Urobiliverdin, a New Bile Pigment Deriving from Uroporphyrin

Eva Benedikt and Hans-Peter Köst

Botanisches Institut, Universität München, Menzingerstr. 67, D-8000 München 19, Bundesrepublik Deutschland

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Semisynthetic Bile Pigments, Urobiliverdin III, Coprobiliverdin III, Biliverdin IX, Uroporphyrin III

5-Aminolevulinic acid is incubated with a crude enzyme extract from *Rhodopseudomonas spheroides*, mutant R 26. The formed porphyrins (main product: uroporphyrin III) are isolated. Incorporation of iron, ring-splitting by coupled oxydation and subsequent iron removal leads to a mixture of pigments, from which urobiliverdin, a new bile pigment with eight carboxylic acid side chains, is isolated. It is characterized by its chromatographic behaviour, chromic acid degradation and UV-vis spectroscopy.

Introduction

Naturally occurring open chained tetrapyrrolic systems (bile pigments) usually bear two carboxylic acid side chains, namely propionic acid side chains. Examples are phycocyanobilin [1–5], phycoerythrobilin [5, 6], phytochromobilin [7, 8], the biliar pigment bilirubin ([9], here older literature) and stercobilin [9] which is encountered in the faeces.

All of these pigments probably derive from protoheme as has been shown experimentally in a number of cases [9], for example for phycocyanobilin [10–19].

It is somehow amazing, that all natural bile pigments should derive from one single type of porphyrin. Theoretically, open-chained pigments could be formed by ring splitting of porphyrins with more than two carboxylic acid side chains.

A new type of bile pigment with four propionic acid side chains has recently been synthesized [20]. A further pigment with four acetic acid side chains and four propionic acid side chains is presented in this paper. The new compound may serve as a reference for the search for natural bile pigments of this type.

Materials and Methods

All solvents used were analytical grade and purchased from E. Merck (Darmstadt, FRG). 5-Aminolevulinic acid was a product of Sigma Chemicals Co. (St. Louis, USA). Crude enzyme extract from the photosynthetic bacteria, *Rhodopseudomonas spheroides*, mutant R 26 was a gift from Prof. H. Scheer, Munich. Marker porphyrins were purchased from Porphyrin products (Logan, USA).

Chromic acid degradation was performed according to the procedure developed by Rüdiger [21]; the degradation products were separated on silicagel 60 coated HPTLC plates (E. Merck, Darmstadt, FRG) using the solvent system chloroform-ethyl acetate-cyclohexane = 6:3:1. The imides formed were identified by comparison with authentic standards.

Absorption spectra were recorded on a Perkin Elmer model 320 UV-vis spectrophotometer. The solvent used was methanol with the additives specified in Table III of this paper. All measurements were carried out under nitrogen to avoid oxidation.

Procedures

Uroporphyrin from ALA

400 mg ALA in buffer (200 ml 0.02 M Tris/HCl, 0.1 M KCl, 0.03 M mercaptoethanol) were incubated at 37 °C with 33 ml of a crude enzyme preparation from *Rhodopseudomonas spheroides* mutant R 26 at pH 8.6 for 5 h. (Protein content of the enzyme preparation: 9.2 mg/ml.) The acidity was adjusted to pH 7.8 and the incubation mixture was allowed to stir for 10 h at 37 °C. 5 ml of glacial acetic acid were added and the pH adjusted to 0.8 with dilute HCl. The protein precipitate was collected by centrifugation. To the supernatant, 0.4 ml of 30% hydrogen peroxide were added. After 1 day, the solution was adjusted to pH 3.5 with dilute NaOH and the precipitated porphyrin was collected by centrifugation, washed with dist. water and dried over calcium chloride; yield: 150 mg.

Reprint requests to Priv. Doz. Dr. H.-P. Köst.

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The dried porphyrin was esterified with BF_3 in methanol (2%, reflux) and worked up with chloroform/water.

Uroporphyrin, the main product, was identified by TLC on silicagel 60 coated HPTLC plates and shown by HPLC (also compare [22]) to consist nearly exclusively of type III. Other porphyrins identified were coproporphyrin, 5-, 6-, 7-carboxylporphyrin.

Prior to carrying out the next reaction steps, iron was introduced according to the method of Fuhrhop and Smith [23] yielding urohemin as reaction product.

Urobiliverdin

Ring splitting leading to the verdohemochrome and subsequent removal of iron was carried out according to the method of Bonnett and McDonagh [24].

