Induced Circular Dichroism and Chiral Discrimination of Racemates Revisited: Bilirubins as Illustrative Examples

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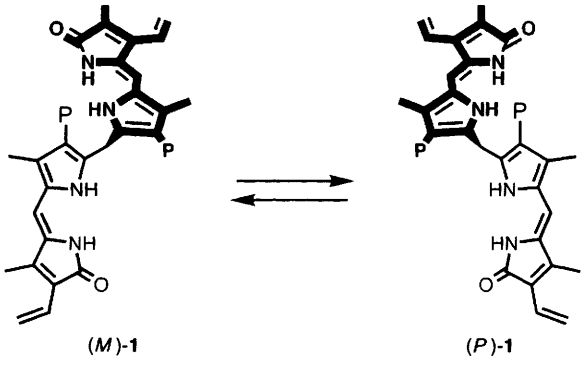
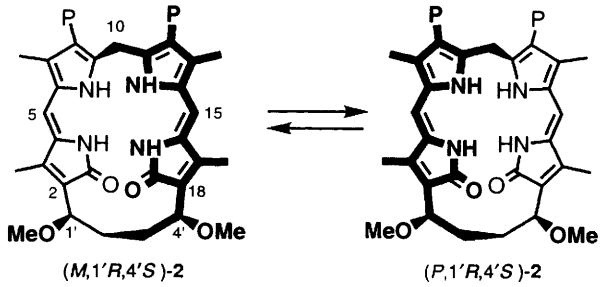
The scope and limitation of the circular dichroism of racemates induced by an optically active co-solute (ICD) are elaborated. Accordingly, the interpretation of ICD spectra depends upon the barrier to interconversion of enantiomers, the degree of complexation, the availability of reference values and population and spectral differences of the species involved. The distinct nature of these factors may occasionally prevent a meaningful application of this technique in the field of molecular and chiral recognition. The relevance of the more general considerations is outlined and eventual implications for the ICD spectra of bilirubin-IXα (**1**) reported in the literature are provided by the bridged analogues **2**, **3a** and **3b**.

Since the discovery of induced circular dichroism (ICD)[1] this technique has become an established method in organic and bioorganic chemistry,[2–26] in particular for the estimation of binding affinities, substrate specifities and host–guest interactions thus contributing to a better understanding of non-covalent binding forces. Many of these studies have been performed by using kinetically labile racemates rather than achiral compounds to which initial studies were mainly devoted. In this case the asymmetric environment may be capable of performing chiral discrimination between the enantiomers. One of the most prominent members within this family comprises bilirubin-IXα (**1**)—a mixture of two rapidly interconverting enantiomers of *M* and *P* helicity.[21,27,28] Bilirubin **1** is a metabolite of heme and has been studied extensively[3,5,12,16,21,27] with the aim of elucidating the binding mode and the localization of the binding sites if complexed with serum albumin which serves as a carrier protein even of other lipophilic substances in the blood stream.[21,27–29] Owing to its amphiphilic properties, **1** has also been used to probe the discriminatory efficiency of cage-like optically active macrocycles.[8,13,14] However, if a more rigorous inspection of ICD applications is made several shortcomings emerge. Firstly, for a reliable interpretation of an ICD spectrum of a racemic optically labile compound in terms of chiral discrimination knowledge about the absolute magnitudes of Cotton effects (CEs) is prerequisite.† Unfortunately, these are in principle unknown but can occasionally be estimated either by drawing a comparison with structurally similar but kinetically stable analogues[21,30] or by calculations using models of optical activity[28] provided that the exact geometry is known. Another approach comprises the systematic search for an upper limit in ICD values by probing a series of discriminators, preferentially joined covalently to the racemic compound.[31,32] For the conformationally most flexible bilirubin **1** appropriate models adopting a fixed ridge-tile shaped geometry are not available. Moreover, computations performed recently[28] suggest a pronounced difference in the CD spectra of energetically similar conformers. Consequently, the relative spatial arrangement of the two dipyrrinone‡ moieties determines the shape and magnitude of the CEs and can be expected to vary more or less unpredictably with the ligand considered depending upon the number, kind and distribution of the binding sites involved. This implies that promotion in ICD intensity of **1** by changing the ligand[10] or other environmental conditions[8] cannot be unambiguously interpreted in terms of a promotion in chiral discrimination. Secondly, ICD studies performed so far neglect the role of eventual differences in CD spectra of coexisting diastereoisomeric complexes by tentatively assuming mirror-image relationships between them.



The increasing application of ICD in exploring molecular and chiral recognition and our own vital interest in this field has prompted us to investigate the background of this method and to elucidate the role of the spectral dissimilarities of species involved in more detail. For this purpose we have chosen the conformationally related but constrained bilirubins **2** (this work), **3a** and **3b**,[33] of [(*M*,1′*R*,4′*S*) and (*P*,1′*R*,4′*S*)], [(*M*,1′*S*)



† Of course, any CD measurement on a mixture of stereoisomers, for which the absolute CD values (reference CEs) are unknown cannot be used to determine the isomer ratio or any quantity related thereto.

