*Photochemistry and Photobiology* Vol. 51, No. 2, pp. 217–222, 1990
Printed in Great Britain. 

0031-8655/90 $03.00+0.00


# PHOTOCHEMICAL RING-OPENING IN *meso*-CHLORINATED CHLOROPHYLLS

A. STRUCK[1], E. CMIEL[2], S. SCHNEIDER[2, 3] and H. SCHEER[1]*
[1] Botanisches Institut der Universität München, Menzinger Str. 67, D-8000 München 19, W. Germany
[2] Institut für Physikalische Chemie der Technischen Universität München, Lichtenbergstr. 4, D-8046 Garching, W. Germany and
[3]Institut für Physikalische und Theoretische Chemie der Universität, Egerland str. 3 D-8520 Erlangen, W. Germany

(*Received* 7 *June* 1989; *accepted* 21 *August* 1989)

**Abstract**—Irradiation of 20-chloro-chlorophylls of the *a*-type with visible light produces long-wavelength shifted photoproducts, which transform in the dark to linear tetrapyrroles (bile pigments). The possible significance for chlorophyll degradation is discussed.

## INTRODUCTION

Chlorophylls (Chl)† and their Mg-less derivatives, the pheophytins† (Phe), are readily and regiospecifically chlorinated at C-20, the free methine-bridge next to the reduced ring D. Chlorinated pheophytins were first obtained (and erroneously described as 17,18-dihydroxy-pheophytins) by H. Fischer's group (Fischer and Lautsch, 1937; Fischer, 1940). The structure was corrected by Woodward and Skariç in 1961, and the electrophilic substitution mechanism at C-20 identified. Selective chlorination of Phe *a'*, the $13^2$-epimer of Phe *a* (**5a**), was observed by Hynninen and Lötjönen (1981). The interest in chlorinated chlorophylls has recently been renewed by isolation of pigments **5b** or **12b** from extracts of various green plants, algae and cyanobacteria (Dörnemann und Senger, 1986; Scheer *et al.*, 1986; Watanabe *et al.*, 1986; Kobayashi *et al.*, 1988), and a suggested relation to photosystem I of oxygenic photosynthesis (Dörnemann and Senger, 1981, 1986). This relation, and hence the involvement in photosynthesis, was met with critical interest but has now been disproved (Fajer *et al.*, 1987; Katoh and Yasuda, 1987; Kobayashi *et al.*, 1988; Senge *et al.*, 1988; Senge and Senger, 1988).

The status of 20-chloro-chlorophylls as natural pigments is still unclear, however. Senge *et al.* (1988) and Kobayashi *et al.* (1988) have demonstrated that chlorination is possible during chromatography on silica plates, and hence concluded that it is an artefact. Kobayashi *et al.* (1988), using a different chromatographic system which avoids such artefactual chlorination, found varying amounts of 20-chloro-chl a (**5b**) in green tissue samples. They have also shown that the status of the natural source strongly affects the quantities of **5b**. It generally increases in ageing tissue, but it is unclear if its origin is enzymatic e.g. by a chloroperoxidase-like enzyme (Champion *et al.*, 1975) or non-enzymatic.

During a systematic investigation of the chemistry of chlorinated chlorophylls, we have now observed a photochemical degradation of these pigments leading eventually to open chain tetrapyrroles, e.g. bile pigments. This raises the possibility that chlorinated chlorophylls may be components of plant tetrapyrrole degradative pathways, about which very little is known to date (see Rüdiger and Schoch, 1988; Hendry *et al.*, 1987).

## MATERIALS AND METHODS

*Pigments.* The $13^2$-hydroxy-20-chloro-methylpheophorbids *a* (**3,4b**) have been synthesized by the method of Woodward and Skariç (1961) from the methyl-pheophorbids **3,4a**, respectively (Scheer *et al.*, 1986). The chlorinated pheophytins **1b** and **2b** were obtained from the pheophytins **1a** and **2a** by a modification using no phase transfer. Unreacted educt was recycled. Insertion of magnesium to yield pigments **5b–9b** was done according to Isenring *et al.* (1975). The yield for both steps was routinely $\geq 50\%$.

