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Geometry Versus Basicity of Bilatrienes: Stretched and Helical Protonated Biliverdins

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Summary. The ease of protonation of bilatrienes at the pyrrolenine nitrogen critically depends on their conformation. The biliverdin **2** being constrained to a helical (*Z*, *Z*, *Z*, *syn*, *syn*, *syn*) geometry by its four link chain is ca. three orders of magnitude less basic than flexible open-chain bilatrienes like biliverdin-IXα dimethyl ester (**1**), which is shown to adopt a stretched conformation in its monoprotonated form. These results are obtained by a comparative investigation of the titrations of **1** and **2** with sulfuric acid in methanol and methanol-water by means of UV-VIS and NMR spectroscopy.

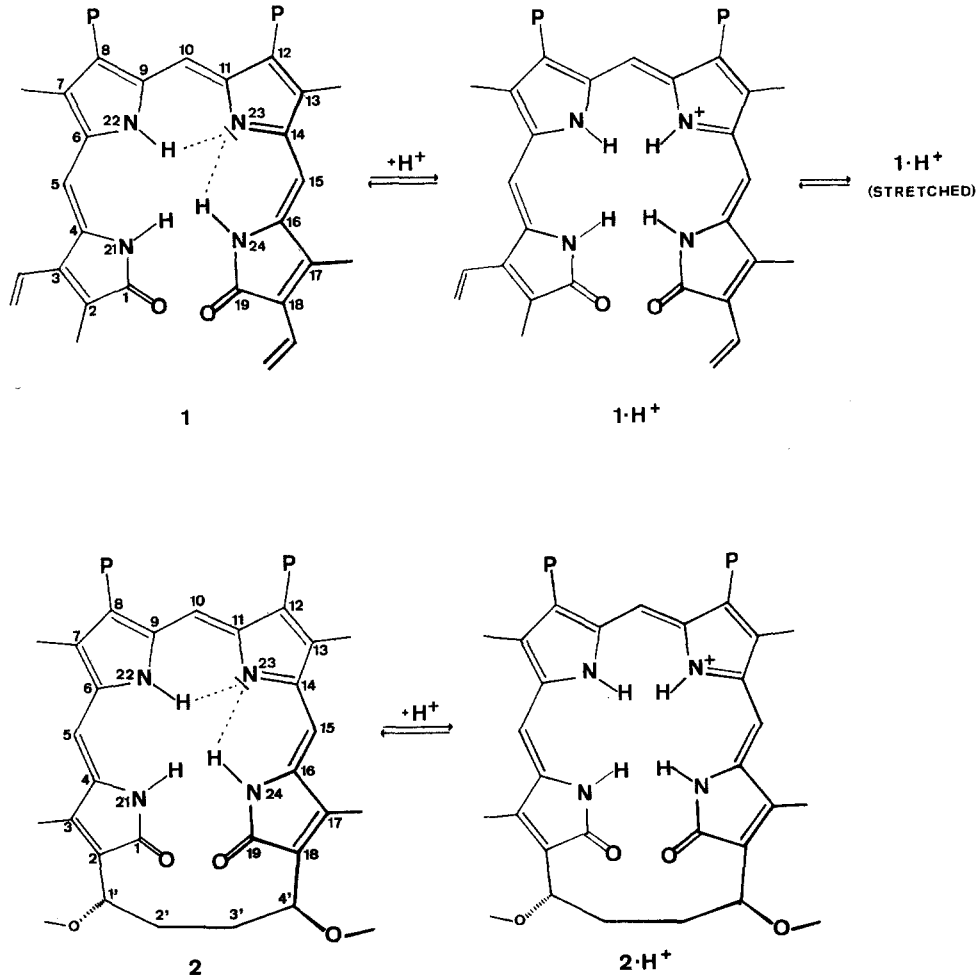
Keywords. Protonated biliverdins; Conformation dependent basicity; Bridged biliverdins; pK_a-Values; UV-VIS; NMR.

Zusammenhang zwischen Basizität und Konformation von Bilatrienen: Gestreckte und helikale protonierte Biliverdine

Zusammenfassung. Die Leichtigkeit der Protonierung von Biliverdinen am Pyrrolenin-Stickstoff hängt von ihrer Konformation ab. Das Biliverdin **2**, das durch eine viergliedrige Brücke in einer helikalen (*Z*, *Z*, *Z*, *syn*, *syn*, *syn*)-Geometrie festgehalten wird, ist um ca. drei Größenordnungen weniger basisch als flexible offenkettige Bilatriene, wie der Biliverdin-IXα-dimethylester (**1**), der – wie gezeigt wird – in seiner monoprotonierten Form eine gestreckte Konformation einnimmt. Diese Ergebnisse werden durch eine vergleichende Untersuchung der Titrationen von **1** und **2** mit Schwefelsäure in Methanol bzw. Methanol-Wasser-Gemischen mithilfe der UV-VIS- und NMR-Spektroskopie erhalten.

Introduction

Linear tetrapyrrols, the prosthetic groups of several chromoproteins (e.g. phycocyanins, phytochrome) are highly flexible molecules, which prefer to adopt a helical (*Z*, *Z*, *Z*, *syn*, *syn*, *syn*) conformation; this arrangement is believed to be stabilized by an efficient system of intra-chromophoric hydrogen bonds (see Scheme 1) [1]. In the native biliproteins, on the other hand, stretched geometries become favoured by the surrounding protein. In general, these stretched conformations are responsible for their distinct biologically important photophysical and photochemical properties [1–3]. In this context also *Z*-*E*-isomerizations and specific protonation-deprotonation equilibria are suspected to play a role [2, 4–7]. To elucidate these hypotheses numerous investigations have been performed on model compounds in recent years [1, 7–19].



