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Chemistry of Bacteriochlorophyll *b*: Identification of Some (Photo)Oxidation Products

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Bacteriochlorophyll, Ethylidene Group, Isomerisation, Oxidation, Photosynthesis

Photoreactions of bacteriochlorophyll *b* have been studied. They all arise from reactions at the $\Delta 8,8^1$ double-bond. The $\Delta 7,8$-isomerisation product **3** is formed under anaerobic conditions. Irradiation in the presence of oxygen leads to three products arising from an autoxidation reaction involving singlet oxygen. The structures of two of them (**4, 5**) have been established from their methylpheophorbides **10** and **11** containing an 8-acetyl and 8-(meth)oxyethyl-substituent, respectively, in addition to the isomerized $\Delta 7,8$-double bond.

Introduction

Bacteriochlorophyll *b* (BCHL *b*) [1, 2] is the photosynthetic pigment of only a few species of photosynthetic bacteria [3–7]. It is the chlorophyll with the absorption at longest wavelengths (1020 nm *in vivo*), and it functions both as light harvesting pigment in the antenna and as photoactive pigment in the reaction center [8–10].

BCHL *b* is rather unstable due to the presence of an exocyclic $\Delta 8,8^1$ ethylidene substituent [1]. It is almost invariably contaminated with by-products absorbing around 680 nm, which are structurally related to the plant chlorophylls. These products can arise from isomerisation and/or from oxidation reactions which both lead from the bacteriochlorin to the chlorin conjugation system. There are examples for both types of reactions in the literature [1, 2], but the chemistry of BCHL *b* is hitherto only insufficiently explored. Here we wish to report the identification of two of these products arising from oxidative isomerisation and present data on the solvent dependence and reaction mechanism.

Abbreviations: BCHL, bacteriochlorophyll; *Rp.*, *Rhodopseudomonas;* Chl, chlorophyll; UV-VIS-NIR, absorption in the ultraviolet, visible and near infrared spectral range; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; DDQ, dichloro-dicyanobenzoquinone.

Reprint requests to Prof. Dr. H. Scheer.

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Materials and Methods

General methods

All solvents were reagent grade or distilled prior to use. HPLC was done as previously published for chlorophylls [7], but with a mixture of methanol/aqueous sodium ascorbate = 93/7. Analytical tlc was carried out on HPTLC-plates. The adsorbents were either silica gel (F 254, Merck, Darmstadt; carbon tetrachloride/acetone = 9/1 as eluent) or RP8 reverse phase silica gel (Merck, Darmstadt; methanol as eluent). Preparative tlc was done on self-coated plates (20 × 20 cm, 0.75 mm layer of silica H, Merck, Darmstadt) with the same eluents. Uv-vis spectra were recorded on a DMR 22 (Zeiss, Oberkochen) or a PE 320 (Perkin-Elmer, Überlingen). The H-NMR spectra were recorded in $CDCl_3$ with TMS as internal standard. Mass spectra were obtained in the electron impact mode on a model CH7 (Varian-Mat, Bremen).

Rp. viridis was grown anaerobically in 15 l flasks on Gloe's medium [11] under incandescent light. The cells were harvested after seven days and the BCHL *b* was isolated by the procedure of Strain and Svec [12], paying attention that the samples were kept in the darkness to inhibit any photoreactions. BCHL *a* was isolated [12] from phototrophically grown *Rp. spheroides* R26 [11] and oxidized to 3-Acetyl-3-devinyl-chl *a* with DDQ [13].

Photochemistry

The pigment was dissolved in acetone or other solvents, and exposed to white light from a tungsten-

halogen lamp (20 mW/cm^2). The reaction was followed spectrophotometrically (decrease of the 792 nm absorption peak of BCHL *b*, increase of a new band around 680 nm). Extinction coefficients of the product mixtures were determined from the extinction differences around 790 and 680 nm, taking into account the absorption of BCHL *b* at 680 nm.

Aerobic experiments were carried out in 1 × 1 cm cuvettes stoppered to prevent solvent evaporation. For anaerobic experiments the sample was dried in a stream of nitrogen in a cuvette with a ground glass joint. This was then attached to a vacuum line and filled under vacuum with the appropriate solvent, which had been deoxygenated by five freeze-pump-thaw cycles.

