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Investigation on Intermolecular Forces between Bile Pigments and Polar Model Compounds Mimicking the Chromophore – Protein Interactions in Biliproteins

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Abstract: A systematic investigation of intermolecular interactions of biliverdin-IXα-dimethyl ester and 2,18-bridged helically fixed verdinoid and rubinoid analogues with a variety of chiral compounds possessing a limited number of donor and/or acceptor sites was performed. To evaluate interaction strengths the concentration dependence of the induced chiral discrimination between *M* and *P* helical species as detected by CD was used. Biliverdin esters show pronounced association only with compounds exhibiting strong hydrogen bonding donor properties. In particular, if the donor of the ligand is provided by a carboxylic acid group defined 1 : 1 complexes are formed but no protonation of the tetrapyrrole backbone takes place. 2,18-bridged helical bilirubins - being monomeric under the conditions employed - behave similarly but interact with acceptors, too. Association constants were determined by Scatchard plot analysis. The quantitative data gained allow to map the non-covalent, polar binding properties of helical biliverdins and bilirubins. The implications of results for the conformation determining interactions in biliverdin peptides and biliproteins are discussed

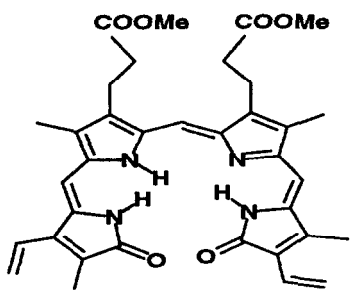
Introduction

Bilatrienes constitute the chromophoric group of several chromoproteins such as C-phycocyanin, phycoerythrin and phytochrome. The conformation of the linear tetrapyrrole backbone and thus the biologically relevant photophysical and photochemical properties are strongly determined by the apoprotein.[1,2] The X-ray analysis of C-phycocyanin has demonstrated that acidic and basic amino acid side chains as well as main chain peptide bonds are involved in the stabilization of the extended (*anti,syn,anti*) conformation of the bilatriene chromophore .[3,4] With the goal to obtain smaller molecules exhibiting similar properties as natural biliproteins several bilatriene-peptides have been prepared.[5-8] It has been shown that peptide esters covalently bound to biliverdin-IXα may induce chiral discriminations up to 100% preserving the helical (*syn,syn,syn*) arrangement [6] or even promote an (*anti,syn,syn*) conformation of the biliverdin backbone.[7] To further improve the peptide sequence controlling the stabilization of *anti* conformers of the cofactor, a better understanding of the associative and conformational pattern of bile pigments in general was needed.

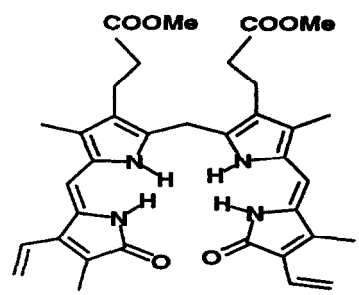
Recently the 2,18-bridged biliverdins **2** [9] and **3** and the bilirubin **4** [10] have been synthesized, which are fixed in a helical ('all *Z*, all *syn*') conformation akin to that of **1** and other open chain bilatrienes-abc. With the aid of these restricted compounds the different homoassociative and conformational patterns of biliverdins and bilirubins have been elucidated.[10] It became obvious that it is the availability of donor

and/or acceptor sites within the tetrapyrrole backbone that largely controlls the molecules' behaviour. Therefore an evaluation of strength and nature of interactions of amino acid residues with the backbone of the bile pigment seems promising in view of the initial goal of our studies. For covalently bound peptides the contacts between the peptidic hydrogen bonding sites and their counterparts at the tetrapyrrole backbone are essential for any conformational influence of the peptide moiety but no quantitative data for this **intra**molecular association can be obtained for principal reasons and therefore no localisation of sites is possible. These restrictions do not apply if **inter**molecular interactions are studied. Several authors have studied the heteroassociation between bile pigments and proteins[11-15] but the large number of potential interaction sites in proteins prohibits the identification of binding partners and the estimation of the corresponding binding energies. The association of optically active amines and bilirubins have been investigated in detail[16,17] but only the interactions with the carboxylic acid groups of the side chains have been considered.

