^{15}N-Coprobiliverdin, a New Model Chromophore

Hans-Peter Köst and Eva Benedikt

Institut für Botanik der Universität München, Menzinger Straße 67, D-8000 München 19

Edmund Cmiel and Siegfried Schneider

Institut für Physikalische und Theoretische Chemie, Technische Universität München, D-8046 Garching

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Natural Bile Pigments, Coprobiliverdin III, Model Chromophore, Spectroscopy, MO-Calculations

The synthesis of ^{15}N-labeled coprobiliverdin III, a bile pigment with four conjugated pyrrole nuclei and four carboxylic acid side chains, is described. Coprobiliverdin is structurally characterized by chemical and various spectroscopic methods (*e.g.* chromic acid degradation, UV-vis-, IR-, mass spectroscopy). The results of ^{15}N NMR studies reveal the potential value of this compound as a probe for structural interactions in biliproteins and as a reference for the future investigation of natural bile pigments deriving from coproheme.

Introduction

Cyanobacteria, rhodophyta and cryptophyta contain special light-harvesting pigments, phycocyanins and phycoerythrins which have been identified as biliproteins with thioether-linked bile pigment chromophores [1–5]. Although there has recently been progress in the elucidation of bile pigment biosynthesis in algae [2, 6–15] the final steps are still unknown. In one case, biliverdin IXα has been found to be an intermediate [15] what previously had been concluded from the biosynthesis in animals.

Although for biochemical investigations the use of radioactive label is widespread, crucial early results in tetrapyrrol biochemistry have been obtained by using stable nuclids, specially ^{15}N.

For example, Gray and Neuberger [17] report the incorporation of ^{15}N into coproporphyrin, uroporphyrin and hippuric acid; incorporation of ^{15}N in stercobilin of normal and porphyric individuals was also reported [18, 19]. The metabolism of ^{15}N-5-aminolevulinic acid has been studied by means of mass spectroscopy [20]. Today, additional methods, specially NMR spectroscopy are available.

Biliproteins with ^{15}N-labeled bile pigment chromophores have the advantage that only four labeled atoms occur in the bile pigment part of the biliprotein molecule. On the other hand, the natural abundance of ^{15}N is rather low.

Reprint requests to H.-P. K. and S. S.

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^{15}N NMR could serve as a special probe for the presence of hydrogen bonds, furnishing at the same time results on the charge of the labeled nitrogen atoms, an information that cannot be obtained from ^{13}C NMR spectra.

For *Cyanidium caldarium*, a red alga, it has been reported [7] that incubation with ^{14}C-labeled 5-aminolevulinic acid leads to a ^{14}C-labeled chromophore within a nearly unlabeled protein moiety. Similar results should be obtained employing respective ^{15}N-labeled compounds. In order to test the feasibility of such a ^{15}N NMR study, it was necessary to synthesize a ^{15}N-labeled model chromophore, which can be obtained by chemical ring opening of a suitable ^{15}N-labeled porphyrin (coproporphyrin III). Coprobiliverdin at the same time might be considered as a reference compound for future studies on natural bile pigments deriving from porphyrins other than protoheme (compare 21). In this paper, we now wish to describe synthesis, purification, properties and some results of ^{15}N NMR spectroscopy of ^{15}N-labeled coprobiliverdin III.

Materials and Methods

All chemicals and solvents were reagent grade and purchased from E. Merck, Darmstadt. Chromatography was carried out on HPTLC plates of the same company. Solvents were redistilled prior to use. Marker porphyrins were purchased from Porphyrin Products, Inc., Logan. The preparation of crude enzymes from *Rhodopseudomonas spheroides*

R-26 was a gift from Prof. Dr. H. Scheer, Munich. ^{15}N-labeled phthalimide potassium (95 atom% purity) was purchased from Merck, Sharp and Dohme, Montreal.

Chromic acid degradation of coprobiliverdin tetramethyl ester was carried out with 2% chromium trioxide in 4 N sulfuric acid according to the method of Rüdiger [22].

