Journal of Inclusion Phenomena **3**, 479–485. 0167–7861/85.15.

Bilirubin Conformational Enantiomer Selection in Sodium Deoxycholate Chiral Micelles

M. REISINGER and D. A. LIGHTNER★
Department of Chemistry, University of Nevada Reno, Nevada 89557–0020 U.S.A.

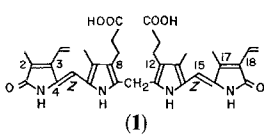
(Received: 25 March 1985)

Abstract. Intramolecularly hydrogen-bonded, bichromophoric tetrapyrrole pigments, bilirubin-IXα and mesobilirubin-XIIIα, adopt either of two enantiomeric conformations which are in dynamic equilibrium in solution. In *p*H 8 aqueous sodium deoxycholate solutions, chiral micelles preferentially select one conformational enantiomer, and the solutions exhibit a bisignate circular dichroism Cotton effect in the vicinity of the bilirubin long wavelength electronic transition. Exciton coupling theory indicates a predominance of the left-handed (or negative) chirality bilirubin conformational enantiomer.

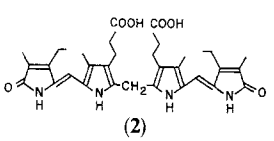
Key words: Sodium deoxycholate, bilirubin-IXα, mesobilirubins-XIIIα and IVα, xanthobilirubic acid, circular dichroism.

1. Introduction

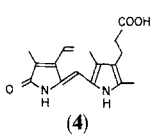
Nearly fifteen years ago Perrin and Wilsey [1] observed that solutions of the tetrapyrrole bile pigment bilirubin-IXα (**1**) in sodium deoxycholate solution exhibited circular dichroism (CD) in the vicinity of the long wavelength electronic transition of the pigment. Maximum values of the bisignate CD Cotton effects (CEs) were found at *p*H 8 and with sodium deoxycholate concentrations above the c.m.c. (4.5×10^{-2} M), but in the absence of sodium deoxycholate no CEs could be observed. This appears to be the first example in which bilirubin has been shown to exhibit circular dichroism induced by chiral micellization. And although the study included the varying of parameters such as sodium deoxycholate concentration (with bilirubin concentration held constant at 3.4×10^{-5} M) and *p*H, the origin of the CD remained unexplained. In the present paper, we extend the CD studies to the symmetric bilirubins, mesobilirubin-XIIIα (**2**) and IVα (**3**) and to their pyrromethenone analog, xanthobilirubic acid



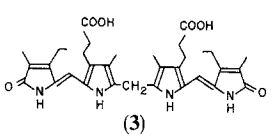
(**1**)



(**2**)



(**4**)



(**3**)

★ Author for correspondence.

(**4**), and we provide an explanation for the induced CD spectra in terms of chiral micelle selection of bilirubin conformational enantiomers.

2. Experimental

Bilirubin-XIα (**1**) was obtained from Sigma and purified by crystallization as previously described [2]. Mesobilirubins-XIIIα (**2**), IVα (**3**) and xanthobilirubic acid (**4**) were prepared by total synthesis [3,4]. Sodium deoxycholate was obtained from Sigma. Buffer solutions, 0.1 M Tris (Trizma, from Sigma), were prepared as described previously [5]. All circular dichroism (CD) spectra were recorded on a JASCO J–40 spectropolarimeter equipped with photo-elastic modulator, and all UV-visible spectra were measured on a Cary 219 spectrophotometer.

3. Results and Discussion

3.1. CONFORMATIONAL ENANTIOMERISM IN BILIRUBINS

Although the constitutional structure of bichromophoric (4*Z*, 15*Z*)-bilirubin-IXα (**1**), the yellow-orange lipophilic and cytotoxic pigment of jaundice, was solved over forty years ago, its conformational strucure was only recently characterized [6] by X-ray crystallography [7,8] and nuclear magnetic resonance spectroscopy [3,9]. Perhaps the most interesting aspect of the structure of **1**, and one with important implications for its biological function, is its ability and marked tendency to form *intra*molecular hydrogen bonds and thereby control its conformation and polarity [10]. The key structural features which collectively govern the shape of **1** include: (i) *syn*-periplanar conformations of its two pyrromethenone chromophores with *Z*-configuration carbon–carbon double bonds at C_4 and C_{15}, (ii) two propionic acid groups located at C_8 and C_{12} – each capable of forming *intra*molecular hydrogen bonds with the opposing pyrromethenone lactam C=O/NH and pyrrole NH groups, and (iii) an

