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A Biomimetic Partial Synthesis of the Red Chlorophyll-a Catabolite from *Chlorella protothecoides*.

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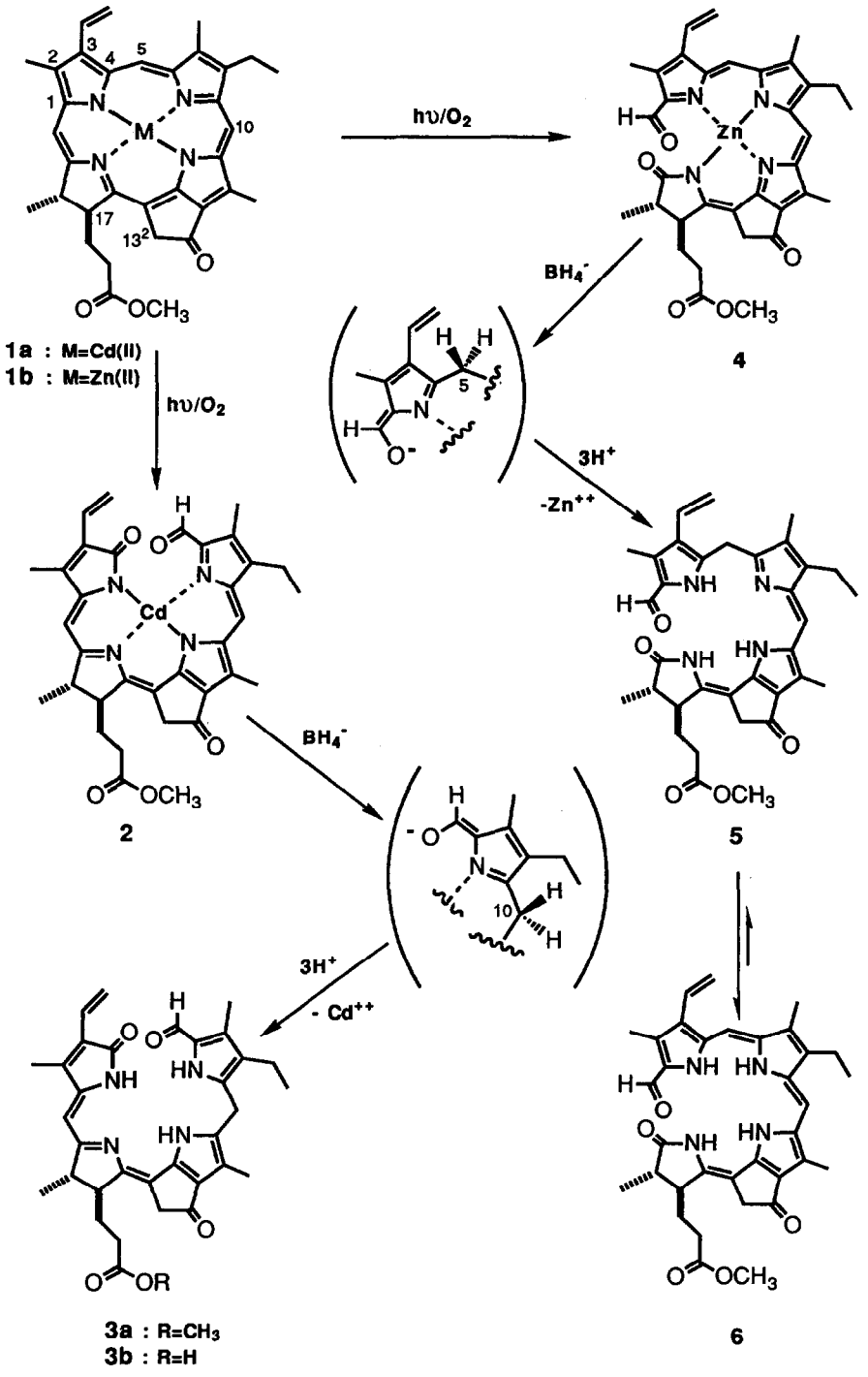
Abstract : The red bilin derivative which is excreted in the culture medium by *Chlorella protothecoides* when this microalgae is grown in a medium rich in glucose but poor in nitrogen has been synthesized in two steps from one of the products of photo-oxidation of the Cd(II) complex of pyropheophorbide-a methyl ester. Under the same conditions, the Zn(II) complex of the latter is transformed into a blue bilin derivative which is an isomer of the foregoing chlorophyll-a catabolite.