Pheophytins of the photoproducts

The products from preparative scale photoreactions were transformed to pheophytins by treating the solutions in diethylether with HCl until the color changes from green to brownish. After work-up the products were separated by preparative TLC. Three major zones were eluted separately with carbon tetrachloride containing 30% acetone in order of decreasing mobility: zone 1: $R_f = 0.55$, zone 2: $R_f = 0.42$, zone 3: $R_f = 0.28$.

UV-VIS in acetone

$R_f = 0.55$: 680 (1.0), 620 (0.13), 545 (0.16), 512 (0.19), 402 (2.31), 380 nm (2.22 relative intensity); $R_f = 0.42$: 674 (1.0), 612 (0.14), sh. 568, 520 (0.24), 434 (2.77), 382 nm (1.09 relative intensity); $R_f = 0.28$: 678 (1.0), 618 (0.18), 542 (0.23), 508 (0.31), 412 (2.26), 380 nm (1.69 relative intensity).

Methylpheophorbides

Methylpheophorbides were prepared by refluxing the pheophytins with 5% methanolic sulfuric acid for 90 min. After work-up the methylesters were chromatographed on silica gel plates. Three zones could again be isolated. The fastest migrating zone contains *3-acetyl-3-devinyl-8-(1-methoxy)ethyl-8-deethyl-methylpheophorbide a* (***10***)*: UV-VIS in chloroform:* 682 (1.0), 625 (0.19), 545 (0.23), 514 (0.29), 415 (2.0), 381 nm (1.5 relative intensity).

H-NMR in CDCl$_3$/TMS: $\delta = 10.14$ (s, 10-H); 10.07 (s, 5-H); 8.83 (s, 20-H); 6.29 (s, 13^2-H); 5.65 (q, 8^1-H); 4.52 (m, 18-H); 4.24 (m, 17-H); 3.93, 3.75, 3.70, 3.62, 3.44, 3.32, 3.23 (7s, 3H each, 2; 3^1; 7; 8^1; 12; 13^4; 17^4-CH$_3$); 1.81–2.38 (m, 17^1, 17^2; CH$_2$–CH$_2$); 2.15 (d, 8^2-CH$_3$); 1.85 ppm (d, 18-CH$_3$).

Mass spectrum M = 652 (100%): M-15 (–CH$_3$, 23%); M-31 (–OCH$_3$, 41%); M-58 (–COOCH$_2$, 45%); M-87 (–CH$_2$CH$_2$COOCH$_3$, 23%); M-147 (–COOCH$_2$,–CH$_2$CH$_2$COOCH$_3$,–2H, 27%); M-176 (–OCH$_3$,–COOCH$_2$,–CH$_2$CH$_2$COOCH$_3$, 27%); M-219 (–OCH$_3$,–COCH$_3$,–COOCH$_2$–CH$_2$CH$_2$COOCH$_3$, 23%).

The second zone contains *3,8-diacetyl-3-devinyl-8-deethyl-methylpheophorbide a* (***11***)*: UV-VIS in chloroform:* 680 (1.0), 619 (0.16), sh. 565, 527 (0.28), 438 (2.63), 380 nm (1.0 relative intensity).

H-NMR in CDCl$_3$/TMS: $\delta = 10.52$ (s, 10-H); 10.24 (s, 5-H); 8.81 (s, 20-H); 6.31 (s, 13^2-H); 4.51–4.59 (m, 18-H); 4.18–4.25 (m, 17-H); 3.91, 3.83, 3.75, 3.68, 3.58, 3.30, 3.18 (7s, 3H each; 2, 3^1, 7, 8^1, 13^4, 12, 17^4-CH$_3$); 1.90–2.48 (m, 17^1-H, 17^2-H; CH$_2$–CH$_2$); 1.62 ppm (d, 18-CH$_3$).