Scheme 1. Protonation equilibria of biliverdins **1** and **2** including the possibility of a change in conformation for $\mathbf{1 \cdot H^+}$. For biliverdin-IXα dimethyl ester (**1**) only one tautomeric species is shown, to which numbering refers in the following. Compound **2** comprises a mixture of two racemic diastereoisomers, rapidly interconverting at room temperature, namely: [(*P*, 1′*R*, 4′*R*) + (*M*, 1′*S*, 4′*S*)]- and [(*M*, 1′*R*, 4′*R*) + (*P*, 1′*S*, 4′*S*)] − 2,18-(1′,4′-dimethoxybutane-1′,4′-diyl)-8,12-bis(2″-methoxycarbonylethyl)-3,7,13,17-tetramethyl-1,19-(21 *H*, 24 *H*)-bilindione. In the following only the stereochemical symbols are used for identification of these isomers; in this Scheme only isomer (*M*, 1′*S*, 4′*S*) **2** is shown

Protonation of linear bilatrienes-abc has been shown to be a very complex process due to involvement of aggregates and mono- and diprotonated species of unknown conformation, their respective populations being dependent on solvent and concentration of biliverdin and acid [12]. In protic solvents, where only monoprotonation occurs, an accompanying conformational transition has been suspected but could not be assessed conclusively up to now since the changes observed in UV-VIS spectra could likewise be due to the formation of the charged bilatriene chromophore itself [1]. To answer this question, bilatrienes with a fixed geometry are needed. Recently we described the synthesis of a macrocyclic biliverdin **2** possessing a (*Z*, *Z*, *Z*, *syn*, *syn*, *syn*) conformation—similar to that of neutral biliverdin-

IXα dimethyl ester (**1**)—which is fixed by its four link chain [16]. The comparative protonation study of these compounds (**1** and **2**) reported here gives evidence that the ease of protonation depends on conformation.

Results and Discussion

The variation of UV-VIS spectra during titration of **1** with sulfuric acid in methanol indicates that only one protonated species is involved if performed at low concentration ($c = 3 \cdot 10^{-6}\,M$) as evident from the appearance of isosbestic points (Fig. 1 a,

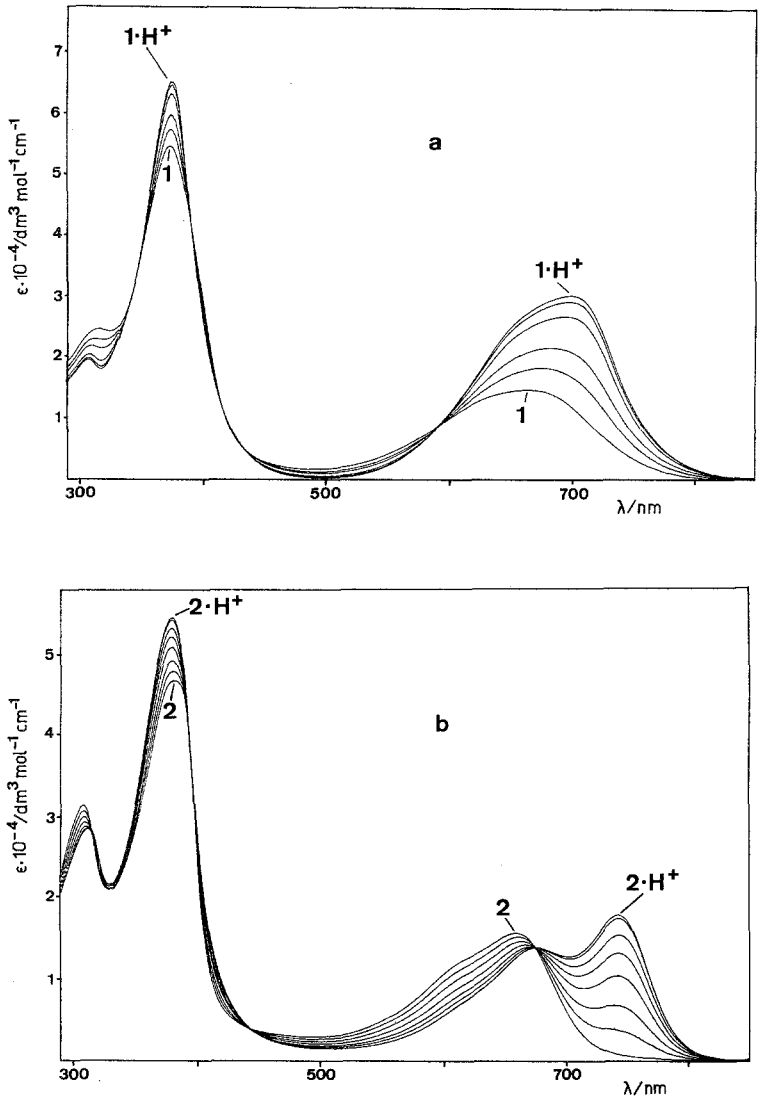


Fig. 1. Spectrophotometric titrations of biliverdins **1** and **2** ($c = 3 \cdot 10^{-6}\,M$) in methanol with sulfuric acid at 298 K: **a** Series of UV-VIS spectra of **1** obtained with increasing acid concentration (0.0, 0.30, 0.34, 0.46, 0.76, and $3.8 \cdot 10^{-4}\,N$); **b** Series of UV-VIS spectra of **2** obtained with increasing acid concentration (0.0, 0.0014, 0.007, 0.014, 0.035, 0.071, 0.25, and 0.53 *N*)