*Spectra.* **5b**: Absorption in ether (relative intensities): 429 (1.00), 534 (0.04), 587 (0.07), 622 (0.12), 663 nm (0.64); in acetone: 432 (1.00), 624 (0.15), 668 nm (0.72); $^1$H-nmr in pyridin-$d_5$:δ [ppm] (proton): 9.91 (5), 10.0 (10), 8.16 ($3^1$), 6.15 ($3^2$a), 6.26 ($3^2$b), 6.67 ($13^2$H), 4.09 (17), 4.32 (18), 3.55 (2 $CH_3$), 3.25 (7 $CH_3$), 1.64 ($8^1$ $CH_3$), 3.71 (12 $CH_3$), 3.81 ($13^2$ $COOCH_3$); in $CDCl_3$: 9.59 (5), 9.60 (10), 7.92 ($3^1$), 6.29 ($3^2$a), 6.17 ($3^2$b), 6.28 ($13^2$), 4.17 (17), 4.82 (18), 3.60 (2-$CH_3$), 3.25 (7-$CH_3$), 3.71 (12-$CH_3$), 3.91 ($13^2$-$COOCH_3$);

**7b**: Absorption in acetone (relative intensities): 432 (1.00), 626 (0.12), 666 (0.63); $^1$H-nmr in pyridin-$d_5$:δ [ppm] (proton) 10.04 (5), 9.83 (10), 8.15 ($3^1$), 6.10 ($3^2$a), 6.19 ($3^2$b), 4.35 (18), 3.19 (2-$CH_3$), 3.60 (7-$CH_3$), 3.79 (12 $CH_3$), 3.80 (8 $CH_2$), 1.65 ($8^1$-$CH_3$);

**8,9b**: ($13^2$-epimer mixture): Absorption in ether (relative intensities): 429 (1.00), 532 (0.02), 583 (0.06), 620 (0.09), 664 nm (0.63); in acetone: 432 (1.00), 622 (0.11), 668 nm (0.68); $^1$H-nmr in pyridin-$d_5$:δ [ppm] (proton): 10.00/10.01 (5), 10.04/10.06 (10), 8.19/8.20 ($3^1$), 6.17 ($3^2$a), 6.28 ($3^2$b), 4.09 (17-H), 4.59 (18-H), 3.58/3.57 (2 $CH_3$), 3.27/3.26 (7 $CH_3$), 3.72/3.74 (12-$CH_3$), 3.61/3.55 ($13^2$-$COOCH_3$);

*To whom correspondence should be addressed.
†*Abbreviations*: Chl, chlorophyll, Phe, pheophytin.

*Photochemistry*. A solution of the pigments in acetone (5–10 μ*M*) was irradiated with a 150 W cold light source (Volpi, W. Germany) at 2 cm distance at ambient temperatures. The reaction was followed spectrophotometrically. After accumulation of the intermediate ($\lambda_{max} \approx 686$ nm), the solution was kept in the dark for 24 h and then worked up. Products were isolated by chromatography on silica gel (toluene with acetone increasing from 10–20%).

*Optical spectra* were recorded with a model 320 (Perkin-Elmer) or model HP 8451 A (Hewlett-Packard) spectrophotometer.

*$^1$H-nmr spectra* were recorded on a AM360 (Bruker) spectrometer. For spectra of the photoproduct, a solution of **5b** was irradiated in the nmr tube in acetone-$d_6$. Spectra were recorded at increasing irradiation times up to a total of 20 min.