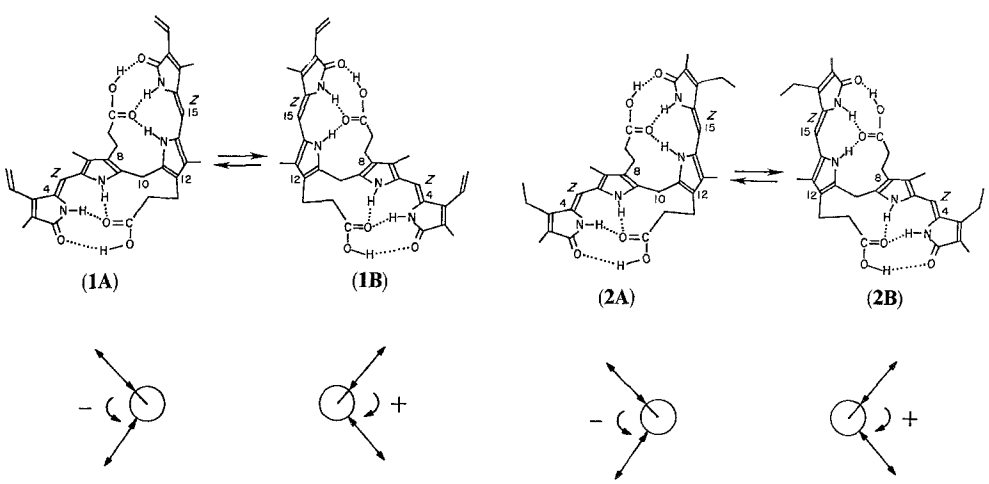


Fig. 1. Interconverting, intramolecularly hydrogen-bonded enantiomeric conformers of bichromophoric (left) (4*Z*, 15*Z*)-bilirubin-IXα and (right) (4*Z*, 15*Z*)-mesobilirubin-XIIIα. The **A** enantiomers have left-handed (–) chirality of the twin pyrromethenone chromophores; the **B** enantiomers have right-handed (+) chirality. The lower part show the relative orientations of the electric dipole transition moments (viewed at C_{10}) of the pyrromethenone chromophores of (left) **1A** and **2A** and (right) **1B** and **2B**.

sp^3 carbon at C_{10} which keeps the two pyrromethenone chromophores, and hence their long wavelength transition electric dipole moments [11], 109° apart. These structural elements allow and even direct **1** to adopt either of the two enantiomeric conformations **1A** and **1B** (shown in Figure 1), which are stabilized by six intramolecular hydrogen bonds.

The enantiomeric conformations have been found in crystalline bilirubin [7, 8] and appear to be equally preferred in solutions of bilirubin in achiral organic solvents, where they interconvert rapidly at room temperature [9, 12]. Remarkably, even when ionization of the propionic acid reduces the number of hydrogen bonds, but probably increases the strength of the remainder, conformers like **1A** and **1B** persist in the crystal [13] and also apparently in solution [14]. This marked preference for (enantiomeric) conformers in which polar groups are intramolecularly hydrogen bonded explains the lipophilic character of bilirubin, that property which prevents its ready excretion across the liver into bile [10]. It also explains why isomers of bilirubin with vinyl groups reduced to ethyl (mesobilirubins), e.g. mesobilirubins-XIIIα (**2**), or vinyl and methyl groups interchanged at C_2/C_3 or C_{17}/C_{18}, e.g. symmetrically substituted bilirubin-IIIα and XIIIα, all exhibit similar solubility properties. However, isomers which have an *E*-configuration carbon–carbon double bond at C_4 or C_{15}, or isomers that do *not* have their propionic groups positioned at C_8 or C_{12}, e.g. mesobilirubin-IVα (**3**), exhibit markedly different chemical and biolgical properties because they cannot fully achieve the intramolecular hydrogen bonding expressed in Figure 1.

