Monatshefte für Chemie 116, 119—131 (1985)

Monatshefte für Chemie
Chemical Monthly

New Aspects of the Protonation of Biliverdins**

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(Received 6 September 1984. Accepted 4 October 1984)

Biliverdins undergo appreciable self association in acidic solutions especially if solvents like benzene or chloroform are employed. The population of aggregates further depends on the concentration of both the acid and the solute. In chloroform and benzene solutions at high acidity a doubly protonated species is formed. This is concluded from a combinatory evaluation of the CD and electronic absorption spectra of chiral optically active biliverdins (**1**–**6**) in benzene, chloroform, and ethanol solutions containing trifluoroacetic acid or hydrochloric acid. The aggregates formed at medium acid concentrations exhibit large *Cotton* effects in the long-wavelength absorption bands and thus dominate the CD spectra. Similarly, agrregates are the main contributors to the *Cotton* effects of achiral, protonated biliverdins in (*S*)-(−)-ethyl lactate (SICD) at usual concentrations. The consequence and relevance of these findings with regard to the recent literature is briefly discussed.

(Keywords: Protonated biliverdins; Association; CD; SICD; Optical activity)

Neue Aspekte bei der Protonierung von Biliverdinen

Biliverdine unterliegen in saurer Lösung einer beträchtlichen Selbstassoziation, besonders dann, wenn Lösungsmittel wie Benzol oder Chloroform verwendet werden. Die Population der gebildeten Aggregate hängt ferner von der Konzentration der Säure und des Biliverdins ab. Bei hohen Säurekonzentrationen kommt es in Chloroform und Benzol zur Ausbildung einer doppelt protonierten Spezies. Dies folgt aus den CD und Absorptionsspektren chiraler optisch aktiver Biliverdine (**1**−**6**) in Benzol, Chloroform und Ethanol bei der Titration mit Trifluoressigsäure bzw. Salzsäure. Die Aggregate, die bei mittleren Säurekonzentrationen auftreten, besitzen starke *Cotton*-Effekte im langwelligen Absorptionsbereich und bestimmen daher das CD Spektrum. Bei üblichen Konzentrationen liefern Aggregate auch den Hauptbeitrag zu den *Cotton*-Effekten achiraler, protonierter Biliverdine (SICD) in (*S*)-(−)-Milchsäureethylester. Die Folgen und die Relevanz dieser Ergebnisse im Hinblick auf die neuere Literatur werden kurz diskutiert.

** Dedicated to Prof. Dr. *Karl Schlögl* on occasion of his 60th birthday.

Introduction

Protonation and deprotonation of bile pigments have aroused considerable interest in recent years, especially in connection with the biological function of chromoproteins[1]. The most thoroughly investigated compounds are biliverdins[2–11]. Their exclusive protonation at the pyrrolenin moiety has been generally accepted. Strikingly, the amount of acid added is ill defined so that studies often refer to appreciably different concentrations. On the other hand, it has been claimed, that different protonated species of biliverdin are present in solutions of different acidity[7]. If this were true some authors would have compared unlike species. The situation becomes even more complex by the finding that, in general, protonated biliverdins in solution constitute a heterogeneous solute[8]. In view of the increasing number of studies concerning structure and conformation of chromoproteins and their chromophores in acidic solutions (see Ref.[1] and Refs. cited therein) the resolution of the dilemma outlined above seems necessary.

The availability of optically active biliverdin derivatives[12] now offers the opportunity to undertake a study on protonated biliverdins by means of circular dichroism (CD). The investigations presented are complemented by results on achiral biliverdins in optically active media (SICD).

