A-α-hydroxymesobiliverdin, a New Bile Pigment

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Bile Pigment, A-α-hydroxymesobiliverdin, Phycocyanobilin

Isolation, identification and characterization of a new bile pigment, A-α-hydroxymesobiliverdin, is described.

Introduction

In a previous paper [1] we discussed biliverdin IXα as an intermediate of phycocyanobilin biosynthesis (also compare [2–4]). The experiments involved the incubation of red algae of the species *Cyanidium caldarium III-D-2* with the tetrapyrrole precursor 5-aminolevulinic acid. After 2–3 days incubation we observed as a main product a rather polar blue pigment of an as yet unknown structure in the medium as well as in the cells themselves, together with porphyrins, phycocyanobilin [5] and biliverdin IXα [1]. In this paper, the structure of the new polar blue pigment is given and its mode of formation is discussed.

Materials and Methods

All solvents and chemicals used were reagent grade and purchased from E. Merck, Darmstadt (FRG). 5-Aminolevulinic acid hydrochloride was prepared from levulinic acid [6]. [4-^{14}C]5-Aminolevulinic acid hydrochloride (53 mCi/mmol) was obtained from Amersham Buchler GmbH & Co KG, Braunschweig. UV-vis spectra were recorded on a Perkin Elmer 320 spectrophotometer. NMR spectra were taken on a Bruker AM 360 equipped with an Aspect 3000 computer. FAB spectra were taken on a MAT 312 machine (Finnigan, Bremen).

Pigment isolation

Cultures of *Cyanidium caldarium,* mutant III-D-2* were grown in Fernbach flasks with 1 l of medium each [7], containing 1% glucose at 27 °C in the dark under shaking. After 8–10 days, the cells were collected by centrifugation under sterile conditions and resuspended in 150 ml glucose-based medium supplemented with 17 mmol/l 5-aminolevulinic acid. For experiments involving ^{14}C-labeling, 10 μCi = 2.22 × 10^6 dpm [^{14}C]aminolevulinic acid were added to 150 ml medium. The cultures were incubated at the same conditions as described above. Pigment excretion started already after 2 days, but the overall yield was considerably higher at prolonged incubation. Therefore, after 5–10 days, the cells were harvested by centrifugation. The blue supernatant was extracted with butanol-l (about 100 ml/150 ml medium), the organic phase washed with water (3 × 100 ml), dried by filtration through cotton wool and brought to dryness with a rotatory evaporator (bath temperature: 40 °C). Esterification was carried out for 1 h at ambient temperature with 4% (v/v) sulfuric acid in methanol. For pigment isolation from the cells, the algae were disintegrated in butanol during 20 min with the aid of a vibrogen cell mill and the butanol treated as described above.

Workup of the esterified product by chromatography was carried out as previously described [1]. Unknown polar blue main product: hRf = 37 (see [1]).

Incubation experiments with phycocyanobilin

50 g spray-dried *Spirulina geitleri** were extracted several times with hot methanol (about 1 l) to remove chlorophyll and carotenoids. For cleaving phy-

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cocyanobilin from phycocyanin, the powder was then boiled with 500 ml of methanol for 8 h. After filtration, the methanol was removed with a rotatory evaporator. About 7 mg crude pigment were dissolved in 10 ml ethanol and added to a culture of 150 ml *Cyanidium caldarium III-D-2,* respective to 150 ml medium. After 5 days of incubation in the dark at 27 °C under shaking, workup was carried out as described above.

^{1}H-NMR spectroscopy of polar blue bile pigment (hRf = 37)

360 MHz ($CDCl_3$): δ = 1.07 (t, J = 7.5 Hz, $H_3C(18^2)$), 1.60 (d, J = 6.7 Hz, $H_3C(3^2)$), 1.93 (s, $H_3C(2^1)$), 2.115, 2.126 (2s, $H_3C(13^1)$ and $H_3C(17^1)$), 2.120 (s, $H_3C(7^1)$), 2.29 (q, J = 7.5 Hz, $H_2C(18^1)$), 2.58 (t, J = 7.6 Hz, $H_2C(8^2)$ and $H_2C(12^2)$), 2.95 (2t, J = 7.6 Hz, $H_2C(8^1)$ and $H_2C(12^1)$), 3.682 and 3.687 (2s, $H_3CO(8^4)$ and $H_3CO(12^4)$), 5.08 (q, J = 6.7, $HC(3^1)$), 6.01 (s, HC(15)), 6.50 (s, HC(5)), 6.87 (s, HC(10)).