A stream of oxygen was introduced into a solution of 25 mg urohemin chloride in 25% aqueous pyridine at 37 °C for 20 min. 300 mg ascorbic acid were added and the solution worked up under nitrogen with ice cold chloroform/water. The chloroform solution was dried and the solvent removed *in vacuo*. This preparation was carried out seven times. The dry combined residues were dissolved in 25 ml of methanol. 2 ml of 2 N methanolic potassium hydroxide were added and after one minute, 25 ml of BF_3 in methanol (14%). The mixture was refluxed for 15 min under nitrogen and worked up with chloroform/water. The solvent was removed *in vacuo* and the product taken up in carbon tetrachloride/ethyl acetate = 1 : 1 and applied to a silicagel 60 column (2.9 × 29 cm) which was developed with the same solvent system. A number of fractions were obtained in the following order: rose (minor), blue (coprobiliverdin), violet (minor), yellow (minor), green (urobiliverdin + yellow contaminent). Urobiliverdin appears after several hours of elution. The green fraction was further purified by chromatography on silicagel 60 coated HPTLC plates (E. Merck, Darmstadt, FRG) yielding a blue main product (≈ 2 mg) (urobiliverdin) and a yellow as yet unknown contaminant.

Results and Discussion

The procedure of urobiliverdin synthesis is summarized in the following scheme (Fig. 1):

5-Aminolevulinic acid (ALA) (**1**) is enzymically converted to uroporphyrin III (**2**). Chemical incorporation of iron leads to urohemin (**3**) which is chemically split to a mixture of four open chained bile pigments urobiliverdin III (α, β, γ, δ) (**4a, b, c, d**).

The new type of bile pigment is separated by thin layer chromatography of the octaethyl esters on

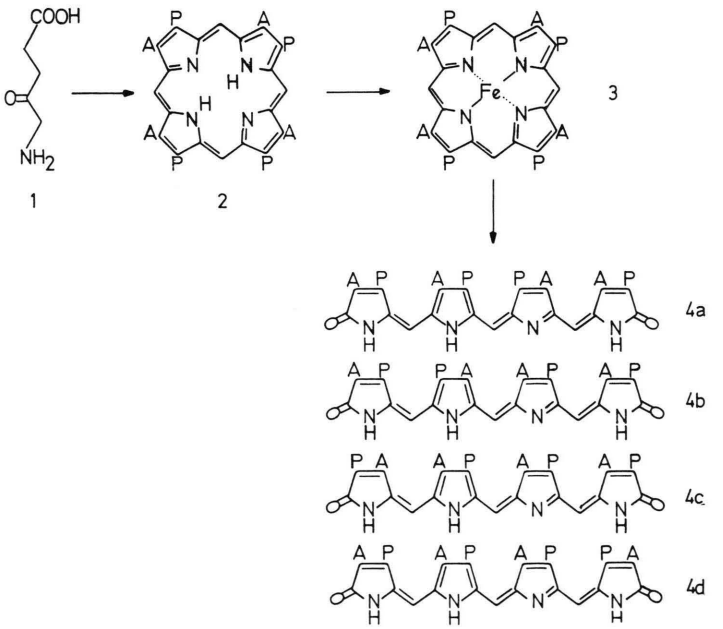

Fig. 1. Scheme of synthesis of urobiliverdin. 5-Aminolevulinic acid (**1**) is enzymically converted to uroporphyrin (**2**). Subsequent incorporation of iron leads to urohemin (**3**) which is split to four isomeric urobiliverdins (**4a–d**) which are difficult to separate. Abbreviations: A = $-CH_2-COOCH_3$; P = $-CH_2-CH_2-COOCH_3$.

HPTLC plates and characterized by chromic acid degradation and UV-vis spectroscopy.

Chromatography of urobiliverdin

The chromatographic data obtained with the solvent system carbon tetrachloride-ethyl acetate = 1:1 are listed in Table I. Obviously, the growing number of carboxylic acid side chains enhances polarity what results in a diminished R_f-value in comparison to the values for coprobiliverdin III (isomers) and biliverdin IX (isomers α, β, γ, δ). The isomerous mixture of biliverdin easily separates into three distinct spots, 2 isomers are nearly inseparable in the system given and tend to comigrate (isomers β and γ). Coprobiliverdin also forms three spots whereas urobiliverdin produces two spots, a main product at $hR_f \approx 7$ and a byproduct at $hR_f \approx 8$, which might be isomers. The distance of separation of the isomers when expressed in hR_f-units is diminished from the isomers of biliverdin via the isomers of coprobiliverdin to the isomers of urobiliverdin (Table I). The following investigations were carried out with the main product ($hR_f \approx 7$).

Table I. Characterization of urobiliverdin III by chromatography on HPTLC plates.

