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Identification of [8-vinyl]-protochlorophyllide *a* in phototrophic prokaryotes and algae: chemical and spectroscopic properties[1]

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Abstract

[8-vinyl]-Protochlorophyllide *a* was isolated from a *Prochloron* sp. associated with the host ascidian, *Lissoclinum patella*. To obtain sufficient amounts for identification of the purified pigment, suitable extraction procedures and HPLC systems were developed. The structure was finally elucidated by UV-VIS and fluorescence spectroscopy, mass spectrometry and NMR (rotating-frame Overhauser enhancement spectroscopy). [8-vinyl]-Protochlorophyllide *a* was originally detected only as an intermediate in chlorophyll biosynthesis. Although its presence as a light-harvesting pigment was previously suggested in some prochlorophytes and eukaryotic algae, this is the first unequivocal demonstration of [8-vinyl]-protochlorophyllide *a* in an oxygenic phototroph. We also show that [8-vinyl]-protochlorophyllide *a* occurs in *Prochloron* species of four other ascidians as well as in *Micromonas pusilla* and *Prochlorococcus marinus*. The possible role of this pigment in photosynthesis is discussed.

Keywords: Evolution; HPLC; MALDI; Photosynthesis; Pheoporphyrin; Pigment extraction; Protochlorophyllide; ROESY; (*Prochloron*)

1. Introduction

Many marine algae and some oxygenic phototrophic prokaryotes contain pigments of the chlorophyll (Chl) *c* family. They are characterized by a fully unsaturated tetrapyrrole ring system, while chlorins (Chls *a* and *b*) and bacteriochlorins (such as bacterio-Chl *a*) have one and two reduced (hydrogenated) pyrrole rings, respectively. With one exception (a phytol-esterified Chl *c* [1]), all Chls *c* are free acids (for recent reviews, see [2,3]).

Although marine algae make a major contribution to world photosynthesis and CO_2 fixation, the Chl *c* group is relatively poorly studied [2]. This might be

Abbreviations: AAS, atomic absorption spectroscopy; Chl, chlorophyll; DV-Pchlide, [8-vinyl]-protochlorophyllide *a*; ESI, electrospray ionization; LHC, light-harvesting complex; MALDI, matrix-assisted laser desorption/ionization; MV, monovinyl; ODS, octadecyl silica; ROESY, rotating-frame Overhauser enhancement spectroscopy

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[1] The nomenclature for chlorophylls is somewhat confusing: [8-vinyl]-protochlorophyllide was known as Mg-2,4-divinyl-pheoporphyrin a_5-monomethyl ester (old Fischer nomenclature). We prefer the name divinyl-protochlorophyllide (DV-Pchlide) which is commonly used in chlorophyll biosynthesis.

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Table 1
Review of some selected algal species in which a DV-Pchlide-like pigment was detected

Algal division	Algal class	Species	Ref.
Chlorophyta	Prasinophyceae	*Prasinoderma coloniale*	[9]
		Prasinococcus capsulatus	[9]
		Bathycoccus prasinos	[37]
		Micromonas pusilla	[9,38]
		Mamiella gilva	[38]
		Mantoniella squamata	[9,39]
		Pseudoscourfieldia marina	[39]
Haptophyta	Prymnesiophyceae	*Emiliania huxleyi*	[40]
		Prymnesium parvum	[40]
Chrysophyta	Pelagophyceae	*Pelagococcus subviridis*	[40]
Cryptophyta	Cryptophyceae	*Cryptomonas maculata*	[41]
		Chroomonas sp.	[41]
Prochlorophyta		*Prochloron* sp.	[11]
		Prochlorococcus marinus	[10]
?[a]		*Acaryochloris marina*	[36]

[a]Since now, there is no conclusive decision about the systematic classification of this Chl *d* containing organism.

due to the fact that Chls *c* occur always in combination with Chl *a* (e.g. in brown algae, Phaeophyceae) or Chl *a* and *b* (e.g. in some Prasinophyceae [4]). However, Chls *c* can contribute a wide range, from less than 1 up to 40%, of the total Chl content [5]. Although the initial separation from the esterified pigments is simple, the further purification of Chls *c* on a quantitative scale is complicated by their polar and oxygen-sensitive character. Hence, a number of members of the Chl *c* family are still only characterized by HPLC, UV-VIS and fluorescence spectra [3], methods which can provide only tentative structures.

The structures of only three members, Chl c_1, c_2 and c_3 are fully elucidated by accepted spectroscopic and chemical methods. A structurally unconfirmed representative of the Chl *c* family is [8-vinyl]-protochlorophyllide *a* (DV-Pchlide, Fig. 1), also known as Mg-2,4-divinyl-pheoporphyrin a_5-monomethyl ester (former Fischer nomenclature). It was originally described in *Rhodobacter sphaeroides* as an intermediate in Chl biosynthesis [6], and investigations on this intermediate were performed with only small amounts resulting in poorly resolved ^{1}H-NMR and mass spectra [7,8].