## RESULTS

Irradiation of **5b** in acetone results in the progressive formation of a red-shifted pigment, which eventually becomes saturated. In the difference spectrum (Fig. 1A), pronounced extrema are observed at 432 and 667 nm (−) and 686 nm (+), with a small net increase in oscillator strength in the red spectral region, and a decrease in the Soret region. If the solution is kept in the dark after saturating irradiation, the red-shifted absorption of the photoproduct disappears, with a concomitant broad absorption increase in the 500–600 nm region (Fig. 1B). The oscillator strength of the red band ($Q_y$) is decreased during this reaction. The spectroscopic changes upon treatment of all other 20-chloro-chlorophylls (**7b** and **8/9b**) were similar and always gave the red-shifted photoproduct first, followed by the formation of a blue pigment with strongly reduced absorption in the visible spectral region. By contrast, the respective pheophorbides **1b**, **2b** and **3/4b** lacking the central magnesium, showed only a slow, irreversible bleaching without the appearance of new absorptions in the visible spectral range.

Chromatography of the final product mixture obtained from the reaction of **5b** yielded (besides the educt and some unidentified material) a single blue band. The isolated pigment(s) show under neutral conditions a weak, broad absorption in the red spectral region, and a more intense band in the blue around 320 nm (Fig. 2, solid line). Upon treatment with acid, the red absorption band is increased, and shifted from 590 to 696 nm (Fig. 2, dashed line); this change is reversed after removal of the acid by washing with water or bicarbonate solution. The pigment is unstable, however, towards prolonged exposure to alkaline conditions, and already partially degraded by the bicarbonate washings.

No fluorescence could be detected from the blue pigment under neutral conditions. The protonated species seems to be weakly fluorescent. The emission spectrum of the acidic solution (not shown) has

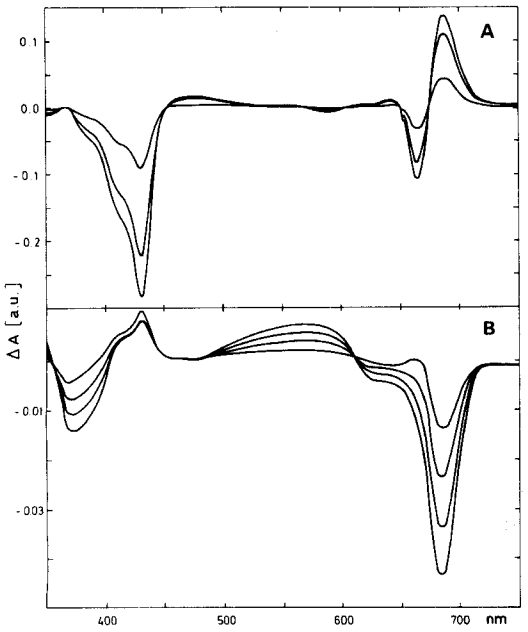



Figure 1. Absorption difference spectra for the photochemical reaction of 20-chloro-chlorophyll *a* (**5b**) (A) and the subsequent dark reaction (B). The solution in acetone was irradiated with white light, with each trace in (A) corresponding to 1 min additional irradiation time. The sample was then kept in darkness, and spectra recorded every 15 min (B).

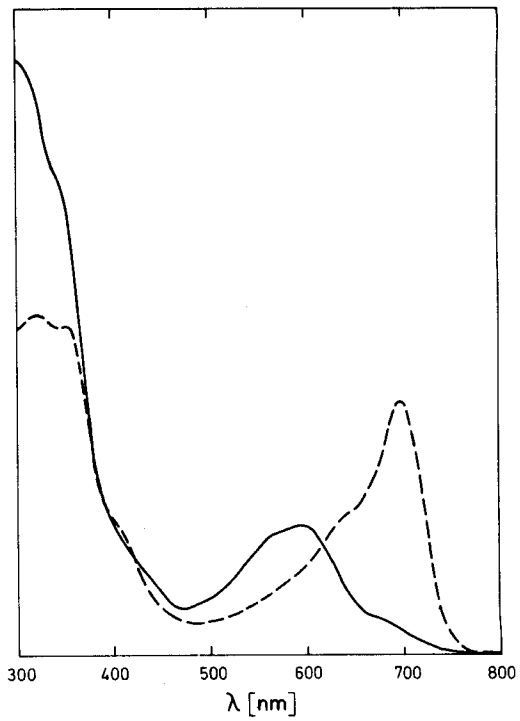



Figure 2. Absorption spectra of the blue product obtained by the light-induced reaction of 20-chloro-chlorophyll *a* (**5b**) in acetone: (—) Spectrum in diethyl ether and (– – –) in the presence of trifluroacetic acid (10% in ether). Both spectra taken with the same concentration of pigment absorption scale 0–0·4 a.u.