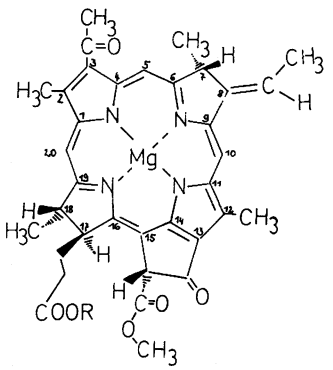
Mass spectrum: M=636 (56%); M-15 (–CH$_3$, 11%); M-31 (–OCH$_3$, 34%); M-58 (–COOCH$_2$, 100%); M-87 (–CH$_2$CH$_2$COOCH$_3$, 27%); M-145 (–COOCH$_2$, –CH$_2$CH$_2$COOCH$_3$, 45%); M-147 (–COOCH$_2$, –CH$_2$CH$_2$COOCH$_3$, –2H, 23%); M-187 (–COOCH$_2$, –CH$_2$CH$_2$COOCH$_3$, –COCH$_2$, 34%).

UV-VIS of the third zone in chloroform: 678 (1.0), 618 (0.22), 550 (0.30), 516 (0.39), 422 (3.09), 380 nm (2.0 relative intensity).

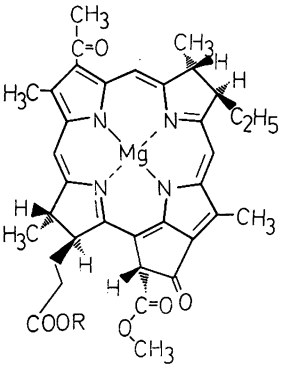
Results and Discussion

Anaerobic photoreaction of BCHL b

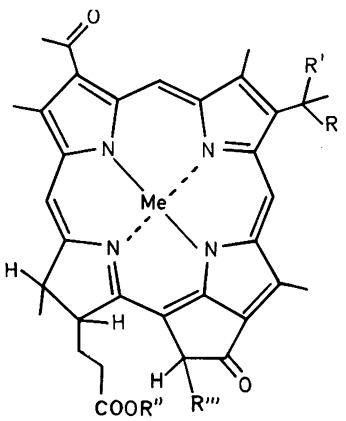
Irradiation of BCHL *b* (**1**) in a thoroughly deoxygenated solution yields essentially a single product. The spectrophotometric traces of the reaction (Fig. 1) give an isosbestic point in the near-IR at about 690 nm. The product was identified as 2-acetyl-2-devinyl-chl *a* (**3**) by comparison with authentic material prepared by quinone oxidation [13] of BCHL *a* from *Rp. sphaeroides* R26 (Scheme 1) and demetalation to the pheophytin **6**. The two pheophytins are identical according to UV-VIS spectroscopy and TLC on both silica gel and reversed phase. These results are in agreement with earlier studies of Brockmann and Kleber [2].



Bchl b (1)



Bchl a (2)



	Me	R	R′	R″	R‴
3	Mg	H	H	Phytyl	$COOCH_3$
4	Mg	H	OCH_3	Phytyl	$COOCH_3$
5	Mg		=O	Phytyl	$COOCH_3$
6	2H	H	H	Phytyl	$COOCH_3$
7	2H	H	OCH_3	Phytyl	$COOCH_3$
8	2H		=O	Phytyl	$COOCH_3$
9	2H	H	H	CH_3	$COOCH_3$
10	2H	H	OCH_3	CH_3	$COOCH_3$
11	2H		=O	CH_3	$COOCH_3$
12	2H	H	OCH_3	CH_3	H
13	2H		=O	CH_3	H

Photooxygenation of BCHL b

Structure of the products

The photoreaction of BCHL *b* is very sensitive to traces of oxygen. Even after saturation with nitrogen only minor amounts of the isomerisation product **3** are obtained. TLC on reversed phase shows instead three new main products which are more polar than **3**.

The UV-VIS-NIR recording of the photooxygenation reaction indicates the formation of the three products in a constant ratio. Their absorption spectra are similar to that of the isomerisation product **3**, and extinction coefficients of 37800 (680 nm), 71200 (442 nm) and 39700 (386 nm) have been determined for the mixture from the spectra shown in Fig. 2.