^{15}N NMR spectra were taken on a Bruker WM 250 Fourier Transform NMR spectrometer (250 MHz); mass spectra were recorded on a CH 5 Varian Instrument (direct sample inlet), ionisation voltage 70 eV, source temperature 230 K. IR-spectra were obtained from a Perkin Elmer 397 spectrometer.

^{15}N-5-aminolevulinic acid

The preparation of ^{15}N-5-aminolevulinic acid was carried out by the method of Shemin [23].

Enzymic synthesis of coproporphyrin [40]

Incubation medium: 400 ml buffer Tris 0.04 M, mercaptoethanol 0.06 M, KCl 0.2 M, 700 mg ^{15}N-5-aminolevulinic acid, 100 ml enzyme extract from *Rhodopseudomonas spheroides* R-26, pH 8.6. The mixture was kept for 5 h under continuous stirring at 37 °C, then further 100 ml of enzyme were added. After additional 1.5 h of incubation, 200 ml of enzyme were added, the pH adjusted to 7.8 and the mixture incubated for 16 h. 10 ml of glacial acetic acid were added and the colour of the solution changed to red. The pH was lowered to pH 0.8 and a protein precipitate was collected by centrifugation. To the clear red supernatant, 1.5 ml of 30% hydrogen peroxide were added. The product was allowed to stand for 1 day in the dark. The solution was brought to pH 3.5 (NaOH) and the precipitated porphyrin collected by centrifugation, washed with dist. water and dried over calcium chloride. Yield: 125 mg. The dry porphyrin was refluxed for 15 min with 20 ml 2% BF_3 in methanol and the methyl ester recovered by chloroform-extraction. Yield: 135 mg. The protein precipitate from above was extracted with 4 × 50 ml of methanolic HCl (5% HCl). After each addition, the sample was left for 16 h at −19 °C. In this way, 339 mg of porphyrin ester were obtained. Total yield: 474 mg = 70.2%.

The formed porphyrin was identified by thin-layer chromatography on silica-gel coated HPTLC plates, solvent system benzene-petrol ether (b.p. 40–60 °C)-methanol-ethyl acetate = 48.5:40.0:10.5:9.0. The ^{15}N-porphyrin was composed of coproporphyrin (main product, $R_f = 0.63$) and minute amounts of other porphyrins (uroporphyrin, 7-, 6-, 5-carboxylporphyrin, protoporphyrin). The obtained coproporphyrin tetramethyl ester was purified by liquid chromatography on silicagel 60, using the solvent as described above.

Analysis of the coproporphyrin by HPLC according to (24) yielded a composition of 97% coproporphyrin III and 3% coproporphyrin I.

Coprohemin

Iron was introduced into the coproporphyrin III according to the method of Warburg and Negelein described by Fuhrhop and Smith [25].

Coprobiliverdin (mixture of the isomers IIIα–IIIδ)

For ring opening, the coprohemin was subjected to coupled oxidation with O_2/ascorbate [26]. The coprobiliverdin tetramethyl ester was taken up in carbon tetrachloride-ethyl acetate = 1:1 and applied to a silica-gel 60 column (2.9 cm ∅ × 29 cm) and developed with the same solvent system. The blue fractions were combined, brought to dryness and used for the experiments described below. Yield: 25 mg (= 12.1%).

Results and Discussion

Preparation and purification of coprobiliverdin (**7a–7d**) (*^{15}N-labeled*)

^{15}N-5-aminolevulinic acid (**3**) is synthesized from 4-oxo-5-chloro pentanoic acid methyl ester (**2**) by Gabriel synthesis using ^{15}N-phthalimide potassium (**1**). (**3**) is enzymatically condensed *via* porphobilinogen (not isolated) to the respective uroporphyrinogen III (**4**) (not isolated) which is subsequently converted *via* coproporphyrinogen to coproporphyrin III (**5**). Iron-insertion yields coprohemin III (**6**). Ring-opening by coupled oxidation with O_2/ascorbate and the final removal of iron and esterification leads to a mixture of coprobiliverdin IIIα, IIIβ, IIIγ, IIIδ tetramethyl ester (**7a–7d**). The procedure applied is briefly summarized in Fig. 1.