‡ 10*H*-Dipyrrin-1-one.[21] **1** can be regarded as a bichromophoric molecule in which two dipyrrinone moieties are bridged by the C-10 methylene.

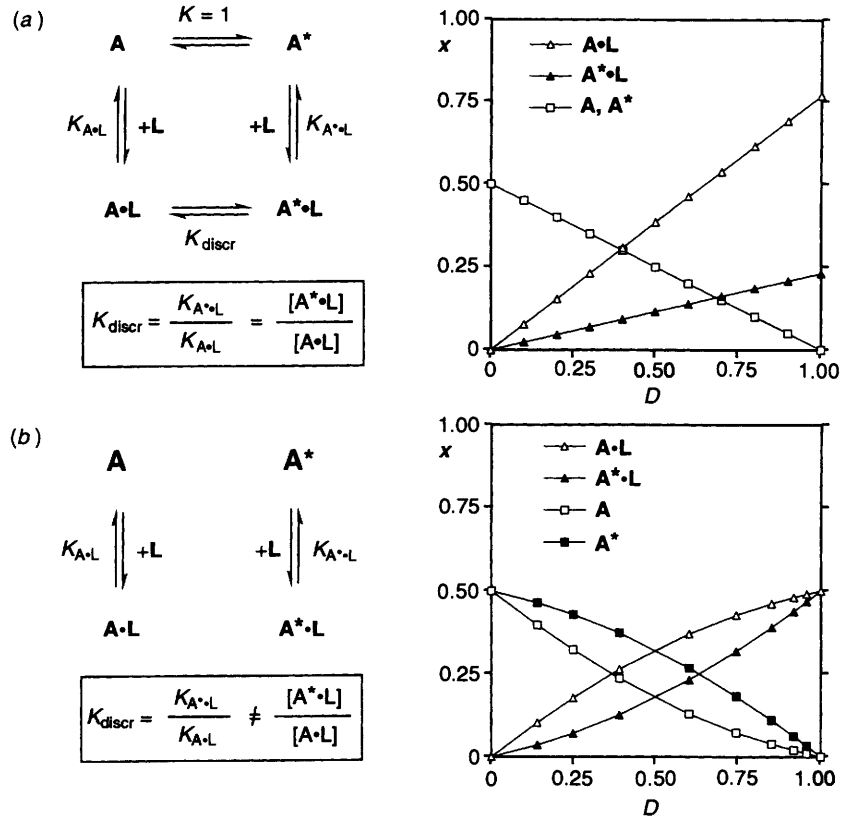
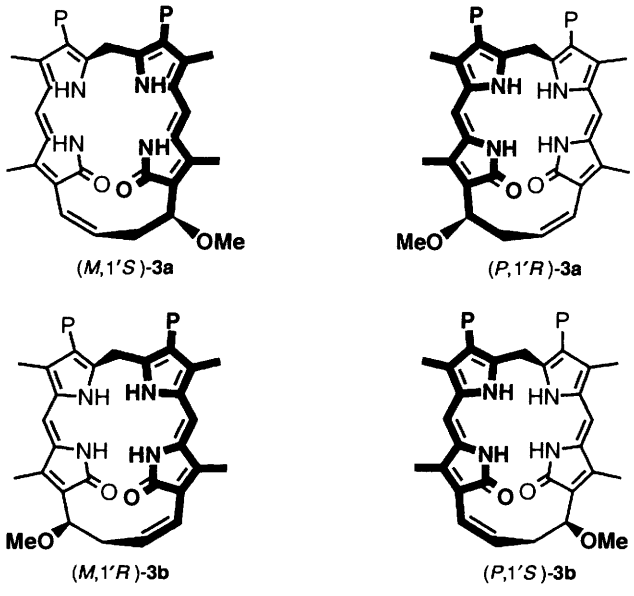


Fig. 1 Equilibria and computed mole fractions x of coexisting species of a kinetically labile (*a*) and stable (*b*) racemate **A**,**A*** on complexation with the enantiomerically pure ligand **L** (1:1 stoichiometry) *vs.* degree of complexation $D = x_{A•L} + x_{A*•L}$ for dilute solutions in an achiral solvent. The values chosen for the point by point calculations were: individual association constants $K_{A•L}$ 10 000 dm^3 mol^{-1}, $K_{A*•L}$ 3000 dm^3 mol^{-1}, K_{discr} 0.3, macroscopic association constant directly accessible only for (*a*) K_{ass} 6500 dm^3 mol^{-1}.



