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Chlorophyll catabolism in *Chlorella protothecoides*

Isolation and structure elucidation of a red bilin derivative

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When grown in a medium rich in glucose but poor in nitrogen, the algae *Chlorella protothecoides* excrete a red pigment the structure of which has been proven to correspond to a product of oxydative ring cleavage of the chlorophyll-*a* chromophore at the C4–C5 methine bridge.

Chlorophyll-*a* catabolism; 19-Formyl-1[21H, 22H]bilinon derivative

1. INTRODUCTION

Despite of its enormous significance both in plant physiology and environmental sciences, the fate of chlorophylls in senescent chloroplasts is still an enigma [1–4]. The major contribution on this research area has been done just recently by Matile et al. [5–9], who have shown that the first steps of chlorophyll breakdown in senescent barley leaves parallel, to some extent, the degradation of haeme in vertebrates. Our interest in chlorophyll catabolism in *Chlorella* emerged from the observation made some time ago by Oshio and Hase, that these green algae, when grown in a medium rich in glucose or acetate but poor in nitrogen (urea), excrete certain red pigments into the incubation medium, the formation of which proceeds simultaneously with the disappearance of chlorophyll from the algal cells [10]. As, to the best of our knowledge, the structures of these red pigments produced during the 'bleaching' of *Chlorella* cells have not been further investigated previously, Hase's work has been reproduced in our laboratory resulting in the isolation of a bilin derivative structurally related to chlorophyll *a*.

2. EXPERIMENTAL

The studies reported in the present work were carried out with material isolated from cultures of the algae *Chlorella protothecoides*, which were a gift from Prof. Horst Senger (Universität Marburg, Germany). The same strain had been used earlier by E. Hase et al. [11], and was originally supplied from the Algal Culture Collection at the University of Indiana (USA) labeled as ACC no. 25. The culture medium employed was essentially the same as described in [10] but modified in order to maintain a constant pH of 6.8 during incubation and to control the level of available nitrogen. Thus, $(NH_4)_2HPO_4$ was used, instead of urea, as nitrogen source, since we found that in sterile filtered preculture media containing urea, rather limited growth of the above strain was detectable. On heat sterilization of the urea containing medium, however, free ammonia, which is traceable with Nessler's reagent, is generated by hydrolysis, thus explaining the normal growth of the cells reported in [10] under these conditions.

The basal medium (BM) used contained per l: K_2HPO_4 (8.7 g), KH_2PO_4 (6.8 g), $MgSO_4 \cdot 7\,H_2O$ (0.3 g), $FeSO_4$ (0.01 M, 1 ml, acidified with 5 ml of 2 N H_2SO_4) and 1 ml of oligoelement solution ARNON A5 [12]. Preculture solutions (100 ml) were prepared by heat-sterilizing the BM together with $(NH_4)_2HPO_4$ (1.0 M, 0.2 ml). After addition of 10% (w/v) aqueous glucose (10 ml), which had been previously sterilized by heating, and of 0.1 ml of Nitsch and Nitsch vitamin solution (Sigma), the preculture medium was inoculated with *C. protothecoides* cells, taken from a standard agar slant, and shaken in the dark at 25°C for 7–15 days. Greening cell cultures were prepared by adding 5 ml of the above preculture to 100 ml of BM containing 1.5 ml of $(NH_4)_2HPO_4$ (1.0 M) and 0.1 ml of vitamin solution. The cultures were shaken at 25°C under illumination with white fluorescent light (4000 lx) for 5–10 days. Thereafter, the cells were centrifuged and washed twice with 50 ml of 0.1 M potassium phosphate buffer (pH 6.8). Bleaching cell cultures were obtained when the washed cells were transferred to 100 ml BM, containing 0.1 ml of vitamin solution and 10 ml of 10% aqueous glucose, and the cultures were shaken at 25°C in the dark. After 50 h the red nutrient broth was separated from the cells by centrifugation and filtration of the supernatant over a thin layer of Cellite. Algae cells can be recycled for the greening process after washing with sterile buffer solution. To isolate the red pigments from the culture medium, an aliquot (1 l) of the above filtrate was shaken with 100 ml of diethyl ether, the organic layer was discarded, and the aqueous phase was extracted repeatedly with diethyl ether (3 × 200 ml), after addition of 10 g of citric acid. The combined organic phases were repeatedly washed with 1% aqueous citric acid (5 × 100 ml), dried shortly over Na_2SO_4 and the solvent was removed on a rotary evaporator. After separation of the mixture of pigments by preparative thin layer chromatrography (PTLC) on silica gel 60 (Merck) using $CHCl_3/CH_3OH$ (95:5) as eluant, the main product (R_f 0.35) was extracted from the stationary phase with ethanol and further purified by repeated precipitation from a conc. $CHCl_3$ solution with a large excess of *n*-hexane. The precipitate was separated by filtration through a cotton plug, washed with *n*-hexane, and dried in vacuo to yield ca. 1 mg of the pure pigment (**1a**) with UV/vis [λ_{max} (rel. intensity) in CH_3OH]: 320 (1), 455 (0.25), 490 (0.38), 525 (0.36), and 580 (0.2).