Table 1. UV-VIS spectra [$\varepsilon_{max}/dm^3 mol^{-1} cm^{-1}$ (λ_{max}/nm)] and ratios of dipole strengths $f = D_{UV}/D_{VIS}$ [a] of biliverdins **1** and **2** at different concentrations in methanol and methanol-water mixtures at 298 K and corresponding data for the protonated species $\mathbf{1}\cdot\mathbf{H}^+$ and $\mathbf{2}\cdot\mathbf{H}^+$ obtained after addition of sulfuric acid [b]

	Methanol[c] $c \sim 1\cdot10^{-2}M$	Methanol $c = 3\cdot10^{-6}M$		Methanol–water 64:36 *n/n* $c = 3\cdot10^{-6}M$		Methanol–water 41:59 *n/n* $c = 3\cdot10^{-6}M$	
	ε (λ)	ε (λ)	*f*	ε (λ)	*f*	ε (λ)	*f*
1	14800 (661)	14900 (664) 55100 (375)	2.65	14800 (668) 55600 (375)	2.67	14900 (669) 56400 (375)	2.76
$\mathbf{1}\cdot\mathbf{H}^+$	s36000 (700)[d] 45400 (662)	30200 (700)[e] s27200 (663) 65600 (375)	1.59	31900 (699)[f] s28700 (663) 69300 (376)	1.60	31400 (701)[f] s28500 (664) 70400 (375)	1.62
2	14700 (658)	14800 (660) 45600 (381)	2.62	15700 (660) 46900 (383)	2.58	16100 (660) 47700 (384)	2.61
$\mathbf{2}\cdot\mathbf{H}^+$	17700 (743)[g] 13200 (677)	17600 (744)[g] 13400 (676) 53600 (379)	2.41	[h]		[h]	

[a] For determination of dipole strengths of the visible bands and the first UV band integration was performed from λ = 850 nm to λ = 500 nm, and from λ = 480 nm to λ = 330 nm (cutoff), respectively

[b] Final spectra obtained during titrations; further addition of acid does not change spectra anymore

[c] Solutions also used for NMR experiments; methanol-d_4 with addition of 25% *v/v* $CDCl_3$ (**1**, $\mathbf{1}\cdot\mathbf{H}^+$) or 10% *v/v* $CDCl_3$ (**2**, $\mathbf{2}\cdot\mathbf{H}^+$) to enhance solubility. UV bands could not be recorded at this high concentration

[d] $c\ (D_2SO_4) = 0.05\,N$

[e] $c\ (H_2SO_4) = 0.0004\,N$

[f] $c\ (H_2SO_4) = 0.03\,N$

[g] $c\ (H_2SO_4, D_2SO_4) = 0.5\,N$

[h] Even in 3.6 *N* sulfuric acid protonation is not completed; it was estimated that ca. 90% $\mathbf{2}\cdot\mathbf{H}^+$ is present under these conditions

Table 1). This protonation process is completed at an acid concentration of $4\cdot10^{-4}N$; further addition of acid does not change spectra anymore. In more concentrated solutions of **1** ($c \geqslant 1\cdot10^{-4}M$) spectra of $\mathbf{1}\cdot\mathbf{H}^+$ become strongly dependent on concentration, indicating the formation of aggregates. This is in accord with earlier CD investigations in which a small but distinct association tendency was suggested for open chain biliverdin derivatives in acidic ethanol ($c = 2\cdot10^{-4}M$) [12].

In the case of the bridged biliverdin **2** [20], on the contrary, UV-VIS spectra are not affected by addition of sulfuric acid up to a concentration, at which **1** is already fully protonated [$c\,(H_2SO_4) = 4\cdot10^{-4}N$]. Even higher acid concentrations are required to cause spectral changes; the process–again characterized by an isosbestic behaviour–is completed only if the solution is ca. 0.5 *N* in sulfuric acid. No concentration dependence is observed up to $2\cdot10^{-2}M$ solutions of **2** (Table 1); the final spectrum displays considerable diversity if compared with the spectrum of $\mathbf{1}\cdot\mathbf{H}^+$, both with respect to band positions and *f*, the ratio of dipole strengths