DV-Pchlide was first postulated in some marine eukaryotic flagellates as an accessory pigment by comparison of the UV-VIS spectrum [9]. During the last 30 years, DV-Pchlide-like pigments have been found in a variety of algae (see review Table 1). Recently, this pigment type was also discovered in prokaryotic prochlorophytes [10,11], but no structural proof was given.

We isolated the DV-Pchlide-like pigment in sufficient quantities from a *Prochloron* sp., where it has

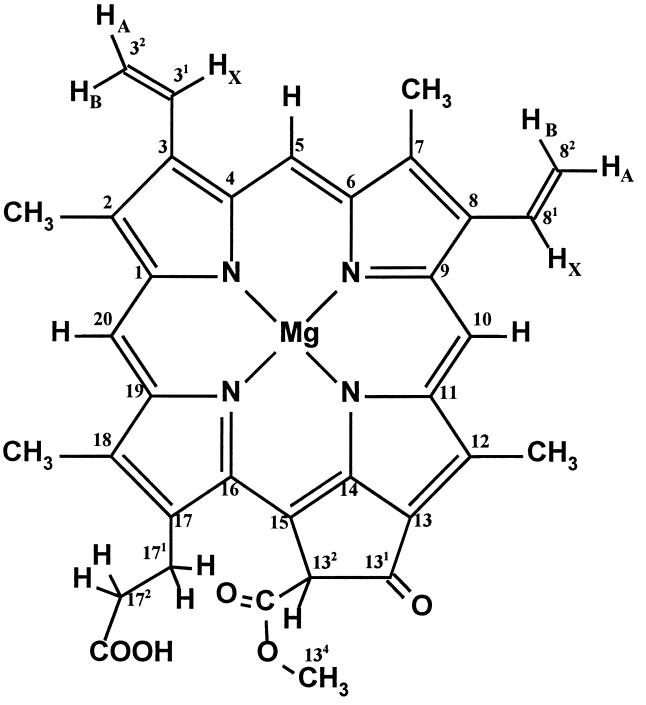

Fig. 1. Structure of [8-vinyl]-protochlorophyllide *a* (DV-Pchlide) showing the IUPAC/IUB numbering system.

been previously shown to take part in photosynthesis [11] and elucidated its structure by mass spectrometry, ^{1}H-NMR, UV-VIS and fluorescence spectroscopy.

2. Materials and methods

2.1. General

All work was carried out under green safety light from commencement of pigment extraction. The solvents were either of HPLC grade or distilled before use. Diethyl ether was refluxed for 2 h over KOH before distillation.

2.2. Algae collection and culturing

Prochloron sp. was harvested from the ascidian *Lissoclinum patella* at Heron Island, Great Barrier Reef, Australia, as described by Larkum et al. [11]. The pigments of *Prochloron* sp. from all small-sized ascidians (also collected at Heron Island) were directly extracted: organisms where first ground in a mortar with sand and a small amount of 1 M Tris buffer (pH 7.8) and then immediately extracted with acetone. After centrifugation, the supernatant was directly applied to HPLC. A frozen sample of *Prochlorococcus marinus* was supplied by Miles Furnas, Townsville, Queensland, Australia, and a freeze-dried sample of *Prochlorothrix hollandica* was obtained from H. Matthijs, Amsterdam, Holland. *Micromonas pusilla* (obtained from S. Jeffrey, Hobart, Tasmania, Australia) was cultured in F2 medium [12] and harvested by centrifugation. *R. sphaeroides* (mutant V3) was obtained from C.N. Hunter (University of Sheffield, UK) and cultured in Medium A [13] supplemented with 100 mM 5-aminolevulinic acid.

2.3. Extraction of pigments

Frozen *Prochloron* cells (80 g wet weight) were extracted with a total of 1.5 l of acetone by grinding with a small amount of sand and liquid nitrogen in a mortar. Acetone was added and the suspension filtered through a sintered glass frit. The residue was re-extracted several times with 80–100% aqueous acetone until the extract was a very light green. Each filtrate was immediately transferred into a separation funnel containing 1 l diethyl ether and 1 l water. The acetone was then removed by washing the organic layer several times with water. This crude extract contained approximately 230 μmol of Chl *a*. To extract the DV-Pchlide according to the method of Schoch et al. [14], the crude extract was diluted with the same volume of petroleum spirit (b.p. 40–60°C) and extracted twice with 150 ml and then twice with 75 ml of cold methanol/10 mM ammonia (4/1, v/v). The combined alkaline methanol extracts were washed with a total amount of 1.5 l of petroleum spirit/diethyl ether (1/1, v/v) and then concentrated on a rotary evaporator to about half of the original volume. The pigment was finally extracted into ethyl acetate, evaporated to dryness and dried for 36 h on a freeze-drier. To prevent oxidation, the DV-Pchlide remained in methanol for the shortest possible time. This crude DV-Pchlide preparation contained approximately 8.3 μmol DV-Pchlide with small amounts of xanthophylls, chlorophyllides and pheophorbides. It was further purified by HPLC.