The enantiomeric conformers (**1A** and **1B**) of bilirubin are in dynamic equilibrium [9, 12], as are those (**2A** and **2B**) of mesobilirubin-XIIIα (Figure 1). They interconvert (**A** $\rightleftarrows$ **B**) by breaking and remaking all six hydrogen bonds, an enantiomeric equilibration process which has potential importance in biological reactions of bilirubin, e.g. enzymic glucuronidation, that probably involve stereoselective complexation [10]. In the absence of special effects, a 1 : 1 mixture of enantiomers **1A** and **1B** (or **2A** and **2B**) is expected, with solutions of **1** and **2** being optically inactive. However, if enantiomers (**A** and **B**) can be perturbed to favor either **A** or **B**, solutions should exhibit optical activity. Such displacement from 1 : 1 conformational enantiomeric equilibrium (**A** $\rightleftarrows$ **B**) might be achieved in a chiral solvent or by selective interaction with a chiral solute. For example, aqueous solutions of bilirubin with albumin [15] or cyclodextrins [16] exhibit circular dichroism associated with the pigment chromophore, and induced circular dichroism has even been observed for bilirubin dimethyl ester in ethyl (*S*)-(-)-lactate and (*R*,*R*)-2,3-butanediol solutions [17]. The picture provided in Figure 1 may serve similarly to explain the circular dichroism of bilirubin in sodium deoxycholate [1].

3.2. BILIRUBIN INDUCED CIRCULAR DICHROISM

In confirmation of the study of Perrin and Wilsey [1], we, too, observe a long-wavelength bisignate circular dichroism (CD) Cotton effect (CE) for bilirubin-XIα (**1**) in *p*H 8 buffered solutions of sodium deoxycholate with $\Delta\varepsilon_{416}^{max} = +8.2$, $\Delta\varepsilon_{467}^{max} = -13.1$ (Figure 2). Interestingly, the associated UV-vis spectrum shows two closely spaced maxima, $\varepsilon_{433}^{max} = 48\,300$, $\varepsilon_{453}^{max} = 48\,800$, which are not exactly coincident with the CD maxima. This behavior is not unique to **1**, as is shown with the induced CD of symmetric mesobilirubin-XIIIα in *p*H 8.0 sodium deoxycholate: $\Delta\varepsilon_{395}^{max} = +7.3$ and $\Delta\varepsilon_{441}^{max} = -11.5$, and its long wavelength UV-vis spectrum: $\varepsilon_{405}^{max} = 45\,700$ and $\varepsilon_{428}^{max} = 45\,900$ (Figure 3). Since *p*H 8.0 aqueous buffered solutions of **1** or **2** alone do not exhibit CEs, (chiral) sodium deoxycholate is clearly the agent inducing the observed CD in Figures 2 and 3. Moreover, the moderately strong bisignate CEs, whose maxima flank, but do not coincide with, the UV-vis λ_{max} are characteristic of exciton coupling [18] (chromophore–chromophore interaction in the excited state). The twin

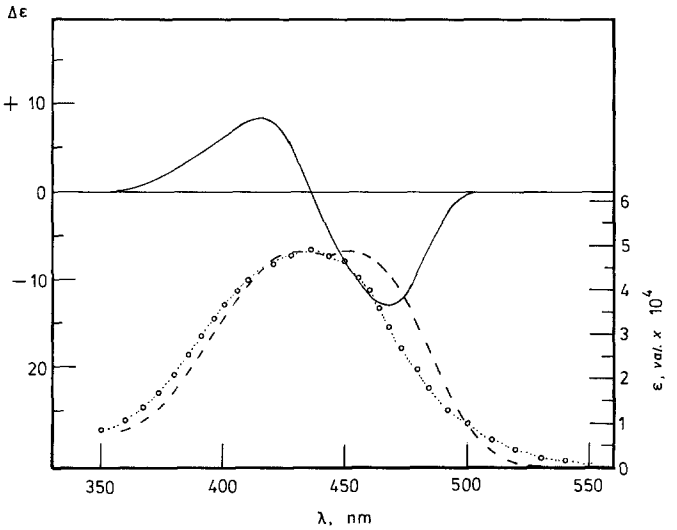


Fig. 2. Circular dichroism (———) and UV-vis (– – – –) spectra of 3.52×10^{-5} M bilirubin-IXα (**1**) in *p*H 8.02 argon-saturated Tris buffer in the presence of 7.24×10^{-2} M sodium deoxycholate at 22 °C. The spectra were recorded within 15 minutes after preparation of the solution and remained essentially invariant for hours at 22 °C. A CD spectrum of the same concentration of **1** without added sodium deoxycholate falls on the $\Delta\varepsilon = 0$ line; a UV-vis spectrum on the line (o···o···o).

pyrromethenone chromophores of **1**, and even the identical pyrromethenone chromophores of **2**, interact to give two electronic transitions, one higher in energy and one lower in energy. The two transitions are not widely separated and show considerable overlap in the UV-visible

