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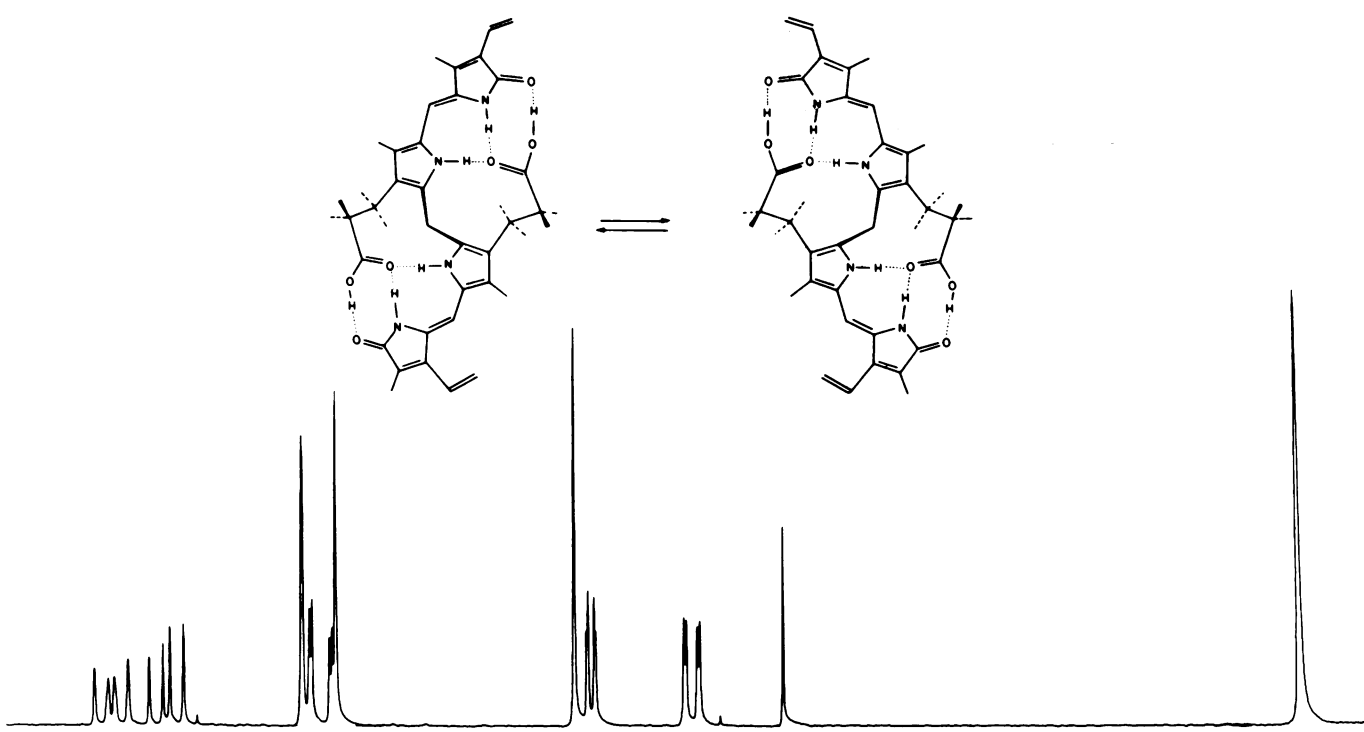
Chemistry and Spectroscopy of the Bile Pigments



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CHEMISTRY AND SPECTROSCOPY OF THE BILE PIGMENTS

Guest Editor: Gil Navon

Isophorcarubin — A Conformationally Restricted and Highly Fluorescent Bilirubin

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Abstract. The biliverdins **2b**, **5** and **1** have been reduced with $NaBH_4$ to the bilirubins **8**, **7** and **6**, respectively. The conformation of these pigments is increasingly extended and the flexibility is increasingly restricted in the sequence given above, which does not influence the ease of reduction. The fluorescence of **6** and **7** has been studied at ambient temperature and 77 K in ethanol and 2-methyltetrahydrofuran. **6** has an unusually high fluorescence yield which has been related to the conformational rigidity of its ring C, D chromophore. Both **6** and **7** show evidence of solvent and temperature dependent inhomogeneities in their fluorescence spectra.

INTRODUCTION*

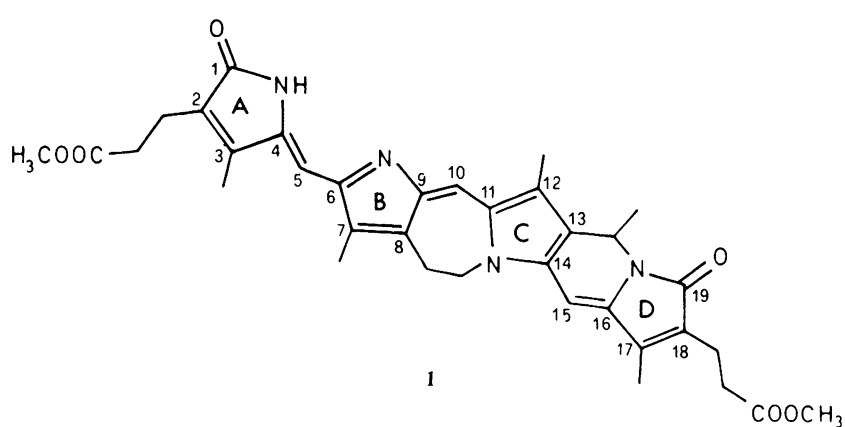
Among the bile-pigments isophorcabilin-DME (**1**) possesses an unusual geometry[1] owing to its unusual structure with three of the four pyrrole rings linked by C_1 or C_2 bridges in addition to the common C_1 bridge.[2] These impose conformational restrictions on the molecule and enforce an extended *Z-anti, E-anti* conformation of the condensed pentacyclic portion. Although such conformations are principally also accessible by, e.g., the conformationally less restricted biliverdins (**2**), they are thermodynamically unfavorable.[3,4] In crystals[5,6] and in solution,[7–9] **2** is present preferentially in the helical *all-Z, all-syn* conformation A (Fig. 1) which has been calculated as being $\geqq 20$ kJ/mol[10] more stable in the isolated molecule than extended conformations such as **B**.