2.4. Chromatography

HPLC apparatus: Waters 600 pump with a quaternary gradient mixer, a Shimadzu 10AV diode array detector (200–800 nm) and a Shimadzu RF-551 fluorescence detector. Column systems: (1) semi-preparative (10×250 mm) or analytical (3.9×150 mm) ODS column, packed with 10 and 3 μm Hypersil ODS (Alltech, Batch 3084), respectively. (2) Semi-preparative (10×250 mm) or analytical columns (4.6×250 mm) were hand-packed with dry polyethylene powder (Type 6560 J-1, Sinclair-Koppers, Port Arthur, TX) as described in [15]. For elution programs see Table 2.

HPLC separation: the DV-Pchlide crude extract was first purified on the semi-preparative ODS column.[2] An aliquot of about 200–300 nmol DV-Pchlide per run was injected in 100% acetone. The

[2] It was important to introduce ammonium acetate into the elution solvent. In the presence of this ion-pair forming reagent, fewer impurities (absorbing below 350 nm) in the crude extract can form unspecific ion-pairs with the free carboxylic acid group of DV-Pchlide: this results not only in a better purification, but also in sharper peaks.

Table 2
Elution programs for HPLC

	Time (min)	% A	% B	% C
Analytical ODS	0	85	15	0
Flow: 0.75 ml/min	15	10	90	0
	25	0	70	30
R_f (DV-Pchlide) = 21.4 min	35	0	70	30
	38	0	100	0
	42	85	15	0
Semi-preparative ODS	0	75	25	0
Flow: 3 ml/min	25	15	85	0
	30	0	85	15
R_f (DV-Pchlide) = 21 min	33	0	70	30
	35	0	100	0
	40	75	25	0
Anal. polyethylene	0	80	20	
Flow: 1 ml/min	25	0	100	
	35	0	100	
R_f (DV-Pchlide) = 11 min	40	80	20	
Semi-prep. polyethylene	0	80	20	
Flow: 4 ml/min	1	80	20	
	20	40	60	
R_f (DV-Pchlide) = 18 min	22	0	100	
	27	0	100	
	30	80	20	

Solvent composition for reverse-phase (ODS) systems: (A) 50% 250 mM aqueous ammonium acetate, 25% methanol, 25% acetonitrile; (B) 50% methanol, 50% acetonitrile; (C) ethyl acetate. Solvent composition for polyethylene systems: (A) 50% 200 mM aqueous ammonium acetate, 50% acetone; (B) 100% acetone.

desired pigment fractions were collected and immediately freeze-dried or extracted into ethyl acetate. The dry fractions were combined by re-dissolving them in a small amount of pyridine and re-chromatographed on polyethylene powder. As the capacity of this system is lower compared to the ODS system, only about 100–150 nmol DV-Pchlide could be separated per run. The purification achieved was checked after each step by both analytical HPLC and mass spectrometry (MALDI). Without special effort to improve the yield, we isolated approximately 3.5 mg of pure DV-Pchlide at this particular purification. Isolated pigments were stored either dry or in pyridine under Ar at 253 K for at least one year without formation of noteworthy amounts of degradation products.

2.5. Spectroscopy

If not otherwise stated, mass spectra were obtained by a MALDI mass spectrometer (Bruker Biflex III, supplied with a N_2 laser) using 5-amino quinoline as matrix. Acid matrices including gentisic acid and sinapic acid give only molecular ions of the demetallated pigment. Some samples were also investigated by ESI mass spectrometry (Finnigan LCQ; HPLC: HP1100; loop injection; eluent: acetonitrile/H_2O, 80/20). Positive ion mode was used for detection in both MS systems.[3]

For NMR sample preparation, approximately 3.75 mg of DV-Pchlide (pool from several preparations) were dried for 2 days in a desiccator over KOH in vacuo. The pigment was dissolved in 650 μl pyridine-d_5 (99.94% D; Cambridge Isotope Laboratories), centrifuged to remove any undissolved crystals and transferred to a 5 mm NMR tube in a final volume of 600 μl. Before sealing with the usual plastic cap, Ar was blown over the surface to prevent oxidation.

NMR spectrometer: Bruker DMX 600 instrument equipped with a self-shielded triple-axis gradient triple-resonance probe-head; acquisition temperature: 300 K. Calibration of the NMR spectra was done using solvent signals: the signal for the proton of pyridine in the meta-position was calibrated to 7.1808 ppm [16].

The one-dimensional proton spectrum was recorded using a standard pulse-sequence. The 90° pulse used for all experiments was determined as 10.7 μs. We recorded 16K complex data-points covering a spectral sweep-width of 11.97 ppm. Allowing a 1.8 s relaxation delay, 256 scans were summed; the spectrum was transformed using resolution optimized exponential filtering to a size of 32K data points.