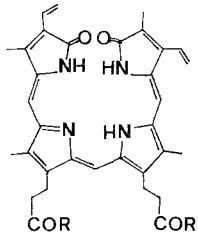
Results

If trifluoro acetic acid (*TFA*) is added successively to a $1.7 \cdot 10^{-4} M$ solution of the alanin derivative **1** in benzene the long-wavelength band[13] of the absorption spectrum ($\lambda = 653$ nm) experiences a slight bathochromic shift accompanied by an increase of absorbance until a *TFA* concentration of $\simeq 3 \cdot 10^{-3} M$ is reached (Fig. 1 *a*). Simultaneously, a shoulder at $\lambda \simeq 730$ nm emerges. Further increasing the acid concentration causes a hypsochromic shift of the main band to $\lambda \simeq 630$ nm with a concomitant increase of absorbance and a decrease of the shoulder at $\lambda \simeq 730$ nm. If the solution is $\simeq 5 \cdot 10^{-2} M$ in *TFA* the absorption spectrum substantially becomes insensitive to additional acid. Qualitatively the same phenomena during titration are observed for chloroform solutions of **1** or if achiral substrates such as biliverdin dimethyl ester (**7**) are investigated in acidic benzene or chloroform.

The corresponding CD spectra are shown in Fig. 1 *b*. If the concentration of *TFA* is close to that of the substrate ($1.7 \cdot 10^{-4} M$) the CD exhibits a positive band at $\lambda \simeq 735$ nm. In more acidic solutions two bands, at $\lambda \simeq 730$ nm and $\lambda \simeq 660$ nm appear. They are opposite in sign and possess similar intensities. Their extrema ($\Delta\varepsilon_{733} = -48$, $\Delta\varepsilon_{657} = +48$) are reached at $\simeq 3 \cdot 10^{-3} M$ *TFA*. Similarly strong bands are

observed for compounds **2–6** (Table 1). On further addition of *TFA* the intensities of these bands synchroneously decrease and a positive CD band at $\lambda \simeq 625$ nm emerges. No further substantial changes occur when the concentration of acid exceeds $\simeq 5 \cdot 10^{-2} M$.

Performing the titration of **1** at the same concentration ($1.7 \cdot 10^{-4} M$) in ethanol-water (5% *v/v*) the VIS absorption band again is red shifted (Fig. 2a). However, in contradistinction to the benzene and chloroform solutions this shift (665 → 685 nm) is more pronounced and, in addition, no shoulder can be observed at $\lambda \simeq 730$ nm. At $\simeq 1 \cdot 10^{-2} M$ *TFA* the



R

1	(S)-AlaOMe	5	(R)-NHCHCH$_2$C$_6$H$_5$ (Me)
2	(S)-LeuOMe	6	(S)-OCHCOOEt (Me)
3	(S)-ValOMe		
4	Gly-(S)-ValOMe	7	OMe

main absorption band reaches its maximum intensity. Further addition of acid does not substantially change the spectrum. The corresponding CD spectra (Fig. 2*b*) are less complex than those obtained for acidic benzene solutions. From $\simeq 3 \cdot 10^{-4} M$ up to $\simeq 1 \cdot 10^{-2} M$ *TFA* the CD is mainly governed by a steady and synchroneous increase of the double band at $\lambda \simeq 730$ nm and $\lambda \simeq 660$ nm. In contradistinction to the situation in benzene the intensity of the bisignate band is not diminished when the acid concentration is further increased. However, its intensity is much lower ($\Delta\varepsilon_{733} = -8$, $\Delta\varepsilon_{655} = +12$) than that obtained at the optimum acidity in benzene or chloroform (Fig. 1*b*, Table). The same phenomena are observed when **1** in ethanol-water solution is titrated with aqueous concentrated HCl.

The shoulder in the absorption spectrum of protonated **1** in benzene and the double bands arising in the CD spectra for acidic benzene *and* ethanol solutions prove to be very susceptible towards dilution[14]. Upon lowering the concentration of **1** from $\simeq 2 \cdot 10^{-4}$ to $\simeq 3 \cdot 10^{-6} M$ at constant acid concentration ($3.3 \cdot 10^{-3} M$ *TFA*) the absorption spectrum