Layer L Solvent S Technique T Bile pigment [a]	hR_f
Urobiliverdin III	
spot 1	7
spot 2	8
Coprobiliverdin III	
spot 1	21
spot 2	23
spot 3	25
Biliverdin IX	
α-isomer	38
β-isomer	43
γ-isomer	42
δ-isomer	31

L: silicagel 60 (E. Merck, Darmstadt, FRG)
S: carbon tetrachloride-ethyl acetate = 1:1
T: ascending, chamber saturation, 25 °C
[a] Pigments as methyl esters.

Table II. Chromic acid degradation of uroporphyrin III octamethyl ester, biliverdin IX dimethyl ester, coprobiliverdin III tetramethyl ester and urobiliverdin III octamethyl ester. For separation conditions of degradation products (imides) and further details see [28].

Compound [a]	Imide formed (hR_f)
Uroporphyrin III	2-Acetic acid-3-propionic acid maleimide [a] (63)
Urobiliverdin III	2-Acetic acid-3-propionic acid maleimide [a] (63)
Coprobiliverdin III	Hematinic acid imide [a] (70)
Biliverdin IX	Hematinic acid imide [a] (70) 2-Methyl-3-vinyl maleimide (81)

[a] Methyl esters (see Fig. 2).

*Chromic acid degradation**

The chromic acid degradation of urobiliverdin should yield 2-acetic acid-3-propionic acid maleimide (**6**) (Fig. 2) as exclusive degradation product. In Table II, imides obtained by chromic acid degradation of biliverdin, coprobiliverdin, urobiliverdin and uroporphyrin are listed. Biliverdin (**1**) yields 3-methyl-2-vinyl maleimide (**2**) and hematinic acid imide (**3**). The degradation product deriving from coprobiliverdin (**4**) is exclusively hematinic acid imide (**3**). The imide deriving from urobiliverdin (**5**) is more polar than the latter; comparison with an authentic standard [b] yielded 3-acetic acid-2-propionic acid maleimide (**6**). Additionally traces of hematinic acid imide are found deriving from decarboxylation which might occur prior to or during degradation what hints to a certain instability of the pigment. Since (**6**) was the only major reaction product, its mother compound urobiliverdin must contain identical pyrrole nuclei as has to be expected from the way of its synthesis.

UV-vis spectra

Biliverdin IX, coprobiliverdin III and urobiliverdin III do not differ in number and arrangement of their double bonds within the tetrapyrrolic system (Fig. 2). However, biliverdin exhibits two extra double bonds with its vinyl side chains. This is expressed in the position of the absorption maxima which is about equal for the long wave bands of urobiliverdin III and coprobiliverdin III (Table III), whereas the absorption maximum of biliverdin IX is bathochromatically shifted (10–20 nm). For other

* For chromic acid degradation, fully esterified pigments were used. The imides mentioned below are therefore esterified (where applicable).

