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2-(1-HYDROXYETHYL)-2-DESVINYL CHLOROPHYLLIDE a: CHARACTERISATION BY NUCLEAR OVERHAUSER ENHANCEMENT PROTON MAGNETIC RESONANCE OF A NOVEL PIGMENT OBTAINED FROM MUTANTS OF RHODOPSEUDOMONAS SPHAEROIDES.

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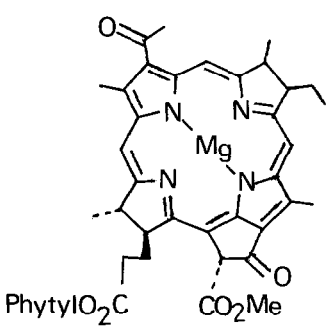
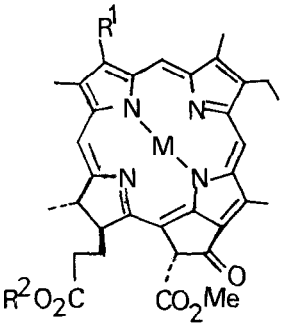
Abstract: The major pigment of mutants of Rp. sphaeroides was isolated and characterised as 2-(1-Hydroxyethyl)-2-desvinyl chlorophyllide a: this compound is a likely intermediate in the biosynthesis of bacteriochlorophyll a.

In the photosynthetic bacterium Rhodopseudomonas sphaeroides the major light harvesting pigment is bacteriochlorophyll a (1) the structure of which closely resembles that of green plant chlorophyll a. (2)[1]. Under conditions of high aeration, however Rp. sphaeroides obtains its energy from oxidation of substrates supplied in the medium. Rp. sphaeroides mutant 01 is unable to synthesise bacteriochlorophyll a but can grow aerobically and excretes a pigment which is a possible intermediate in bacteriochlorophyll biosynthesis. The visible spectrum is identical to a pigment accumulated when bacteriochlorophyll synthesis by Rp. sphaeroides is inhibited by 8-OH quinoline[2] or in a mutant of Rp. sphaeroides[3]. The pigment from these sources has never been fully characterised; its structure has always been proposed with the assumption that the substitution pattern resembles chlorophyll a, but the precise substitution sequence has not been determined. In this paper we describe a detailed examination of the structure of this pigment and find that it fits logically in a biosynthetic sequence leading from chlorophyllide a to bacteriochlorophyll a. In support of this view it has been found that production of pigment by 01 is greatly stimulated when haem synthesis is partially inhibited by N-methyl

protoporphyrin-IX, and protoporphyrin-IX is diverted to the magnesium branch of tetrapyrrole pigment biosynthesis[4].

Cells were grown under limited aeration at 30°C in the dark on the medium of Sistrom[5]. At the end of the log phase of growth, the cells were removed by centrifugation and the green supernatant was neutralised with $NaHCO_3$ and extracted twice with diethyl ether. The ether extract was washed with water, concentrated by evaporation, and the contaminating copro- and protoporphyrin removed by washing twice with an equal volume of 5% (w/v) HCl. The pigment was removed from the organic phase by extracting twice with an equal volume of 20% (w/v) HCl. After buffering the aqueous layer with aqueous saturated sodium acetate solution, the pigment was extracted into ether, and washed with water to remove traces of the acid. The ether was evaporated to dryness and was then esterified with 5% H_2SO_4 in methanol (w/v, 2h., 4°C). The chlorin ester was purified by column chromatography on alumina (grade IV, neutral), eluting with methylene chloride. Two fractions were obtained: a minor fraction identified as methyl phaeophorbide a, and the major, more polar fraction, the novel pigment (ca. 1mg).





(1)

(2) M = M_g ; R^1 = CH = CH_2 ; R^2 = Phytyl
(3) M = H_2 ; R^1 = CH = CH_2 ; R^2 = CH_3
(4) M = M_g ; R^1 = $CH(OH)CH_3$; R^2 = H

The isolated chlorin was analysed by UV-visible spectroscopy, and gave the following absorbances: (CH_2Cl_2 solution) λ max 659,602,531,502 and 407nm. The intense band at 659nm occurs at a shorter wavelength than the corresponding band of methyl phaeophorbide a (667nm) indicating that the chlorin did not contain a vinyl group. The spectrum also showed the chlorin is metal-free, magnesium being removed by the acid extraction.

The mass spectrum did not show a molecular ion (m/z 624), however significant ions at: m/z 609 (33%, M-15), 606 (6%, M-18), 548 (100%, M-76) were observed. The presence of a hydroxyl group was confirmed by acetylation and dehydration studies[2].