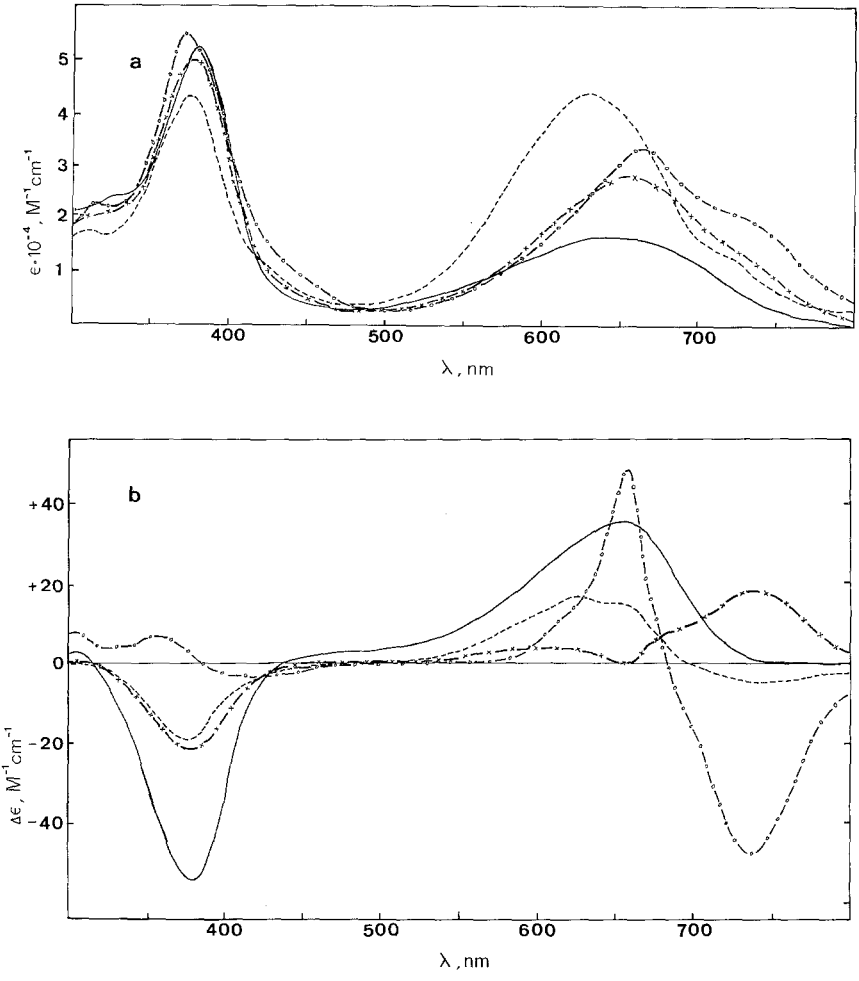


Fig. 1. *a* UV-VIS absorption spectra and *b* CD spectra of **1** ($1.7 \cdot 10^{-4} M$) in benzene at different concentrations of *TFA*: 0 *M* (———), $1.6 \cdot 10^{-4} M$ (– × –), $3.3 \cdot 10^{-3} M$ (– ○ –), and $5.0 \cdot 10^{-2} M$ (– – –) at 293 K

in benzene experiences a decrease of the shoulder around $\lambda \simeq 730$ nm with a concomitant hypsochromic shift and increase of intensity of the main absorption band (Fig. 3). The corresponding CD spectrum shows a pronounced decrease of the double band (Fig. 3).

A similar experiment at higher acid concentration ($5.0 \cdot 10^{-2} M$ *TFA*) shows a decrease of the shoulder at $\lambda \simeq 730$ nm, a further enhancement of absorption and a hypsochromic shift of the main band (635 → 627 nm) in