a band at 663 and a smaller band around 720. We interpret the 663 nm band to represent the fluorescence of a trace of pheophytin (not seen in absorption), and, at most, part of the long-wavelength fluorescence to the product.

The formation of the photoproduct was followed by *in situ* proton nmr spectroscopy. During successive irradiations, the product spectrum gradually grew into the original spectrum of **5b**. This formation was much slower than in the optical cuvette due to the increased concentration and unfavorable geometry, and reached an optimum of 15–20% (Fig. 3). The most informative changes occurred in the low-field region. The two methine signals are shifted to higher fields ($\Delta\delta$ = 0.86 and 0.76 ppm), and a somewhat smaller shift ($\Delta\delta$ = 0.36 ppm) is observed for the $3^1$ vinyl proton signal ($H_x$). The changes in the other regions were similar (not shown), with decreasing $\Delta\delta$ for protons experiencing decreasing ring-current shifts.

## DISCUSSION

### *Structure of products*

From its absorption properties, the photoproduct still appears to be a cyclic-conjugated tetrapyrrole.

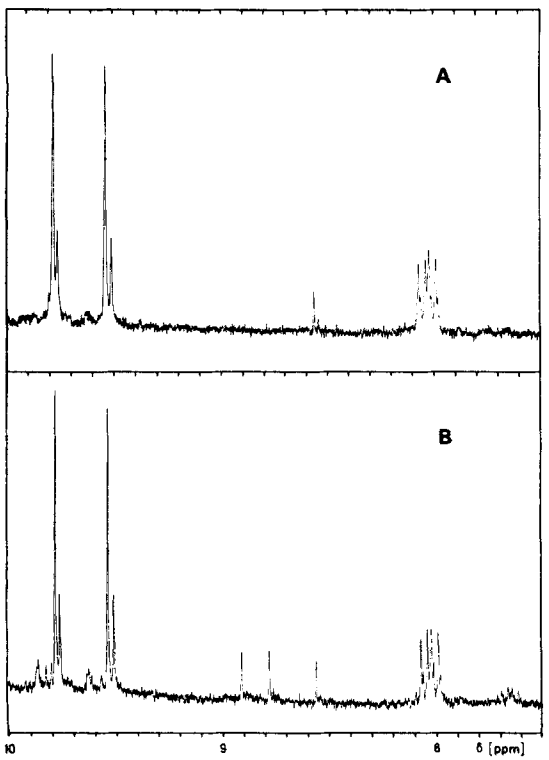
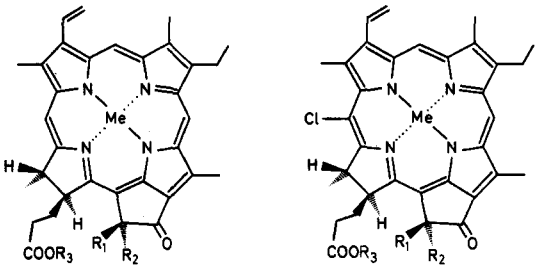



Figure 3. $^1$H-nmr spectra (low field region) of **5b** (A) and of its photoproduct in acetone-$d_6$ after irradiation with white light for 20 min (B). The satellite signals at lower field are due to the prime pigments present in equilibrium with the common $13^2$(R)-epimers.