The unusual geometry of **1** is reflected by a characteristic UV-vis spectrum.[11,12] It further substantiates theoretical calculations which commonly predict an increased visible to near-UV absorption ratio for extended conformations such as **B**.[13–19]

Another class of bile pigments exhibiting the same type of spectrum as **1** are the biliproteins, e.g., phycocyanin (**3**) and phytochrome in its P_r form (**4**).[20] For this reason a similarly extended geometry has been suggested for their chromophores.[21] Non-covalent chromophore–protein interactions, which can be (reversibly) uncoupled by unfolding the protein, have been suggested to bring about the extended geometry in the biliproteins.[21,22] There is evidence, however, that extended conformers are also present to a certain extent in fluid solutions of free pigments like **2**.[23]

The chromophores of native biliproteins exhibit some other unusual spectroscopic and chemical properties, both when compared to the chromophores of the respective denatured pigments and to free bile pigments with structures similar to **2**. One such difference is the stability towards reducing agents. Native biliproteins react only slowly and incompletely,[22,24] whereas denatured biliproteins as well as free pigments are readily converted to compounds of the rubin spectral type.[22,24–27] One possibility is that this change in reactivity is also related to the extended conformation of native biliprotein chromophores and this has now been investigated by reduction studies of bile pigments forced into extended conformations, e.g., **1**.

**Abbreviations*: MTHF = 2-methyltetrahydrofuran; 9,10-DPA = 9,10-diphenylanthracene; MeOH = methanol; EtOH = ethanol; DMSO = dimethylsulfoxide; DME = dimethylester; (HP)TLC = (high-performance) thin layer chromatography.



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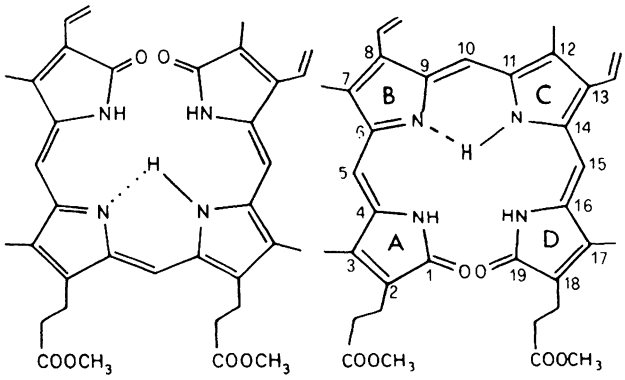
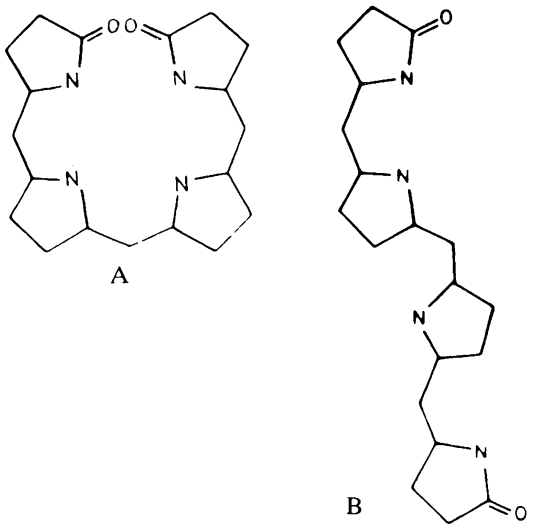
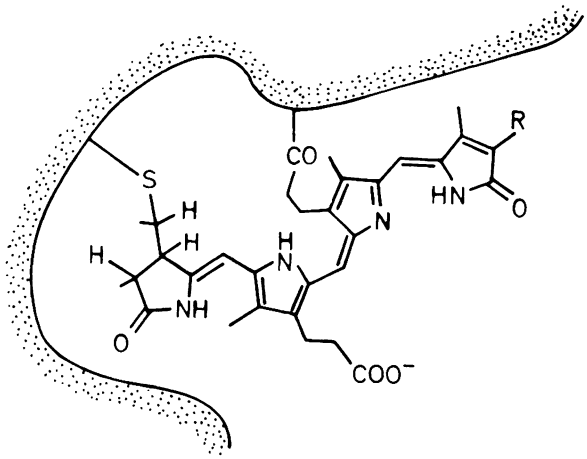


2a 2b



A B

Fig. 1. Helical *all-Z, all-syn* (A) and extended type conformations (B) of the bile pigment skeleton. Free biliverdins like **2** are generally present in conformation A, whereas isophorcabilin (**1**) and isophorcarubin (**6**) are restricted to conformations of type **B** (see text).