The two-dimensional ROESY experiment was recorded using a standard pulse sequence as described in the literature [17]. A spectral width of 11.97 ppm

[3] The purification grade of DV-Pchlide has to be very high for both systems, as even minor impurities can lead to erroneous mass peaks: the free acid pigment is able to carry along impurities from the crude extract by forming ion-pairs. These adducts can easily dissociate in the mass spectrometer: however, most of these compounds are more sensitive to the ionizing method than the pigment itself and therefore give markedly stronger peaks.

was recorded in both dimensions. 2K complex data points in t_2 and 256 complex data points in t_1 where recorded by averaging 48 scans. The relaxation delay was set to the same value as in the one-dimensional experiment. A mixing time of 300 ms and a field-strength of 2.5 kHz was employed as a continuous-wave spin-lock. As window functions, we employed $2\pi/3$ shifted squared sine windows in both dimensions. The data where Fourier-transformed to a size of 1K×4K data points.

UV-VIS spectra were measured at room temperature on a double beam spectrophotometer (UV 2401 PC, Shimadzu) in either pure pyridine or dry diethyl ether and acetone: the latter two containing 1% pyridine.

The molar extinction coefficient (ε) in pyridine was calculated using atomic absorption spectroscopy (AAS) to determine pigment concentrations. Due to the relatively strong binding of Mg in DV-Pchlide, the method of Porra et al. [18] had to be modified: to avoid solubility problems, DV-Pchlide was completely dissolved in pyridine to give an approximate extinction of 8.6 at 638 nm. After recording UV-VIS spectra of several diluted samples between 350 and 750 nm, five samples containing each 300–500 μl, representing about 100–200 nmol pigment were dried in Eppendorff tubes by blowing off the solvent with Ar in a heat block at 333 K. After re-dissolving the pigment in 50 μl pyridine, 50 μl of twice distilled water and 150 μl of HCl (37%, AAS grade) were added. The tubes were kept at room temperature for 48 h to ensure complete demetallation. The samples were adjusted with more bi-distilled H_2O to a final volume of 1000 μl and subjected to AAS. Control samples were prepared with Chl c_1, c_2 and without any pigment (blank control). The ε values obtained for the reference pigments were lower by a factor of 1.5 compared to reported values in the literature [19]. Normalizing the ε values determined for DV-Pchlide by multiplying by the factor obtained for the Chls *c*, we calculated a millimolar extinction coefficient of 24 for the absorption maximum at 638 nm in pyridine.

Extinction coefficients in solvents other than pyridine were determined indirectly: 30 μl of a pigment solution in pyridine (with $E_{445} = 50$ in the Soret band) were added to a cuvette containing 2970 μl of either pyridine, acetone or dry diethyl ether. The averaged spectra of 4–5 measurements in each solvent were compared by using the previously determined extinction coefficients in pyridine.

Fluorescence emission spectra were recorded at both room and liquid nitrogen temperature (77 K) in 99% diethyl ether/1% pyridine on a Hitachi F-4500.

3. Results and discussion

3.1. Extraction and purification

The initial studies on the pigments of *Prochloron* species revealed two major and several minor carotenoids together with Chl *a* and *b* [20–22]. The Chl *c*-like pigment was not detected in these studies. It was probably masked by co-elution with the easily formed breakdown product chlorophyllide *a*. Using a HPLC system, Larkum et al. [11] have shown at least one Chl *c*-like pigment in a *Prochloron* sp. which was isolated from the ascidian *L. patella*. The extraction of a pigment which is only about 5% of the total Chl content (and even less of the total pigment content), needs an effective extraction and initial purification step before further purification. It was possible to separate this polar pigment from all unpolar pigments by a simple extraction of all free acids from a crude pigment preparation in ether using slightly alkaline aqueous methanol. Besides DV-Pchlide, this extract only contained breakdown products of Chl *a* and *b* (i.e. chlorophyllide and pheophorbide derivatives) and some very polar xanthophylls of not determined structure.

The DV-Pchlide crude extract was chromatographed on a reverse-phase ODS column. A remaining pheophorbide derivative, co-eluting with DV-Pchlide, was completely removed by the polyethylene column.

The only by-product formed during purification or prolonged standing of pure DV-Pchlide was a more polar pigment which by its identical UV-VIS spectrum could be the 13^2-hydroxy derivative. This probably corresponds to the second Chl *c*-like pigment detected in *Prochloron* in a previous study [11].