This study deals with the heteroassociation of bile pigments with polar compounds possessing only **one** or a **few** donor and/or acceptor sites in a systematic way and is focussed on the localisation of interaction sites **within the backbone** of the chromophores. To gain detectable effects in spite of the limited number of potential binding sites solvents such as benzene and chloroform were used to minimize



1



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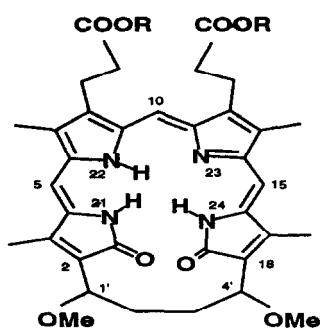
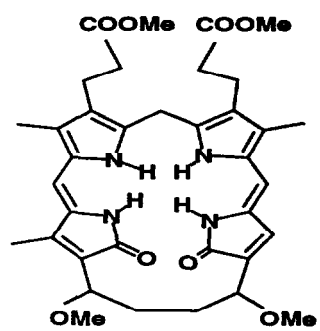




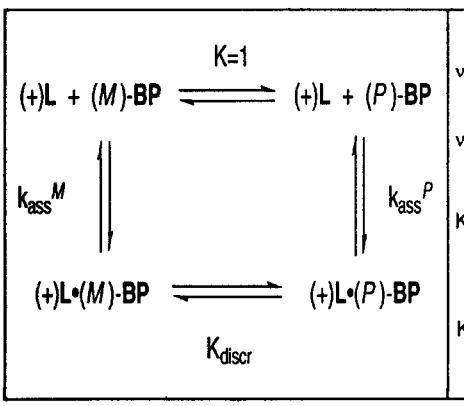
R = Me	H		
2a	3a	[(*P*, 1'*R*, 4'*R*) + (*M*, 1'*S*, 4'*S*)]	4a
2b	3b	[(*M*, 1'*R*, 4'*R*) + (*P*, 1'*S*, 4'*S*)]	4b

interferences of the solvent. As models biliverdin-IXα-dimethyl ester (**1**), bilirubin-IXα-dimethyl ester (**5**), and the 2,18-bridged analogues **2-4** were used. Since the restricted compounds **2-4** all possess an obligate helical geometry they are particularly suited for a comparative study of heteroassociation. Furthermore, interferences by protonation,[18] deprotonation,[19] nucleophilic attack at C-10[19,20] or homoassociation[10] eventually occurring with open chain bilatrienes-abc and biladienes-ac can largely be excluded. As observable the chiral discrimination detected by CD was chosen; its dependence from the concentration of the optically active ligands will in turn allow to estimate association energies. An indication that even small compounds may interact efficiently with biliverdins was given by the successful resolution of **2** upon addition of (*R*)-(–)-phenylhydroxyacetic acid (PHA)[21] and (*R*)-(–)-cyclohexyl-hydroxyacetic acid (CHA).[10] The molecular basis of this phenomenon still awaits elucidation, since salt formation can be excluded.[18]

Results and Discussion

Methods

The chiral discrimination between *M* and *P* helical species of the biliverdins **1, 2, 3** and **4** mediated by an optically active cosolute is determined by both its associative and its discriminating ability (Scheme). To separate the two equilibria the dependence of the CD on the concentration of the ligand must be investigated since the position of the discrimination equilibrium (K_{discr}) – being only a property of the complex – is independent from concentration. For bile pigments possessing only the helix as chirality element (e.g. **1**) the Scheme fully applies. The obligate helical tetrapyrroles **2-4**, on the other hand, each comprise a mixture of two rapidly interconverting chiral diastereoisomers **a** and **b**. Accordingly two systems of equilibria similar to that given in the Scheme (but generally K≠1) - for the species with configurations (1'*R*,4'*R*) and (1'*S*,4'*S*), respectively - must be considered and thus K_{discr} cannot be determined by CD spectroscopy alone. Two different association constants (K_{ass}^{RR} and K_{ass}^{SS}) and thus curved Scatchard plots can be expected to occur. But actually the influence of the chirality centre is small, since a linear plot yielding a mean K_{ass} ($\approx K_{ass}^{RR} \approx K_{ass}^{SS}$) is obtained.



$$v = \frac{\Delta\varepsilon}{\Delta\varepsilon_{ass}} = \frac{[L\bullet(M)\text{-}BP] + [L\bullet(P)\text{-}BP]}{[BP]_{total}} \quad (1)$$

$$v / [L]_{free} = K_{ass} - v\ K_{ass} \quad \text{(Scatchard plot)} \quad (2)$$

$$K_{discr} = \frac{1 + h.e._{ass}}{1 - h.e._{ass}} = \frac{k_{ass}^{P}}{k_{ass}^{M}} \quad (3)$$

$$K_{ass} = \frac{[L\bullet(M)\text{-}BP] + [L\bullet(P)\text{-}BP]}{[L]\ \{[(M)\text{-}BP] + [(P)\text{-}BP]\}} = \frac{(k_{ass}^{P} + k_{ass}^{M})}{2} \quad (4)$$