Recently we reported that the regioselectivity of the photo-oxidative ring cleavage of the chlorophyll macrocycle depends on the complexed metal ion.[1] Thus, whereas irradiation of the Zn(II) complex of pyropheophorbide-a methyl ester (**1b**), in the presence of oxygen, leads chiefly to the corresponding 19,20-dioxo-19,20-*seco*-derivative **4**, the corresponding Cd(II) complex **1a** yields a mixture of bilins, in which the product of ring cleavage at the C(4)-C(5) bond (i. e. **2**) preponderates.

Simultaneous success in the isolation and characterization of **3b** as a primary product of chlorophyll catabolism excreted into the culture medium by *Chlorella protothecoides*, when this microalgae is grown in a medium rich in glucose but poor in nitrogen [2], stimulated the present work, in which the transformation of the above mentioned Cd(II) chelate **2** into the red bilin derivative resulting from enzymatic breakdown of the chlorophyll-a macrocycle is described. This synthetic approach is particularly attractive since it possibly mimics the biological pathway of chlorophyll-a breakdown both in *Chlorella* and in dark-bleached chloroplasts of barley leaves, from which a pigment analogous to **3b** (so-called RP-14) has been recently characterized.[3]

The transformation of **2** into **3b** involves three reactions : hydrogenation of the C(10) methine bridge, demetallation of the chelate and hydrolysis of the methyl ester. As, however, earlier attempts to isolate metal free formylbilinones derived both from porphyrins and chlorins failed [4,5], the hydrogenation step has been carried out first, expecting that the reduced metal chelate should be easier to become demetalled.


hυ/O2
BH4-
1a : M=Cd(II)
1b : M=Zn(II)
hυ/O2
4
3H+
-Zn++
2
BH4-
5
3H+
- Cd++
3a : R=CH3
3b : R=H
6

In order to reduce the chromophore of **2**, $NaBH_4$ was chosen since biliverdins can be smoothly transformed into bilirubins with this reagent, without affecting other functional groups present in the molecule.[6,7] The regioselectivity of this reaction has been rationalized in terms of the atomic orbital coefficients in the LUMO which are larger at the central than at the external methine bridges [8-10] thus rendering the former proner for nucleophilic attack.[11] In default of corresponding theoretical data for partially reduced bilinone chromophores of the type present in **2**, a prediction of the regioselectivity of hydride ion addition to **2** is not straightforward. Nevertheless, preferred reduction at the C(10) methine bridge may be anticipated for two reasons : i) In the chlorin series, the π-electron density at the methine bridges which are adjacent to the pyrroline ring is enhanced with respect to the other bridge positions [12,13] and ii) at least in the resonance structure represented by formula **2** the C(9)=C(10) bond is conjugated to the formyl group, the electron-withdrawing effect of which renders the C(10) bridge position the most electrophilic in the molecule. In fact, reaction of **2** with $NaBH_4$ in tetrahydrofuran as solvent, followed by acidification, led to **3a** in 65% yield after purification. Fortunately, the aldehyde function is not reduced in the short time required for the completion of the reaction, probably because in presence of Cd^{++} ions in the solution $NaBH_4$ is significantly less reactive towards aldehydes (cf. ref. [14]).