and (*P*,1′*R*)] and [(*M*,1′*R*) and (*P*,1′*S*)] chirality,§ respectively, which form defined associates with carboxylic acids in a similar fashion to other bile pigments.[15] Besides, their geometry is less susceptible towards environmental changes if compared with **1**. The distinguishing feature of macrocycles **2** and **3** is their kinetic stability being labile [**2**, half life ($t_{\frac{1}{2}}$) *ca.* 2 s, 293 K] and stable (**3a** and **b** $t_{\frac{1}{2}}$ *ca.* 9 h, 265 K), respectively, but these properties can be conveniently controlled with temperature. At 248 K the enantiomeric conformers of **2** become sufficiently stable to allow for a CD performance even if the discriminating influence is withdrawn. On the other hand, *M*–*P* interconversion of **3a** and **b** can be accomplished at 293 K (ref. 33) and equilibration between diastereoisomers takes place. Accordingly, since in addition the CD spectra of bilirubins for the cyclic closed (4*Z*,15*Z*,5*syn*,9*syn*,10*syn*,14*syn*) conformation as adopted by **2** and **3** are available from natural optical activity,[34] both the macroscopic association constants, $K_{ass} = (K_{A*•L} + K_{A•L})/2$ and the discrimination constants, K_{discr}, as defined in Fig. 1, can be estimated. In this report we stress attention on the ambiguities in the interpretation of ICD spectra of both kinetically labile and stable racemates, which occasionally may arise and which have not been considered fully hitherto. Our considerations are of general interest and go beyond the range of application to bilirubins.

§ The carbons C-1′ and -4′ of **2** are heterochiral but constitutionally equivalent. Since *M*–*P* interconversion of the biladiene backbone of one stereoisomer thus always produces the enantiomeric counterpart, **2** can be regarded as chiral mesoform. For convenience the chirality centres of **2**, **3a** and **3b** will be omitted here.

Results and Discussion

Consider a kinetically labile racemate consisting of the enantiomers **A** and its mirror-image **A*** which undergo association (1:1 stoichiometry) with one enantiomer of the ligand **L** [Fig. 1(*a*)]. At low overall concentrations as used in our study remote interactions can be neglected and to a first approximation the equilibrium between enantiomers **A**,**A*** will not be influenced by the chiral environment. Therefore, even if **L** may discriminate between **A** and **A*** the equipopulation will be

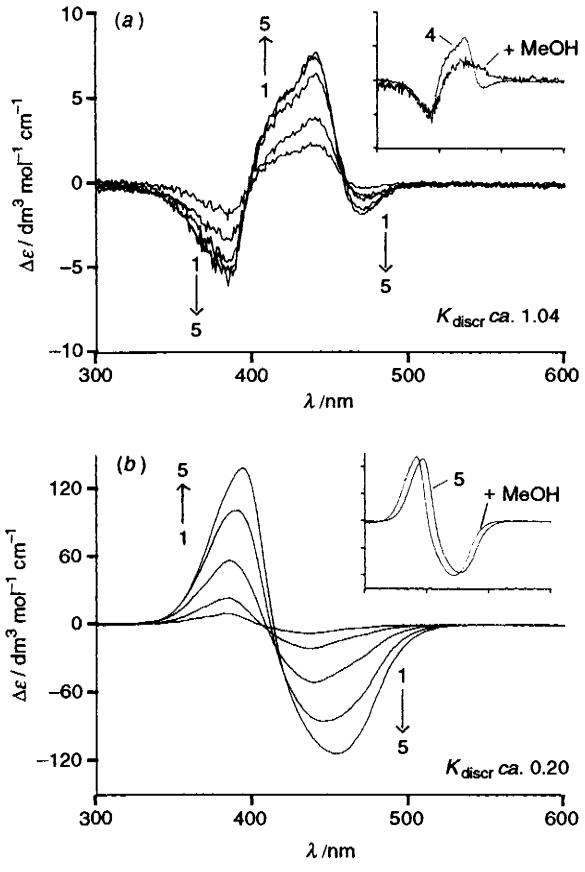


Fig. 2 ICD of the kinetically labile racemate **2** [*c* 0.02 mmol dm^{-3} (*a*), *c* 0.04 (*b*)] in toluene–chloroform (17% v/v) at 293 K on addition of (−)-PBA (1: 0.76 mmol dm^{-3}, 2: 1.50, 3: 3.80, 4: 7.60, 5: 11.40 (*a*) and (−)-CHA (1: 0.07 mmol dm^{-3}, 2: 0.21, 3: 0.55, 4: 1.52, 5: 4.57; no final spectrum, see Experimental) (*b*). Insets: CD before and after removal of the corresponding discriminator by dilution with methanol at 248 K.