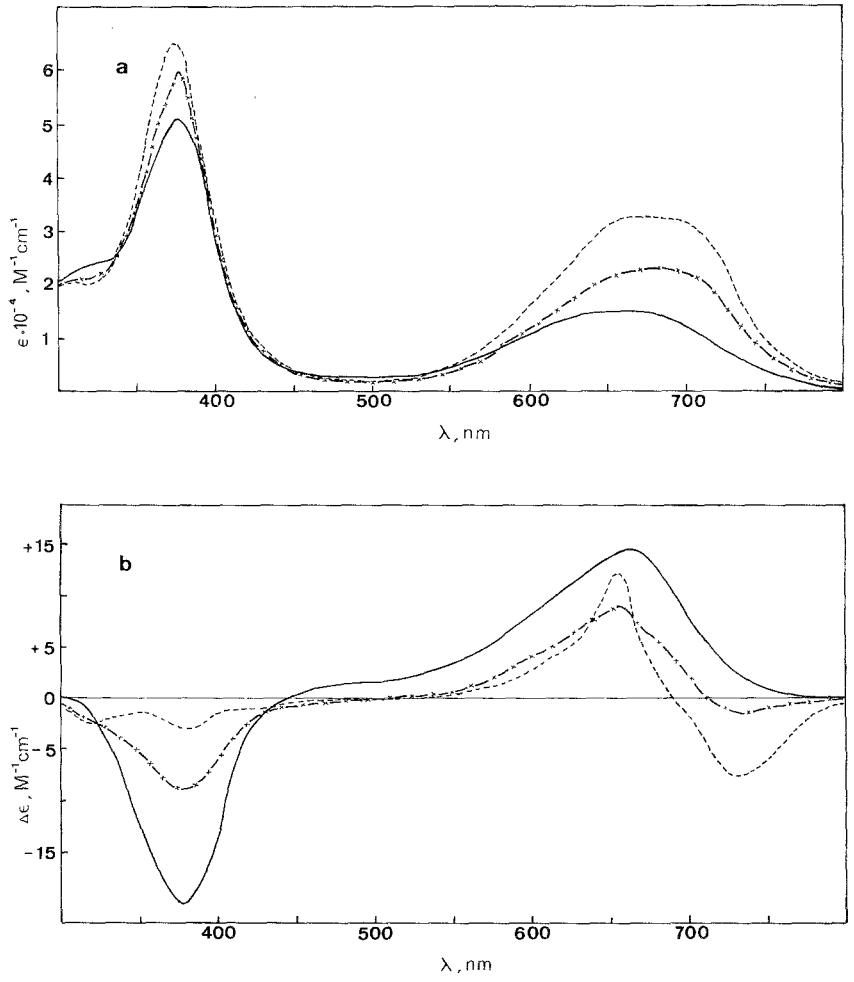


Fig. 2. *a* UV-VIS absorption spectra and *b* CD spectra of **1** ($1.7 \cdot 10^{-4} M$) in ethanol-water (5% *v/v*) at different concentrations of *TFA*: 0 *M* (———), $3.4 \cdot 10^{-4} M$ (— × —), and $1.2 \cdot 10^{-2} M$ (— — —) at 293 K

the absorption spectrum during dilution (Fig. 4). In the CD spectrum the double band ($\lambda \simeq 730$ and 660 nm) vanishes and the *Cotton* effect at $\lambda \simeq 625$ nm becomes larger.

The dilution experiment performed in ethanol-water/HCl/NaCl ($pH^* = 2.7$) is shown in Fig. 5. While the absorption spectra show close similarity at the two concentrations chosen, the CD spectrum of the less concentrated solution ($\simeq 8 \cdot 10^{-6} M$) exhibits a weak band at $\lambda \simeq 660$ nm

Table 1. *CD data of optically active protonated biliverdins* **1–6** ($c \simeq 1.7 \cdot 10^{-4} M$) at 293 K *in various solvents;* λ, nm ($\Delta\varepsilon$, $M^{-1}\,cm^{-1}$)

Solvent	**1**	**2**	**3**	**4**	**5**	**6**
benzene[a]	733 (−48) 657 (+48)	735 (−150) 657 (+110)	738 (−90) 660 (+80)	735 (+70) 657 (−70)	735 (−140) 657 (+125)	730 (−23) 656 (+21)
chloroform[b]	730 (−52) 657 (+50)	730 (−107) 657 (+94)			728 (−75) 655 (+71)	
ethanol[c]	733 (−8) 655 (+12)	730 (−7) 655 (+12)			730 (−7) 654 (+6)	

[a] The maximal $\Delta\varepsilon$ values are reached at $\simeq 3 \cdot 10^{-3} M$ *TFA* (**1–3, 5**) and $\simeq 8 \cdot 10^{-3} M$ *TFA* (**4, 6**), respectively.
[b] The maximal $\Delta\varepsilon$ values are reached at $\simeq 8 \cdot 10^{-3} M$ *TFA*.
[c] The maximal $\Delta\varepsilon$ values are reached at $\simeq 1 \cdot 10^{-2} M$ *TFA* or HCl.