Scheme. Association and discrimination equilibria for a 1:1 complex between a chiral ligand [e.g. (+)L] and a bile pigment (**BP**) and equations used for evaluation of the equilibrium constants compiled in Tables 1 and 2 ($\Delta\varepsilon_{ass}$ refers to the CD of the associate and $h.e._{ass}$ to its helical excess).

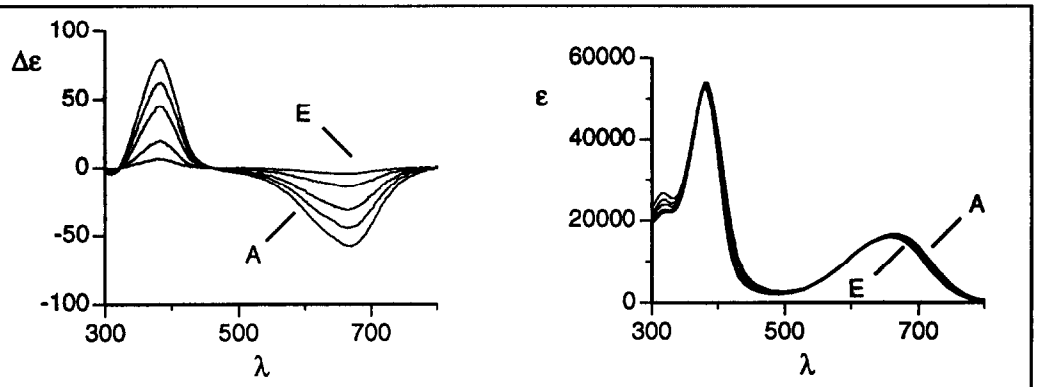


Fig. 1. CD ($\Delta\varepsilon$/cm^{-1} M^{-1}; λ/nm) and UV-VIS spectra (ε/cm^{-1}M^{-1}; λ/nm) of **1** (concentration range from 0.2 mM to 0.026 mM) in the presence of decreasing amounts of CHA (A, 4.5 mM; B, 1.8 mM; C, 0.71 mM; D, 0.20 mM; E, 0.05 mM) in benzene at 293 K.

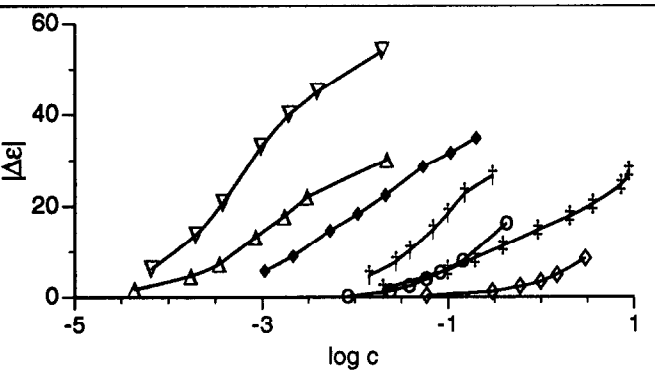


Fig. 2. CD ($\Delta\varepsilon_{max}$/cm^{-1} M^{-1}; benzene, 293 K) of **1** vs. concentration (log c) of selected optically active additives: ∇, CHA; Δ, Z-Pro-Val-OH; ♦, 2-phenylbutyric acid; †, Z-Pro-Leu-Val-OtBu; o, Z-Pro-Val-OtBu; ‡, 2,3-butandiol; ◊, camphor (the $\Delta\varepsilon$ values for the last four ligands have been multiplied by a factor 2).

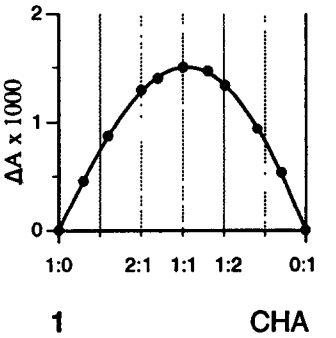


Fig. 3. Job-diagram for the complex formation of **1** with CHA detected by CD (ΔA).