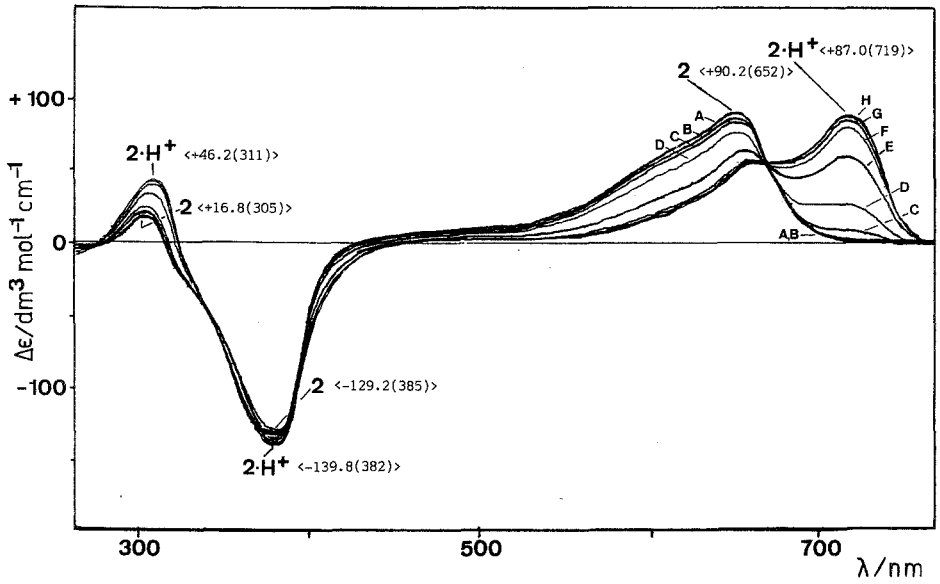


Fig. 2. Series of CD spectra obtained during titration of a mixture of (*P*, 1′*R*, 4′*R*) and (*M*, 1′*R*, 4′*R*) **2** ($c = 3 \cdot 10^{-5}$ *M*) in methanol with sulfuric acid at 293 K [20]. Concentrations of acid were: *A*, 0.0 *N*; *B*, 0.0005 *N*; *C*, 0.0016 *N*; *D*, 0.0064 *N*; *E*, 0.032 *N*; *F*, 0.064 *N*; *G*, 0.16 *N*; and *H*, 0.64 *N*. Maxima and minima [$\Delta\varepsilon_{max}(\lambda_{max})$] for the neutral (**2**) and the fully protonated species (**2** · H^+) are given in brackets

Table 2a. ^{1}H NMR parameter (250 MHz) (δ/ppm) of **1** and **1** · H^+ [a] in methanol-d_4 containing 25% *v*/*v* $CDCl_3$ at 303 K for $6 \cdot 10^{-3}$ *M* solutions and change in chemical shifts (Δδ/ppm) occurring in the course of protonation

	1 δ	**1** · H^+ δ	Δδ
10-H (1 H)	6.87 s	8.11 br s	+1.24
3-*Vn*-H_X (1 H)[b]	6.64 m	6.72 m	+0.08
18-*Vn*-H_X (1 H)	6.45 m	6.27 br	−0.18
5-H (1 H) / 15-H (1 H)	6.09 s / 6.06 s	6.50 br	~ +0.4
18-*Vn*-H_M (1 H)	5.98 m	~5.7 br	~ −0.3
3-*Vn*-H_B (1 H)	~5.6 m	5.81 m	~ +0.2
3-*Vn*-H_A (1 H)	~5.6 m	5.66 m	~ 0
18-*Vn*-H_A (1 H)	5.35 m	5.07 br	−0.28
COO*Me* (6 H)	3.61 s	3.62 s	+0.01
8-,12-CH_2 (4 H)	2.91 m	~3.3 m	~ +0.4
CH_2 − COO (4 H)	2.55 m	2.78 m	+0.23
17-*Me* (3 H)	2.14 s	2.17 s	+0.03
7-,13-*Me* (3 + 3 H)	2.08 s / 2.05 s	2.31 br s / 2.29 br s	~ +0.25
2-*Me* (3 H)	1.78 s	1.61 br s	−0.17

[a] Final spectrum obtained during titration with sulfuric acid-d_2; *c* (D_2SO_4) = 0.05 *N*

[b] *Vn* is used as abbreviation for the vinyl substituents of **1**

Table 2b. ^{1}H NMR parameter (250 MHz) (δ/ppm) of the two thermally interconvertible diastereoisomers [20] [(*P*, 1′*R*, 4′*R*) + (*M*, 1′*S*, 4′*S*)][a] and [(*M*, 1′*R*, 4′*R*) + (*P*, 1′*S*, 4′*S*)][b] of **2** and **2**·H^+ [c] in methanol-d_4 containing 10% *v/v* $CDCl_3$ at 303 K for $2 \cdot 10^{-2}$ *M* solutions and change in chemical shifts (Δδ/ppm) occurring in the course of protonation

	[(*P*, 1′*R*, 4′*R*) + (M, 1′*S*, 4′*S*)]			[(*M*, 1′*R*, 4′*R*) + (*P*, 1′*S*, 4′*S*)]		
	2 δ	**2**·H^+ δ	Δδ	**2** δ	**2**·H^+ δ	Δδ
10-H (1 H)	7.44 s	8.16 s	+0.72	7.26 s	7.99 s	+0.73
5-,15-H (2 H)	6.63 s	6.72 s	+0.09	6.42 s	6.48 s	+0.06
1′-,4′-H (2 H)	4.14 d	4.17 d	+0.03	4.42 br s	4.49 br s	+0.07
COO*Me* (6 H)	3.64 s	3.68 s[d]	+0.04	3.65 s	3.68 s[d]	+0.03
1′-,4′-O*Me* (6 H)	3.18 s	3.21 s[d]	+0.03	3.34 s	–[d]	–
8-,12-CH_2 (4 H)	3.13 t	3.27 t	+0.14	3.06 t	3.20 t	+0.14
CH_2–COO (4 H)	2.68 t	2.77 t	+0.09	2.64 t	2.74 t	+0.10
3-,17-*Me* (6 H)	2.36 s	2.40 s	+0.04	~2.4 s	~2.4 s	~0
7-,13-*Me* (6 H)	2.29 s	2.38 s	+0.09	2.21 s	2.32 s	+0.11
2′-,3′-H (4 H)[e]	–	–		–	–	