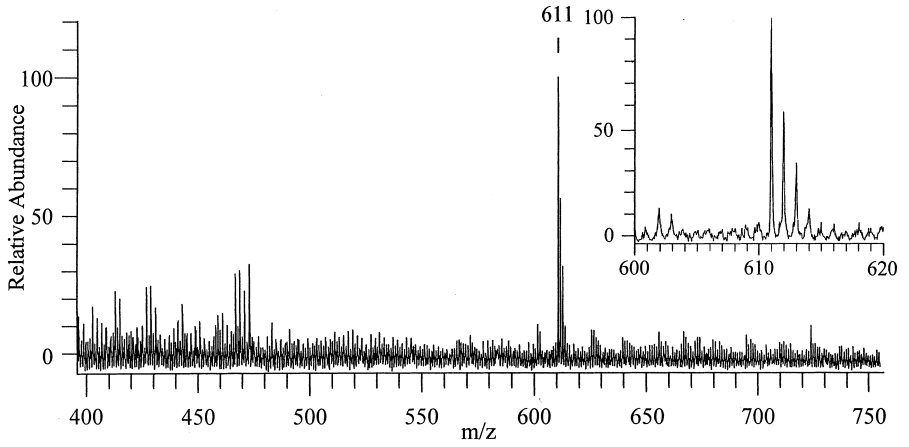

Fig. 2. MALDI mass spectrum of DV-Pchlide ($C_{35}H_{30}N_4O_5Mg$). The choice of matrix was essential for obtaining a correct molecular peak. The present spectrum was obtained with 5-amino quinoline as a matrix (inset: molecular peak region). Usage of acid matrices resulted in demetallation of the pigment to give [8-vinyl]-protopheophorbide *a* ($C_{35}H_{32}N_4O_5$; +H = 589 mu).

3.2. Mass spectra

Because of the chemical instability of monomeric chlorophylls, caused by a labile magnesium atom and the tendency to form allomerization products, they require a mass spectrometry technique with a soft ionizing method. We used MALDI and ESI. The latter gave a molecular peak at $m/z = 589$ which is consistent with the demetallated, protonated DV-Pchlide (= [8-vinyl]-protopheophorbide *a*; $[C_{35}H_{32}N_4O_5+H]^+$). Although there was no acid used in the HPLC eluent, it was not possible to obtain a spectrum of the Mg complex: comparable chlorin-type pigments did not show such a propensity for demetallation, although their demetallation rates in acids are generally higher [23].

The MALDI technique combined with an alkaline matrix prevented demetallation and gave a mass peak of $m/z = 611$ $[M+H]^+$ (Fig. 2). No fragmentation was observed under the conditions used. The calculated isotopic pattern for $C_{35}H_{30}N_4O_5Mg+H$ was compared with the measured molecular peak (Fig. 2, inset): m/z (% calculated; % found) = 611 (100; 100), 612 (54; 56), 613 (28; 34), 614 (9; 13). The complete protonation of the molecular ion is different to previously measured protochlorophyllide derivatives in the fast atom bombardment technique [24].

3.3. NMR spectra

The one-dimensional ^{1}H-NMR spectrum of DV-Pchlide (Fig. 3A) testifies the high purification grade of the pigment. Only several signals in the low field and some minor signals in the high field region (marked with asterisks) are either due to solvents, remaining column material (polyethylene; broad signals), or algal impurities. An unambiguous assignment of all protons was straightforward with the help of ROESY. A net of through-space correlation peaks can be found, starting at the proton of C-13^2 and going clockwise around the molecule to the methyl group of C-12 (see Table 4 below). The four multiplets at 3.4 and 4.5 ppm (Fig. 3B) can be assigned to the protons of the 17-propionic acid chain: two multiplets within each set are fairly symmetrical. Although the carbons 17 and 18 are not asymmetric as in chlorins, the protons of the pro-

Table 3
Coupling constants for the ABCD spin system of the propionic acid side chain, obtained by simulation of the coupling pattern with NUMARIT [42]

Proton pair	Coupling constant ± S.D.
J_{A1B1}	15.2 ± 0.2
J_{A1A2}	9.6 ± 0.2
J_{A1B2}	6.3 ± 0.2
J_{B1A2}	6.3 ± 0.2
J_{B1B2}	9.8 ± 0.2
J_{A2B2}	16.4 ± 0.2