Hydrolysis of the methyl ester **3a** under basic conditions afforded the corresponding carboxylic acid **3b** in 86% yield, after purification. All analytical data of **3b** and **3a** agree with those of the red bilin isolated from *Chlorella protothecoides* and its methyl ester, respectively.[2] Thus, the foregoing described procedure confirms the structure proposed in ref.[2] for the natural compound by its six-step synthesis from chlorophyll-a as the starting material. Particularly, the geometry of the C(15)=C(16) bond, which remained uncertain in the ^{1}H-NMR spectrum of the natural compound, has been now unequivocally established to be Z by NOE correlations between $CH_2(13^2)$ and CH(17) as well as $CH_2(13^2)$ and ($CH(17^1)$ in both the carboxylic acid **3b** and its methyl ester **3a**.

Under the same conditions used for **2** the 19,20-*seco*-derivative **4** was reduced with $NaBH_4$. Surprisingly, however, the obtained demetalled pigment is blue instead of red, as it would be expected for the same chromophore as in **3a**. Though the parent peaks in the mass spectra of the blue pigment and of **3a** (m/z 582) correspond to the same molecular formula for both compounds, fragment ions at m/z 446 and 150 (both arising from the loss of the formylpyrrole ring) are present in the mass spectrum of **3a**, whereas the corresponding fragment ions at m/z 448 and 148 are virtually absent in the mass spectrum of the reduction product of **4**. Moreover, the presence of two singlets at δ 5.51 and 5.69 ppm in the^{1}H-NMR spectrum of the blue pigment points out that **6**, rather than its tautomer **5**, is the actual structure of the reduction product of **4**. In $CDCl_3$ solution, indeed, the ^{1}H-NMR spectrum reveals the presence of two species of similar structure (cf. Exp. Part). As NOE experiments manifest a transference of saturation between pairs of corresponding resonances, the duplicity of signals may be explained by the presence of two diastereomeric helical conformations of the molecule **6** which are mutually in equilibrium. In acetone as well as in a mixture of $CDCl_3$

and CD_3OD the ^1H-NMR spectrum becomes simpler although the resonance signals are broader, thus indicating that the mutual transformation of both conformers is faster in these solvents.

In order to find out which methine bridge of **4** is primarily reduced in the course of its transformation into **6**, a reduction experiment was carried out with $NaBD_4$. In the ^1H-NMR spectrum of the pigment obtained after quick work up of the reaction mixture the intensity of the singlet assigned to CH(5) was decreased by approximately 62%. When the reaction mixture was allowed to stand for a longer time in the presence of acid, exchange of D for H was virtually complete. Both the ready exchange of the H atoms on C(5) and the preference of tautomer **6** in relation to **5** in neutral medium parallel the transformation of protonated biladienes-*ac* (10,23-dihydro-(21H)-bilins) into bilatrienes (22,24-dihydro-(21H)-bilins) which is well-documented in the literature.[15]

Contrarily, reduction of **2** with $NaBD_4$ leads, after demetallation, to the red pigment **3a** with a ratio of CH(10) to CD(10) of 1:1, so that, as expected from the structure of the chromophore, no subsequent exchange occurs under acidic conditions. On the other hand, the unequal distribution of deuterium label between the two diastereotopic H atoms on C(10) (approx. 3 : 2) indicates that asymmetric control of the approach of BD_4^- by the chiral part of the molecule takes place to some extent.

Although not yet characterized as a catabolite of chlorophyll-a, **6** may be present in the mixtures of pigments extracted both from *Chlorella* cultures [16] and dark-bleached chloroplasts of barley leaves.[17-19] On the other hand, the synthesis and characterization of different products of ring cleavage of chlorophylls is of fundamental interest because it should improve the actual insufficient knowledge of chlorophyll breakdown in living systems.