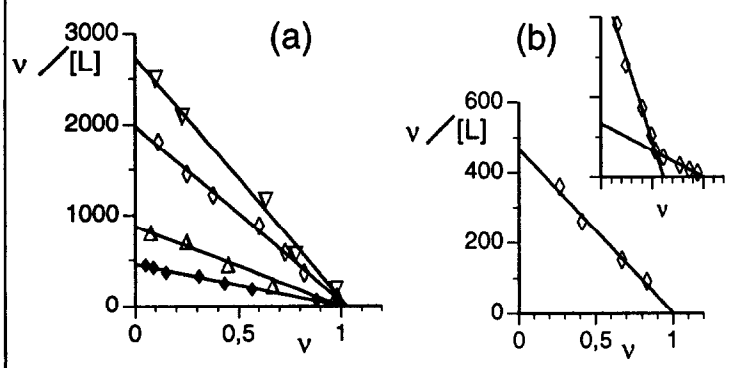


Fig. 4. Scatchard plots ν/[L] (M^{-1}) vs. ν for the association of **1** with (a) CHA in chloroform (♦, 293 K) and benzene (◊, 293 K; Δ, 308 K; ∇, 286 K) and (b) 2-phenylbutyric acid in benzene (293 K). In the latter case a biphasic Scatchard plot is obtained (see insert) which - after decomposition - gives a linear plot for the first association step.

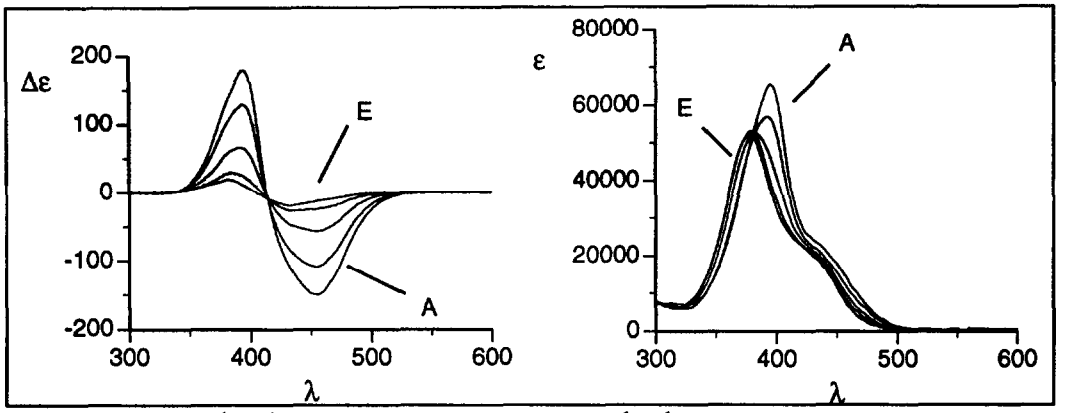


Fig. 5 CD ($\Delta\varepsilon$/cm^{-1} M^{-1}; λ/nm) and UV-VIS spectra (ε/cm^{-1}M^{-1}; λ/nm) of the 2,18-bridged bilirubin **4** (c in the range of 0.16 mM to 0.014 mM) in the presence of decreasing amounts of CHA (A, 4.2 mM, B; 1.7 mM; C, 0.67 mM; D, 0.19 mM; E, 0.07 mM) in benzene at 293 K.

To exclude interferences with other processes like protonation[19,22] or conformational changes[7] UV–VIS spectra were taken parallel to CD measurements. The CD is largely dominated by chiral discrimination. An occasional contribution of induced CD – the CD in the absence of chiral discrimination (i.e. *M*:*P* = 1:1) – could be shown to be smaller than 5-10% (see Experimental and ref. 23). The stoichiometry of associates was determined according to Job's method[24]; in the case of small K_{ass}-values, the linearity of a plot $\nu/1-\nu$ vs. $[L]_{total}$ was taken as proof for a 1 : 1 stoichiometry. For well defined complexes linear Scatchard plots[25] were obtained which were fitted with respect to the CD of the complex ($\Delta\varepsilon_{ass}$) if this value could not be determined directly, yielding K_{ass} and K_{discr}. The log $c_{1/2}$ values, corresponding to the concentration at which about one half of the final discrimination is achieved, are inversely proportional to the association tendency and particularly suited for a comparison of all ligands.