Results

Characterization of polar blue bile pigment (hRf = 37)

UV-vis spectroscopy

UV-vis spectra were recorded in neutral chloroform, in acidic methanol and in methanol containing NH_3/Zn^{2+}. The spectra obtained (Fig. 1) were typical for *Z,Z,Z*-configurated bile pigments with a fully conjugated tetrapyrrole system. Table I summarizes the absorption maxima in the UV and visible region. Whereas in acidic methanol the long-wave maximum at 683 nm differs only slightly from that of phycocyanobilin (PCB), a clear difference of about 20 nm can be seen in neutral chloroform (PCB: 620 nm, polar blue pigment: 643 nm). Differences can also be seen comparing the zinc complex salts (blue polar pigment: 715 nm, PCB: 665 nm).

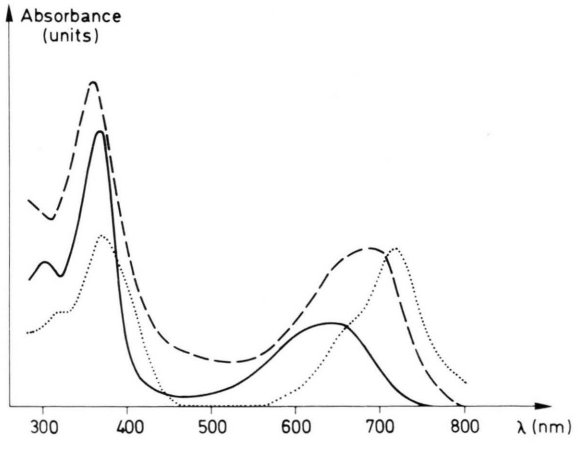


Fig. 1. UV-vis spectra of polar blue bile pigment. —— In chloroform; --- in methanol + 0.15% HCl (hydrochloride salt); ··· in methanol + Zn^{2+} + NH_3 (zinc complex salt).

Table I. Absorption maxima of polar blue pigment.

Solvent	λ^{UV}_{max}	λ^{vis}_{max}
chloroform	370	643
methanol + 0.15% HCl (hydrochloride salt)	356	683
methanol + Zn^{2+} + NH_3 (zinc complex salt)	373	715

Table II. Chromic acid degradation.

Bile pigment ester	Yield of imides MESI	HSE	MEM	MVM
polar blue bile pigment	–	8.1%	7.7%	–
phycocyanobilin	1.0%	11.0%	8.5%	–
biliverdin IX_α	–	11.5%	–	6.7%

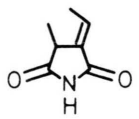
Chromic acid degradation

The chromic acid degradation of esterified material yielded two imides* which cochromatographed with hematinic acid imide methyl ester and methyl ethyl maleimide (Fig. 2), whereas phycocyanobilin dimethyl ester furnished E-methyl ethylidene succinimide additionally. To achieve good TLC separation, only a limited amount of product can be applied. This may prevent the visualization of inherently weak spots.

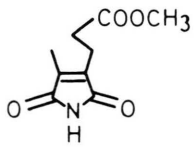
This difficulty may be overcome by radioactive labeling which enables the quantitative determination of imide yields as previously shown by Troxler *et al.* [8]. The results obtained are summarized in Table II. The ratio of hematinic acid imide methyl ester to methyl ethyl maleimide is in satisfactory agreement with the results obtained from the degradation of phycocyanobilin. Other imides are not observed in

* The chlorine benzidine positive spot at R_f = 0.36 visible upon chromic acid degradation with the crude pigment mixture [1] does not appear when the pure product is degraded.

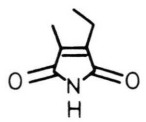
methyl ethylidene succinimide MESI



hematinic acid imide methyl ester HSE



methyl ethyl maleimide MEM



methyl vinyl maleimide MVM

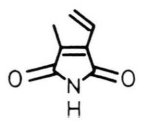


Fig. 2. Imides, obtained by chromic acid degradation.

contrast to the latter. This leads to suspect that one of the outer pyrrole rings is destroyed during chromic acid degradation possibly due to side chains susceptible to oxidation by chromic acid.