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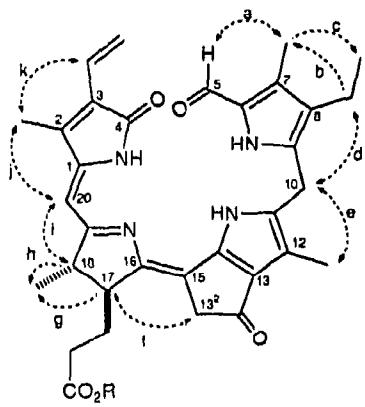
The purified pigment was transformed into the corresponding methyl ester as follows: to a solution of **1a** in dry tetrahydrofuran (2 ml) carbonyldiimidazole (50 mg) was added. The mixture was allowed to stand at 25°C for 24 h and, thereon, 2 ml of CH_3OH, containing 3 drops of a 1% solution of CH_3ONa in CH_3OH, were added. After 5 h, the reaction mixture was diluted with 30 ml of diethyl ether and shaken with 50 ml of 5% aqueous citric acid. The organic phase was washed with water, dried over Na_2SO_4, and the residue obtained after evaporation of the solvent under reduced pressure was purified by PTLC as above. The product (R_F 0.7) was extracted from silica gel with ethanol and dried in vacuo to yield **1b** with UV/vis [λ_{max} (rel. intensity) in CH_3OH]: 315 (1), 450 (0.33), 483 (0.39), 525 (0.33) and 580 (0.19).

3. RESULTS

The structures of the red pigment from *C. protothecoides* and its methyl ester (**1a** and **1b**, respectively) were elucidated by spectroscopic analysis. A first hint was given by the pattern of the ^{1}H-NMR spectrum of **1a** in CD_3OD as solvent, which shows all characteristic signals for the peripheric substituents of pyropheophorbide-*a*. However, all signals are high field shifted with respect to those of the latter, thus suggesting that π-electron delocalization in the macrocycle has been interrupted either by reduction of bridging double bond(s) or by cleavage of the macrocycle at one of the methine bridges. Accordingly, a Soret band at λ_{max} ca. 400 nm is absent in the UV/vis spectrum of **1a**. Therefore, the down field singlet at δ 9.36 ppm has to be assigned to an aldehydic H-atom rather than to a methine proton. Although in $CDCl_3$ as solvent the ^{1}H-NMR spectrum is significantly less resolved, three additional broad singlets corresponding to NH groups appear at δ 9.7, 10.0, and 10.5 ppm. Moreover, the diastereotopic H atoms at C10, which in CD_3OD give rise to a singlet at δ 4.05 ppm, are manifested by an AB-system with J = 15.6 Hz in $CDCl_3$. The presence of a carboxylic acid group in the molecule of **1a** is evidenced by the fact that the pigment can be dissolved in aqueous Na_2CO_3 and extracted, after acidification with citric acid, with an organic solvent. As expected, after esterification a singlet at δ 3.56 ppm is present in the ^{1}H-NMR spectrum of **1b**. The bulk structure of **1a** was convincingly confirmed by the high resolution FAB mass spectrum of the corresponding methyl ester (**1b**), which established the molecular formula to be $C_{34}H_{38}N_4O_5$ (calc. for $[C_{34}H_{39}N_4O_5]^+$: 583.2915; found: m/z 583.2926 $(MH)^+$; Xe at 8 kV in a glycerol/1-thioglycerol matrix). Thus, compared with methyl pyropheophorbide-*a* ($C_{34}H_{36}N_4O_3$), the molecular formula of **1b** contains *two* atoms of oxygen and *two* atoms of hydrogen more. The number of H-atoms corresponds to that found in the ^{1}H-NMR spectrum. Evidence concerning the site at which the putative biogenetic precursor of **1a**, pyropheophorbide-*a*, has been oxidatively cleaved was furnished by the characteristic pattern of the ABX system assigned to a vinyl group adjacent to a lactam carbonyl in the bile pigment series [13]. Moreover, the same pattern has been observed in the 9,10-dehydro derivative of **1b**, which has been characterized recently (as the Cd complex) in our laboratory as



1a R = H
b R = CH_3
Fig. 1.