currently restored as the degree of complexation *D* increases, and the corresponding dependencies of the mole fractions *x* from *D* are linear and coincide. Thus, the diastereoisomer ratio **A*·L** : **A·L** is independent of the amount of **L** present in solution and always reflects K_{discr}. As *D* approaches unity only **A·L** and **A*·L** are populated.¶ Accordingly, if titration of the racemate **A,A*** with **L** is monitored by CD, spectra should be distinguished in their magnitude but not in their shape. Ideally, an isodichroic point—if any—should appear at the baseline. These expectations are essentially fulfilled if (*M* and *P*)-**2** (**A,A***) is subjected to titration with (*R*)-(−)-2-phenylbutyric acid [(−)-PBA] and (*R*)-(−)-cyclohexylhydroxyacetic acid [(−)-CHA] (**L**), respectively, at 293 K (Fig. 2).‖ However, while the spectra in Fig. 2(*b*)** are characteristic of bilirubins showing two bands, opposite in sign, due to exciton coupling of the two chromophores, in Fig. 2(*a*) more complex ICD spectra occur and their shapes strongly suggest a superposition of two components. Besides, the distinctly different phenotypes displayed in Figs. 2(*a*) and (*b*) seem to be related to the efficiency of the chiral discriminator, which is poor [(−)-PBA] and pronounced [(−)-CHA], respectively. Clearly, both sets of ICD spectra must be compounded from two components [Fig. 1(*a*)] with a more or less imperfect mirror-image relationship but it is K_{discr} which determines their individual contributions. Accordingly, differences in CD spectra between **A·L** and **A*·L** will come increasingly into play if K_{discr} approaches unity. In this limiting case the ICD observed reflects the differences in CD spectra of a 1:1 mixture of the diastereoisomer complexes **A·L** and **A*·L** rather than a difference in the population of bound species. Therefore, the signs of the corresponding CEs may not even reflect the helicity of the preferentially bound enantiomer, and chiral recognition may be simulated. The shape of such ICD spectra may either be 'normal', that is resemble those expected for the enantiomers, if CD spectra of the two diastereoisomers differ only in magnitude, or more complex, if spectra are shifted relative to each other. Thus, the ICD spectra due to **2** and (−)-PBA [Fig. 2(*a*)] originate from two intense (|Δε| 200–250 dm^3 mol^{-1} cm^{-1}) exciton couplets with inverted signs but slightly differing in magnitudes (5–10%), band positions (± 5 nm) and band widths. At 248 K *M*–*P* interconversion of the enantiomers of **2** becomes slow and if the chiral influence is withdrawn at this temperature by injecting a toluene–chloroform solution of **2** and (−)-PBA into precooled methanol the composite CD is replaced by the usual exciton couplets [Fig. 2(*a*), inset] of exceptionally low intensity corresponding to K_{discr} 1.04.†† Performing the same experiment on the corresponding (−)-CHA complexes spectral changes are confined to a hypsochromic shift by about 10 nm [Fig. 2(*b*) inset]. This implies, that if chiral discrimination is sufficiently large the ICD allows for a direct access to K_{discr} provided that reference CEs are available.

Evidence in support of the relevance of our considerations, in particular, of our interpretation of the complex CEs in Fig. 2(*a*) is provided by the ICD exhibited by the kinetically stable bilirubins (*M* and *P*)-**3a** or (*M* and *P*)-**3b** (**A,A***) in the presence of (−)-CHA and (−)-PBA (**L**), respectively, at 265 K. Since enantiomers **A** and **A*** do not interconvert the interdependent equilibria in Fig. 1(*a*) are split and decoupled from each other yielding those delineated in Fig. 1(*b*). Irrespective of the discriminating power of **L** the ratio **A*·L**/**A·L**—which no longer reflects the actual K_{discr}—approaches unity if complexation is driven to completion (*D* = 1). Hence differences in CD spectra of diastereoisomeric complexes **A·L** and **A*·L** are obtained directly. This is equivalent to a simulation of K_{discr} = 1 for an optically labile racemate. The ICD spectra obtained for **3b**‡‡ on association with (−)-PBA and (−)-CHA, respectively (Fig. 3), which necessarily each comprises an envelope of two CD spectra, closely resemble each other although the actual K_{discr} amounting to 1.04 and 0.30, respectively (see Experimental section), are quite different and their composite nature is reminiscent of the ICD obtained for kinetically labile **2** on addition of (−)-PBA [Fig. 2(*a*)]. Thus, even if it might be tempting to interpret the spectra in Figs. 2(*a*), 3 and 4 in terms of chiral discrimination or any severe conformational hetero-

¶ This limiting case is closely related to the discrimination between the moieties **A** and **A*** by a covalently bound optically active substituent **L**.

‖ In toluene and chloroform non-bridged bilirubins such as **1** and its diester do not associate with the acids considered here, for different reasons. Firstly, the diacid **1** forms strong intramolecular hydrogen bonds between the propionic side chains and the C=O and N–H entities of the tetrapyrrol backbone. On the other hand for the diesters of **1** similarly strong but intermolecular hydrogen bonding between like molecules takes place and all donor and acceptor sites become occupied. Neither (−)-CHA nor (−)-PBA can efficiently compete for the bilirubin sites involved in these two kinds of hydrogen bonding networks, resulting in vanishing CD at least within the concentrations in acids applied in this study. For geometric reasons these kinds of hydrogen bondings are prohibited in cyclic bilirubins.[33]

** The slight spectral shift even observed in the corresponding UV–VIS spectra can be attributed to a medium effect.

†† If (−)-PBA discrimination is calculated from the ICD spectra 4 or 5 at λ 398 nm, K_{discr} becomes 1.04, too. However, this value rests on the wrong experiment, the agreement is accidental and wavelength dependent.