3 $R = C_2H_5$ **4** $R = C_2H_3$

Yet another difference between native biliproteins, on the one hand, and denatured or free pigments, on the other hand, is the high fluorescence yield of the former. Free biliverdins generally only have a low fluorescence yield (see however Ref. 23) and there are pronounced indications of heterogeneous composition in solution.[9,25] Bilirubins show a similar heterogeneity in their fluorescence, which is as yet only partly understood.[28,29] Studies of conformationally restricted bilirubin should yield information on the origin of this heterogeneity.

EXPERIMENTAL

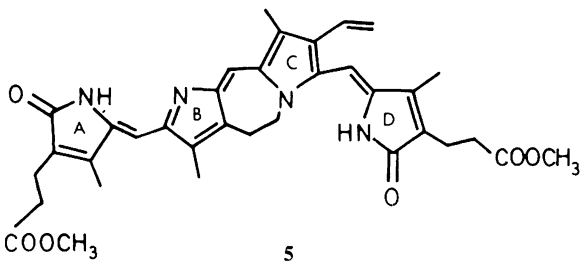
General Methods

UV-vis spectra were recorded on spectrophotometers of either DB-GT type (Beckman, München) or of a 320 E type (Perkin-Elmer, Konstanz). NMR-spectra were recorded using either a CFT-80 (Varian, Palo Alto) or HFX 90 spectrometer (Bruker, Karlsruhe) in FT mode with C^2HCl_3 or acetone-$^2H_6$ as solvent and tetramethylsilane as internal standard. Analytical TLC was carried out on (HP) TLC silica plates (Merck, Darmstadt) with $CHCl_3$/acetone 80:20 as eluent.

The extinction coefficients of the rubins described here were determined from absorption spectra obtained by titration of the corresponding verdins (concentration range: $2–3 \times 10^{-5}$M) with $NaBH_4$ (concentration range: 0–0.1 mg/ml) in MeOH. Spectra were taken about 10 min after the addition of the $NaBH_4$ aliquots, when no further spectral changes were observed. The spectra were corrected for dilution and in the case of absorption values of the rubins, also for the absorption of the residual verdin in this spectral region. The reaction of verdins with $NaBH_4$ to form rubins may proceed even further to colorless products in the presence of too large an excess of the reagent.[24] The existence of two isosbestic points in each of the spectra (486/343; 458/350; 490/385 nm) for the conversions of isophorcabilin, phorcabilin and biliverdin-IXγ-DME, respectively, and especially the linear extinction difference diagrams[30] calculated from the spectra, do not indicate further conversions of the rubins formed during the titration experiments by the aforementioned side reactions.

Materials and Methods

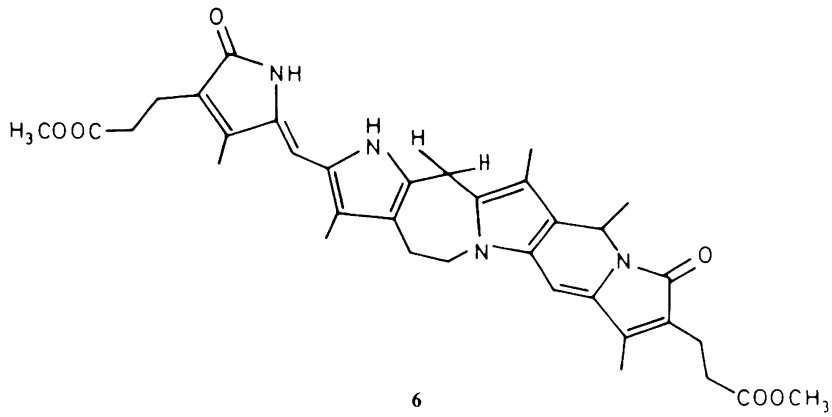
The chemicals were reagent grade unless otherwise stated. Biliverdin IXγ-DME (**2b**), phorcabilin-DME (**5**) and isophorcabilin-DME (**1**) were prepared from hemin (Serva, Heidelberg) by a series of known reactions. Hemin was first subjected to coupled oxidation with hydrazine, followed by esterification with BF_3/MeOH to yield the four isomeric biliverdin DME's.[31] The IXγ isomer was purified by column chromatography (4×20 cm) (silica gel, Merck, Darmstadt) with carbon tetrachloride/acetone 9:1 as the eluent (mobility decreasing from the β-, γ-, α- to the δ-isomer). It was then purified by TLC (20×20 cm plates, 0,75 nm silica H, Merck, Darmstadt) with a heptane/ethylmethylketone/acetic acid = 10:5:0.5 eluent[32] (yield, 7%). **2b** was converted to phorcabilin-DME (**5**) (56% yield) and then to isophorcabilin-DME, (**1**) (54% yield).[2,33,34]



5

Isophorcarubin-DME (6). A solution of **1** (1.4 mg, 2.3 μmol) in 100 ml MeOH was heated to 40°C under a steady stream of N_2. Portions of $NaBH_4$ (about 100 mg $\hat{=}$ 2.6 mmol) were added until the color had changed to yellow. The reaction was followed spectrophotometrically by decrease of the 605 nm absorption band of **1**.