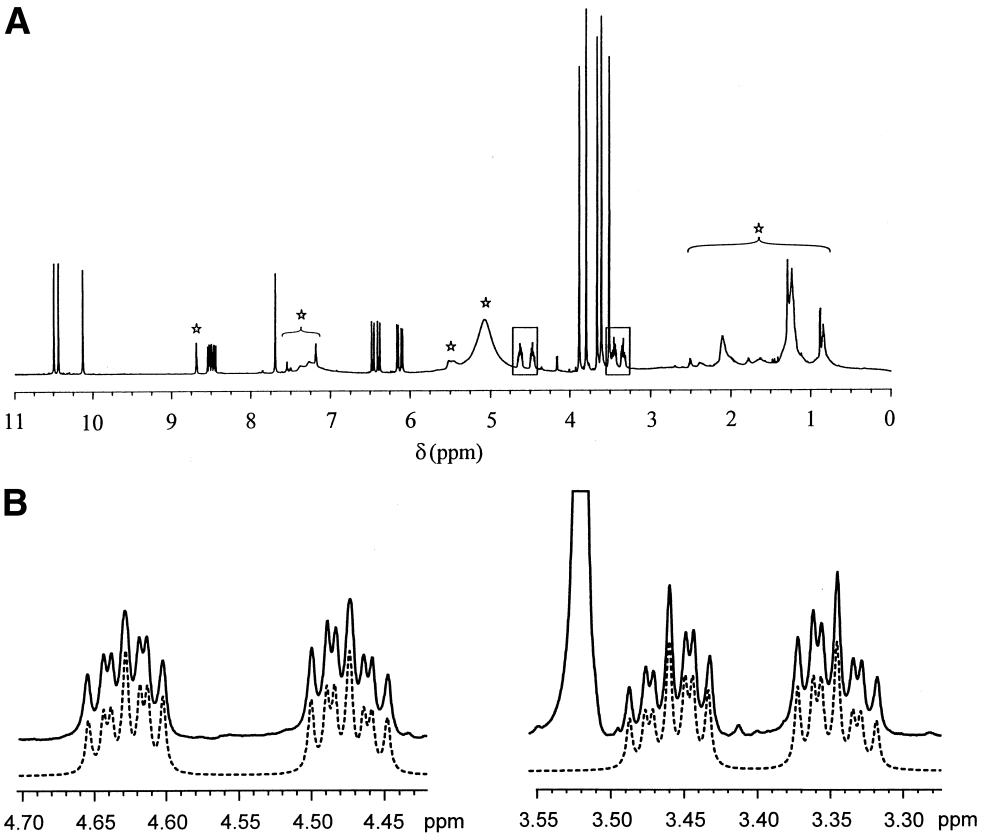

Fig. 3. (A) ^{1}H-NMR spectrum of DV-Pchlide in pyridine-d_5 at 300 K. Signals marked with an asterisk are either solvent signals (pyridine, water) or remaining impurities (e.g. column matrix). (B) Measured (——) and simulated (- - -) ^{1}H-NMR spectrum for the 17-propionic acid side chain (see boxes in panel A) to establish the coupling constants of the ABCD spin system (see Table 3).

pionic acid group still form an ABCD system due to the chiral centre at C-13^2. To work out coupling constants, the coupling pattern of the two signals was simulated (Fig. 3B). The results are given in Table 3 and show the expected large geminal coupling constants (15–16 Hz) and two sets of vicinal coupling constants between 6 and 9 Hz.

The ROE correlations (Table 4) clearly show that the 3-vinyl protons occur at lower field than the corresponding 8-vinyl protons. Considering the proximity of the 13^2-oxo group, Wu and Rebeiz [8] erroneously assigned the 8-vinyl protons of DV-Pchlide to lower field; thus, it is more likely that differences in the electron density of the pyrrole rings A and B influence the magnetic ring current which, in turn, accounts for the differences in the chemical shifts. Another explanation could involve a difference in the average conformation of the vinyl groups to the macrocycle. A detailed conformation analysis of the vinyl groups (together with the propionic acid side chain) will be published elsewhere. The following can be stated without further analysis: firstly, the integrated ROE signals of both vinyl groups are very similar which makes it unlikely that the orientation of the vinyl groups accounts for their differences in the chemical shifts. Secondly, the vinyl groups are both orientated towards the corresponding methyl groups (as drawn in Fig. 1). This is in agreement with NMR data of heme [25], but in contrast to the X-ray data of ethyl chlorophyllide, where the 3-vinyl group is orientated in the opposite direction [26]. Whether these conformation differences are due to the structural differences or whether they are an effect of the solvent cannot be decided here.

3.4. UV-VIS and fluorescence spectra

UV-VIS spectra of Chls *c* are very solvent sensitive [19]. A second difficulty arises from the low solubility of extensively purified porphyrins in usually well-

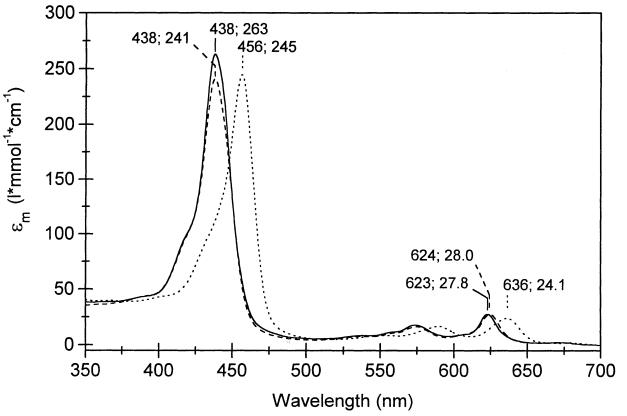

Fig. 4. Quantitative UV/Vis spectra of DV-Pchlide measured in diethyl ether/pyridine (99/1; ——), acetone/pyridine (99/1; - - -), and pure pyridine (···) at room temperature.

suited solvents for Chls including diethyl ether, tetrahydrofuran and acetone. We therefore used pyridine, as recommended by Jeffrey [19], as the standard solvent. Because pyridine is regarded as an unsafe solvent in marine laboratories, we also measured spectra in acetone and dry diethyl ether, containing each 1% of pyridine which is necessary to ensure a quantitative comparison between the three solvents. The spectra (Fig. 4) exhibit typical porphyrins with low intense Q bands and strong B (Soret) bands. The 438 and 623 nm absorption maxima in diethyl ether containing 1% pyridine are identical to those of DV-Pchlide determined in the pure solvent [6,7]. Compared to the monovinyl (MV) derivative, the maximum of the B band is shifted by 7 nm to longer wavelengths [27]. This bathochromic shift is typical for the substitution of an ethyl group versus a vinyl group as can be observed for the pigment pairs Chl c_1/Chl c_2 (4 nm) and MV-Chl *a*/DV-Chl *a* (7 nm), respectively [3]. The use of pure pyridine as a solvent for DV-Pchlide causes a bathochromic shift of the absorption maxima. This can be explained most probably not only by a general solvent effect but also by a change in the coordination number of the central Mg from 5 to 6 [28,29].