Absorption maxima and in particular the intensity of the red band show only small differences from those of the starting material. The nmr spectrum indicates a reduced ring-current. There is a general high-field shift of all signals affected by the ring current, and this incremental shift $\Delta\delta$ is proportional to the latter. Since only two methine signals appear in both the educt and the photoproduct, and the subsequent ring opening yields a bile pigment carrying a reduced end-ring (see below), the C-20 position lacks a hydrogen substituent in the product. It could still contain the Cl-substituent, but the spectra are better compatible with a 20-oxonia-chlorophyll (type-structure **10**) in which the C-20 is replaced by oxygen. Pigments of this type still have a pronounced, albeit reduced, ring current if compared to porphyrins (Fuhrhop *et al.*, 1977). Their optical spectra, too, are similar to those of the parent tetrapyrroles. Pigments of unknown structures with red-shifted absorption spectra (up to 697 nm) of the chlorophyll-type have been described in work related to chloro-chlorophylls (Groß, 1986; Senge, 1987), but they appear to be much more stable than the intermediate observed here.

The final product of the subsequent dark reaction is a bile pigment. The reversible spectral changes upon protonation-deprotonation demonstrate the presence of a ring-opened system and the absence of a liganding $Mg^{2+}$, and the wavelengths of the maxima are indicative of an A-dihydrobilindione chromophore (Scheer, 1976). The optical spectrum of the neutral pigment is compatible with that of the bilin (**11**, $R_1 = R_2 = H$) described by Huster and Smith (1988) as product of a non-photochemical reaction sequence from Chl *a*. The nmr spectrum of the product is complex and shows many split lines (not shown). It indicates that several isomers(?) are formed, e.g. by reactions of the ring E still present in **11**. Formula **11** therefore only represents a type-structure for the final product(s) obtained in the photochemically induced reaction sequence. The ring-opening of oxa-porphyrins to bilins is well documented (Fuhrhop and Krüger, 1977).

The only other photochemical reaction converting a chlorin to an open-chain tetrapyrrole is the photooxygenation of the bacteriopheophytins or bacteriomethylpheophorbides *c* and *e*, which are methylated at C-20 (Brockmann and Belter, 1979; Brown *et al.*, 1980; Risch *et al.*, 1984; Hendry *et al.*, 1987). The common structural feature of the latter and the 20-chloro-chlorophylls is the presence of a bulky substituent at the C-20 methine bridge. In fact, the absorption characteristics of chlorophyll-RC1 ($13^2$-hydroxy-20-chloro-chlorophyll *a*, **12b**) were the first indication of its being substituted at C-20 (Scheer *et al.*, 1984). The effect of such a substituent is an increased steric hindrance (Smith *et al.*, 1982; 1983), which may also be responsible for the facilitated cleavage at this bridge. That the 20-Cl substituent and no other part of the molecule is responsible for



a b

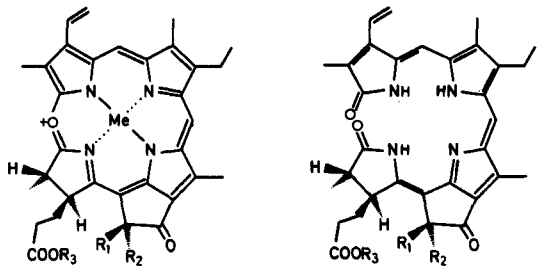
**Pheophorbides (Me - $H_2$)**

| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 1 | $COOCH_3$ | H | Phytyl |
| 2 | H | H | Phytyl |
| 3 | $COOCH_3$ | OH | $CH_3$ |
| 4 | OH | $COOCH_3$ | $CH_3$ |

**Chlorophylls (Me - Mg)**

| | | | |
|---|---|---|---|
| 5 | $COOCH_3$ | H | Phytyl |
| 6 | H | $COOCH_3$ | Phytyl |
| 7 | H | H | Phytyl |
| 8 | $COOCH_3$ | H | $CH_3$ |
| 9 | H | $COOCH_3$ | $CH_3$ |
| 12 | $COOCH_3$ | OH | Phytyl |