The reaction mixture was partitioned between 100 ml $CHCl_3$ and 100 ml water. The organic phase was exhaustively washed with water until neutral, dried over NaCl and evaporated to dryness giving a yield of 1.95 μ mol $\hat{=}$ 1.2 mg (85% of the theoretical yield, determined spectrophotometrically); TLC:



6

$R_f = 0.17$ (R_f of **1** = 0.18); UV-vis for free base λ [nm] and $\varepsilon \times 10^{-3}$: (MeOH) 405 (31.5) shoulder, 435 (40.2), 455 nm (36.3) shoulder; ($CHCl_3$) 432, 413 and 450 nm shoulders (Fig. 2); (EtOH) 460 shoulder, 431 nm; (MTHF) 452 shoulder and 420 nm; NMR: 10.88, 10.18 (2 × NH, br); 6.16, 6.07 (s, 5-, 15-H); 5.36 (q, 13^1-H); 4.32 (m, 8^2-H_2); 4.14 (s, br, 10-H_2); 3.65 (s, 2^3-, 18^3-OCH_3); 2.99 (m, 8^1-H_2); 2.65(m,8H, 2-, 18-CH_2CH_2); 2.16, 2.07, 2.01, 1.96 (s, 3-, 7-, 12-, 17-CH_3); 1.36 (d, 13^1-CH_3); fluorescence: see text and Figs. 3 and 4.

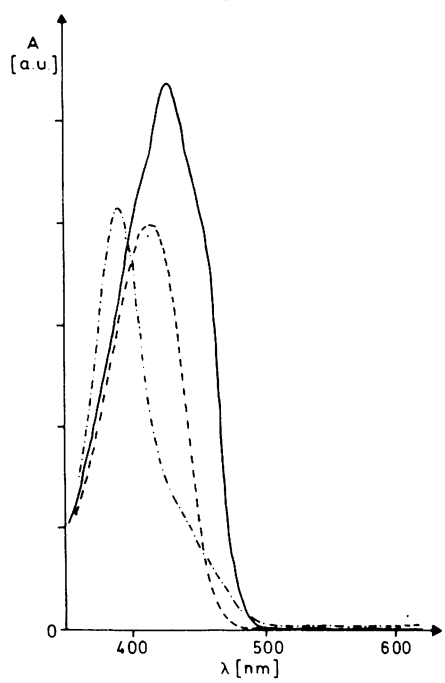


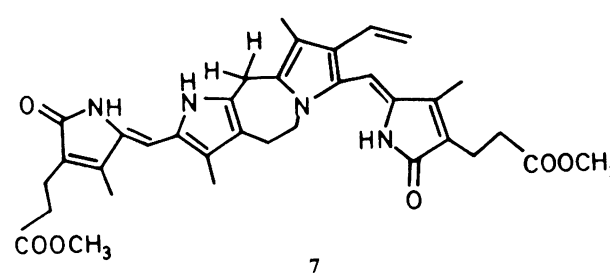
Fig. 2. UV-vis absorption spectra of isophorcarubin-DME (**6**) (——), phorcarubin-DME (**7**) (– – –) and bilirubin IXγ DME (**8**) (– · – · –) in $CHCl_3$. The absorption and fluorescence spectra of **6** and **7** in MTHF and EtOH are shown in Figs. 3–6. The absorption maximum of **8** is solvent dependent and shifted to 402 nm in methanol (see Experimental).

Phorcarubin-DME (7). This was similarly prepared from 1.6 mg of **5** with a yield of 89%; TLC: $R_f = 0.21$ (R_f of **5** = 0.29); UV-vis for free base: (MeOH) 417 (52.7); ($CHCl_3$) 416 (see Fig. 2); (EtOH) 418; (MTHF) 409, broad peak with possible overlap of two bands; NMR: (acetone-$^2H^*_n$) 6.18, 6.08 (s, 5-, 15-H); 5.36–5.15 (m, 13^2-H_2); 4.3 (m, br, 8^2-H_2); 4.06 (s, 10-H_2); 3.6 (s, 2^3, 18^3-OCH_3). A singlet at 7.36 (~0.7 H) was assigned to residual $CHCl_3$. The CH_2-signals were hidden under the solvent resonance: fluorescence: see text and Figs. 5 and 6.

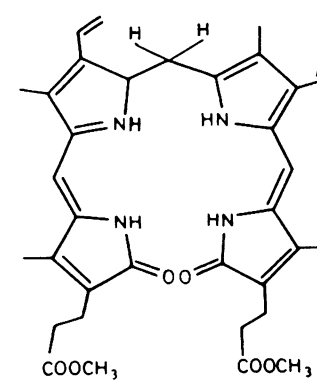
Bilirubin IXγ-DME (8). This was prepared in a similar way to **7** from **2b** with a yield of 78%; TLC: $R_f = 0.08$ (R_f of **2b** = 0.86). The R_f value seems to be concentration dependent; with larger amounts of substance applied tailing occurred, which makes exact determination of R_f values impossible; UV-vis: (MeOH) 402 (46.2); 424 (44.6) shoulder; ($CHCl_3$) 393, 440 shoulder; NMR: 10.41, 10.36, 10.28 (s, 4 × NH); 6.89, 6.68, (dd, 8^1-H, 13^1-H); 6.12, 6.01 (s, 5-, 15-H); 5.34, 5.16 (2 × dd, 2H each, 8^2-, 13^2-H_2); 4.13 (s, 10-H_2); 3.46 (s, 2^3-, 18^3-OCH_3); 2.17 (s, 14H, 2-, 18-CH_2CH_2; 7-, 12-CH_3); 1.94, 1.92 (s, 2 × 3H, 3-, 17-CH_3).

Reoxidation of rubins. A few drops of a solution of 2,3-dichloro-5,6-dicyanobenzoquinone in DMSO were added to samples of **6**, **7** and **8** dissolved in DMSO, until the yellow colour had changed to blue violet or green. After addition of $CHCl_3$ the organic phase was exhaustively washed with water, dried over NaCl and applied on to a TLC plate.