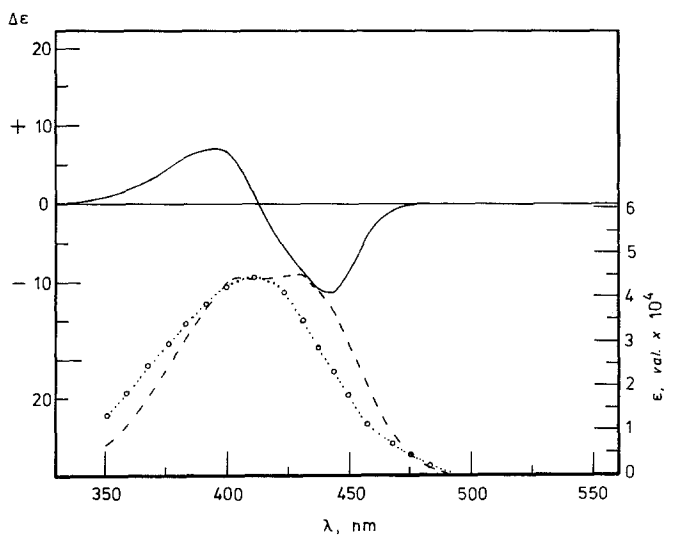


Fig. 3. Circular dichroism (———) and UV-vis (– – – –) spectra of 3.14×10^{-5} M mesobilirubin-XIIIα (**2**) in *p*H 7.98 argon-saturated Tris buffer in the presence of 7.52×10^{-2} M sodium deoxycholate at 22 °C. The spectra were recorded within 15 minutes after preparation of the solution and remained essentially invariant for hours at 22 °C. A CD spectrum of the same concentration of **2** without added sodium deoxycholate falls on the $\Delta\varepsilon = 0$ line; a UV-vis spectrum on the line (o···o···o).

spectra, where the electronic transitions have the same sign. (Their separation energy depends on the nature of the chromophores and the relative orientation of their electric dipole transition moments.) In the CD spectra, however, where the close-lying transitions have *oppositely signed* CEs, the net observed CD spectra, with widely separated maxima flanking the UV-vis λ_{max}, result from considerable cancellation in the region of overlap [19]. This behavior parallels that observed for bilirubin complexed with proteins [15] and cyclodextrins [16], where characteristic bisignate induced CEs are seen. And the presence of the exciton coupling phenomenon in **1** and **2**, depending as it does on the electronic interaction of two loosely coupled pyrromethenone chromophores, cannot be seen with sodium deoxycholate solutions of xanthobilirubic acid (**4**), which is the pyrromethenone chromophore of one-half of **2**. In fact, no induced CD ($\Delta\varepsilon \leqslant \pm 0.1$) is observed, although evidence for interaction of the pigment with the micelle can be seen in the UV-vis λ_{max} shift of 3.3×10^{-5} M **4**: $\lambda_{max} = 425$ nm in *p*H 8 sodium deoxycholate solution, $\lambda_{max} = 411$ nm in *p*H 7.7–8.0 buffer alone.

We suggest that sodium deoxycholate chiral micelles [20], in which hydrophobic bonding dominates [21], select preferentially, but probably not exclusively, one of the (lipophilic) enantiomeric, intramolecularly hydrogen bonded conformers (**A** or **B**) of **1** and **2**. The net mole fraction of **A** or **B** enantiomers is thus no longer 0.5, and the pigment exhibits CD. In support of this rationale, solutions of mesobilirubin-IVα (**3**), which cannot assume the hydrogen bonded conformations of Figure 1, in sodium deoxycholate are only weakly dichroic and have bisignate CEs with *signs opposite* to those observed for **1** and **2** (Figure 4). These data are important because they reinforce the notion that exciton coupling is seen only when the pyrromethenone chromophores are covalently linked together (recall that **4** does not exhibit CD), and they show that only the pigments capable of intramolecular hydrogen bonding (Figure 1) give rise to induced CD with moderately intense bisignate CEs which have a (−)