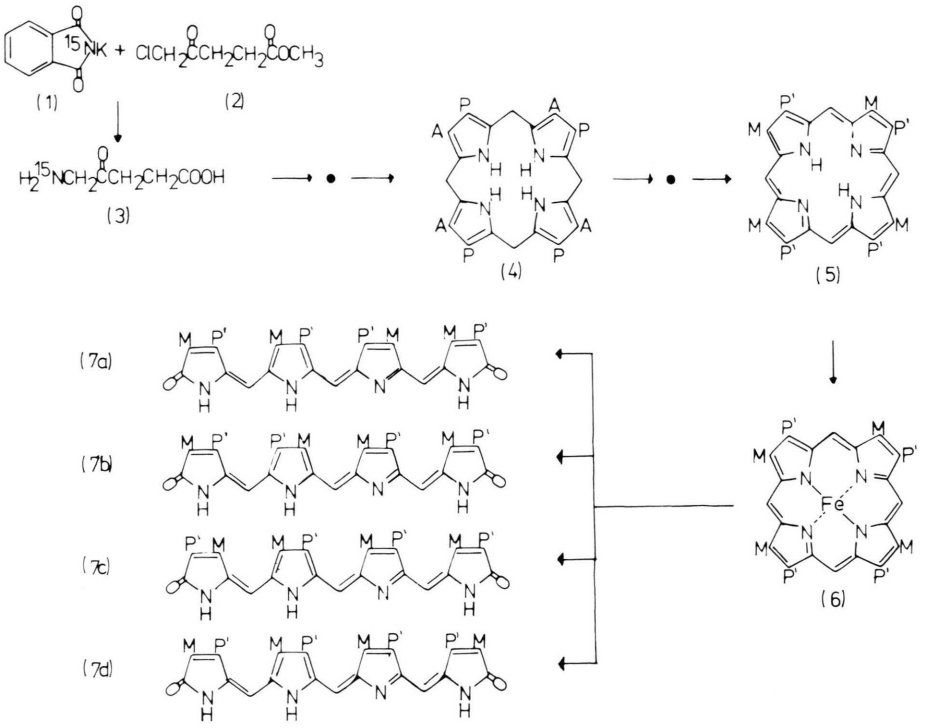

Fig. 1. Principal scheme of synthesis of fully ^{15}N-labeled coprobiliverdin (**7a–7d**). For details see text. A = $-CH_2COOH$; P = $-CH_2CH_2COOH$ respective P′ = $-CH_2-CH_2-COOCH_3$.

Characterization of coprobiliverdin by chromic acid degradation

Chromic acid degradation [22] of (**7a–7d**) yielded hematinic acid methyl ester as the only reaction product detectable by thin layer chromatography; $R_f = 0.51$, solvent system chloroform-ethyl acetate-cyclohexane = 6:3:1 on silica-gel coated HPTLC plates.

This finding proves the presence of one methyl- and one propionic acid side chain at each of the four unsaturated rings, thus confirming structure (**7a–7d**).

Mass spectrometry

The successfull use of ^{15}N-labeled compounds for the study of natural porphyrins and bile pigments has been demonstrated for coproporphyrin and stercobilin [17, 18]. James and Abbott [27] in a similar study investigated the formation of ^{15}N-hemins and ^{15}N-stercobilin from ^{15}N-glycine. In order to analyse for isotope content, the authors converted the bound nitrogen to $^{15}N_2$ which was analyzed in a mass spectrometer.