Typical examples for CD and UV-VIS spectra, titration curves, Job-diagrams and Scatchard plots as observed with the open chain biliverdin **1** are presented in Figures 1-4. Figure 5 shows the CD and UV-VIS spectra for the titration of the helically fixed bilirubin **4** with CHA. The open chain bilirubin **5** displayed no heteroassociation in solutions of benzene and chloroform. In general, only negligibly small changes in UV-VIS spectra are observed during titrations even with the open chain biliverdin **1**. The ratio of dipole strengths ($f = D_{UV}/D_{VIS}$) of the two main bands of **1** - a very sensitive probe for any conformational change[7] - remains almost invariant, i.e. the helical arrangement is preserved (Fig. 1). For the helically fixed bilirubin **4** the changes in absorption spectra with increasing population of the associate are more pronounced (Fig. 5) owing to the larger flexibility of bilirubins[10] which becomes apparent even if the global conformation is fixed. The data computed for the heteroassociation of compounds **1-4** are compiled in Tables 1 and 2.

Discussion

The results clearly demonstrate that carboxylic acids form the by far strongest heteroassociates with compounds **1-4** – their association constants being all of the same order (K_{ass} = 500 - 3000 M^{-1}). Interestingly, the stoichiometry is found to be always 1:1. As expected association constants K_{ass}

Table 1. Data for the association and discrimination equilibria of biliverdin-IXα dimethyl ester (**1**) with chiral additives at 293 K.

Ligand (L)	S[a]	log $c_{1/2}$	St[b]	Corr[c]	$\Delta\varepsilon_{ass}$[d]	K_{discr}[e]	K_{ass}[f]
(*R*)-2-Phenylbutyric acid 1st step	B	–2.5	1 : 1	–0.992	–22	0.64	470
2nd step	B	≈–1.3		g			
(*S*)-2-Chloropropionic acid [h]	B	–2.5	1 : 1	–0.990	–11	0.80	≈600
(1*S*,4*S*)-Camphanic acid	B	–2.8	1 : 1	–0.998	–35	0.48	1100
PHA	B	–3.4	1 : 1	–0.994	–33	0.50	3000
CHA	B	–3.1	1 : 1	–0.996	–56	0.29	2000
CHA	C	–2.5	1 : 1	–0.994	–44	0.39	460
CHA	E	≈–1.0			i		
(*S*)-Lactic acid	C	–2.5	1 : 1	–0.994	+12	1.27	450
Z-Ala-OH	B	–3.0	1 : 1	–0.997	–18	0.69	1700
Z-Ser-OH	B	–3.3	1 : 1	–0.998	–11	0.80	2300
Z-Ala-Ala-OH	B	–3.4	1 : 1	–0.997	–34	0.49	2400
Z-Ala-Ala-OH	C	–2.8	1 : 1	–0.987	–12	0.79	1100
Z-Pro-Val-OH	B	–3.0	1 : 1	–0.996	–33	0.50	1700
Z-Ala-Ala-OMe	B	–0.8		g			
Z-Pro-Val-OtBu	B	–0.8		g			
Z-Pro-Leu-Val-OtBu	B	–1.2	1 : 1	–0.996	–19	0.68	10
(*R*,*R*)-2,3-Butandiol 1st step	B	–1.0	1 : 1	–0.992	–9	0.84	5
2nd step	B	≈+0.5		g			
(+)-Camphor	B	≥+0.5		g			

[a] Solvents: B,benzene; C, chloroform; E, ethanol. [b] Stoichiometry of the associate [c] Correlation factor of the linear regression analysis performed in Scatchard plots. [d] $\Delta\varepsilon_{max}$ ($cm^{-1}\ M^{-1}$) of the band at λ ca. 650 nm extrapolated for the pure associate; error ± 10%. [e] Discrimination constant (see Scheme); error ± 5-20%. No effort has been made to interpret K_{discr} in terms of the chirality and the geometry of the ligands, as a solution to this question would not contribute to the scope of this study. [f] Association constant (M^{-1}); error ± 10%. [g] Curved Scatchard plot. [h] Protonation of **1** starts at medium concentrations; data of the associate are extrapolated. [i] No Scatchard plot could be performed since the chiral discrimination was too small ($\Delta\varepsilon_{ass} \leq 5$).

pronouncedly decrease with increasing temperature and increasing polarity of the solvent [Figure 4(a) and Table 1]. For acceptors such as camphor or brucin no significant association tendencies with the biliverdins **1** and **2** can be detected in contrast to the bilirubin **4** (Tables 1 and 2). Obviously the tetrapyrrole backbone of bilatrienes-abc interacts only with hydrogen-bonding donor groups. This implies, that only acceptor sites are accessible at all. The formation of 1:1 complexes with carboxylic acids would suggest a participation of the pyrrolenine nitrogen. But the similarity of association constants for the interaction of CHA with the biliverdin **2** and the corresponding bilirubin **4** (Table 2) clearly demonstrates that it is the pyrrolinone carbonyl group which is involved, since bilirubins lack further acceptor sites. This is in accord with the observation that **1** and the restricted biliverdin **2** exhibit similar K_{ass} values although the basicity of N-23

Table 2. Data for the association equilibria of the bile pigments **2**, **3** and **4** with chiral additives at 293 K.