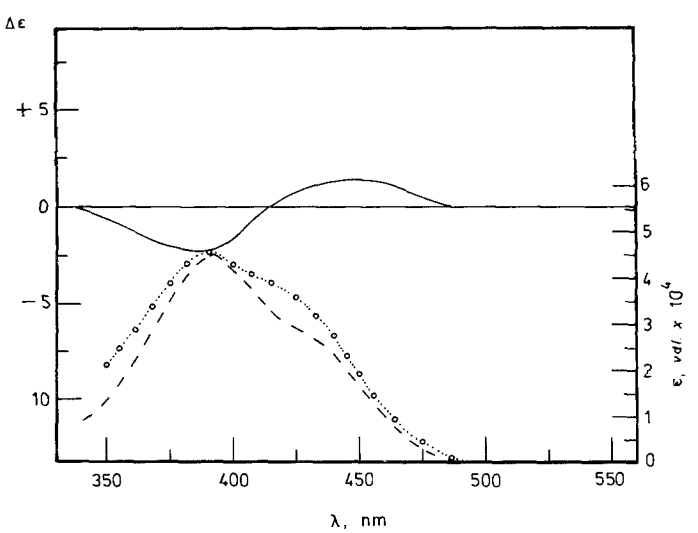


Fig. 4. Circular dichroism (———) and UV-vis (– – – – –) spectra of 2.94×10^{-5} M mesobilirubin-IVα (**3**) in *p*H 8.02 argon-saturated Tris buffer in the presence of 7.26×10^{-2} M sodium deoxycholate at 22 °C. The spectra were recorded within 15 minutes after preparation of the solution, remained essentially invariant for hours at 22 °C, and are identical with those CD spectra at *p*H 7.4 and 10.0. A CD spectrum of the same concentration of **3** without added sodium deoxycholate falls on the $\Delta\varepsilon = 0$ line; a UV-vis spectrum on the line (o···o···o). When the sodium deoxycholate concentration falls well below 6.2×10^{-4} M (the c.m.c.), **3** shows $\Delta\varepsilon_{380}^{max} = -0.9$, $\Delta\varepsilon_{440} < +0.1$. The UV-vis spectra do not change shape over the pigment concentration range 8×10^{-4} to 1×10^{-6} M; however, λ_{max} shifts from 381 nm ($\varepsilon^{max} = 39100$) to 394 nm ($\varepsilon^{max} = 39400$), respectively.

component at long wavelengths. The behavior of **3** is very much akin to that of **1** and **2** in solutions where the sodium deoxycholate concentrations fall considerably below the c.m.c., e.g. where the weak (+) long wavelength, (−) short wavelength bisignate CD CEs are induced by univalent deoxycholate [1]. In this connection, it is important to note that the signed order of the induced bisignate CD of 3 remains invariant over the *p*H range 7.4–10.0 and also over a range of sodium deoxycholate concentrations from that well above the c.m.c. to that well below (Figure 4). The CE magnitudes are independent of *p*H in the cited range, but they decrease considerably at sodium deoxycholate concentrations below the c.m.c. Consequently, the selection of a chiral conformation in **3** appears in no way to depend on chiral micelles and is due, presumably, to an as yet undetermined interaction between the steroid and the pigment.

Exciton coupling theory points the way for the assignment of absolute configuration of the chiral bilirubins. The handedness or screw sense that the electronic transition moments of the coupled pyrromethenone chromophores make with each other (Figure 1) correlates with signed order of the bisignate CD CEs [18]. A right-handed screw sense (positive chirality) of the transition moments leads to a (+) longer wavelength CE followed by a (−) shorter wavelength CE, and with a left-handed screw sense (negative chirality) the CE signs are inverted: (−) at the longer wavelength and (+) at the shorter wavelength component of the bisignate CE. Since the direction of the electric dipole transition moment in the pyrropethenone chromophore has been determined from theoretical studies [11] to lie along the longitudinal axis of the planar conjugated π-system, the exciton model can predict the expected CEs of the enantiomers **A** and **B** in Figure 1. In the intramolecularly hydrogen bonded conformations of **1** and **2** (Figure 1), the relative orientations of the two pyrromethenone electric dipole moments constitute a left-handed chirality for **A** and a right-handed chirality for **B**. Since the induced bisignate CDs of both **1** and **2** show (−) long wavelength CEs followed by (+) short wavelength CEs, theory predicts a predominance of enantiomer **A** in sodium deoxycholate micelles for these substances.