Table I

^{1}H-NMR signals of compound **1a** assigned by ^{1}H{^{1}H}-NOE difference experiments

Irradiated signal[a]	Enhanced signal[b]	% Enhancement	Assignment in formula **1a**[c]
9.36	2.25 (*s*, 3 H-7^1)	3.7	a
6.63	2.19 (*s*, 3 H-2^1)	2.7	k
6.03	2.19	5.3	j
	2.86 (*qd*, *J* = 7.3 & 1 Hz, H-18)	3.3	i
4.05	2.03 (*s*, 3 H-12^1)	2.3	e
	2.42 (*q*, *J* = 7.6 Hz, 2 H-8^1)	1.3	d
3.35	2.63 (*dm*, *J* = 9 Hz, H-17)	small	f
2.86	1.19 (*d*, *J* = 7.3 Hz, 3 H-18^1)	2.5	h
	6.03 (*s*, H-20)	3.3	i
2.63	1.19	2.5	g
	3.35 (*d*, *J* = 20.4 Hz, $H_{pro\text{-}R}$-13^2)	small	f
2.42	2.25	1.0	b
	4.05 (*s*, 2 H-10)	0.9	d
2.25	0.95 (*t*, *J* = 7.6 Hz, 3 H-8^2)	2.5	c
	9.36 (*s*, H-5)	2.6	a
2.19	6.03	1.4	j
	6.63 (*dd*, *J* = 17.6 & 11.5 Hz, H-3^1)	2.7	k
2.03	4.05	0.6	e

[a] δ in ppm (CD_3OD, 360.13 MHz) referred to CHD_2OD (δ = 3.30) as internal standard.

[b] The other resonance signals were assigned on the base of their multiplicity and chemical shifts: 6.32 (*dd*, *J* = 17.6 & 2.1 Hz, H_{trans}-3^2), 5.51 (*dd*, *J* = 11.5 & 2.1 Hz, H_{cis}-3^2), 3.60 (*d*, *J* = 20.4 Hz, $H_{pro\text{-}S}$-13^2), 2.23 (*m*, 2 H-17^2), 1.93 and 1.64 (2*m*, 2 H-17^1).

[c] Corresponding signals for **1b**: 9.48 (*s*, H-5), 6.61 (*dd*, *J* = 17.7 & 11.6 Hz, H-3^1), 6.35 (*dd*, *J* = 17.7 & 2.4 Hz, H_{trans}-3^2), 6.05 (*s*, H-20), 5.53 (*dd*, *J* = 11.6 & 2.4 Hz, H_{cis}-3^2), 4.05 (*s*, 2 H-10), 3.65 (*s*, OCH_3), 3.55 (*d*, *J* = 20.5 Hz, $H_{pro\text{-}S}$-13^2), 3.35 (*d*, *J* = 20.5 Hz, $H_{pro\text{-}R}$-13^2), 2.83 (*qd*, *J* = 7.2 & 1.5 Hz, H-18), 2.63 (*ddd*, *J* = 9.4, 3.4 & 1.5 Hz, H-17), 2.43 (*q*, *J* = 7.6 Hz, 2 H-8^1), 2.37 (*dd*, *J* = 7.8 & 7.0 Hz, 2 H-17^2), 2.26 (*s*, 3 H-7^1), 2.21 (*s*, 3 H-2^1), 2.04 (*s*, 3 H-12^1), 1.98 (*ddt*, *J* = 14.0, 7.8 & 3.4 Hz) and 1.68 (*ddt*, *J* = 14.0, 9.4 & 7.0 Hz) (2 H-17^1), 1.19 (*d*, *J* = 7.2 Hz, 3 H-18^1), 0.97 (*t*, *J* = 7.6 Hz, 3 H-8^2).

the main product of the in vitro photooxidation of the cadmium chelate of methyl pyropheophorbide-*a* [14]. At last, structure **1a** has been confirmed by a series of NOE correlation experiments which are depicted on formula **1** (cf. Table I). Despite substantial efforts on NOE measurements, however, an interaction between H-13^2 and H-17 could not be conclusively established (cf. ref. [15]). Nevertheless, considering both the probable biogenesis of **1a** and the unequivocal correspondence of the chemical shifts assigned to the ^{1}H-atoms in the proximity of the C15-methine bridge of **1b** with those of the Cd complex of its 9,10-dehydro derivative [14], the geometry of the bridging olefinic bond between C15 and C16 is assumed to be Z. On the other hand, the same absolute configurations found in chlorophylls [16,17] are tentatively assigned to the asymmetric atoms C17 and C18.

4. DISCUSSION

Structure **1a** of the red biline derivative excreted into the incubation medium by 'bleaching' *Chlorella protothecoides* cells resembles that of a chlorophyll catabolite recently isolated from chloroplasts of senescent barley leaves [18]. This similarity is particularly interesting as both on the basis of theoretical considerations [19] and of the structure of some luciferins isolated from bioluminiscent plancton [15,20] electrophilic attack at the methine bridges *adjacent* to the reduced pyrrole ring had been predicted as the initial step of enzymatic chlorophyll breakdown (cf. ref. [21]). Though almost 30 years ago, in the porphyrin series, it was claimed that a formylbilinone analogous to **1a** had been obtained by cleavage of the haeme chromophore by a liver enzyme [22,23], the structure of the isolated product could not be confirmed later [24]. Thus, compound **1a** together with the chlorophyll-*a* catabolite reported in ref. [18] represents the first examples of natural products, whose structures correspond to those of the photooxidation products of porphyrins and chlorins in vitro [24].

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