[a] 80% *n/n* throughout titration with sulfuric acid

[b] 20% *n/n* throughout titration with sulfuric acid

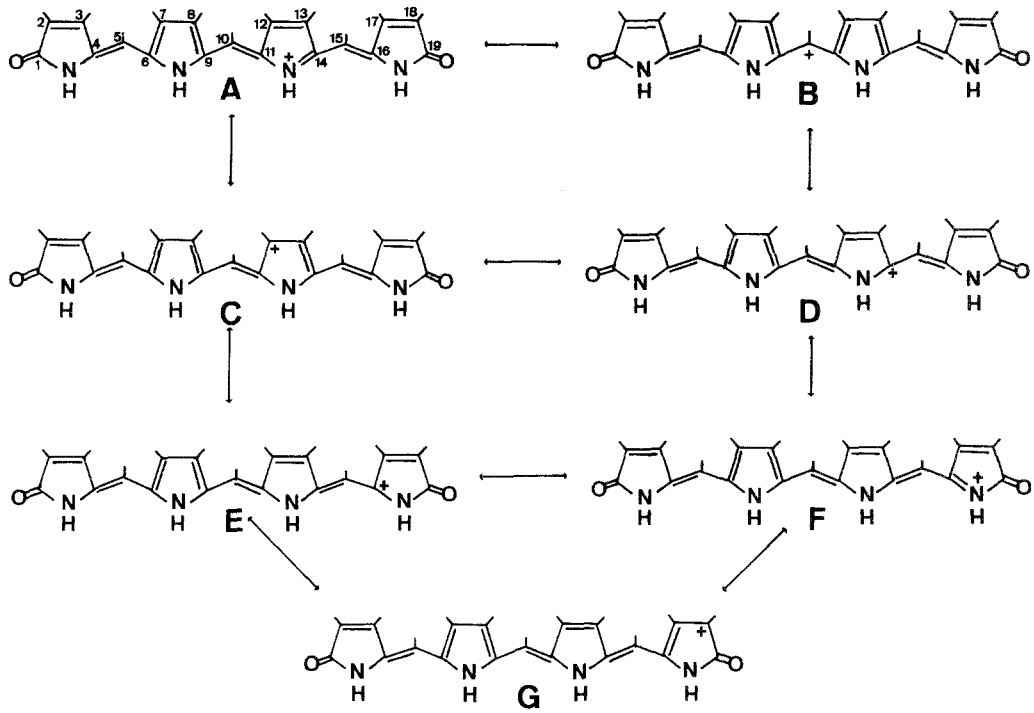
[c] Final spectra obtained during titration with sulfuric acid-d_2; *c* (D_2SO_4) = 0.5 *N*

[d] Due to exchange with CD_3OD the intensity of this signal gradually was diminished during titration; eventually the remaining signal could not be detected anymore in the final spectrum

[e] Not assigned (δ 2.5–1.0 ppm)

of the first UV and the visible absorption bands $f = D_{UV}/D_{VIS}$ (Table 1). Using optically active **2** of configuration (1′*R*, 4′*R*) at the chirality centres, resolved by first-order asymmetric transformation [17], the titration could also be followed by CD spectroscopy (Fig. 2) [21]. The final CD spectrum essentially correlates with the UV-VIS spectrum with respect to band positions. It exhibits positive visible bands and a negative first UV band for the predominating *P*-helical diastereoisomer: that means that the predictions concerning the sign of VIS- and UV-CD absorptions deduced for the *P* and *M* helix of neutral bilatrienes equally apply for the corresponding protonated helical chromophores [22].

Titrations of **1** and **2** ($c = 3 \cdot 10^{-6}$ *M*) performed with sulfuric acid in methanol and monitored by UV-VIS spectroscopy indicate that in each case only two species are involved in the spectral changes observed; but the striking result is the considerable difference in the acid concentrations necessary to produce these changes. Several explanations could provide for these findings: (i) Protonation of **2** takes place at the same acid concentration as for **1** but is not reflected by spectral changes since the conformation of **2** is fixed; the process observed with a large excess of acid would then represent a di-protonation step that does not occur with **1** under these conditions. (ii) Alternatively, the spectral changes observed for both **1** and **2** would be due to mono-protonation, that might take place at the same or (iii) different sites of the molecules. Interpretations (ii) and (iii) imply that protonation of **2** is considerably hindered.



Scheme 2. Presentation of all mono-charged mesomeric structures possible for a protonated bilatriene-abc chromophore

To answer these questions, titrations were simultaneously monitored by UV-VIS and NMR spectroscopy. To yield reliable NMR spectra these investigations had to be performed at higher concentration [23]. The most pronounced shift of ^{1}H-NMR absorption signals observed during titration of **1** concerns 10-H ($\Delta\delta = +1.2$ ppm), all other changes in chemical shifts being less than $|0.4|$ ppm (Table 2 a). These results are in accord with protonation at the pyrrolenine nitrogen N(23) [24] as has been generally suggested for bilatrienes-abc [1, 7, 9, 10, 13]. In this case a mesomeric species with the positive charge located at C(10) (Scheme 2, **B**) should contribute to the electronic structure of $\mathbf{1}\cdot\mathbf{H}^+$ thus causing the pronounced shift of 10-H to lower field. During titration of **2** changes in ^{1}H-NMR spectra exclusively occur parallel to the changes in UV-VIS spectra (i.e. at high acid concentration). Again it is 10-H which experiences a considerable downfield shift ($\Delta\delta = +0.7$ ppm); all other differences in signal positions being smaller than $|0.2|$ ppm (Table 2 b).

