> and zinc(II) porphyrin derivatives<sup>181-183</sup> which require multi-step syntheses. Efficiencies have recently reached >13 % conversion through careful molecular engineering of the dye<sup>183</sup> but realizing large scale production of such pigments is not very promising due to high costs and the use of toxic metals. Studies into the use of Chls derived from natural sources and derivatives such as chlorin-*e*<sub>6</sub> have shown that through appropriate device fabrication and chemical manipulations, *e.g.*, transesterification of the phytol chain have escalated the device performance using Chl dyes to 8 %.<sup>184</sup> The increase in performance with respect to other Chl and pheophorbide derivatives has been attributed to the suppression of the backward charge recombination between the TiO<sub>2</sub> and the electrolyte. Investigations into the co-sensitization of Chl derivatives have also shown promise. Here it was hoped to mimic natural photosynthetic light-harvesting systems *via* the introduction of multiple dyes. Enhanced performance of the DSSC was observed using co-sensitization of a Chl a derivative along with a Chl *c*<sub>2</sub> analog. An 8.1 % solar energy conversion efficiency was observed for this DSSC due to enhanced light-harvesting capabilities and improved electron injection efficiency.<sup>185</sup> Additionally, Chl incorporation into gold

nanoparticles for water-based DSSCs has shown promising energy conversion efficiencies for a more environmentally friendly cell (with the use of water as the solvent).<sup>186</sup> Recently, a Cu(II) chlorin-*e*<sub>6</sub> dye was investigated in DSSCs and although a low efficiency was achieved (<2 %) it shows that simple natural dyes can be useful for solar energy conversion.<sup>187</sup> Natural Chls could provide a sustainable solution to future solar energy conversion technologies as they are available in large quantities, are cheaper to isolate and modify and are environmentally friendly, with respect to other synthetic dyes used in DSSCs.

### Materials science chemistry of chlorophylls

One of the neglected areas of chlorophyll chemistry is their use as new materials. Their main disadvantage, low stability, can in part be circumvented through materials preparations. Notably, their photochemical stability can be increased by adsorption on solid phases, *e.g.*, silica or polymer supports, smectite conjugates.<sup>188-192</sup> Such mesoporous silica adsorbed materials have been shown to be able to generate hydrogen gas through photo-reduction when coupled with appropriate electron carriers through which Chl molecules interact in a similar fashion to that in a living leaf,<sup>189</sup> while an amide bond coupled PEG-chlorophyllide conjugate was able to function in the coupled generation of glutamate from NADP<sup>+</sup> photoreduction.<sup>193</sup> A highlight in the medical area for future developments is the use of amphiphilic pheophorbides to construct 'porphosomes', *i.e.* nanovesicles formed from self-assembled chlorin bilayers.<sup>194</sup> In effect liposomal formulations, they combine the properties of nanomaterials with the functionality of individual photosensitizer or imaging molecules.

Despite these promising results, only very few groups are active in this area. A similar situation persists with regard to applications in solar energy conversion. While this has been the catchword in almost any modern publication and grant application dealing with chlorophyll or porphyrin chemistry, only very few recent studies actually deal with the use of isolated chlorophyll derivatives in photovoltaic cells.<sup>195-197</sup> While initial steps have been taken here, major research efforts are required to advance to useful applications. However, if successful, these would provide the necessary demand to further stimulate this whole line of research.

### Conclusions

Currently, chemically pure Chl derivatives are not in large scale commercial use. Very few specialized applications exist which require only small amounts of material (multi-gram to kg scale), which can easily be obtained from a dedicated source, *e.g.*, *Spirulina*. On the other hand, if we take into account the whole area covered by pyrrole-based dyes, there are well-established major industrial uses of tetrapyrroles. However, their use as coloring agent in inks, paints, or for plastics and rubber, are exclusively based on non-natural technical pigments. Should the reader elect to print out a copy of this article with a laser printer then this text most likely will put on paper with participation of phthalocyanines (5,10,15,20-azaporphyrins) as photoconductor or dyes. Due to their many uses, the annual production of phthalocyanines is about 80000 metric tons.<sup>198</sup> In fact, the technically most important green pigment in industrial chemistry is "Phthalo green", a chlorinated copper phthalocyanine. Phthalocyanine dyes are derived from simple and cheap starting materials in few steps. Thus, it is doubtful if

any bioresource derived pigments can ever compete with bulk organic pigments from established synthetic chemistry and this situation has not changed since our initial assessment.<sup>199</sup>

How to proceed? Initially, more quantitative data on the content and composition of the dye fractions derived from green biorefineries are needed. These then need to be assessed with regard to extraction processing conditions which could lead to improved work-up conditions for the “green fraction” and its practical use. This might open the use of the green fraction for the industrial scale production of chlorophyllin, the only Chl derived material immediately marketable. The development of more cost effective and environmentally benign reactions for the transformation of the crude chlorophyll fraction from the “green biomass fraction” into homogenous and pure materials could then allow their use as fine chemicals. Ultimately, it might still be more prudent to use blue-green algae with their higher Chl content in a biotechnological set-up. Here an economic and environmental impact study of both strategies is needed. Many applications exist for utilizing natural Chls, however most of these are very much so at a developmental stage, deterring any real attempts for large-scale isolations from biomass. Realizing breakthroughs such as HPPH for PDT, conjugates of such for theranostics and the co-sensitization of Chl derivatives for improved DSSC efficiencies would further enhance the applicability of these natural compounds. More industrial applications for natural tetrapyrrole pigments must be sourced and developed to provide an economic stimulus for this area. This will require a more thorough assessment of translational aspects of chlorophyllin<sup>200</sup> and its economic potential. Only then will “green” be truly a part of green chemistry.

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#### Biographical Sketches



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