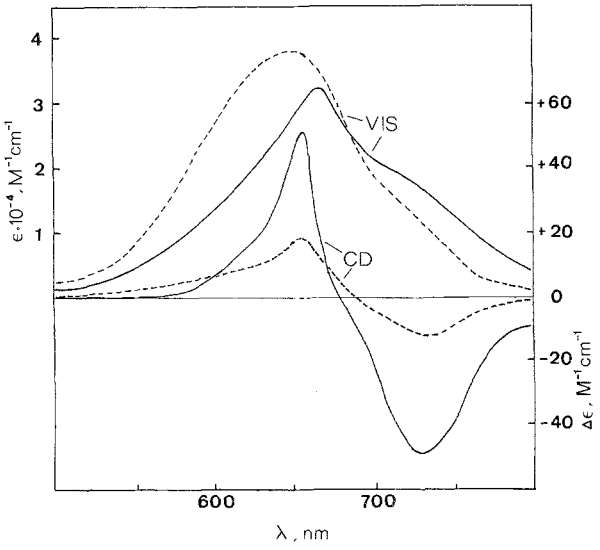


Fig. 3. VIS absorption and CD spectra of **1** ($1.9 \cdot 10^{-4}\,M$, ——— and $2.7 \cdot 10^{-6}\,M$, – – –) in benzene at constant concentration of *TFA* ($3.3 \cdot 10^{-3}\,M$) at 293 K

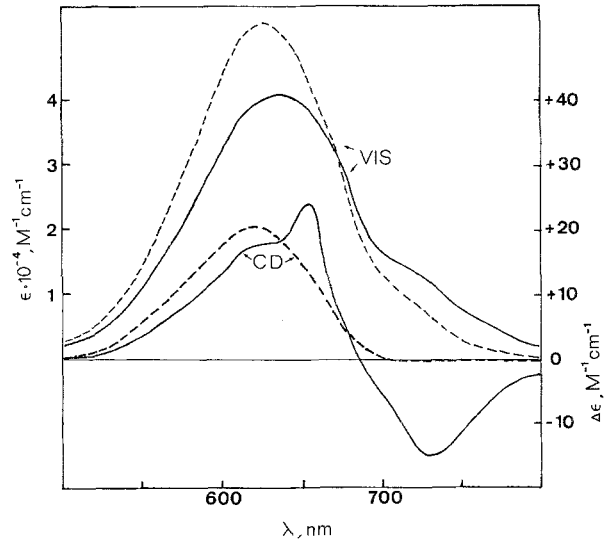


Fig. 4. VIS absorption and CD spectra of **1** ($1.6 \cdot 10^{-4}\,M$, ——— and $3.2 \cdot 10^{-6}\,M$, – – –) in benzene at constant concentration of *TFA* ($5.0 \cdot 10^{-2}\,M$) at 293 K

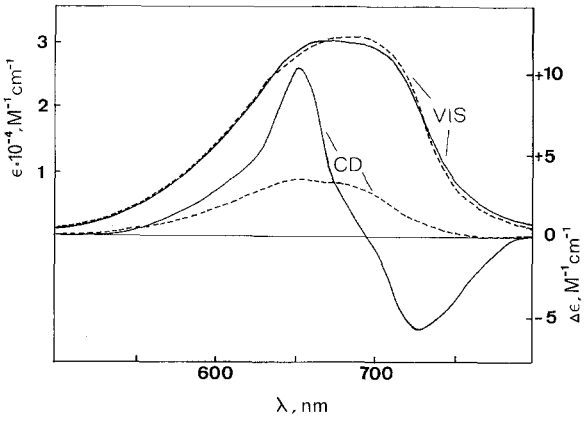


Fig. 5. VIS absorption and CD spectra of **1** ($1.9 \cdot 10^{-4}\,M$, ——— and $7.6 \cdot 10^{-6}\,M$, — — —) in aqueous ethanol (90% *v/v*) – NaCl ($4.8 \cdot 10^{-2}\,M$) – HCl ($1.2 \cdot 10^{-2}\,M$) ($pH^* = 2.7$) at 293 K