The computer-assisted mass spectrometry of today allows a more rapid characterization of compounds within much shorter time than previously possible and furnishes additional information what is essential for the characterization of natural bile pigments. Labeling with stable isotopes thereby enables the followup of biosynthetic pathways, a task that today is mainly achieved using radioactive isotopes. The results of mass spectrometry of ^{15}N-coprobiliverdin tetramethyl ester are summarized in Fig. 2. **5** (mass peak, $m/e = 734$, 5.4%) yields fragments **6** ($m/e = 376$, 14.1%) and **7** ($m/e = 362$, 100%) (fragmentation at the middle methine bridge). The methine bridge is probably first reduced by disproportionation in the mass spectrometer. The methylene bridge is than fragmented [28]. Further fragments are **8–12** (Fig. 2). A cleavage of the propionic acid side chain leads to compounds with mass numbers diminished by 31 ($-OCH_3$), 59 ($-COOCH_3$), 73 ($-CH_2COOCH_3$) and 87 ($-CH_2CH_2COOCH_3$).

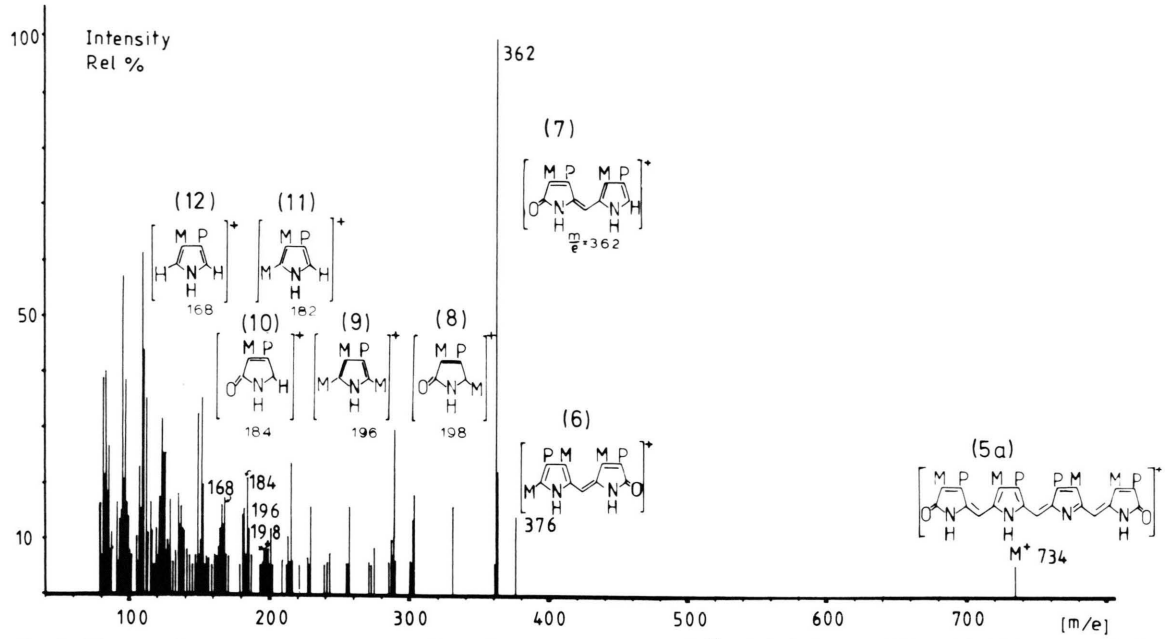

Fig. 2. Electron impact mass spectrum of an isomeric mixture of ^{15}N-labeled coprobiliverdin III tetramethyl ester (**7a–7d**). Mass peak $m/e = 734$, basis peak (100% rel. intensity) = 362, direct sample inlet. Formula of one isomer and fragments thereof are shown. M = $-CH_3$, P = $-CH_2CH_2COOCH_3$.

The fragmentation pattern of coprobiliverdin tetramethyl ester (Fig. 2) is in good agreement with the fragmentation pattern of biliverdin dimethyl ester [29, 30] and mesobiliverdin dimethyl ester [29]. These spectra exhibit a basis peak at $m/e = 610$ resp. $m/e = 614$. Fragments with structures similar **6** and **7** occur with relative intensities of 20–40% (biliverdin) and 60–80% (mesobiliverdin). For the spectrum of mesobiliverdin, a fragment with the mass number $m/e = 123$ (ca. 20%) is described [29] to which a structure similar to **10** can be attributed. In all spectra, a similar successive fragmentation of the propionic acid side chain can be observed.