Another control degradation was carried out with biliverdin IXα dimethyl ester. Whereas the yield of hematinic acid imide methyl ester is comparable with that obtained from polar blue pigment, methyl vinyl maleimide is only found in small percentage which demonstrates the great instability of this imide.

Fast atom bombardment (FAB) spectrum

FAB spectra of bile pigments are especially well suited to obtain the molecular weight of the parent molecule. In our case, the spectrum showed the mass peak M+1 = 631 (8%) and a fragment with 614 mass units (4%). This mass difference of 17 fits to a bile pigment carrying an OH-group at that pyrrole ring which is destroyed during chromic acid degradation. If the M+1-peak of 631, the fragment with 631−17 mass units and the results of chromic acid degradation are looked at in concert, as a working hypothesis, the molecule can be considered as a hydroxylated mesobiliverdin. Further evidence for this structure was obtained by NMR-spectroscopy.

^{1}H-NMR-spectroscopy

The 360 MHz ^{1}H-NMR spectrum of Fig. 3 exhibits 3 well-separated singulets of the methine bridges at 6.87, 6.50 and 6.01 ppm. The resonances of the two expected propionic acid methyl ester side chains can be seen at 3.687 and 3.682 ppm (s, $2\times OCH_3$), 2.95 (2t, $2\times CH_2$; $-\underline{CH_2}CH_2OCH_3$) and 2.58 ppm (t, $2\times CH_2$; $-CH_2\underline{CH_2}OCH_3$). The quartet at 2.29 ppm (2H) and the triplet at 1.07 ppm (3H) can be assigned to an ethyl side chain as shown by decoupling. Singulets are found at 2.115, 2.120, 2.126 and 1.93 ppm ($4\times CH_3$). By comparing with other spectra of the verdin type [9, 10], the methyl group at 1.93 ppm can be assigned to position 2 near the lactam oxygen. Additionally, a quartet at 5.08 ppm (1H) and a doublet at 1.60 ppm (3H) can be seen. The connection between these resonances was confirmed by decoupling at 5.08 ppm. These results fit perfectly to an α-hydroxy-ethyl side chain.

The position of the hydroxy-ethyl side chain and the sequence of the pyrrole rings were determined by NOE difference spectroscopy (Fig. 4).

Irradiation of the proton at 5.08 ppm induced NOE's on 1.60 ppm (CH_3 of the hydroxy-ethyl side chain), on the methine proton at 6.50 ppm and the

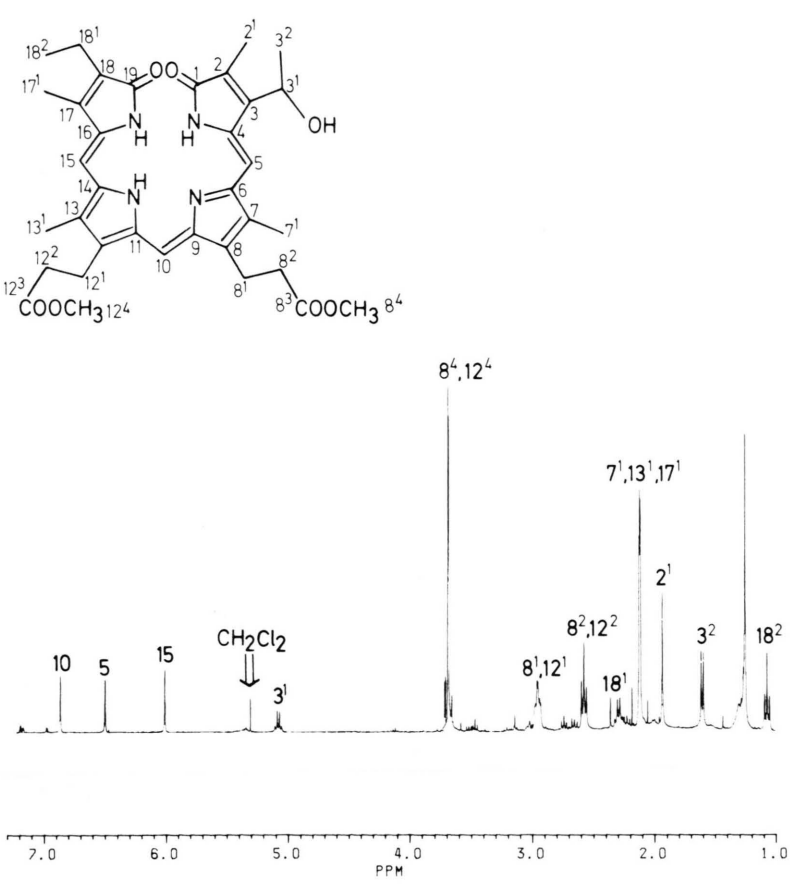


Fig. 3. ^{1}H-NMR spectrum (360 MHz, in $CDCl_3$) of A-α-hydroxymesobiliverdin dimethyl ester.