Compound	Ligand (L)	S[a]	log $c_{1/2}$	St[b]	Corr[c]	$\Delta\varepsilon_{ass}$[d]	K_{ass}[e]
2	(*R*)-2-Phenylbutyric acid 1st step	B	–2.5	1 : 1	–0.992	–22	500
	2nd step	B	≈–1.4		f		
	(*S*)-2-Chloropropionic acid	B	–2.5	1 : 1	–0.996	–16	550
	PHA	B	–3.0	1 : 1	–0.995	–87	1800
	CHA	B	–3.0	1 : 1	–0.999	–97	1600
	CHA	C	–2.8	1 : 1	–0.993	–48	900
	(2 *S*-2-Aminobutanol-1	B	–1.5		f		
	(-)-Brucin	B	g				
	(+)-Camphor	B	≥+0.5		f		
3	CHA	C	–2.8	1 : 1	–0.990	–77	700
	(2*S*)-2-Aminobutanol-1	C	–3.8	1 : 1	–0.994	–23	7000
	(–)-Brucin	C	–4.3	1 : 1	–0.990	–6	≈20000
4	PHA	B	–3.1	1 : 1	–0.990	+160	1300
	CHA	B	–3.0	1 : 1	–0.990	+210	1000
	(–)-Brucin	B	–1.0		f	h	
	(+)-Camphor	B	≈–0.5		f		

[a] Solvents: B,benzene; C, chloroform [b] Stoichiometry of the associate. [c] Correlation factor of the linear regression analysis performed in Scatchard plots. [d] $\Delta\varepsilon_{max}$ ($cm^{-1}\ M^{-1}$) of the band at λ ca. 650 nm (**2** and **3**) or λ ca. 400 nm (**4**) extrapolated for the pure associate; error ± 10%. [e] Mean association constant (M^{-1}); error ± 10%. [f] Curved Scatchard plot. [g] No chiral discrimination even in solutions 0.1 M in brucin. [h] $\Delta\varepsilon \approx +25$ is observed for a solution ca. 70 mM in brucin.

of **2** is ca. three orders of magnitude lower.[18] The acceptor N-23 must be strongly involved in the intrachromophoric hydrogen-bonding network. Hence the corresponding donor sites (pyrrole and pyrrolinone N-H) should not be accessible for ligands neither. This accounts for the failure to detect heteroassociates of the biliverdins **1** and **2** with camphor or brucin possessing acceptor sites only (Tables 1 and 2). The preservation of this intrachromophoric hydrogen bonding network is also reflected in the preservation of the (*syn,syn,syn*) geometry of **1** upon heteroassociation. This helical geometry in turn obviously impedes binding of the second carbonyl group to the ligand. In helical tetrapyrroles the propionic acid groups do not interact intramolecularly with the lactam C=O groups, since the association tendencies of the dimethyl ester **2** and the corresponding diacid **3** with CHA for chloroform solutions are very similar (Table 2). This observation fits the expectation derived from the inspection of molecular models.

If on the other hand the properties of the ligands are considered, it emerges that the largest K_{ass} are observed with α-hydroxy- and α-amido-carboxylic acids (see Table 1). Obviously two donor sites are most effective in interaction with biliverdins like **1** and **2**. The association tendency of non–acidic donor ligands such as peptide esters is less pronounced and occasionally curved Scatchard plots (especially concave-down)[25] occur, which implies that there exist at least two interdependent associates. The peptide

ester Z-Pro-Leu-Val-OtBu, however, forms a well defined complex with 1 suggesting that the peptide adopts a well defined secondary structure leaving only one preferred possibility for interaction. A change of the helical conformation of 1 on complexation is not observed in contradistinction to covalently linked peptides of similar sequence[7] or of sequence -Pro-Val-OH[19] which provoke a stretching of the chromophore.