The structures of the two less polar products **4** and **5** have been determined by conversion to the methylpheophorbides **10** and **11**. Demetalation of the crude products to the pheophytin mixture (**7**, **8**, and others) is followed by transesterification (accompanied by methyl-ether formation in the case

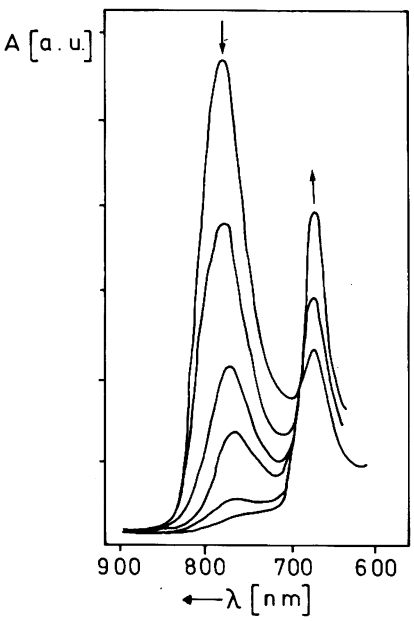
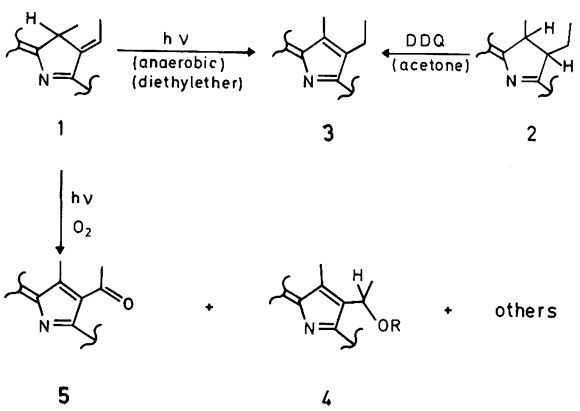


Fig. 1. UV-VIS-spectra of the photoreaction of BCHL *b* (5.2 μM) under anaerobic conditions. Irradiation in diethylether.



Scheme 1

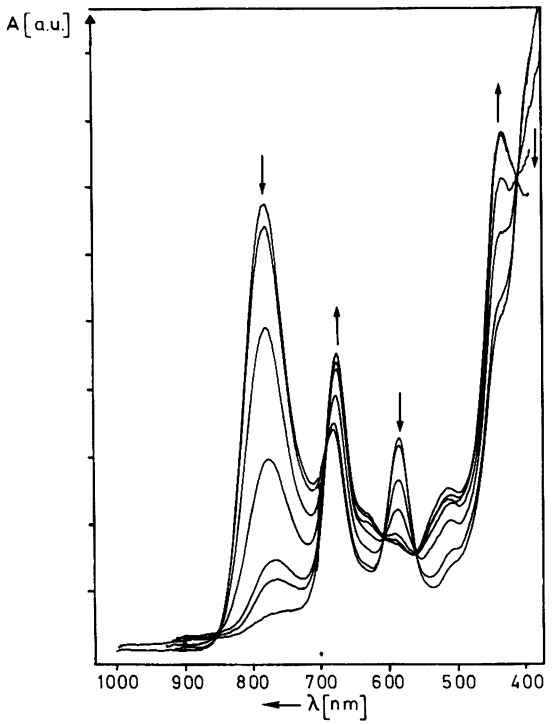


Fig. 2. UV-VIS-spectra of the photoreaction of BCHL *b* (8.1 μM) under aerobic conditions. Irradiation in chloroform with incandescent light (107 mW/cm^2). The individual traces correspond to irradiation times of 0, 3, 8, 13, 18, 28, 120 s. Isosbestic points are at 692, 612, 560 and 412 nm.