‡‡ In all experiments the behaviour of **3a** closely resembles that of **3b** (see Experimental section) and discussion will be restricted to one of them (**3b**).

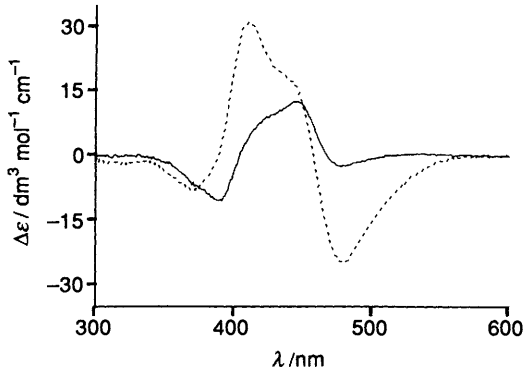


Fig. 3 ICD of equipopulated diastereoisomeric associates [(*M*)-**3b**·(−)-PBA and (*P*)-**3b**·(−)-PBA] (——) and [(*M*)-**3b**·(−)-CHA and (*P*)-**3b**·(−)-CHA] (– – – –) in toluene–chloroform (17% v/v) at 265 K. Solutions were 0.02 mmol dm^{-3} in **3b**, and 7.85 and 4.80 in (−)-PBA and (−)-CHA, respectively ($D > 0.9$).

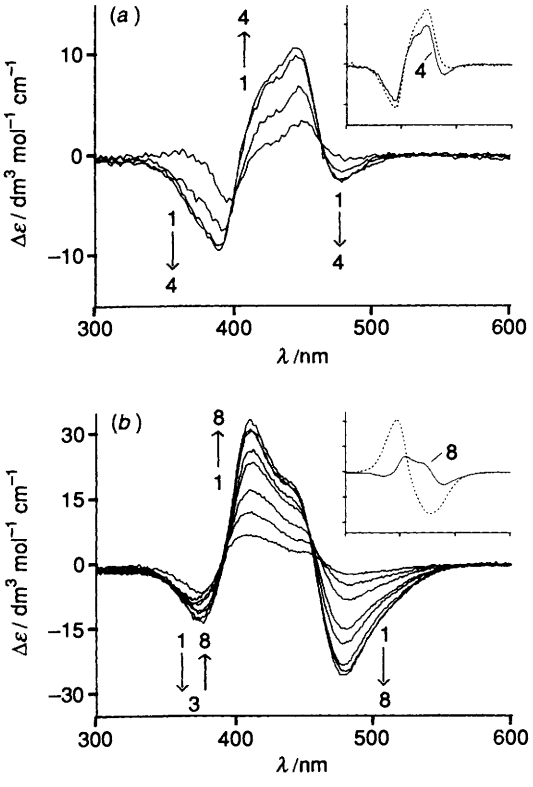


Fig. 4 ICD of the kinetically stable racemate **3b** (*c* 0.02 mmol dm^{-3}) in toluene–chloroform (17% v/v) at 265 K on addition of (−)-PBA (1: 0.33 mmol dm^{-3}, 2: 1.96, 3: 7.85, 4: 15.70) (*a*) and (−)-CHA (1: 0.03 mmol dm^{-3}, 2: 0.06, 3: 0.12, 4: 0.30, 5: 0.60, 6: 1.20, 7: 2.40, 8: 4.80) (*b*). Insets: ICD spectra before and after equilibration at 293 K.

geneity they are simply due to essentially equipopulated diastereoisomeric complexes. The *x vs. D* dependencies for the diastereoisomers **A**·**L** and **A***·**L**, and the enantiomers **A** and **A*** are now non-linear and lens-shaped and the latter no longer coincide [Fig. 1(*b*)] except for $D = 1$. In general, curvatures increase with discrimination energy. Only if $K_{discr} = 1$ two straight lines, one for **A**·**L** and **A***·**L**, and one for **A** and **A*** with identical slopes appear which implies that the shape of ICD spectra should not change with *D*. This expectation is approximately fulfilled for racemic **3b** and (−)-PBA (K_{discr} *ca.* 1.04) [Fig. 4(*a*)]. However, if discrimination becomes pronounced a remarkable general singularity of ICD spectra of kinetically stable racemates emerges which follows from the computed changes in the population of species with *D* [Figs. 1(*b*) and 5]. Accordingly, different from the behaviour of a