Conclusions

A mapping of the polar interaction sites located at the tetrapyrrole backbone of helically shaped bile pigments has been performed. The results are in accord with those obtained from the aggregation behaviour of bilirubins and biliverdins.[10] Bilirubin-IXα-dimethyl ester (5) which readily dimerizes in certain solvents, shows no tendency towards heteroassociation, since the equilibrium constant of homoassociation is larger by far and the dimer formed represents a closed structure. Similar considerations should hold true for any open chain biladiene-ac, which cannot adopt an intramolecularly stabilized structure. Thus the conformational heterogeneity of bilirubin-IVß reported in ref. 16 might be rationalized in terms of a considerable population of non-ridge-tile like, helical dimeric aggregates. Clearly, for geometric reasons the propionic acid side chains in bilirubin-IVß are less suited to stabilize the ridge-tile like monomer. This would additionally account for the poor chiral discrimination observed on addition of optically active amines.[16] If homoassociation is prohibited as in the bridged helical bilirubins 4 both acceptor (pyrrolinone C=O groups) and donor sites (pyrrole, pyrrolinone N-H) interact with the complementary sites of the ligands. This implies that amines will form complexes with bilirubins even if acidic side chains are absent. Consequently this kind of interaction should additionally be considered for the mechanism of chiral discrimination of bilirubin-IXα by optically active amines.[16,17] This might explain the comparable intensities of the CD spectra of the ester 4 (Table 2) and the acid bilirubin-IXα[16] in the presence of brucin under similar conditions.

In helical bilatrienes-abc only the lactam C=O groups, at least one of them, are accessible for intermolecular interactions, all other hydrogen bonding sites being involved in the intrachromophoric network which stabilizes the helical geometry. It has been shown that the helical geometry is destabilized if covalently bound donors are specifically oriented to interact with the pyrrolenine nitrogen, as in biliverdin peptides of sequence -Pro-X-Y-OR. The present study further corroborates previous suggestions[7] for the design of biliverdin peptides with *anti* conformations of the bilatriene backbone. In particular the synthesis of covalently bound peptides with a carboxylic acid group appropriately situated to interact with N–23 seems promising.[19] Consequently also the donor groups should become accessible, as found for the bilirubin 4, so that a further stabilization of non–helical conformations by interaction of the lactam groups with corresponding peptide bonds may become possible. The importance of the interaction with the pyrrolenine nitrogen has been demonstrated by the X-ray analysis of C-phycocyanin, where in all three chromophores an aspartic acid side chain is occupying this site.[3] Although the present study shows that carboxylic acids in general are too weak to protonate the bilatriene-abc chromophore unless a very high excess is present if the helical conformation is adopted, it seems still likely, that the phycocyanin chromophores are protonated in the native biliprotein, since it has been predicted[18] and verified[20] that biliverdins are much more basic if present in a stretched conformation.

Experimental

M.p.s were determined with a Kofler-Reichert hot-stage apparatus. ^{1}H NMR spectra were recorded at 250 MHz with a Bruker AC 250 AF instrument. Optical rotations were obtained with a Perkin Elmer 241 polarimeter (10 cm path length, solvents used were of Loba p.A. quality, EtOH contained 5% H_2O). CD- and UV-VIS spectra were taken with a Jobin-Yvon CD6 circulardichrograph and a Perkin Elmer Lambda 7 spectrometer (equipped with data station 3600), respectively, using quartz cuvettes (0.01 - 2 cm path length). All optical measurements were carried out in thermostatted cell compartments (20±1°C). As solvents spectroscopic grade benzene, methanol, ethanol, and dichloromethane (all Merck, Uvasol) and chloroform (Merck, LiChrosolv) were used. Benzene and chloroform were chromatographed on alumina prior to use. Solutions of the bilirubins **4** and **5** were deaerated by at least two freeze-pump-thaw cycles and kept under argon atmosphere and protected from light.