The ^{13}C-NMR investigation of the titration of **2** provides further evidence for the site of protonation (Table 3). The relative shifts observed for the carbons of the bilatriene backbone are in accord with the mesomeric structures possible for a biliverdin protonated at N(23) (Scheme 2). Carbons which acquire a positive charge in a mesomeric structure are generally shifted downfield ($\Delta\delta = +2$ to $+5$ ppm) [e.g. C(10, 12, 16, and 18)], whereas carbons adjacent to these centres of partial positive charge are shifted upfields ($\Delta\delta = -3$ to -5 ppm) [C(11, 15)] [25]. Protonation at any other possible site like CO(1) or 1'-OCH_3 can be safely excluded

Table 3. ^{13}C NMR parameter (62.9 MHz)[a] (δ/ppm) of the major diastereoisomer [20] [(*P*, 1′*R*, 4′*R*)+(*M*, 1′**S**, 4′*S*)][b] of **2** and **2**·H^{+}[c] in methanol-d_4 containing 10% *v/v* $CDCl_3$ at 303 K for $2 \cdot 10^{-2} M$ solutions and change in chemical shifts (Δδ/ppm) occurring in the course of protonation

	2 δ	**2**·H^{+} δ	Δδ
CO−OCH_3	174.79	174.31	−0.48
CO(1,19)	169.27	170.56	+1.29
C(6,14)	150.69	149.05	−1.64
C(3,17)	149.24	150.39	+1.15
C(9,11)	142.62	137.46	−5.16
C(4,16)	139.55	144.72	+4.2 to +5.3
C(8,12)	139.42	143.75	
C(2,18)	131.07	133.04	+1.97
C(7,13)	130.26	132.38	+2.12
CH(10)	116.00	119.77	+3.77
CH(5,15)	100.55	97.49	−3.06
CH(1′,4′)	76.62	76.38	−0.24
1′-, 4′-OCH_3	56.47	56.68[d]	+0.21
COO−**CH_3**	52.16	52.30[d]	+0.14
CH_2−COO	36.32	35.39	−0.93
CH_2(2′,3′)	29.65	29.44	−0.21
8-,12-CH_2	20.87	20.94	+0.07
3-,17-CH_3	9.89	10.01	+0.12
7-,13-CH_3	9.46	9.43	−0.03

[a] For the methods used for assignment of signals see Experimental
[b] See footnote [a] to Table 2b
[c] See footnote [c] to Table 2b
[d] See footnote [d] to Table 2b

by this method since the relative shifts Δδ of these carbons or proximate ones are small ($\Delta\delta \leqslant |1.3|$) (Table 3).

The NMR investigations thus clearly demonstrate that the changes observed in UV-VIS spectra during titrations of **1** and **2** in methanol with strong acid reflect monoprotonation at N(23). As already mentioned, this fact implies that the basicity of **2** is considerably lowered as compared with **1**. For quantification of that finding pK_a-values were determined for both compounds at low concentrations ($c = 3 \cdot 10^{-6} M$) in mixtures of methanol and water. However, in these solvent systems containing water **2** cannot be completely protonated even with 3.6 *N* sulfuric acid (10% *v/v*); thus the ratio of protonated (BVH^{+}) to non-protonated biliverdin species (*BV*) c_{BVH^+}/c_{BV} was calculated from the UV-VIS spectrum of **2**·H^{+} obtained in pure methanol as reference. Hill plots of $\log(c_{BVH^+}/c_{BV})$ vs. pH^* gave linear relationships with slopes near −1.1. The acidity constants thus determined are listed in Table 4. The pK_a-value for **1**·H^{+} agrees fairly well with one already published [9] and is close to the values determined for other open chain bilatrienes-abc [10]. The basicity of the bridged biliverdin **2**, on the other hand, is at least three orders of magnitude smaller!

Table 4. pK_a-values of $\mathbf{1}\cdot\mathbf{H}^+$ and $\mathbf{2}\cdot\mathbf{H}^+$ for different methanol-water mixtures as determined from spectrophotometric titrations of $3\cdot10^{-6}\,M$ solutions (of **1** and **2**, respectively) with sulfuric acid at 298 K by Hill plots of $\log(c_{BVH^+}/c_{BV})$ vs. pH^* [a]. Mean pK_a-values with their respective error ranges are estimated [b]

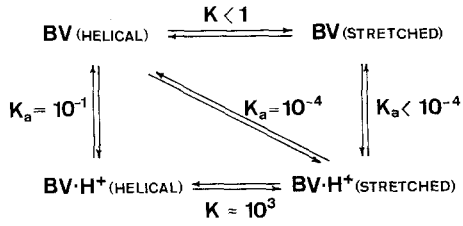
	pK_a	
	$\mathbf{1}\cdot\mathbf{H}^+$	$\mathbf{2}\cdot\mathbf{H}^+$
Methanol – water (64 : 36 *n/n*)	3.76	0.30
Methanol – water (51 : 49 *n/n*)[c]	3.73	0.45
Methanol – water (41 : 59 *n/n*)	3.89	0.55
Mean value	3.8 ± 0.2	0.4 ± 0.4

[a] Linear regression analysis was done for 7–14 points covering the entire accessible range. Correlation factors were −0.998 or −0.999 for all plots; slopes ranged from −1.0 to −1.1 for $\mathbf{1}\cdot\mathbf{H}^+$ and from −1.1 to −1.2 for $\mathbf{2}\cdot\mathbf{H}^+$