IR-spectrum

The isotopic shift of the N–H-stretching frequency upon substitution of ^{14}N by ^{15}N has a value of about 7.5 cm^{-1}. No drastic differences can, therefore, be expected in the region around 3400 cm^{-1}. In contrast to McDonagh and Palma [31], who observed in the biliverdin spectrum two weak, broad absorption bands at 3420 and 3200 cm^{-1}, which they assigned to free and hydrogen bonded N–H-stretching vibrations, we find only one extremely broad and completely unstructured band with a maximum at 3400 cm^{-1} (Fig. 3). This value coincides with the frequency of the very intensive N–H-stretching mode in pyrrole. This observation could indicate that the intra- and intermolecular hydrogen bonding of the various N–H-groups depends both on the gross molecular shape as well as on the substitution pattern (see below). The broad absorption extending upward from about 2500 cm^{-1} is interpreted as being due to hydrogen bonded carboxylic C–H-stretching vibrations [31]. In the spectrum of coprobiliverdin, three clearly resolved absorption peaks (2825, 2895, 2925 cm^{-1}) are superimposed (Fig. 3a). Their origin must be the C–H-stretching vibrations of methyl-, methylene- and methine-groups. In ^{14}N-coprobiliverdin, the most intense maximum (Fig. 3b) appears around 1620 cm^{-1}, whereas only a moderate strong peak is observed in ^{15}N-coprobiliverdin. Two new peaks can be observed, however, in the latter spectrum at 1570 and 1670 cm^{-1}. At 1670 cm^{-1}, there is, in contrast, a pronounced minimum in the spectrum of the ^{14}N-species. Since one must conclude from the model calculations (see Fig. 4) that the double bonds in the three pyrrole rings are

essentially isolated double bonds, the isotope insensitive part of the absorption around 1620 cm^{-1} must be attributed to the C=C-stretching mode of these quasi isolated double bonds. The isotopic sensitive vibrations should correspond to the C=N=C-stretching and the ring-breathing mode of ring C, respectively. In accordance with the biliverdin spectrum, we interpret the strong band at 1715 cm^{-1} to be the C=O-stretching mode of the carboxylic groups and the lactam fragment. Furthermore, we find pronounced absorption peaks at 1425 and 1155 cm^{-1}, respectively. An interpretation of these and the other peaks of smaller intensity is, however, not possible.

Quantum mechanical model calculations and UV-vis-spectra

Quantum mechanical model calculations

Quantum mechanical model calculations in the π-electron approximation of Pariser and Parr [32] and Pople [33] have been performed using a set of parameters developed earlier [34]. With respect to the gross molecular shape, three conformers were considered: a cyclic, slightly non-planar, helical form and two planar forms, a semi open one and a fully extended form (for more details see [35]). The calculated transition energies and oscillator strengths for transitions with 270 nm are summarized in Table I. Furthermore, we have included the net charges at the positions of the heteroatoms for the ground state and the first excited state in Table II. Since the differences in π-bond order of the conformers are small, only those of the fully extended conformation are given in Fig. 4.

UV-vis-spectra

Information on the conformational state of model pigments like coprobiliverdin have to be compared with data of natural bile pigments in order to evaluate their model character. In our paper we compare coprobiliverdin with mesobiliverdin and biliverdin. The UV-absorption spectrum of the