EXPERIMENTAL

Melting points (m.p.) were determined on a Kofler hot stage apparatus (C. Reichert AG. Vienna) and are uncorrected. UV and visible spectra were recorded with a Hewlett Packard 8452 A Diode Array spectrophotometer using CH_2Cl_2 solutions ; λ_{max} are quoted in nm and band intensities in parentheses, as log ε. ^1H-NMR spectra were performed by F. Fehr at 360.13 MHz with a Bruker AM-360 instrument equipped with an Aspect 3000 data system. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane, as internal standard, and coupling constants (J) in Hertz. Spin multiplicities are indicated by symbols *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), and *m* (multiplet). ^{13}C-NMR spectra were measured in a Varian Gemini-200 instrument at 50.3 MHz in $CDCl_3$ as internal standard (δ = 77.0 ppm). ^1H- and ^{13}C-NMR assignments are based on NOE correlations and Attached Proton Test (APT) and/or chemical shifts. Mass spectra (MS) were obtained by F. Nydegger with a Vacuum Generator Micromass 70 70E instrument equipped with a DS 11-250 data system

using the fast atom bombardment (FAB) ionization technique in 3-nitrobenzyl alcohol with Ar at 8 kV, at an acceleration voltage of 6 kV. All chemicals were reagent grade and solvents were distiled prior to use. Thin layer chromatography (TLC) aluminium sheets precoated with silica gel 60 for analytical purposes and silica gel 60 $PF_{254+366}$ for preparative TLC (1.25 mm thick, 20 x 20 cm) were purchased from E. Merck (D-6100 Darmstadt).

10,22-Dihydro-4,5-dioxo-4,5-secopyropheophorbide-a Methyl Ester (**3a**) : To a solution of **2**[1] (5 mg) in 1 ml of CH_2Cl_2 an excess of $NaBH_4$ (1-2 mg) was added, and the mixture was diluted with 10 ml of tetrahydrofuran. When the colour of the solution changed from greenish to blue it was poured into water (200 ml), acidified with aq. HCl (32%, 0.5 ml) and extracted with CH_2Cl_2. The organic layer was separated and the residue obtained after evaporation of the solvent *in vacuo* was chromatographed on silica gel plates using CH_2Cl_2/ acetone (8 : 2) as eluant. The main reddish component was extracted with acetone and, after evaporation of the solvent, the residue was crystallized from CH_2Cl_2/n-hexane to yield 2.9 mg (65%) of **3a**. M.p.: 128-129 °C ; MS : 583 (M+H); UV/vis : 312 (4.40), 462 (3.78), 496 (3.90), 534 (3.89), 582 (3.64) ; ^{1}H-NMR (8.8 x 10^{-3}M in $CDCl_3$): 10.37, 10.17 and 9.27 (NH), 9.38 (s, CHO), 6.42(*dd*, *J*=17.5, *J*=11.2, $CH(3^1)$), 6.26 (*dd*, *J*=17.5, *J*=2.5, $CH(3^2)_{trans}$), 5.66 (*s*, CH(20)), 5.46 (*dd*, *J*=11.2, *J*= 2.5, $CH(3^2)_{cis}$), 3.96 and 3.91 (2 x *d*, *J*=16.1, $CH_2(10)$), 3.64 (*s*, OCH_3), 3.50 and 3.32 (2 x *d*, *J*=20.0, $CH_2(13^2)$), 2.69 (*dq*, *J*=7.3, *J*=1.7, CH(18)), 2.60 (*dm*, *J*=8.8, CH(17)), 2.38 (*q*, *J*=7.6, $CH_2(8^1)$), 2.28 (*s*, $CH_3(12^1)$), 2.26 (*m*, $CH_2(17^2)$), 2.20 (*s*, $CH_3(7^1)$), 2.12 (*s*, $CH_3(2^1)$), 1.96 and 1.70 (2 x *m*, $CH_2(17^1)$), 1.21 (*d*, *J*=7.3, $CH_3(18^1)$), 0.96 (*t*, *J*=7.6, $CH_3(8^2)$). ^{13}C-NMR: 192.8 ($CO(13^1)$) 176.4 (CHO), 173.2 ($C(17^3)$), 176.9, 171.2, 152.9, 150.2, 145.7, 139.1, 134.8, 134.5, 131.7, 129.4, 128.7, 128.5, 124.9, 122.3 (13 quat. C's and CO(4)), 125.1 ($C(3^1)$), 116.3 ($C(3^2)$), 111.5 (C(15)), 98.2 (C(20)), 51.7 (CH_3O), 49.9 and 47.5 (C(17) and C(18)), 44.7 ($C(13^2)$), 30.9 ($C(17^2)$), 28.5 ($C(17^1)$), 23.0 (C(10)), 18.8 ($C(18^1)$), 17.0 ($C(8^1)$), 15.1 ($C(8^2)$), 9.6, 9.3, 8.8 ($C(2^1)$, $C(7^1)$ and $C(12^1)$).