[b] For the error range the doubled standard deviation ($2\sigma_{n-1}$) was taken; for $\mathbf{2}\cdot\mathbf{H}^+$ an enhanced error, caused by *pH*-measurements in the range from *pH* 1 to *pH* 0.2 in methanol-water mixtures was additionally taken into account

[c] The UV-VIS data for this solvent mixture are not given in Table 1; they are similar to those in methanol-water 64 : 36 *n/n*

For an explanation of this remarkable finding a conformational analysis of the protonated species $\mathbf{1}\cdot\mathbf{H}^+$ and $\mathbf{2}\cdot\mathbf{H}^+$ was performed. Since the helical geometry of **2**—being quite similar to that of neutral open chain biliatrienes like **1** [16]—is invariant due to the constraints imposed by the four link chain the spectral properties of $\mathbf{2}\cdot\mathbf{H}^+$ are representative for a mono-protonated helical (*Z*, *Z*, *Z*, *syn*, *syn*, *syn*) bilatriene chromophore thus rendering the possibility to draw a comparison by UV-VIS spectra between protonated biliverdins with respect to conformation [26]. Remarkable is the occurrence of a long wavelength absorption band around $\lambda = 740$ nm shifted by approximately 80 nm with respect to that of the neutral species (Fig. 1) [27]. The *f*-value, on the other hand, experiences only a slight change from $f = 2.65$ (**2**) to $f = 2.41$ ($\mathbf{2}\cdot\mathbf{H}^+$) (Table 1) being within the range of variations for different solvents (e.g. $f = 2.41$ for **2** in benzene [18]). Thus, the quotient of dipole strengths *f* turns out to be only weakly influenced by protonation of the bilatriene chromophore if the conformation is preserved. This suggests that the UV/VIS criterion (*f*) [28] may be equally applied to detect conformational changes in protonation equilibria as has been done for neutral systems [1, 18]. The bathochromic shift of the long wavelength band observed for $\mathbf{1}\cdot\mathbf{H}^+$ is less pronounced but the UV/VIS ratio strikingly changes on protonation: whereas the *f*-value of neutral **1** ($f = 2.62$) is similar to that of **2** ($f = 2.65$), protonation diminishes this value to $f = 1.59$ ($\mathbf{1}\cdot\mathbf{H}^+$) (Table 1). This dramatic decrease in *f* indicates a changed conformation for $\mathbf{1}\cdot\mathbf{H}^+$. Parallel to predictions of theory developed for neutral bilatrienes [28], it is concluded that the protonated biliverdin-IXα dimethyl ester ($\mathbf{1}\cdot\mathbf{H}^+$) adopts a "stretched" geometry by rotation about at least one of the single bonds at C(5, 10, or 15). Since UV-VIS spectra of most protonated open chain bilatrienes-abc strongly resemble that of $\mathbf{1}\cdot\mathbf{H}^+$, this finding can be generalized.



Scheme 3. Protonation and conformation equilibria of a flexible bilatriene (schematic). Speculative estimation of corresponding equilibrium constants as derived from the results of this study

Conclusions

From the results reported here hindrance of protonation of **2** can be explained in terms of a dependence of this process on conformation. The flexible biliverdin-IXα dimethyl ester (**1**) is much more easily protonated and concomitantly changes its conformation from a helical to a more stretched one. Since for neutral bilatrienes the helical geometry is generally more stable [1] it follows from Scheme 3 that a stretched biliverdin should be more easily protonated than a helical one. The $\Delta pK_a = pK_a(\mathbf{1}\cdot\mathbf{H}^+) - pK_a(\mathbf{2}\cdot\mathbf{H}^+) = $ ca. 3 roughly reflects the energy difference between helical and stretched protonated bilatrienes-abc. Bearing in mind that protonation at the pyrrolenine nitrogen N(23) destroys the hydrogen bonding system based on this nitrogen acceptor, this study presents further evidence that the stretched conformation is generally more stable than the helical one if the intra-chromophoric hydrogen bonds are weakened or disrupted. This finding is reminiscent of recent investigations demonstrating that stretched conformations become relevant if compensation for the intra-chromophoric hydrogen bonds is provided by other optimally oriented donor groups as present in covalently bound peptides of the sequence Pro-X^1-X^2 [18].

The study presented now further supports earlier findings [12] showing that a meaningful investigation of the protonation process of bilatrienes should be restricted to polar solvents and low concentrations. In a previous paper [16] the question has been raised, whether the minor spectral changes observed for **2** at medium concentrations of trifluoroacetic acid in benzene or chloroform would be indicative of a protonation or not. Now—with the knowledge of the UV-VIS spectrum of $\mathbf{2}\cdot\mathbf{H}^+$—a protonation can be excluded. Even in 1 *M* trifluoroacetic acid only a small amount of $\mathbf{2}\cdot\mathbf{H}^+$ can be detected. The changes thus observed might be produced by association phenomena or even a reversible reaction occurring in these aprotic solvents. On the other hand, stronger acids such as methanesulfonic or *p*-toluene sulfonic acid (for pK_a-values of these acids see Ref. [29]) yield a high extent of protonation at relatively low acid concentrations in chloroform, as demonstrated by UV-VIS spectra similar to that of $\mathbf{2}\cdot\mathbf{H}^+$ in methanol [30]. Accordingly, not only solvent and concentration but also the strength of the acid used influences results obtained in comparative protonation studies of bilatrienes since their basicities might vary in a wide range.

Experimental

Compounds **1** [31] and **2** [16] were prepared according to the literature. Resolution of **2** was performed by asymmetric transformation as described in Ref. [17].