Fluorescence. The PDP11 computer-driven Spex Fluorolog spectrofluorimeter, equipped with a photon-counting detector



7



8

*Dissolution of **7** in the best grade of commercially available C^2HCl_3 resulted in a rapid destruction and color change from yellow to blue. **5** has been identified among other oxidation products in the resulting mixture. This is most likely due to the presence of heavy metals. Both **6** and **7** autoxidize readily to **1** and **5**, respectively, e.g., in the presence of Zn^{2+}.

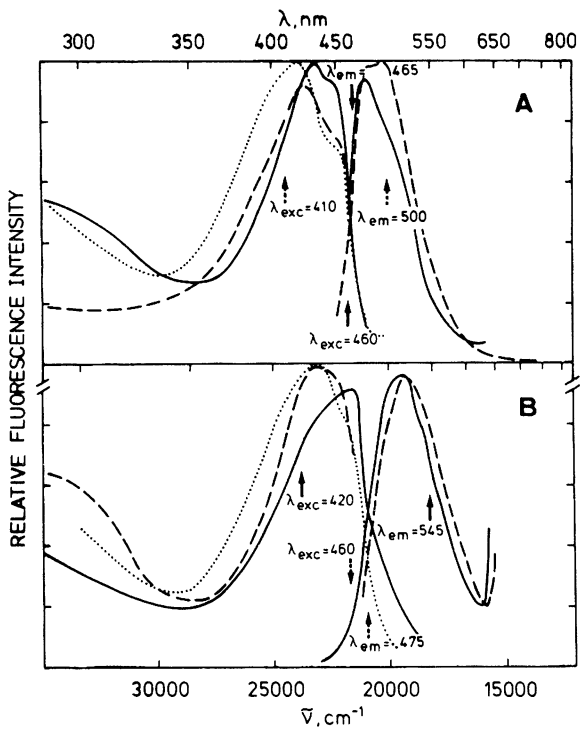


Fig. 3. Corrected and normalized room temperature fluorescence and fluorescence excitation spectra of isophorcarubin DME (**6**) in MTHF (A) and in EtOH (B). The excitation and emission wavelengths are indicated by arrows (solid and dashed arrows for the solid and dashed curves, respectively). The absorption spectra in the above solvents at room temperature are also given (dotted line).

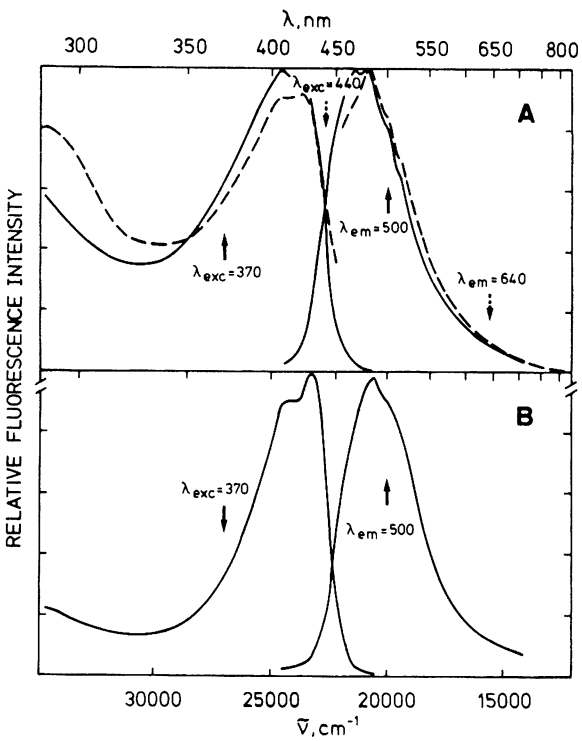


Fig. 4. Corrected and normalized 77 K fluorescence and fluorescence excitation spectra of isophorcarubin-DME (**6**) in MTHF (A) and in EtOH (B). The excitation and emission wavelengths are indicated by arrows (cf Fig. 3).

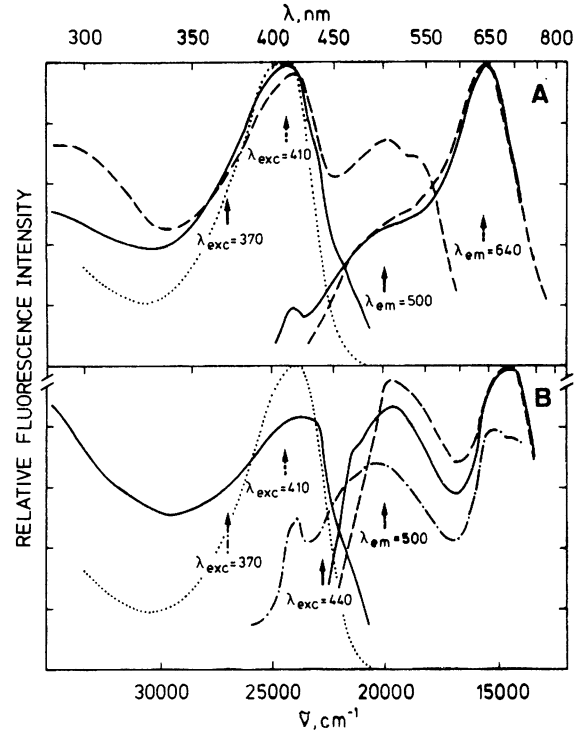


Fig. 5. Corrected and normalized room temperature fluorescence and fluorescence excitation spectra of phorcarubin-DME (**7**) in MTHF (A) and in EtOH (B). The excitation and emission wavelengths are indicated by arrows (cf Fig. 3). The emission bands with maxima at 640 nm and above are caused by contamination by phorcabilin DME. The absorption spectra in the above solvents at room temperature are also given (dotted line).