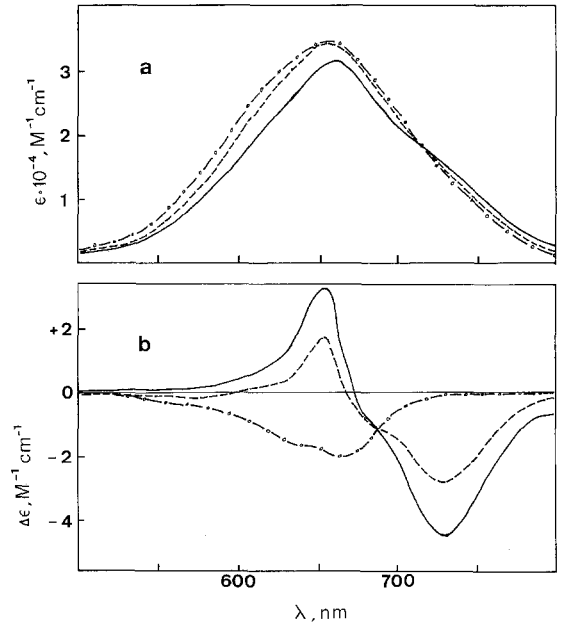


Fig. 6. *a* VIS absorption spectra and *b* SICD spectra of **1** ($4.3 \cdot 10^{-4}\,M$, ———; $8.5 \cdot 10^{-5}\,M$, — — —; and $8.5 \cdot 10^{-6}\,M$, – ○ –) in (*S*)-(–)-ethyl lactate at constant concentration of *TFA* ($5.0 \cdot 10^{-2}\,M$) at 293 K

instead of the more intense double band observed at higher concentration ($\simeq 2 \cdot 10^{-4} M$) of **1**.

Qualitatively the same spectroscopic results are obtained for the chiral derivatives **2–6**.

The spectral changes observed for biliverdin dimethyl ester (**7**) in (*S*)-(−)-ethyl lactate on successive addition of *TFA* are very similar to the behaviour of **1** in ethanol-water. In close relation, the CD spectrum phenomenologically changes on dilution. Thus the bisignate band ($\Delta\varepsilon_{730} = -4.5$, $\Delta\varepsilon_{655} = +3.2$) is replaced by a single band ($\Delta\varepsilon_{665} = -2.0$) (Fig. 6).

The spectral phenomena observed are fully reversible on addition of triethylamine.

Discussion

The course of titrations of biliverdins with acid turns out to be very complex especially when carried out in benzene or chloroform solutions[13]. Apparently, there exist equilibria between different species. The position of these equilibria is dependent on the concentration of the solute, the concentration of the acid, and the nature of the solvent. Four species can be detected. If the *pK* values for biliverdins given in the literature[3–6] are reliable, our results then imply the existence of a monoprotonated monomeric species, charged aggregates, and a diprotonated species. This will be outlined in some detail below.

The shoulder around $\lambda \simeq 730$ nm in the VIS absorption spectrum and the double band in the CD spectrum of **1** in acidic benzene synchroneously increase or decrease with the concentration of both *TFA* (Fig. 1) and the solute **1** (Fig. 3). Hence, both spectral phenomena are due to the same species (species **A**). From the dilution experiment (Fig. 3) it follows that species **A** constitutes a protonated aggregate. Since at that stage of titration **1** has been protonated to a large extent, the aggregate **A** most likely consists of monoprotonated molecules[15]. The $\Delta\varepsilon$ values ($\Delta\varepsilon_{733} = -48$, $\Delta\varepsilon_{657} = +48$) due to species **A** of **1** obtained in benzene/*TFA* are similar to those obtained for the other chiral derivatives **2–6** investigated. The $\Delta\varepsilon$ values for the leucine derivative **2** may even be as high as −150/+110 (Table). The large rotational strengths of the two bands might be due to chromophoric coupling. Since species **A** represents an aggregate its population is markedly decreased in solvents capable of hydrogen bonding. Thus, the characteristic shoulder on the long-wavelength edge of the main absorption band observed for benzene solution is scarcely detectable in ethanol-water (Fig. 2 *a*). Furthermore, the $\Delta\varepsilon$ values of the double band of the corresponding CD spectrum are quite smaller (Fig. 2 *b*, Table). In more dilute solutions this double band completely vanishes (Fig. 5). Hence, at usual concentrations even in hydroxylic solvents the