As no extinction coefficients were available for DV-Pchlide, we determined values using the magnesium concentration, determined by AAS, to establish pigment concentrations. As outlined in Section 2, we can only offer tentative extinction coefficients for DV-Pchlide. Nevertheless, as our calculated ε_{mM} value in acetone at 624 nm is close to 30 (see Fig. 4), it would be quite similar to 30.4 for the MV derivative in 80% acetone [30]. It should be mentioned that the ε_{mM} values for MV-Pchlide also vary from 22.0 to 35.6, a disagreement which has been discussed in detail by Porra [31].

Fluorescence emission spectra (Fig. 5) are not mir-

Table 4
^{1}H-NMR assignments and ROE correlations for DV-Pchlide

Proton	δ (ppm)	ROE cross signal with protons
2^1	3.61	20, 3^1, 3^2 (A,B)
3^1 (H_X)	8.52[a]	2^1, 3^2 (A,B), 5
3^2 (H_B, *trans*)	6.47[a]	2^1, 3^1, 3^2 (A), 5
3^2 (H_A, *cis*)	6.16[a]	2^1, 3^1, 3^2 (B), 5
5	10.44	3^1, 3^2 (A,B), 7^1
7^1	3.67	5, 8^1, 8^2 (A,B)
8^1 (H_X)	8.46[b]	7^1, 8^2 (A,B), 10
8^2 (H_B, *trans*)	6.39[b]	7^1, 8^1, 8^2 (A), 10
8^2 (H_A, *cis*)	6.10[b]	7^1, 8^1, 8^2 (B), 10
10	10.50	8^1, 8^2 (A,B), 12^1
12^1	3.80	10
13^2	7.69	13^4, 17^1, 17^2
13^4	3.89	13^2, 17^1, 17^2[c]
17^1	4.47, 4.63	13^2, 13^4, 17^2, 18^1
17^2	3.34, 3.45	13^2, 13^4[c], 17^1, 18^1[d]
18^1	3.52	17^1, 20
20	10.13	2^1, 18^1

[a] J_{AX} = 11.5 Hz, J_{BX} = 17.8 Hz, J_{AB} = 1.53 Hz.
[b] J_{AX} = 11.4 Hz, J_{BX} = 17.8 Hz, J_{AB} = 1.53 Hz.
[c] The 17^2 proton at lower field shows a markedly stronger cross peak to the 13^4 methyl protons.
[d] Visible only after large expansion of the region close to the diagonal.

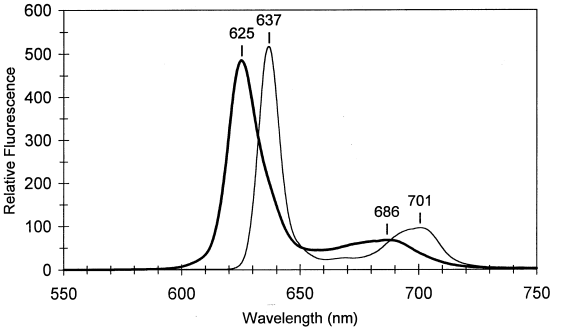

Fig. 5. Room temperature (293 K; bold line) and low temperature (77 K; thin line) fluorescence emission spectra (λ_{exc} = 439 nm) of DV-Pchlide in diethyl ether/pyridine (99/1). The low temperature spectrum renders the far-red emission band more clearly which is accompanied by a markedly bathochromic shift.

ror images of the absorption spectrum, suggesting that more than one transition is involved to form the Q band. The low-temperature (77 K) spectrum is characterized by the typical line-sharpening effect combined with a distinct bathochromic shift. The latter may be again an evidence for a change from coordination number 5 to 6, as the higher coordination number is preferred at low temperatures. Both effects render the far-red emission band (686 and 701 nm, respectively) more clearly. Excitation spectra at these emission wavelengths were identical to those measured at the emission wavelengths of 625 and 637 nm, proving that the far red band is not based on a Chl *a* impurity. This band might play a key role for the function of Chls *c* as light-harvesting pigments: excited energy could be transferred via this emission band to Chl *a*. The fluorescence quantum yields of Chls c_1 and c_2 in organic solvents have been found to be higher than those of Chl *b* [19].

3.5. DV-Pchlide from other sources

In addition to *L. patella*, we have screened several other organisms for DV-Pchlide by HPLC (Table 5).