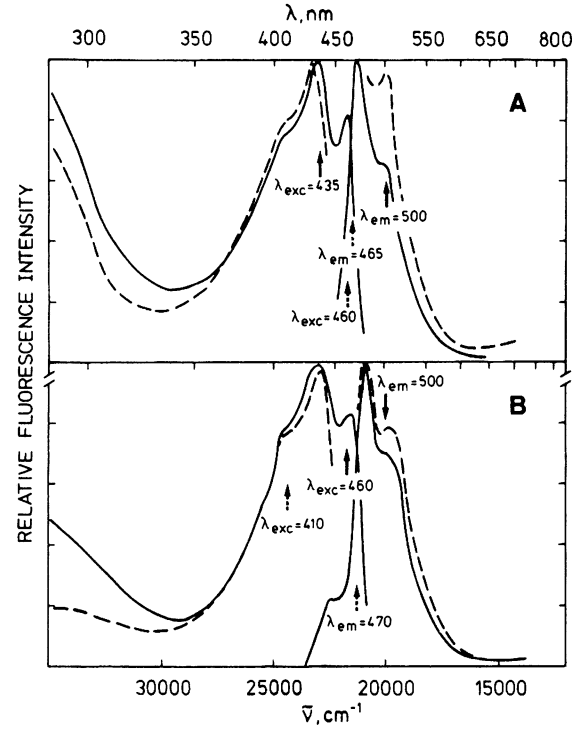


Fig. 6. Corrected and normalized 77 K fluorescence and fluorescence excitation spectra of phorcarubin-DME (**7**) in MTHF (A) and in EtOH (B). The excitation and emission wavelengths are indicated by arrows (cf Fig. 3). In EtOH the same fluorescence spectrum is also obtained with λ_{exc} = 410 nm and λ_{exc} = 440 nm.

anod the correction procedure used to measure the fluorescence anod fluorescence excitation spectra, are described in Refs. 9 and 25.. Fluorescence yields have been measured relative to degassed solutions of 9,10-DPA in EtOH with matched absorbances at the respective excitation wavelengths (λ_{exc} for 9,10-DPA = 372 nm). The quantum yield of 9,10-DPA is reported as 1.0.[35] The relative lamp intensities at the respective excitation wavelengths were measured with a Rhodamine B quantum counter solution (10 g/L in MeOH). All solutions were degassed by 3 freeze-pump-thaw cycles. The concentrations of the rubins were ca 1×10^{-6} mol/L.

RESULTS

Structure of Reduction Products

The three biliverdins **2b**, **5** and **1** react smoothly with $NaBH_4$. The reaction of the three pigments proceeds to completion at about the same rate when a constant excess of the reagent is added. The UV-vis spectra of the three products **8**, **7** and **6**, respectively, are indicative of the formation of bilirubin-type pigments. They are shown in Fig. 2. The somewhat more pronounced structure in the spectrum of extended **6**, as compared to other free bile pigments, is noteworthy. The reduction at the central methine bridge (C-10) is borne out by the ^{1}H-NMR spectra of the bilirubins **8**, **7** and **6**. The C-10 one-proton singlet at $\delta = 6.7$ to 7.3[1,34] is replaced by a two-proton singlet around $\delta = 4.1$, whereas the other signals are essentially unaffected. This is similar to the changes observed upon reduction of other biliverdins to the respective bilirubins.[37]

It is noteworthy that the AB spectra of the anisochronous C-10 methylene protons of **6** and **7** are coalesced to broadened singlets at room temperature, which is obviously due to the rapid conformational motion of the central azacycloheptene ring. The bilirubin structures of **6–8** are finally confirmed by reoxidation to the corresponding biliverdins **1**, **5** and **2b** with 2,3-dichloro-5,6-dicyanobenzoquinone.[38] An interesting observation is the similar TLC mobility of the rubins **6**, **7** and **8**. The corresponding verdinoid pigments **1**, **5** and **2b** show rather different mobilities. This may reflect a similar (extended) geometry in the former and rather different geometries in the latter group of compounds.

Fluorescence Measurements

Figure 3 shows the room temperature fluorescence emission and excitation spectra of isophorcarubin-DME (**6**) in EtOH and MTHF. The spectra are recorded at different excitation and emission wavelengths. The band positions are compiled in Table 1. Both in EtOH and MTHF the room temperature fluorescence yields are

Table 1. Emission and Excitation Spectra of Isophorcarubin-DME (**6**) and Phorcarubin-DME (**7**)

Substance	Solvent	Temperature, K	λ_{exc}, nm	λ_{em}, nm	Emission, λ_{max}, nm	Excitation, λ_{max}, nm
Isophorcarubin-DME	MTHF	77	435		470, 500sh	
			460		502	
				500		460, 434
				465		430
Isophorcarubin-DME	EtOH	77	410		480, 510	
			460		479, 510sh	
				500		465, 434, 405sh
				470		438,405sh
Isophorcarubin-DME	MTHF	300	410		480sh, 500	
			460		476, 501sh	
				460		433, 451sh
				500		455sh, 424
Isophorcarubin-DME	EtOH	300	420		516	
			460		518	
				545		464, 430sh
				475		450, 432
Phorcarubin-DME	MTHF	77	370		467, 480	
			440		462sh, 482	
				500		423, 407
				640		423, 406
Phorcarubin-DME	EtOH	77	370		485, 512sh	
				500		430, 412
Phorcarubin-DME[a]	EtOH	300	370		495	
			410		505, 540sh	
			440		512	
				500		435, 410sh
Phorcarubin-DME[a]	MTHF	300	370		490[a]	
			410		480[a]	
				500		470sh, 410
				640		538, 505, 417

a. Emission bands at 640 nm and above arise from contaminating phorcabilin-DME.

high (Table 2). In all solvents measured there are strong indications of the presence of at least two emitting species.