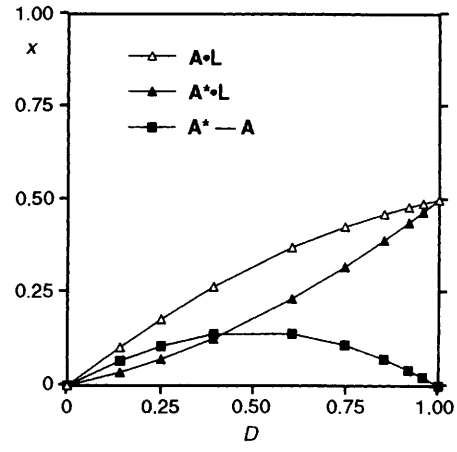


Fig. 5 Computed mole fractions *x* of species contributing to the ICD of **3b** in the presence of (−)-CHA *vs. D*. Point by point calculations [parameters as for Fig. 1(*b*)] correspond with conditions for ICD spectra 1–8 in Fig. 4(*b*).

kinetically unstable racemate even unbound enantiomers contribute to the ICD spectra as long as complexation is incomplete ($D < 1$).§§ This is due to the fact that enantiomers **A** and **A*** are differently involved in complexation, and equilibration between them is hindered. Therefore ICD spectra are compounded from three differently participating components arising from the diastereoisomeric associates **A**·**L** and **A***·**L** and one enantiomer, **A** or **A***, in excess. This is illustrated for the optically stable racemic bilirubin (*M* and *P*)-**3b** (**A**,**A***) on titration with (−)-CHA (**L**) (K_{discr} *ca.* 0.3) [Fig. 4(*b*)]. In principle no isodichroic point appears since all proportions of species must change with *D*. Fig. 5 shows the change in mole fractions *x* of species contributing to the ICD with increasing complexation. At low acid concentration, species **A**·**L** and an excess population of unbound **A*** determine the ICD. The relative contribution of unbound **A*** in excess is largest at this initial stage even if the maximum is reached at $D = 0.5$. In the vicinity of $D = 1$, the contribution from unbound **A*** becomes unimportant and the population of **A***·**L** approaches that of **A**·**L**. Clearly, the phenotype of ICD spectra now does depend on *D* and this singularity accounts for the decrease and increase of the CE around λ 370 nm with increasing concentration in (−)-CHA [Fig. 4(*b*)]. Since $x_{A\cdot L} + x_A$ equals $x_{A^*\cdot L} + x_{A^*}$ within the whole range of *D*, mixtures of **3b** with (−)-CHA and (−)-PBA, respectively, become instantaneously CD inactive if the influence of the acid is withdrawn by mixing with methanol. On the other hand, if initial solutions are allowed to equilibrate by warming up to 293 K for 1 h (insets Fig. 4) prior to methanol addition CEs are obtained from which K_{discr} values were evaluated (see Experimental section).

Conclusions

Many of the ICD studies performed on kinetically labile racemates intuitively imply congruence of CD spectra of bound and unbound enantiomers and mirror-image relationship between the diastereoisomeric associates. As long as chiral discrimination is pronounced this approximation is feasible and does not interfere with interpretations. However, if chiral

§§ In NMR spectroscopy no distinction is made between **A** and **A**·**L** and **A*** and **A***·**L**, respectively, as long as ligand exchange is rapid. This is due to the different timescale of NMR if compared with electronic absorption CD spectroscopy and comprises the basis for the determination of enantiomeric purity (ee) of optically stable or fairly stable compounds by NMR which is also possible if complexation is incomplete ($D < 1$).

discrimination becomes poor even small spectral dissimilarities come into play and may lead to misinterpretations because then even the lack of enantioselectivity of an optically active ligand is not necessarily associated with vanishing CD within the absorption region of the racemate. On the other hand the proof whether K_{discr} is 'sufficiently' pronounced can hardly be derived from ICD spectra as long as reference CEs are not available. This comprises an additional shortcoming in the application of ICD on kinetically labile racemates. Fortunately, the aforementioned factors do not interfere with the direct evaluation of the macroscopic association constants K_{ass} for which the CD response *per se* is relevant rather than its origin provided that the stoichiometry of associates is known and interconversion between enantiomers is rapid. The most striking feature of the ICD of a kinetically stable racemate in the presence of a chiral discriminator comprises the contribution from unbound enantiomers as long as complexation is incomplete. In this case the ICD technique comprises no adequate means for the determination of K_{ass}. On the other hand if changes in the CD spectra of the optically active ligand are used, as observable, eventual differences in the CD spectra of diastereoisomeric associates might still prohibit a reliable determination of K_{ass} of a kinetically stable racemate. If additionally CD and ICD overlap the situation becomes obscure. The latter two factors might provide a rationale for the 'less precise values' obtained in recent binding studies[19] between an aromatic host and some racemic but likewise aromatic guests. Using non-chiroptical methods like UV–VIS spectroscopy as reported for a racemic host studied in ref. 4 an eventual excess population becomes irrelevant but diastereoisomeric species may still exhibit different spectra. Therefore chiral discrimination between the enantiomers of a kinetically stable racemate by an optically active compound can be regarded as a potential perturbing factor in the evaluation of K_{ass} by any optical method. Analogous problems as summarized in the above may also arise if solvent induced CD is considered.