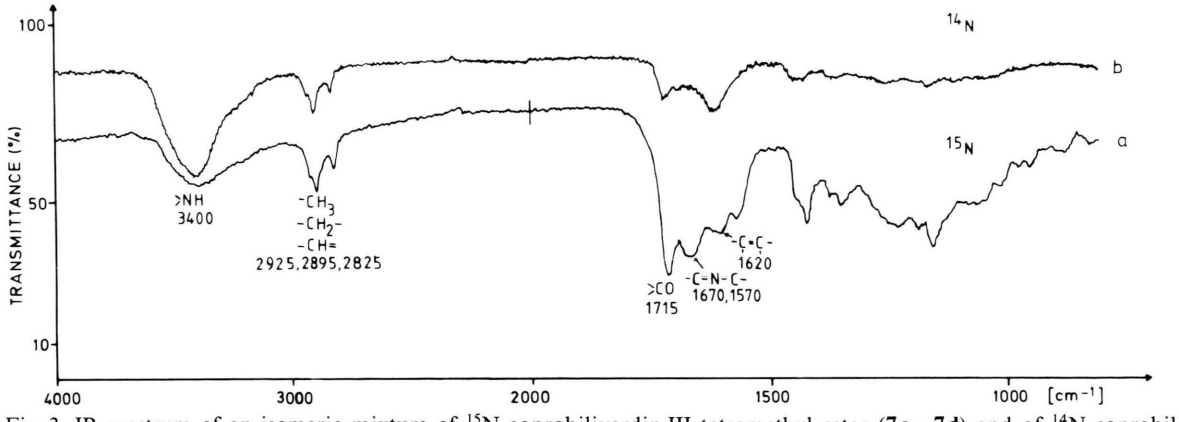

Fig. 3. IR-spectrum of an isomeric mixture of ^{15}N-coprobiliverdin III tetramethyl ester (**7a–7d**) and of ^{14}N-coprobiliverdin III tetramethyl ester. Note bands marked with arrows.

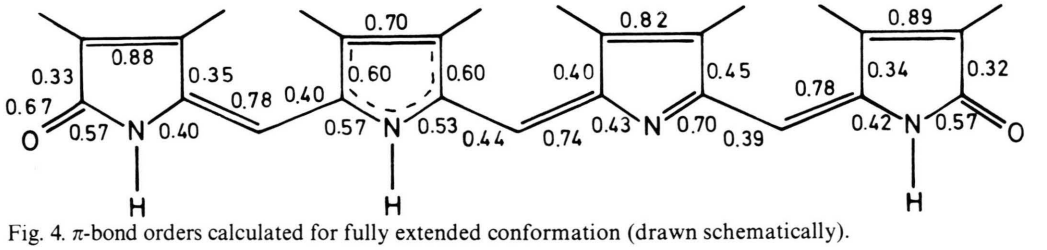

Fig. 4. π-bond orders calculated for fully extended conformation (drawn schematically).

Table I. MO-calculations: oscillator strengths (f) for various electronic transitions $S_0 \rightarrow S_1$; $S_0 \rightarrow S_2$; ... of model structure **4**. ΔE is given as a function of wavenumber in kK (and wavelength in nm, respectively).

Electronic transitions	Cyclic form			Semi-open form			Open form		
	$\bar{\nu}$ [kK]	λ [nm]	f	$\bar{\nu}$ [kK]	λ [nm]	f	$\bar{\nu}$ [kK]	λ [nm]	f
$S_0 \rightarrow S_1$	14.3	697	0.08	16.2	617	0.71	16.9	591	2.02
$S_0 \rightarrow S_2$	15.7	638	0.01	22.4	447	0.92	23.3	429	0.21
$S_0 \rightarrow S_3$	21.9	458	0.20	24.4	409	0.18	23.9	418	0.02
$S_0 \rightarrow S_4$	24.2	414	0.09	26.4	379	0.22	27.0	370	0.13
$S_0 \rightarrow S_5$	26.0	384	0.73	30.1	333	0.43	29.4	340	0.04
$S_0 \rightarrow S_6$	28.5	350	0.43	30.2	331	0.62	31.1	321	0.14
$S_0 \rightarrow S_7$	30.0	333	1.12	31.3	320	0.07	34.0	294	0.09
$S_0 \rightarrow S_8$	31.0	323	1.71	36.4	275	0.19	37.0	270	0.28
$S_0 \rightarrow S_9$	32.4	309	0.24						
$S_0 \rightarrow S_{10}$	33.9	295	0.10						

Table II. Net charges (in atomic units) at the positions of the hetero-atoms N_1, N_2, N_3, N_4 (belonging to rings A, B, C, D in this order), O_1 and O_2 (from rings A and D) and the methine bridge atom C-10 (in methine bridge between rings B and C). The values are given for the ground state S_0 and the first excited state S_1.