NMR spectra (^{1}H-NMR at 250 MHz, ^{13}C-NMR at 62.9 MHz and 100.6 MHz) were recorded with Bruker WM 250 and AM 400 instruments with $SiMe_4$ as reference, using $CDCl_3$ (chromato-

graphed on alumina prior to use), methanol-d_4 and sulfuric acid-d_2 (all Sigma) as solvents. Assignment of ^{13}C signals was done by comparison with estimated shift values using the "CSEARCH"-database [32] and 2D-NMR experiments (100.6 MHz ^{13}C frequency) using the standard software of Bruker. Especially a standard chemical shift correlation ($^1H/^{13}C$) via one bond C, H coupling and three $^1H/^{13}C$ chemical shift correlation experiments via long range coupling (COLOC) with delays optimized for coupling constants of 3.5 Hz, 5.0 Hz, and 7.5 Hz were performed (4 K × 128 W).

UV-VIS spectra were recorded with a Perkin-Elmer Lambda 7 spectrometer equipped with data station 3600. As solvents chloroform (LiChrosolv, Merck), benzene, methanol (Uvasol, Merck), and bidistilled water, as well as the solvents used for NMR experiments were applied. Benzene and chloroform were chromatographed on alumina prior to use. The acids for titration experiments were sulfuric acid (Merck, p.A.), trifluoroacetic acid and methanesulfonic acid (Fluka, distilled), and *p*-toluenesulfonic acid (Fluka, puriss.). CD measurements were performed with a Jobin Yvon Mark III instrument. For all optical measurements thermostatted (20° or 25° ± 1°C) quartz cuvettes ($d = 0.1 - 10$ cm pathlength) were used.

pK determinations were carried out at room temperature by adding sulfuric acid (of suitable concentrations) to 100 ml of a $3 \cdot 10^{-6}$ *M* solution of **1** or **2** in the respective methanol-water mixture using a digital *pH* meter WTW *pH* 96 with a WTW E 50 glass electrode calibrated against appropriate buffer solutions. The set of UV-VIS spectra recorded exhibits isosbestic points. The ratio c_{BVH^+}/c_{BV} was calculated from extinction coefficients at 720, 700, and 375 nm (for **1**) or 740 and 600 nm (for **2**). The plot of $\log(c_{BVH^+}/c_{BV})$ vs. *pH** (Hill plot) gave straight lines with excellent correlation coefficients (Table 4). Since no spectra for $\mathbf{2} \cdot \mathbf{H}^+$ could be obtained for these solvent mixtures containing water, the extinction coefficients of the spectrum in methanol were taken as reference; this assumption also gave the best correlation in the Hill plots. At low *pH* in methanol-water mixtures partial hydrolysis of the methyl esters of **1** and **2** occurs. But this process did not affect UV-VIS spectra detectably. In these cases full reversibility could only be demonstrated after re-esterification.

Generally, solvents were deaerated before use and acidic solutions protected from light. All acid dependent processes observed (UV-VIS, NMR) were fully reversible upon neutralization.

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[20] Compound **2** comprises a mixture of thermally interconvertible diastereoisomers, i.e. [(*P*, 1′*R*, 4′*R*)+(*M*, 1′*S*, 4′*S*)] and [(*M*, 1′*R*, 4′*R*)+(*P*, 1′*S*, 4′*S*)] whose UV-VIS spectra are very similar. In NMR experiments the individual spectra of the two diastereoisomers are observed [16]
[21] The ratio of thermally interconvertible diastereoisomers of **2**, i.e. (*P*, 1′*R*, 4′*R*)/(*M*, 1′*R*, 4′*R*) (80/20 *n*/*n*) is not affected by addition of sulfuric acid (NMR). Thus, a constant helical excess (h.e. = 60%) of *P*-helix is maintained throughout titration
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[23] Self-association occurring with $\mathbf{1} \cdot \mathbf{H}^+$ at this concentration ($c = 6 \cdot 10^{-3}$ *M*) leads to considerable broadening of some resonance absorptions but only minor shifts in ^{1}H-NMR spectra. ^{13}C-NMR spectra revealed none of the carbon signals of the bilatriene backbone due to broadening and hence provide no valuable information
[24] Here and in the following numbering refers to one of the two tautomeric species possible for **1** (see Scheme 1)
[25] Of course not all mesomeric structures of Scheme 2 are equally contributing to the electronic structure of $\mathbf{2} \cdot \mathbf{H}^+$. Since no relative shifts $\Delta\delta$ (Table 3) exceed |6| ppm, mesomer **A** seems to be of major importance. The negative relative shift of C(6, 14) can be explained taking into account that the C(14)=N(23) bond responsible for the shift of C(14) in **2** [1], is now directly involved in the protonation process
[26] The UV-VIS spectrum of a N(21)-N(24) methanobridged biliverdin in acidic chloroform [19] does not serve as suitable reference since (i) the helix of this compound is severly flattened if compared with open chain neutral bilatrienes and (ii) it is not clear if and what kind of protonation took place in this aprotic solvent. The biliverdinium salts synthesized by the group of J. Lugtenburg [8] are all modified (by bridging or N-methylation) at the nitrogens (22, 23). Thus their UV-VIS spectra do not serve as suitable references in this context neither
[27] This change in band positions phenomenologically resembles that observed for the transition of the two forms of phytochrome $P_r \rightleftharpoons P_{fr}$ [2, 5]. This result demonstrates that protonation might provide for spectral shifts of this magnitude (compare Ref. [6])
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