From our study eventual implications for the ICD studies performed on the kinetically labile racemic bilirubin-IXα **1** become evident. We have shown that spectral differences between diastereoisomeric bilirubin associates may become quite large and may give rise to complex spectra or even asymmetric exciton CEs, if chiral discrimination is poor. This offers an alternative to explain some of the anomalous ICD spectra reported[3,5,12,13] for **1**. Owing to its pronounced conformational flexibility **1** can be expected to fit its geometry to the ligand offered so that the corresponding hypothetical reference CEs may vary between $|\Delta\varepsilon|$ *ca.* 250 dm^3 mol^{-1} cm^{-1}, or even higher,[28,32] and an unknown lower limit. Consequently, the reliability of ICD spectra of bilirubins decreases with decreasing $|\Delta\varepsilon|$-values. In particular, those of low intensity ($|\Delta\varepsilon| < 10$ dm^3 mol^{-1} cm^{-1})[8,10,14] do not necessarily reflect the helicity of the preferentially bound enantiomer and indicate—if at all—chiral discrimination to an unknown extent.

Experimental

General.—Mps were determined with a Kofler-Reichert hot-stage apparatus. ^{1}H NMR spectra (250 MHz) were run with a Bruker AC 250 AF instrument at 297 K for *ca.* 10 mmol dm^{-3} solutions. Molecular masses have been determined by fast atom bombardment (3-nitrophenylmethanol, Xe) MS using a Finnigan MAT 900 instrument. UV–VIS spectra and CD spectra were measured with a Perkin-Elmer Lambda 7 spectrometer and a CD6 circular dichrograph (I.S.A. Jobin-Yvon), respectively, in spectroscopic grade (Uvasol, Merck) toluene, chloroform (both chromatographed on alumina prior to use) and methanol using thermostatted (± 1 °C) quartz cuvettes (0.1–1.0 cm path pength). TLC was performed on Kieselgel 60 precoated plates (0.25 mm, Merck). The optically active acids (−)-PBA (Sigma) and (−)-CHA (Fluka) showed satisfactory optical rotation and were used as purchased. For syntheses methanol (p.A., Loba) and sodium borohydride (zur Synthese, Merck) were used. The bridged bilirubins **2** (this work) and **3a** and **b**[33] are particularly sensitive towards oxygen and light, therefore the precautions as delineated[33] were taken into account. All measurements reported here refer to freshly prepared solutions of freshly prepared compounds. Since ageing of solutions is accelerated by the presence of the acids used here measurements were performed with the minimum delay. However, even after 1 h at room temperature decomposition products did not exceed 5% as revealed by TLC and ^{1}H NMR spectroscopy.

[(P,1′R,4′S) *and* (M,1′R,4′S)]-(4Z,15Z)-2,18-(1′,4′-*dimethoxybutane*-1′,4′-*diyl*)-8,12-*bis*(2″-*methoxycarbonylethyl*)-3,7,13,17-*tetramethyl*-10,23-*dihydro*-1,19(21H,24H)-*bilindione* (**2**). —This compound was obtained from the corresponding racemic biliverdin[35] (13.5 mg, 0.02 mmol) by sodium borohydride reduction in analogy to ref. 33 in almost quantitative yield (12.1 mg, 90%). Yellow crystals, mp (from benzene–pentane) 140–145 °C (decomp.), *m/z* (positive FABMS) 674 (M^+); $\lambda_{max}(CHCl_3)$/nm 425sh (ε 16 700 dm^3 mol^{-1} cm^{-1}) and 379 (42 100); (MeOH) 425sh (21 000) and 386 (57 700); (toluene) 430sh (15 000) and 378 (41 900); δ_H([$^2H_5$]-pyridine) 10.65, 10.49, 10.38 and 10.36 (1 H × 4, s, 21-, 22-, 23-, 24-H), 6.43 and 6.33 (1 H × 2, s, 5-, 15-H), 4.76 (1 H, d, *J* 3, 1′- or 4′-H), 4.10 (1 H, d, *J* 11, 1′- or 4′-H), 4.06 and 3.96 (2 H, AB, J_{AB} 19, 10-H_2), 3.61 and 3.60 (3 H × 2, s, CO_2Me), 3.26 and 3.15 (3 H × 2, s, 1′-, 4′-OMe) 2.9–1.5 (4 H, m, 2′-, 3′-H_2), 2.5–2.2 (8 H, m, 8-, 12-CH_2–CH_2–CO_2), 2.62, 2.19, 2.08 and 2.02 (3 H × 4, s, 3-, 7-, 13-, 17-Me). The barrier to *M–P* interconversion was determined as described in detail for the (1′*R*,4′*R*) and (1′*S*,4′*S*) stereoisomers[33] to give ΔG_{248} = 73.3 kJ mol^{-1} (*k* 1.9 × 10^{-3} s^{-1}, $t_{\frac{1}{2}}$ 6 min).