CD spectra of optically active biliverdins mainly reflect the properties of the aggregate **A** rather than those of the monomeric monoprotonated species (species **B**). Expectedly, the changes of the absorption spectrum on dilution are minute and uncharacteristic because species **B** predominates and the population of aggregates is small *a priori*. Thus, even if the determinations of the *pK* values of biliverdins in protic solvents by spectrophotometric titration[4–6] have been carried out at concentrations higher than 10^{-6} *M* the values given in the literature can be regarded as fair approximations.

Remarkably, the population of the aggregate **A** in benzene solution increases and decreases during titration (Fig. 1 *b*). On the other hand, in ethanol-water solutions a corresponding decrease cannot be observed even if the *TFA* or HCl concentration is high (Fig. 2 *b*). Clearly, the acid strength of *TFA* is quite larger in benzene than in ethanol-water. Therefore, the aggregate **A** is destroyed in benzene and chloroform solutions by double protonation[16]. This process is less likely in ethanol and ethanol-water[17]. The diprotonated species (species **C**) possesses absorption and CD maxima at $\mu \simeq 625$ nm. It exhibits a positive *Cotton* effect ($\Delta\varepsilon \simeq +21$) and a large absorbance in the long-wavelength region (Fig. 4).

In benzene solution a fourth species (species **D**) occurs when the acid concentration is smaller or equals that of the solute **1**. It can be easily detected by its positive CD band at $\lambda \simeq 735$ nm which is different from those obtained for neutral **1**, species **A**, **B**, and **C** (Fig. 1 *b*). Most probably it constitutes an aggregate formed of neutral and monoprotonated **1**.

The results obtained for the derivatives **2**–**6** closely resemble those of the alanin derivative **1** with regard to the occurrence of species **A**, **B**, **C**, and **D**[15]. Likewise, the achiral biliverdin dimethyl ester (**7**) in benzene and chloroform solution shows similar phenomena during titration when monitored by electronic absorption spectroscopy. Unfortunately, UV-VIS spectra alone turn out to be less suited for the detection of the heterogeneities under consideration. For example, in acidic ethanol-water solutions the absorption spectra of biliverdin dimethyl ester (**7**) and those of the chiral derivatives do not give any hint for the coexistence of an aggregate at higher concentrations. The changes observed during dilution are only small and uncharacteristic (Fig. 5). Nevertheless, the protonated species observed for the chiral compounds **1**–**6** should be characteristic for biliverdins in general.

Aggregation is also responsible for the occurrence of the double band in the long-wavelength region of the CD of achiral biliverdin dimethyl ester (**7**) in (*S*)-(−)-ethyl lactate (Fig. 6). As observed for the CD of the chiral compounds **1**–**6**, the SICD of **7** is likewise very sensitive to dilution. If the solution is $\simeq 8 \cdot 10^{-6}$ *M* in **7** a single, weaker band at $\lambda \simeq 660$ nm appears[18].

Concluding Remarks

The relatively large *Cotton* effects of optically active *Z,Z,Z*-configurated biliverdins in neutral solutions have been shown to reflect an excess population of one helical conformer[12]. Similar conclusions have been arrived at for the CD of achiral biliverdins in (*S*)-(−)-ethyl lactate[8,19,20]. In acidic solvents, however, the occurrence of aggregates prevents a meaningful application of CD methods to conformational analysis. Hence, the relation between conformation and CD data gained for neutral biliverdins cannot simply be extended to acidic solutions as has been done in Ref.[21]. The conditions for the exclusive occurrence of the monomeric monoprotonated state of biliverdins in acidic solutions are only met at very low concentrations using solvents providing effective hydrogen bonding sites. Aggregation in solution may also occur in other protonated bile pigments. Therefore, the monomeric state of protonated bilatrienes and chromopeptides should be assessed before drawing stereochemical conclusions from CD data.