Figure 4 shows the low temperature (77 K) fluorescence emission and excitation spectra of **6** in the same solvents (Table 1). In a similar way to the room-temperature data, these indicate the presence of more than one emitting species in these solvents. The fluorescence yields are again very high (Table 2).

The emission and excitation spectra of phorcarubin-DME (**7**) at room temperature and 77 K are shown in Figs. 5 and 6, respectively. At room temperature, fluorescence and fluorescence excitation spectra depend again on the excitation and emission wavelengths, thereby indicating the presence of more than one emitting species of **7**. The situation is complicated, however, owing to strong fluorescence bands at 690 nm (EtOH) and 640 nm (MTHF) due to some contamination by phorcabilin-DME (**5**). Nevertheless, owing to the large separation of the rubin- and bilin-type emission maxima the excitation bands can be unequivocally assigned. The fluorescence yields of **7** at room temperature are drastically decreased in comparison to the corresponding data of isophorcarubin-DME (**6**) (Table 2). At 77 K these yields are again considerably increased, reaching values similar to those of **6**. Interestingly, the emission spectrum of **7** in EtOH is now independent of the excitation wavelength, i.e., the same fluorescence spectra are obtained with $\lambda_{exc} = 370$ nm (EtOH, Fig. 6A), 410, and 440 nm. A different result is obtained, however, in MTHF. In this solvent both excitation and emission spectra are wavelength dependent also at 77 K, thus indicating the presence of two different emitting species of **7**. No fluorescence from contaminant phorcabilin-DME (**5**) is observed at 77 K in these solutions, in contrast to the room-temperature results, although the concentration ratios should remain unchanged. This is in agreement with the vastly different temperature dependences of the fluorescence yields of the rubins and the verdin-type chromophores which are known (in the neutral unprotonated molecule).[12,25] The band positions and fluorescence yields for **7** under various different conditions are also summarized in Tables 1 and 2, respectively.

Table 2. Fluorescence Quantum Yields of Compounds **6** and **7**[a]

Substance	Solvent	Temperature, K	ϕ_F
Isophorcarubin-DME **6**	EtOH	298	0.04
Isophorcarubin-DME **6**	EtOH	77	0.81
Isophorcarubin-DME **6**	MTHF	298	0.075
Isophorcarubin-DME **6**	MTHF	77	0.14[b]
Phorcarubin-DME **7**	EtOH	298	0.0015
Phorcarubin-DME **7**	EtOH	77	0.37
Phorcarubin-DME **7**	MTHF	298	0.0035
Phorcarubin-DME **7**	MTHF	77	0.29[b]
Bilirubin-IXα-DME[c]	EtOH	298	0.00076
Bilirubin-IXα-DME[c]	EtOH	77	0.31

a. All yields refer to emission spectra recorded with $\lambda_{exc} = 410$ nm. b. These values represent a lower limit, since they are based on the room temperature extinction coefficients of compounds **6** and **7**. Unfortunately no 77 K absorption spectrum was available. c. Data taken from Ref. 25 for comparison.

DISCUSSION

Isophorcabilin (**1**) is the only well characterized[1,2,11,12,33] bile pigment with an extended conformation. The UV-vis spectroscopic similarities between **1** and native biliproteins like **3** or **4** are substantial and thus similarly extended chromophore structures have been assigned to the latter two. The aim of this work was to determine whether any other unusual properties of the native biliprotein chromophores could be related to the conformational change from helical to extended geometries. There are two distinctly different factors involved, however, when conformational changes in bile pigments are considered. One is the geometric change from A to B (Fig. 1). The other is the conformational flexibility within either geometric form, i.e., the ease of (small) conformational changes around any local potential minima. As will be discussed below, both factors can be separated, and both are important with regard to the native structure of biliprotein chromophores.

In the series **2–5–1** the tetrapyrrole skeleton is forced into increasingly extended conformations. It has previously been shown that the nucleophilic addition of dithionite at C-10 of bilindiones proceeds equally well with the predominantly helical biliverdins **2a**, **b** and with the more extended **5** and **1**.[24] Obviously, their different geometries do not significantly influence their reactivity towards dithionite. The results with $NaBH_4$ reported here corroborate these findings. There is again no significant difference in the reactivity of the four pigments. The decreased reactivity of the chromophores of native biliproteins with these reagents thus appears to be unrelated to their extended conformation per se. The conformational flexibility in the series **2–5–1** requires a more detailed discussion. Free bile pigments are generally rather flexible[3,9,20,21] (see however Ref. 23). The broad and unstructured absorption bands have been related to this flexibility.[20] They are interpreted as arising from the presence of several conformers with similar potential energies but different excitation energies. This interpretation is supported by Ramachandran,[4,18] force field calculations (potential energy),[3] and MO-calculations (excitation energy).[15,17–19] The broad unstructured absorption bands and thus the conformational flexibility are retained in the extended pigments **1** and **5**. The absorption bands acquire some structure, when biliverdins are reduced to bilirubins. The structure is especially pronounced in isophorcarubin-DME (**6**), which exhibits a weakly structured absorption spectrum already at ambient temperatures (Fig. 2).