Scheme 1



10 11

the photochemistry observed, is supported by the same reaction sequence found in all the 20-Cl chlorophylls investigated, irrespective of the substituents present at ring E. A puzzling fact is the influence of the central metal. In the 20-chlorinated pigments, the Mg-complexes are the reactive species, and the metal-free pheophorbides are unreactive; the situation is just opposite in the 20-methylated-pigments. Here, the pheophorbides react smoothly, but not the chlorophylls (Mg-complexes) (Brockmann *et al.*, 1979; Brown *et al.*, 1980; Risch *et al.*, 1984). These different reactivities indicate subtle substituent effects, which may be related to the differences observed in Raman spectra of C-20 substituted chlorophylls (T. M. Cotton, private communication, 1989).

*Significance to chlorophyll breakdown*

The degradation of chlorophylls has been hitherto only poorly understood. Several pigments of the cyclic tetrapyrrole-type have been identified in ageing or darkened photosynthetic organisms, including chlorophyllides lacking the esterifying C-17[4] alcohol, pheophorbides lacking the central Mg, pyrocompounds lacking the 13[2] carbomethoxy-group, and pigments showing a combination of the above modifications (see Hendry *et al.*, 1987 and Rüdiger and Schoch, 1988 for leading references). Although all these pigments are clearly chlorophyll derived, it is unclear at present if any of them are intermediates of natural chlorophyll breakdown, e.g. in leaves before shedding in the fall. Firstly, enzymes catalyzing such conversions have been identified for the first two reaction types, e.g. de-phytylation (see e.g. Michalski *et al.*, 1988; Schoch and Brown, 1986; Shioi and Sasa, 1986; Terpstra, 1982) and removal of Mg (Kowalewska *et al.*, 1987; Owens and Falkowski, 1982; Ziegler and Schanderl, 1969), but their natural substrates are yet unknown. Secondly, none of the aforementioned products has been identified under conditions of 'natural' chlorophyll-breakdown, e.g. in fall. Thirdly, there is no chemical evidence which qualifies these pigments as intermediates preparing the macrocycle for degradation. All of the pigments are chemically or photochemically at least as stable as the parent chlorophylls. Finally, all these pigments are highly fluorescent and hence potential sensitizers for photodynamic action, the accumulation of any one of them could therefore be critical to the cells if it is not protected as effectively from them as from the chlorophylls in their native pigment/protein environment.

Few further chlorophyll breakdown products are known in plants and they are structurally only partially characterized (Jen and MacKinney, 1970; Matile *et al.*, 1987). The only natural bile pigments known to be derived from chlorophylls are found in some bioluminescent marine organisms (Shimomura *et al.*, 1988).

Chlorinated chlorophylls have not been identified in senescent plants, but they have been reported for 'ageing' green cells (Kobayashi *et al.*, 1988; Watanabe *et al.*, 1986). These pigments would principally have certain advantages, however, over the aforementioned macrocyclic derivatives. Their fluorescence yield is lower, hence photodynamic effects are expected to be reduced as well. In the light of the present study, a particular advantage would be the easy photodegradation of these pigments, e.g. chlorination would at least be a chemically reasonable preparatory step for further degradation. Chlorinating enzymes are known (Champion *et al.*, 1975), and peroxidases generally exhibit chloroperoxidase activities as side reactions (Thomas *et al.*, 1970; Harrison and Schultz, 1976). Enzymatic and chemical reactions (*vide supra*) generating posi-

tively charged chlorine-species are expected to generate 20-chlorophylls, which has been verified by Senge and Senger (1989). We therefore suggest a search for such pigments *in situ* in spite of their negative correlation with PSI. Since chlorine-containing compounds are abundant in marine plants (Moore, 1978), chlorination may also be involved in the generation of bile pigments involved in bioluminescence (Shimomura, 1988).

*Acknowledgements*—This work was supported by the Deutsche Forschungsgemeinschaft, Bonn (SFB 143) and the Fonds der Chemie, Frankfurt. A. Struck is grateful for a stipend from the Hanns-Seidel-Stiftung, München. We acknowledge the skillful technical assistance of I. Katheder.

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