From the investigations presented it becomes obvious that the absorption, resonance *Raman,* and fluorescence spectra of biliverdin dimethyl ester (**7**) in chloroform solutions of different acidity reported in Ref.[7] reflect an acidity dependent population of monoprotonated and diprotonated species including aggregates rather than the properties of pure species, designated as "Cation I" and "II". This is because the spectra have been obtained at high concentration in acidic chloroform solutions.

In the light of our findings the "far red" and "red" emitting species of protonated biliverdin dimethyl ester (**7**) in ethanol found in Ref.[8] can be assigned to an aggregated and a monomeric species, respectively. However, it is unlikely that the "red" emitting species detected in ethanol and (*S*)-(−)-ethyl lactate solution at high acid concentrations should be identical with that postulated for chloroform or toluene solutions.

Note Added in Proof

An influence of an external point charge on the dihydrobilatriene-abc system could not be observed [*Falk H., Zrunek U.,* Monatsh. Chem. **115**, 1071 (1984)]. The phenomena observed in Ref.[10] on the bilatriene-abc system may partly be attributed to association equilibria similar to those described above. However, an unequivocal decision can only be made by additional careful studies.

Experimental

The preparation of compounds **1**–**3**[12], **4**[22], **5**[12], **6**[12], and **7**[19] has been described previously.

Electronic absorption spectra were taken at 20 °C with a Cary 15 spectrometer (0.1–2.0 cm quartz cuvettes). The CD spectra were recorded with a Jobin Yvon Mark III instrument carrying cylindrical quartz cuvettes (0.05–2.0 cm) at 20 °C.

The CD spectra have been corrected for their baselines. Spectroscopic grade (Uvasol, Merck) ethanol, benzene, and chloroform (freed from ethanol by chromatography over alumina oxide) were used. (*S*)-(−)-ethyl lactate (Fluka) was twice distilled through a *Vigreux* column, $[\alpha]_D^{20} = -10.3°$ (neat). Water refers to bidistilled water.

Titrations were carried out at 20 °C by adding increasing amounts of concentrated *TFA* to stock solutions of the appropriate biliverdin. All solutions used have been degassed by several freeze-pump-thaw cycles immediately after preparation and were protected from light.

Acknowledgements

Financial support by the *Hochschuljubiläumsstiftung der Stadt Wien* and the *Fonds zur Förderung der wissenschaftlichen Forschung* (Project 3033) is gratefully acknowledged.

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[13] For brevity, we restrict our discussion to the long-wavelength band.
[14] The concentration dependence of the CD and UV-VIS spectra of protonated biliverdins is in contradistinction to the neutral forms[12].
[15] Our notation of different species **A**, **B**, **C**, and **D** refers to the state of aggregation and the degree of protonation only and *not* to conformation. We cannot exclude a conformational heterogeneity of the single species.
The interconversion of the species under consideration must be rapid at room temperature. Upon monitoring the titration of **1** (CD_2Cl_2, *TFA*) by ^1H NMR (250 MHz) only the chemical shifts are changed but all spectra reveal virtual homogeneity (*Krois D., Lehner H.*, unpublished results).
[16] From our experiments the second protonation site of the bilatriene cannot be derived.
[17] Thus, absorption and CD spectra in acidic benzene and chloroform solutions are particularly sensitive towards addition of traces of ethanol and water.

[18] The position of equilibria and the nature of the species of protonated biliverdins in chiral optically active transparent solvents most probably depends also on the substituents directly bound to the bilatriene backbone. As an example, on dilution the SICD of protonated octaethylbilindion in (*S*)-(−)-ethyl lactate *increases* and the sign of bands is opposite to the SICD of protonated biliverdin dimethyl ester (**7**) (*Krois D., Lehner H.*, unpublished results).

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