Materials.- Compounds 1^{26}, 2^9, 3^7, 4^{10} and 5^{27} were synthesized as described in the literature. The optically active substances purchased from Fluka and Sigma showed satisfactory optical rotations : (*R*)-(–)-2-phenylbutyric acid { Sigma, $[\alpha]_D^{20}$ = –93.2° (c 1, toluene)}, (*S*)-(–)-2-chloropropionic acid {Sigma, $[\alpha]_D^{20}$ = –13.9° (neat)}, (1*S*,4*S*)-(–)-camphanic acid {Fluka, $[\alpha]_D^{20}$ = –19.2° (c 1, dioxane)}, (*R*)-(–)-phenylhydroxyacetic acid (PHA) {Fluka, $[\alpha]_D^{20}$ = – 146.3° (c 5, H_2O)}, (*R*)-(–)-cyclohexylhydroxyacetic acid (CHA) {Fluka, $[\alpha]_D^{20}$ = – 22.8° (c 1, acetic acid)}, (*S*)-(+)-lactic acid {Fluka, $[\alpha]_D^{20}$ = –12.9° (c 2.5, 1.5 M NaOH) contained 33 % lactide (by titration)}, (2*R*,3*R*)-(–)-2,3-butandiol {Fluka, $[\alpha]_D^{20}$ = –12.7° (neat)}, (*S*)-(+)-2-aminobutanol-1 {Fluka, $[\alpha]_D^{20}$ = +11.5° (c 2, EtOH)}, (+)-camphor {Fluka, $[\alpha]_D^{20}$ = +43.0° (c 10, EtOH)} and (–)-brucin {Fluka, $[\alpha]_D^{20}$ = –113.0° (c 2.5, $CHCl_3$)} Amino acid and peptide derivatives were synthesized from (*S*)-amino acids according to standard procedures for peptide synthesis in solution[28]; their purity was assessed by ^{1}H NMR spectroscopy; Z-Ala-OH {m.p. 85-86°C, $[\alpha]_D^{20}$ = –14.0° (c 2, acetic acid)}[28], Z-Ser-OH {m.p. 117-118°C, $[\alpha]_D^{20}$ = +5.7° (c 6, acetic acid)}[28], Z-Ala-Ala-OH {m.p. 150-151°C, $[\alpha]_D^{20}$ = –25.9° (c 1, EtOH)}[29], Z-Pro-Val-OH {m.p. 135-136°C, $[\alpha]_D^{20}$ = –49.4° (c 1, EtOH)}[30], Z-Ala-Ala-OMe {m.p. 105-108°C, $[\alpha]_D^{20}$ = –44.5° (c 1 EtOH)}, Z-Pro-Val-OtBu {m.p.111-115°C, $[\alpha]_D^{20}$ = –63.5° (c 1, EtOH)} and Z-Pro-Leu-Val-OtBu {m.p. 108-112°C, $[\alpha]_D^{20}$ = –87.1° (c 1.5, EtOH)}.

Procedures

For the 2,18-bridged bile pigments **2-4**, the effective chiral discrimination could be determined for each solution by diluting the sample with MeOH at –20°C (where helix inversion is sufficiently hindered) and taking a CD spectrum immediately thereafter. Under this condition the influence of the chiral additive is abolished and the CD is exclusively determined by the chiral discrimination still persisting, which then fades away according to the respective barrier of helix inversion[10]. In the case of the open chain biliverdin-IXα-dimethyl ester (**1**) this method cannot be applied to exclude the influence of the induced CD but the phenotype of CD spectra (i.e. the correspondence of band positions in CD and UV-VIS spectra)[23] and the comparison of results obtained with the analogous bridged biliverdin **2** demonstrate the predominance of chiral discrimination.

Two principal methods were applied to determine the concentration dependence of the chiral discrimination observed by CD. *A. Titrations.*-A concentrated solution of the bile pigment in the respective solvent was added to solutions containing varying concentrations of the ligand or a concentrated solution of the ligand was successively added to a diluted solution of the bile pigment. *B. Dilution-experiments with the associate.*- This method was only applied in the case of larger association constants. 2-5 equivalents of the ligand dissolved in MeOH were added to a solution of the biliverdin in dichloromethane. The solvents were evaporated in vacuo and the residue was dissolved in benzene or chloroform so that the final concentration of biliverdin was ca 1 x 10^{-2} M. This solution was then appropriately diluted and the CD spectra were measured using cuvettes with suitable path lengths. With bilirubins (**4** and **5**) only the first variant of procedure *A* was used, because the stability of bilirubins in acidic solutions is rather low. For biliverdins analysis of the data obtained by both methods (*A* and *B*) gave the same results. As a measure for the chiral discrimination the $\Delta\varepsilon_{max}$ values of the two main CD bands (at λ 660 and 380 nm for **1-3** and at λ ca. 440 and 390 nm for **4**) were taken yielding similar results. The $h.e._{ass}$ of **1** is computed from $\Delta\varepsilon_{ass}$: $h.e._{ass} = \Delta\varepsilon_{ass}$ (660)/100.[6] The concentration of free ligand ($[L]_{free}$) was evaluated from its total concentration considering the amount bound in the associate.

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