CD-Measurements.—Unless changes due to *M–P* helix interconversion occurred all ICD and CD spectra were independent from time indicating rapid ligand exchange.

CD monitored titrations of **2**, **3a** *and* **3b** *with* (−)-*CHA and* (−)-*PBA, respectively.* Stock solutions (0.5 cm^3) of **2**, **3a** and **3b** (each 5–7 mmol dm^{-3}) in toluene were stored at −70 °C and used within 2 days for the following measurements. 5–10 solutions (2 cm^3) each containing increasing amounts (0.03–16.0 mmol dm^{-3}) of the corresponding optically active acid in toluene–chloroform (17% v/v) were prethermostatted (293 and 265 K for titrations of **2** and **3**, respectively) in 1 cm quartz cuvettes and provided for the respective baseline correction between λ 300–600 nm. Then 10–25 mm^3 (1 mm^3 = 1 μl) of the corresponding bilirubin stock solutions were, one after another, added by injection followed by an immediate CD performance. Each of the ICD titration spectra displayed in Figs. 2 and 4 corresponds to a freshly prepared solution. Synchronously, UV–VIS absorption spectra were taken to check eventual errors in concentrations. After measurements solutions were concentrated and compared with an authentic sample by TLC. The 1:1 stoichiometry of the associates between the bilirubins and acids used was assessed by Job's method[36] as described.[15]

Determination of the macroscopic association constants K_{ass} *and discrimination constants* K_{discr}. (*a*) Compound **2**. The K_{ass} values were determined directly from the CD monitored titrations at 293 K (see above) by using the Scatchard method.[15,37] In all cases straight line graphs were obtained and the constants calculated from changes in ICD spectra were independent of wavelength to afford K_{ass}[**2**/(−)-CHA] 950 ± 100 and K_{ass}[**2**/(−)-PBA] 470 ± 100 dm^3 mol^{-1},

respectively. Due to the limited solubility of (−)-CHA in toluene–chloroform (17% v/v) the final ICD spectrum [λ_{max}/nm 394 ($\Delta\varepsilon$ +167 dm^3 mol^{-1} cm^{-1}), 453 (−136)] was obtained by extrapolation ($1/\Delta\varepsilon$ *vs.* $1/c$).[17] The determination of the K_{discr} values was accomplished by injecting a solution of **2** in toluene (220 mm^3; 0.2 mmol dm^{-3}) containing the respective highest acid concentration as used for titrations into precooled (248 K) methanol (2 cm^3) followed by a CD recording (Fig. 2, insets). After correction for volume contraction the magnitudes of the bisignate CEs were correlated with the reference CEs ($|\Delta\varepsilon|$ 250 ± 10 and 200 ± 20 dm^3 mol^{-1} cm^{-1} at λ *ca.* 390 and 440 nm, respectively)[34] to afford the K_{discr} values given in Fig. 2.

(*b*) Compounds **3a** and **b**. K_{ass} values were determined by allowing the corresponding mixtures as used for direct titrations at 265 K to equilibrate at 293 K (*ca.* 1 h) followed by an additional CD performance (Fig. 4, insets). In this case equilibration through *M–P* interconversion of the tetrapyrrol backbone is associated with a **3a–b** transition and the K_{ass} obtained for (−)-PBA (1200 ± 200 dm^3 mol^{-1}) and (−)-CHA (1100 ± 100 dm^3 mol^{-1}) are mean values. However, the K_{ass} values of isomers must be very similar to each other and the corresponding K_{discr} values, too, as can be concluded from all the experiments performed on pure isomers **3a** and **b** exhibiting almost superimposable ICD spectra if the same concentrations in acids are applied. The mean K_{ass} values of **3** and (−)-PBA (7000 ± 1500) and (−)-CHA (6500 ± 1000 dm^3 mol^{-1}) at 265 K were then obtained by extrapolation using $\Delta S^{\ddagger}$ −80 J K^{-1} mol^{-1}.[15] The mean K_{discr} values were evaluated by injecting a solution (220 mm^3) of an equilibrated mixture **3a–b** in toluene containing the respective optically active acid (15.7 and 4.8 mmol dm^{-3}, respectively) into prethermostatted (265 K) methanol (2 cm^3). The bisignate CEs obtained after CD performance [λ_{max}/nm 386 ($\Delta\varepsilon$ −4.5 dm^3 mol^{-1} cm^{-1}), 442 (+4.0) for the (−)-PBA discrimination and λ_{max}/nm 385 ($\Delta\varepsilon$ +130 dm^3 mol^{-1} cm^{-1}), 439 (−105) for the (−)-CHA discrimination] were then correlated with the reference CEs given above to afford K_{discr} 1.04 and 0.30, respectively. Between 283 and 303 K constants were within experimental error suggesting the same value to be valid for 265 K. An estimate of (−)-CHA discrimination can likewise be accomplished more directly by using the ICD parameters of equilibrated acidic solutions.

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