The peak assignments of the ^{1}HNMR spectrum (fig. 1.) were initially made by comparison with the spectrum of methyl phaeophorbide a (3)[6]. Decoupling studies confirmed the presence

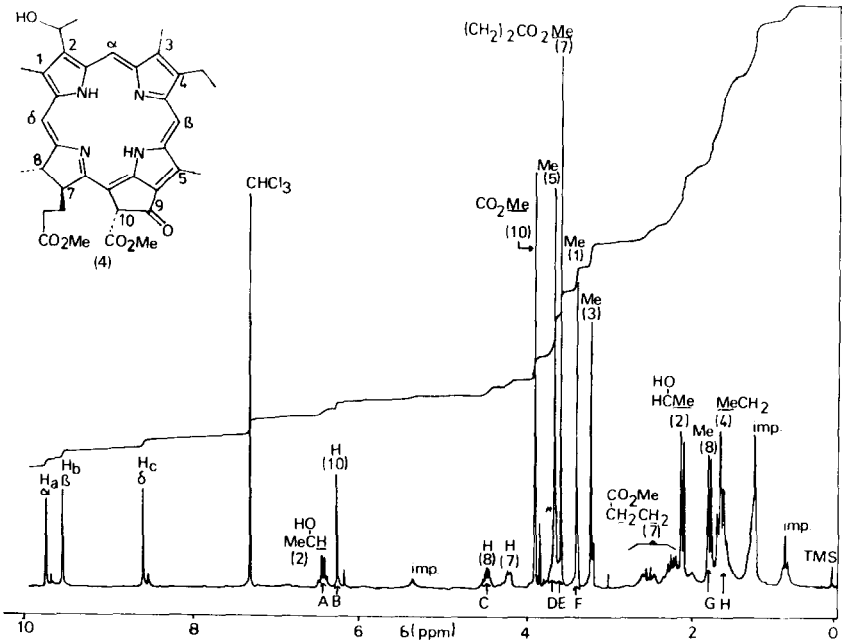


Fig. 1 ^{1}HNMR of the isolated chlorin showing peak assignments, and sites of irradiation for nOe studies (designated A_1B_1....H; see Table 1). The 4-CH_2CH_3 quartet coincides with the methyl signals.(*). (imp. = impurity)

Table 1: nOe (% increases) for meso protons of the mutant pigment 01.

Irradiated Site*	Group* (position)	% Enhancement # H_a	H_b	H_c	Comment
A	$CH_3CH(OH)$	7.5	-	-	H_a flanked by hydroxyethyl moiety
B	$HCCO_2CH_3$	-	-	-	Used as an internal blank
C	H(8)	-	-	4.6	H_c is the δ-meso H
D	$CH_2CH_3(4)+CH_3(5)$	-	3.2	-	H_b is the β-meso H
E	$CH_3(1)$	-	-	4.1	Confirms assignment of CH_3 at position 1 Fig. 1). A small enhancement (ca. 0.5%) 0.5%) of $CH_3CH(OH)$ fixes the hydroxyethyl at position 2, and fixes H_a at position α.
F	$HCCO_2CH_3(10)$	-	-	-	Used as an internal blank
G	$CH_3(8)$	-	-	2.5	Confirms irradiations C and E.
H	CH_2CH_3	-	0.5	-	Confirms irradiation D.

*See Fig. 1 # ^{1}HNMR nOe difference spectra were obtained using a Bruker WH400 NMR.

of the 2-(1-hydroxyethyl), 4-ethyl, 7-propanoate, 8-H and 8-methyl functionalities. The sequence of the substituents around the macrocycle was determined by ^{1}HNMR nuclear Overhauser enhancement (nOe) difference spectra (Table 1). Previous studies have shown that irradiation of ring-attached CH_3, CH_2 or CH substituents gives rise to enhancements of the adjacent meso (bridge) proton signal[e.g.7].

The unambiguous identification of the δ-meso proton and the assignment of the hydroxyethyl group to position 2 (Fig. 1; Table 1), allowed the isolated chlorin to be unambiguously assigned as (4) although the absolute configuration of the hydroxyethyl group was not determined. The results were confirmed by comparison with spectroscopic data for a racemic mixture of the hydroxyethyl derivative synthesised by the method of Richards and Lascelles[3].

The pigment has a similar substitution pattern to chlorophyll a (2), and does not bear any unusual alkyl substituents (e.g. n-propyl or iso-butyl). This is in contrast to bacteriochlorophylls c and d which also contain the 1-hydroxyethyl group at position 2[8]. The pigment occurs in the cell as the magnesium complex, 2-(1-Hydroxyethyl)-2-desvinyl chlorophyllide (5). It seems likely that this pigment, the major product of magnesium porphyrin synthesis in the mutant 01, is a true intermediate of the bacteriochlorophyll a synthesis pathway in wild-type Rp. Sphaeroides.

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