10,22-Dihydro-4,5-dioxo-4,5-secopyropheophorbide-a (**3b**) : KOH (100 mg) was added to a solution of **3a** (5.1 mg) in 40 ml of methanol-water (3:1) and the mixture was stirred in the dark during 5 h. Then, it was poured into water (200 ml) and shaken with CH_2Cl_2 The organic layer was separated and the dark red residue obtained after evaporation of the solvent under reduced pressure was purified by prep. TLC using CH_2Cl_2/acetone/ methanol (20 : 4 : 1) as eluant. The main reddish band was extracted with acetone/methanol (7 : 3) and the residue obtained after evaporation of the solvent was precipitated from CH_2Cl_2 with hexane to yield 4.3 mg (86%) of pigment **3b**, the analytical data of which were identical with those reported earlier for the natural product.[2]

21,23-Dihydro-19-20-dioxo-19,20-secopyropheophorbide-a Methyl Ester (**6**) : was obtained by reduction of **4**[1] (10 mg) under the same conditions used for **3a**. On the chromatography plate the main blue product (4.3 mg ; 51%) is followed by a red-violet trail. After extraction of both products with acetone the red-violet pigment was re-chromatographed

under the same conditions to yield additional 0.9 mg of **6**, so that the total yield amounted to 62%. M.p.>150 °C (dec.) ; MS : 583 (M+H) ; UV/vis : 314 (4.45), 388 (4.36), 586 (4.35). The ^{1}H-NMR spectrum reveals two isomers present in equilibrium in a ratio of 2 : 1. Major component : δ_H : 11.36, 11.93, 11.68 and 9.3 (4*s*, NH), 8.60 (*s*, CHO), 6.56 (*m*, $CH(3^1)$), 5.69 (*s*, CH(10)), 5.51 (*s*, CH(5)), 5.32 (*m*, $CH_2(3^2)$), 3.73 (*s*, OCH_3)), 3.47 and 3.26 (2 × *d*, *J*=19.8 ($CH_2(13^2)$), 2.53 (*m*, $CH_2(8^1)$ and CH(17)), 2.34 (*s*, $CH_3(12^1)$), 2.0-2.3 (*m*, $CH_2(17^2)$ and CH(18)), 2.07 (*s*, $CH_3(7^1)$), 1.98 (*s*, $CH_3(2^1)$), 1.85 and 1.62 (2 × *m*, $CH_2(17^1)$), 1.24 (*t*, *J*=7.3, $CH_3(8^2)$), 1.06 (*d*, *J*=7.4, $CH_3(18^1)$). Minor component : δ_H : 12.12, 12.10, 11.71 and 9.82 (4*s*, NH), 8.65 (*s*, CHO), 6.56 (*m*, $CH(3^1)$), 5.68 (*s*, CH(10)), 5.45 (*s*, CH(5)), 5.32 (*m*, $CH_2(3^2)$), 3.63 (*s*, OCH_3)), 3.39 and 3.25 (2 × *d*, *J*=19.4, $CH_2(13^2)$), 2.53 (*m*, $CH_2(8^1)$ and CH(17)), 2.30 (*s*, $CH_3(12^1)$), 2.0-2.3 (*m*, $CH_2(17^2)$ and CH(18)), 2.05 (*s*, $CH_3(7^1)$), 2.00 (*s*, $CH_3(2^1)$), 1.85 and 1.62 (2 × *m*, $CH_2(17^1)$), 1.22 (*t*, *J*=6.7, $CH_3(8^2)$), 0.65 (*d*, *J*=7.4, $CH_3(18^1)$).

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