All these observations can be related to the conformational properties of the additional rings introduced into the tetrapyrrole skeleton of the pigments **1**, **5**, **6** and **7**. All four possess a central azacycloheptene ring fusing the pyrrole rings B and C. This ring type is known to be rather flexible,[39] which is supported by the apparent coalescence of the C-10 methylene proton signals in the NMR spectra of **6** and **7**. The situation is different for the azacyclohexadiene ring linking the pyrrole rings C and D of **1** and **6**. This ring is rather rigid and conveys rigidity to one of the two chromophores of **6**, which is also the compound with the most pronounced structure in its absorption bands. The rigid ring C,D-fragment is present in both **1** and **6**, but while in **1** the chromophore extends over the entire tetrapyrrole system, including the flexible ring A,B,C-moiety, the C,D-fragment of **6** represents one of two isolated chromophores. Based on this interpretation, the unusually high fluorescence yield of the

free bile pigment **6** at room temperature and the increased fluorescence and decreased reactivity of native billiprotein chromophores can be rationalized as well.

Conformational flexibility is known to be an important factor in other chromophoric systems in causing radiationless deactivation of excited states.[25,40-42] **6** is the most effficiently fluorescing bilirubin in fluid solution* and we suggest that this originates from the rigid C,D chromophore. The second chromophore of **6** comprises rings A and B. It is similar in its flexibility to bilirubin and, thus, a similarly low quantum yield would be expected from the A,B chromophore. The contribution of the A,B chromophore to the total observed fluorescence of **6** must therefore be small at room temperature. The fluorescence yield of the isolated ring C,D unit should then in fact be almost twice as high as the measured total fluorescence yield of 4–7.5% (Table 2). For the native biliproteins, one would postulate a rigid fixation of the entire chromophore, viz all four rings in structures **3** and **4**. The narrow absorption bands of the individual chromophores in these pigments are in agreement with this interpretation (cf Ref. 20).

A rigid fixation of the biliprotein chromophore in a way which is favorable for sp^2 but unfavorable for sp^3 hybridization at C-10 could finally be advanced as an explanation for the decreased reactivity at C-10 of the chromophore in these systems. This situation could be brought about by fixation of the entire chromophore and of the region around C-10, in particular, in an essentially planar conformation. Since addition of nucleophiles or reduction requires a rehybridization of C-10 from sp^2 to sp^3, the reaction would become thermodynamically unfavorable. In isophorcabilin-DME (**1**), the extended conformation is similar to that of the biliprotein chromophores, but the flexibility at C-10 is less affected since it is part of a (flexible) azacycloheptene ring.

The fluorescence and excitation spectra of both **6** and **7** indicate a heterogeneous composition of these solutes under all conditions except for (**7**) in EtOH at 77 K. This is reminiscent of a similar situation reported for bilirubin IXα-DME.[28] For this compound different conformers, tautomers (lactams vs. lactims) and also dimers have been discussed as possible a priori causes for this heterogeneity[28] and wavelength-dependent photochemistry.[29] It had been argued that under the experimental conditions used[28] associative phenomena could be excluded as an explanation. Such associative phenomena do occur, however, at higher concentrations in polar solvents.[43] In this study no indications were found for the occurrence of dimers of **6** and **7** under the experimental conditions used for fluorescence and absorption measurements. In particular no deviations from Beer's law were observed for concentrations in excess of ca 10^{-5} mol/L, i.e., more than tenfold the concentration used for the fluorescence measurements.

The presence of significant amounts of lactim tautomers in solutions of pyrromethenones and rubins has been excluded by other workers,[43,44] and we have also not found any evidence in favour of such tautomers. We are thus left with the postulate that the pronounced heterogeneity of compounds **6** and **7** is due to different conformers. Comparison of the fluorescence data of **6** and **7** with those of bilirubin IXα-DME[28] reveals a remarkable similarity of the fluorescence spectra of the three compounds with respect to heterogeneity, except for some blue shift in the emission bands of **6** and **7**. Interestingly, rigid fixation of the ring C,D chromophore in **6** does not result in a removal of the heterogeneity.

Different relative geometries of rings A and B can hardly be held responsible for the heterogeneous emissions, at least at room temperature, since this part of the structure contributes negligibly to the total emission of **6**, as has been discussed above. We therefore prefer to interpret the heterogeneity of compounds **6** and **7** as arising from conformers with different geometries around the central carbon C-10. In other words, the relative orientation of the two sub-chromophores is different. Wavelength shifts may thus be brought about by geometry dependent electronic coupling of the two halves of these molecules. This suggestion has been put forward earlier for bilirubin IXα-DME based on the fact that its spectral properties could not be extrapolated in a straightforward manner from those of the individual partial chromophores.[28] The blue fluorescence of phytochrome has been discussed very recently in terms of a dipyrromethenone partial structure of its chromophore.[45] Although it is now part of a ring-system, conformational changes around C-10 are still possible in **6** and **7** and evidence has been presented from NMR data that such changes do indeed occur on an NMR time scale. Accordingly, conformational heterogeneity within the rings A, B and C, D chromophores contributes to a much lesser extent to the observed spectral heterogeneity in fluorescence.

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**Note added in proof.* Koek, von Es and Lugtenburg have recently also observed a high fluorescence in a bridged pyrromethenone.

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