Atom designation	Cyclic form		Semi-open form		Open form	
	S_0	S_1	S_0	S_1	S_0	S_1
N_1	0.48	0.50	0.43	0.47	0.44	0.50
N_2	0.55	0.55	0.56	0.51	0.64	0.57
N_3	−0.51	−0.49	−0.35	−0.38	−0.43	−0.47
N_4	0.56	0.60	0.46	0.56	0.46	0.55
O_1	−0.57	−0.51	−0.59	−0.55	−0.58	−0.54
O_2	−0.48	−0.50	−0.58	−0.51	−0.57	−0.52
C_{10}	0.04	0.03	0.11	−0.02	0.16	0.00

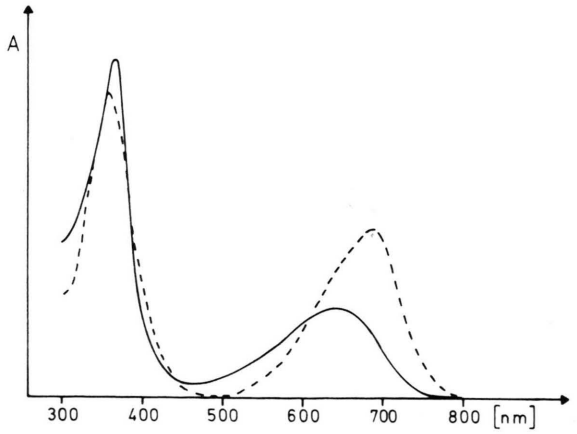

Fig. 5. UV-vis-spectrum of isomeric coprobiliverdin III tetramethyl ester (**7a–7d**), free base (recorded in methanol, solid line) and hydrochloride (recorded in 0.15% HCl in methanol, broken line).

^{15}N-enriched compound is, as has to be expected, identical to that of the normal ^{14}N-coprobiliverdin. It furthermore resembles very much those of mesobiliverdin and biliverdin, both with respect to the position of the absorption maxima as well as with respect to the intensity ratio of red/UV-transition (see Fig. 5). From this observation we conclude that the methyl- and propionic acid methyl ester side chains do not influence the π-electron system of the tetrapyrrole chromophore neither by electronic interaction nor by means of steric hindrance. In acidic solutions, the long wavelength band shifts bathochromic. This is typical for bile pigments.

As in the case of biliverdin dimethyl ester [36] and other tetrapyrrole derivatives [35] we must assume that the electronic absorption spectrum is a superposition of the spectra of various conformers in thermodynamic equilibrium. In accordance with this statement, the observed ratio $D_{UV}/D_{vis} = 1.83$ cannot be explained by the calculated results for one conformer only. The cyclic form has a strong transition around 27 kK, but only two very weak transitions below 20 kK. In the extended forms, on the other hand, the ratio D_{UV}/D_{vis} is roughly the inverse, with the red band being the most intense one. The change in π-electronic density upon excitation at the heteroatoms is small. The effect of hydrogen bonding and protonation on the absorption spectrum should, therefore, be minor as long as the geometrical parameters are unchanged. The observed increase of the long wavelength absorption upon acidification could, therefore, be indicated for a transformation from the helical to the more extended forms.

Nuclear magnetic resonance spectra (NMR)

Since no ^{15}N NMR spectrum could be obtained from chlorophyll *a* enriched with 95% ^{15}N despite of two days signal averaging [37], it was not at all clear whether the nuclear Overhauser effect (NOE), too long relaxation times and similar disadvantageous effects would prevent the recording of ^{15}N spectra of coprobiliverdin (**7a – 7d**).