methyl group at 1.93 ppm. As this methyl group was assigned to position 2, the hydroxy-ethyl group is attached to C(3). The chemical shift of the proton at C(5) is therefore 6.50 ppm. By irradiation of this methine proton, 2 NOE's were induced, one as expected at 5.08 ppm and one at 2.120 ppm. Hence, this methyl group has to be placed to position 7. Irradiation of the methylene protons of the ethyl side chain was performed (2.29 ppm). Only one NOE for the resonance of the methyl group at 1.07 ppm was observed. The absence of the NOE to any of the methine signals proved that the ethyl side chain has to be at C(18). The resonance of the methyl group at C(17) could not be observed because the signal was too near to the irradiation peak. By irradiation of the proton at 6.01 ppm, enhancement of 2 methyl groups at 2.125 and 2.114 ppm was observed. Therefore, the signal at 6.01 ppm has to be attributed to HC(15). The methyl groups at C(13) and C(17) have to resonate at 2.125 and 2.114 ppm, respectively. By irradiation of the proton at 6.87 ppm, only the NOE at 2.95 ppm ($\underline{CH_2}CH_2OCH_3$) was induced. This means that the propionic acid side chains are located at position 8 and 12. The chemical shift of the methine proton at C(10) is 6.87 ppm. Furthermore, the NOE's obtained by irradiation of the methine pro-

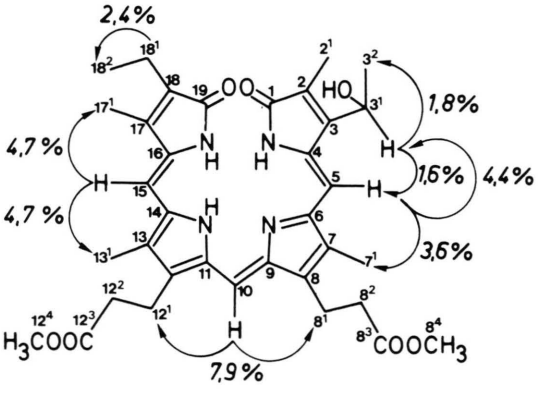


Fig. 4. ^{1}H-NMR (360 MHz in $CDCl_3$) NOE correlations for A-α-hydroxymesobiliverdin dimethyl ester.

tons also show that the configuration of the bile pigment is really *Z,Z,Z*.

Discussion

Upon incubation with 5-aminolevulinic acid, the red algae *Cyanidium caldarium* excrets large amounts of coloured compounds into the medium. After appropriate workup (extraction, esterification and TLC chromatography) a polar blue bile pigment could be isolated and identified as A-α-hydroxymesobiliverdin.

Interestingly, another naturally occurring hydroxylated mesobiliverdin has been described as constituent of the ovaries of the turban snail *Turbo cornutus* [11, 12].* Since the snail feeds on algae which contain biliproteins, this pigment (A-β-hydroxymesobiliverdin) might be a degradation product of the latter.

Although the chemical structure of the polar pigment from *Cyanidium caldarium* has been unequivocally identified as A-α-hydroxymesobiliverdin, its mode of formation remains somewhat unclear. On the one hand we observed that after two days incubation the newly identified product is the main component as well in the medium supernatant as in the cells themselves. After a period of about 5 days, however, *E*-phycocyanobilin and biliverdin become the predominant bile pigment species. Judging from the order of appearance, the hydroxy-pigment might be considered as a biochemical precursor. However, it is known that biliverdin in fact is incorporated into phycocyanin [2, 3] or *in-vitro*-transformed in cell-free systems to phycocyanobilin [4]. On the other hand, control incubations showed a conversion of phycocyanobilin to the hydroxy-pigment, although in relatively bad yield.

Further work is in progress to elucidate the biochemical relevance of the new compound.

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