The pigment composition of acetone extracts from four other *Prochloron*-hosting ascidians, namely *Diplosoma virens* and *Diplosoma similis*, *Tridemnum cyclops* and *Tridemnum miniatum*, were qualitatively very similar; however, the DV-Pchlide content varied between 3 and 15% of the total Chl content. Interestingly, we were able to detect traces of pheophorbide *d*, the demetallated and hydrolyzed breakdown product of Chl *d*, in two rigorously extracted ascidians, namely *D. similis* and *T. cyclops*. In those ascidians where the algae where carefully squeezed out of the carbohydrate test, we could not detect any traces of this pigment.

Table 5
Extracts from several other organisms were screened for DV-Pchlide by HPLC (conditions see Section 2)

Species	Presence of DV-Pchlide
Diplosoma virens	+
Diplosoma similis	+
Tridemnum cyclops	+
Tridemnum miniatum	+
Prochlorococcus marinus	+
Prochlorothrix hollandica	−
Micromonas pusilla	+
Rhodobacter sphaeroides V3	+

The Prochlorophyte, *P. marinus*, was also reported to contain DV-Pchlide [10]. Analysis with our HPLC systems and also by co-chromatography with the authentic standard confirmed this report. Because we knew about the existence of DV-Chl *a* and *b* in *P. marinus*, we re-examined the esterified Chls of *Prochloron* by HPLC, UV-VIS and ^{1}H-NMR spectroscopy: however, we unambiguously confirmed the presence of only the monovinyl form for both Chl *a* and *b*. As a representative of the third genus of Prochlorophytes, *P. hollandica* was examined, but no DV-Pchlide could be detected in this species.

We have also isolated a DV-Pchlide-like pigment from *M. pusilla*. This pigment co-eluted with that from *Prochloron* and had identical UV-VIS and mass spectra. It confirms that DV-Pchlide also occurs in a member of the Prasinophyceae.

We finally isolated DV-Pchlide from *R. sphaeroides* where it was first described [6]. An acetone extract of the mutant V3, which accumulates DV-Pchlide, revealed several intermediates of Chl-biosynthesis; however, the most abundant pigment on the chromatogram was identified as DV-Pchlide by co-chromatography with DV-Pchlide isolated from *Prochloron*.

4. Conclusions

We have unequivocally demonstrated for the first time the occurrence of DV-Pchlide in *Prochloron* sp.; previously, only tentative and inconclusive suggestions of its occurrence in this organism have been reported [11]. Although the complete structure elucidation was performed only on the pigment isolated from *Prochloron* sp., it is now very likely that DV-Pchlide-like pigments of many other algae are of the same nature. We have shown this for *M. pusilla* and *P. marinus* by co-chromatography with authentic DV-Pchlide, using specially developed HPLC systems, and also by mass spectrometry. Considering the great diversity of the Chl *c* family, a structure other than DV-Pchlide cannot be excluded in those algae where DV-Pchlide was postulated only on the basis of UV-VIS spectra and HPLC retention times.

The existence of DV-Pchlide in these disparate

groups of algae poses interesting questions. In *Prochloron* it has been shown not only that DV-Pchlide is located in the light-harvesting Chl protein but also is active in light harvesting [11]. This light-harvesting property for DV-Pchlide has also been shown in *Pseudoscourfieldia marina* [32] and *Mantoniella squamata* [4]. Thus, this pigment is not only an intermediate in Chl biosynthesis but also serves a light-harvesting function in some algae. Raven [33] has demonstrated theoretically that Chl *c*-type pigments can make an appreciable contribution to the light-harvesting capacity even for organisms which also contain Chl *b*. Thus the question arises: Why is DV-Pchlide (or any other Chl *c*) absent in concentrations required for light harvesting in *P. hollandica* which is a member of the prochlorophyte family, and from green algae in general except for the Prasinophyceae, to which *M. pusilla*, *M. squamata* and *P. marina* belong?

For light-harvesting purposes, DV-Pchlide must be diverted from Chl biosynthesis before hydrogenation by either 8-vinyl-reductase or protochlorophyllide-oxidoreductase, as this pigment is a good substrate for both enzymes [34]. MV-Pchlide, the most abundant intermediate in Chl *a* biosynthesis, has not been detected in algae in amounts comparable to DV-Pchlide. This suggests the presence of a transport/binding mechanism which carries a portion of the DV-Pchlide intermediate from its site of formation to its location as a light-harvesting pigment in the LHC proteins.

Attempts to culture the symbiotic prochlorophyte *Prochloron* without its host (*L. patella*) have generally failed [35]. Recently, Miyashita et al. [36] obtained a new algal species while trying to culture *Prochloron* from the same ascidian. This alga, *Acaryochloris marina*, contains Chl *d* as the major pigment. As we could find traces of this long-wavelength-absorbing pigment in those extracts where we mortared the entire ascidian in the presence of acetone, we conclude that *A. marina* or another Chl *d*-containing organism is situated at the more inaccessible inner surface of the carbohydrate test of the ascidian. Even with a layer of *Prochloron* on top, there will be sufficient light in the far red region for the alga. Interestingly, also *A. marina* contains a DV-Pchlide-like pigment [36].

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