At 300 K, however, the spectrum actually shows three peaks: a broad peak with the maximum at – 175 ppm (rel. to ext. NO_3^-) and two sharp ones at – 240.7 ppm and – 251.7 ppm, respectively (Fig. 6). The chemical shift of the first resonance compares favorably with a resonance observed by several authors for porphyrins at higher temperature. Due to fast proton exchange, only one resonance is observed [37, 38]. At lower temperatures, Gust and Roberts [38] observed two new resonances, one at – 132 ppm (≙ ^{15}N) and one at – 241 ppm (≙ ^{15}NH). If no proton decoupling is applied, the coupling constant J_{NH} is 100 Hz [39]. Since in our experiment, a broad band proton decoupling was applied, the singlet resonances around – 250 ppm must be assigned to the two nitrogens in the rings A and D, respectively. Since the calculated asymmetry in net charges on the nitrogen atoms in rings A and D is rather small (see Fig. 4) and also the change in charge, distribution upon a conformational change, we cannot decide without further experimental evidence, whether the appearance of two different resonances in the region around 240 ppm resembles the molecular asymmetry or the mixture of stereo- and substitutional isomers. Intringuingly enough, there is also a pronounced difference in linewidth for this two resonances. We are unable at this point to give an explanation for the apparent structure in the – 175 ppm ^{15}N-resonance. Experiments are on the way, to separate the substitutional isomers produced by the ring opening reaction. We hope that by this precaution and in combination with temperature studies, an answer to the above quoted questions will be possible.

The experiments described prove the clear feasibility of a study of biliprotein biosynthesis with

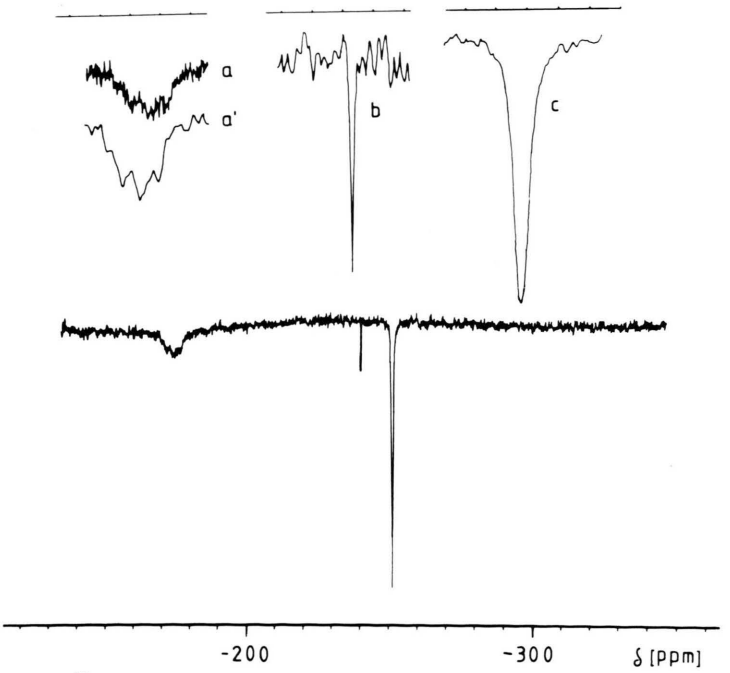

Fig. 6. ^{15}N NMR spectrum of isomeric coprobiliverdin III tetramethyl ester labeled with 95 atom% ^{15}N. The spectrum was taken on a Bruker WM 250 Fourier transform NMR spectrometer at 25.3 MHz with NO_3^- as external standard. a: enlargement of peak found at – 175 ppm; 1 unit on scale = 100 Hz; a′: like a, with artificial line broadening (10 Hz) revealing possible peak fine structure; b: enlargement of peak at – 240.7 ppm; 1 unit on scale = 25 Hz; c: enlargement of peak at – 251.7 ppm; 1 unit on scale = 25 Hz. For further explanations see text.

^{15}N-labeled 5-aminolevulinic acid. Incubation of *Cyanidium caldarium* with this compound should yield a ^{15}N-labeled chromophore within a nearly unlabeled protein moiety, a situation uniquely suited to furnish information on the charge of the pyrrole nitrogens and the presence of hydrogen bonds which to a certain degree might be responsible to fix the chromophore in its extended state.

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