** Reference imides may be obtained by degradation of uroporphyrin octamethyl ester (Fig. 1, (**2**)).

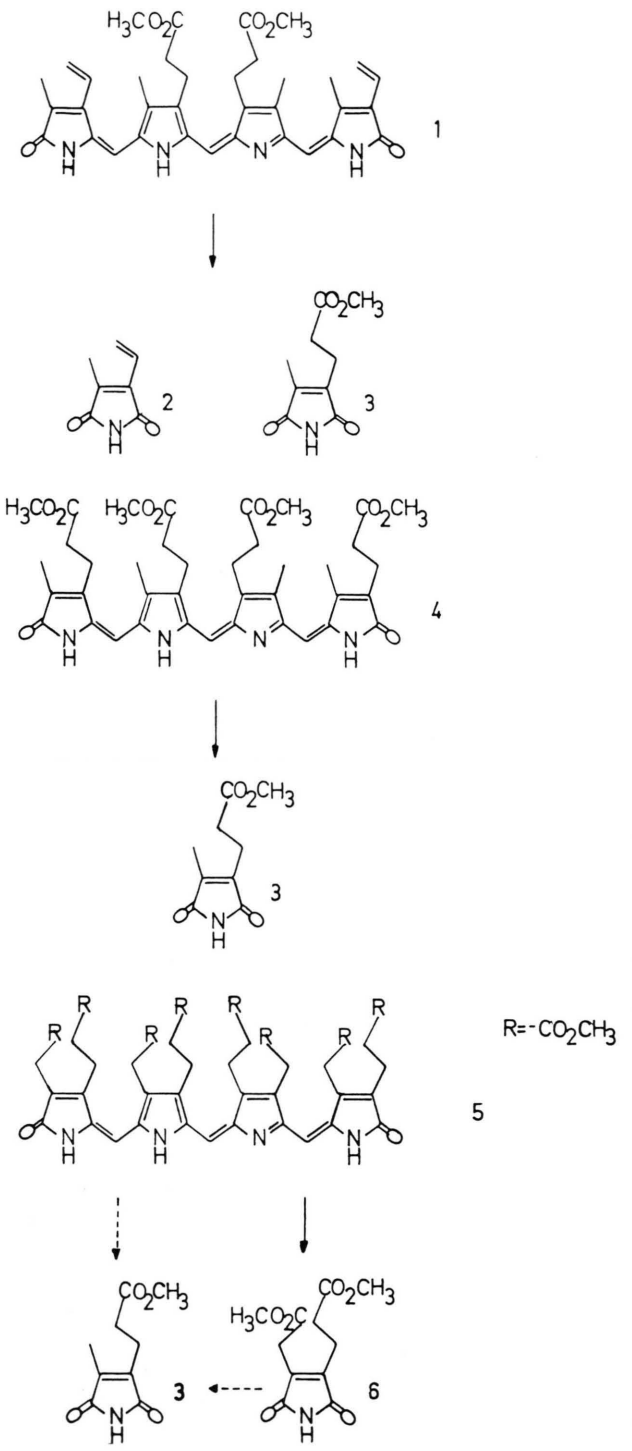

Fig. 2. Characterization of biliverdin (**1**), coprobiliverdin (**4**) and urobiliverdin (**5**) by chromic acid degradation. Whereas (**1**) yields the imides 2-methyl-3-vinyl maleimide (**2**) and hematinic acid imide (**3**), (**4**) furnishes (**3**) as only reaction product and (**5**) yields the imide 2-acetic acid-3-propionic acid maleimide (**6**) with traces of hematinic acid imide (**3**) (deriving probably from spontaneous decarboxylation). (All degradations were carried out with the fully esterified bile pigments. However, some saponification may occur during degradation and facilitate decarboxylation, see above.)

pigments (*e.g.* phycocyanobilin) the ratio of long and short wave absorption maxima (*e.g.* $A_{683\,nm}/A_{368\,nm}$) has been considered as an indicator for the conformation present [25, 26]. In our case, these values increase with growing amount of carboxylic acid side chains (from 0.29 for biliverdin ester to 0.41 for urobiliverdin ester in methanol). This increase is even more pronounced in an acidic environment (0.15% HCl in methanol, Table III). These values indicate a more cyclic conformation of biliverdin IX which may be changed to a more semi-open structure of urobiliverdin III.

Concluding remarks

In this paper a new bile pigment with eight carboxylic acid side chains is described and compared with pigments with four (coprobiliverdin) and two carboxylic side chains (biliverdin). Although the new pigment is semi-synthetical, there is a possibility that it may be found in nature as a degradation product of uroporphyrin resp. uroheme.

One as yet hypothetical mode of its formation might be based on the fact, that free porphyrins are poisonous to some organisms (*e.g.* due to their photosensitizing effect [9]) what requires their rapid elimination. This elimination can be achieved by oxydative ring opening leading to bile pigments which may subsequently be altered (reduced, conjugated) and excreted.

In fact, an excretion of bile pigments parallel to that of porphyrins is observed in cells of *Cyanidium caldarium* which have been fed high concentrations of 5-aminolevulinic acid [27, 28]. The bile pigments excreted are phycocyanobilin, its precursor biliverdin [28] and some as yet unknown polar pigments.

Although our experiments have shown that ring splitting is becoming increasingly difficult with growing number of carboxylic side chains (due to sterical hindrance or charge effects) it does not appear unlikely that naturally occurring bile pigments with four or eight carboxylic acid side chains will soon be identified.

Table III. Absorption maxima of urobiliverdin III, coprobiliverdin III and biliverdin IX in the visible (long wave) and UV region. The spectra were recorded from the "neutral" pigments (solvent methanol), the hydrochloride salts (methanol with 0.15% HCl) and the zinc complex salts (29). The absorbance ratio at the wavelengths indicated is given. Since zinc complex salts are very labile these data have been omitted. All pigments are present as the fully esterified compound.

Pigment	λ_{max}^{UV}	λ_{max}^{vis}	$\frac{A^{vis}}{A^{UV}}$	λ_{max}^{UV}	λ_{max}^{vis}	$\frac{A^{vis}}{A^{UV}}$	λ_{max}^{UV}	λ_{max}^{vis}
Urobiliverdin III	372	640	0.41	368	683	0.67	400	720
Coprobiliverdin III	364	640	0.27	357	684	0.56	375	716
Biliverdin IX[a]	376	663	0.29	376	694	0.44	395	736
	in methanol			in methanol with 0.15% HCl			(zinc complex salts)	

[a] Compare also [30, 31].

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Note added in proof:

Since the typesetting of the present publication a description of a newly dicovered natural verdinoid bile pigment "bactobilin" with eight carboxylic acid side chains has been published (32) which probably is identical to urobiliverdin III. This stresses the necessity and biological relevance of further studies on "semisynthetic" bile pigments.

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