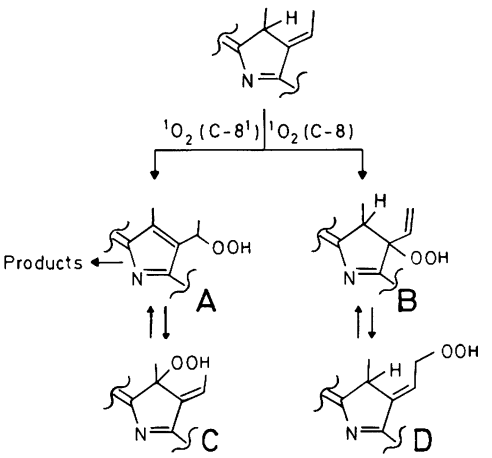
of **10**) to the methylesters **10** and **11**, which are then purified by preparative TLC. The fastest moving zone contains 3-acetyl-3-devinyl-8-(1-methoxy)ethyl-8-deethyl-methylpheophorbide *a* (**10**). The second moving zone yielded 3,8-diacetyl-3-devinyl-8-deethyl-methylpheophorbide *a* (**11**). The spectroscopic results obtained for **10** and **11** are comparable to the data given by Inhoffen *et al.* [14] for the respective pyromethylpheophorbides **12** and **13** lacking the 13^2-COOCH$_3$ group. The UV-VIS spectra of **10** and **12** are identical. The NMR-spectrum of **12** lacks one methyl singlet in the 3–4 ppm range corresponding to the 13^2-COOCH$_3$ group, and the methine singlets of **10** occur at slightly lower field. This may be due to the much lower concentrations of our samples, because aggregation of methylpheophorbides is known to produce upfield shifts (see Scheer and Katz [15] for references). The differences are somewhat more pronounced for the diacetyl compounds **11** and **13**. The Soret band is proportionally stronger in the pyro-derivative **13**. Most of the NMR-signals show a downfield shift which is most pronounced (about 0.7 ppm) for the 5-(10.24 *vs.* 9.76 ppm) and the 10-methine signal (10.52 *vs.* 9.86 ppm). Dilution shifts of this magnitude have been reported by Brockmann *et al.* [16] for the methylpheophorbide of a bacteriochlorophyll *d*, where they are related to strong aggregation *via* the 3^1-OH substituent. This would indicate a similarly strong aggregation tendency of the diacetyl-pigments **11** and **13** as compared to the mono-acetyl derivatives **10** and **12**, which has not been further elaborated here due to a lack of material.

Mechanism of the photooxidation

If judged from TLC, HPLC and UV-VIS-NIR analysis the same three oxidation products are obtained from the oxidative isomerisation of BCHL *b*, irrespective of the solvent used. The speed of the reaction is on the other hand, dependend on the solvent (Table I). Of the solvents studied, the reaction is fastest in 1,2-dichlorobenzene and slowest in petrol ether, where it is decreased by more than an order of magnitude. This appears to be a special solvent effect, for neither the polarity, nor the in first approximation similar solubility of oxygen can be related in a straightforward way to this effect. Since many tetrapyrrol pigments are excellent sensitizers for the formation of singlet oxygen [17], it is conceivable that this species is involved in the reaction. We have supported this in three different ways: A first indication was obtained from the addition of β-carotene to the reaction mixture. The conversion of BCHL *b* is slowed down by a factor of 5.7

Table I. Photoreactivity of BCHL *b* in various solvents. Halflife τ(1/2) (in s) of BCHL *b* (7.8 μM) under irradiation with white light from a 150 W tungsten halogen lamp (107 mW/cm^2):

Solvent	τ(1/2)
1,2-Dichlorobenzene	25
Petrolether	256
Methanol	50
Acetone	48
Chloroform	33
Benzene	30
i-Octane	145



Scheme 2

and 6.2 in the presence of an equimolar amount and a four-fold excess of β-carotene, respectively. Tlc analysis of the reaction mixture confirmed, that the same products are formed under these conditions. On the other hand the reaction rate is increased by a factor of 3.5 when adding a 50-fold excess of the singlet oxygen sensitizer methylene blue. Direct support comes from a test developed by Schenck *et al.* [18]. It allows a positive identification of singlet oxygen by the quantitative analayis of the reaction products of limonene, which is added in excess to the reaction mixture. We obtained the same six products in the expected ratios by illumination with white light (which excites BCHL *b* as well as its photoproducts) and also with far red light ($\lambda \leqq 740$ nm), which excites selectively BCHL *b*. This proves the formation of singlet oxygen during the photoreaction of BCHL *b*. Further support comes finally from the structure of the products. The diacetyl derivative **5** is the product expected from the "ene-reaction" (Scheme 2). There are two primary peroxides (A, B), and two further ones (C, D) which can arise from isomerisation. If judged from the exclusive formation of products containing the Δ7,8 double bond, the 8^1-substituted secondary peroxide A is the predominant isomer formed. The 8^1-hydroxyethyl-product **4** is somewhat unusual [17] but can derive from the same hydroperoxide A by reduction (disproportionation?). The latter, or another side reaction could also be responsible for the third, yet uncharacterized product.

Acknowledgements

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