Similar conclusions favoring predominance of the left-handed chiral conformation (**A**, Figure 1) are in accord with: (1) the previously reported induced CD bisignate CEs [$\Delta\varepsilon_{466}^{max} = -6.5, \Delta\varepsilon_{405}^{max} = +3.4$] and [$\Delta\varepsilon_{435}^{max} = -9.8, \Delta\varepsilon_{386}^{max} = +6.6$] of 3.4×10^{-5} M **1** and **2**, respectively, in *p*H 8 buffered solutions of 4.5×10^{-2} M β-cyclodextrin [16], and (2) the CD data of Harmatz and Blauer [22] for selected albumin–bilirubin complexes, e.g. the powerful bisignate CE [$\Delta\varepsilon_{467}^{max} = -80, \Delta\varepsilon_{415}^{max} = +21$] for 2.4×10^{-5} M bilirubin-IXα in 5.0×10^{-5} M goat albumin at *p*H 9.8. However, a predominance of a right-handed chiral conformation appears to be indicated [bisignate ICD CE: $\Delta\varepsilon_{460}^{max} = +53, \Delta\varepsilon_{410}^{max} - 33$] for bilirubin-IX$\alpha$ (2.5×10^{-5} M) bound to human serum albumin (5.0×10^{-5} M) at *p*H 9.8. The order of magnitude larger bisignate CEs associated with the protein-bound bilirubin suggests a relatively larger enantiomeric excess of pigment in the aqueous albumin solutions than in sodium deoxycholate solutions. The stereochemical facets of the interaction of bilirubins and pyrromethenones with protein (chiral solute), and cyclodextrin solutions are currently under further study in our laboratory.

Acknowledgements

We thank the National Institutes of Health (HD–17779) for generous support of this work and Dr G. L. Landen for preliminary studies of xanthobilirubic acid in sodium deoxycholate solution.

References

1. J. H. Perrin and M. Wilsey: *J. Chem. Soc. Chem. Commun.* 769 (1971).
2. D. A. Lightner, T. A. Wooldridge and A. F. McDonagh: *Proc. Natl. Acad. Sci. U.S.A.* **76**, 29 (1979).
3. F. R. Trull, J.-S. Ma, G. L. Landen and D. A. Lightner: *Isr. J. Chem.* **23**, 211 (1983).
4. D. A. Lightner, J.-S. Ma, R. W. Franklin and G. L. Landen: *J. Heterocycl. Chem.* **21**, 139 (1984).
5. D. A. Lightner, A. Cu, A. F. McDonagh and L. A. Palma: *Biochem. Biophys. Res. Commun.* **69**, 648 (1976).
6. For leading references see D. A. Lightner: *Structure, Photochemistry and Organic Chemistry of Bilirubin* (Bilirubin, vol. I, ed. K. P. M. Heirwegh and S. B. Brown) chapter 1, CRC Press, Boca Raton, FL (1982).
7. R. Bonnett, J. E. Davies, M. B. Hursthouse and G. Sheldrick: *Proc. R. Soc. Lond.* **B202**, 249 (1978).
8. G. LeBas, A. Allegret, Y. Mauguen, C. DeRango and M. Bailly: *Acta Crystallogr.* **B36**, 3007 (1980).
9. For leading references see D. Kaplan and G. Navon: *Isr J. Chem.* **23**, 177 (1983).
10. D. A. Lightner and A. F. McDonagh: *Acc. Chem. Res.* **17**, 417 (1984).
11. G. Blauer and G. Wagnière: *J. Am. Chem. Soc.* **97**, 1949 (1975).
12. P. Manitto and D. Monti: *J. Chem. Soc. Chem. Commun.* 122 (1977).
13. A. Mugnoli, P. Manitto and D. Monti: *Acta Crystallogr.* **C39**, 1287 (1983).
14. J.-S. Ma and D. A. Lightner: unpublished observations.
15. G. Blauer: *Isr. J. Chem.* **23**, 210 (1983).
16. D. A. Lightner, J. K. Gawroński and K. Gawrońska: *J. Am. Chem. Soc.* **107**, 2456 (1985).
17. S. E. Braslavsky, A. R. Holzwarth and K. Schaffner: *Angew. Chem. Int. Ed. Engl.* **22**, 656 (1983).
18. For examples and leading references see N. Harada and K. Nakanishi: *Circular Dichroic Spectroscopy – Exciton Coupling in Organic Stereochemistry*, University Science Books, Mill Valley, CA (1983).
19. This phenomenon has been explained prevously. K. M. Wellman, P. H. A. Lauer, W. S. Briggs, A. Moscowitz and C. Djerassi: *J. Am. Chem. Soc.* **87**, 66 (1965).
20. A. R. Campanelli, S. Candeloro de Sanctis, E. Giglio and S. Petriconi: *Acta Crystallogr.* **C40**, 631 (1984).
21. J. H. Perrin and P. Idsvoog: *J. Pharm. Sci.* **59**, 1525 (1970).
22. D. Harmatz and G. Blauer: *Arch. Biochem. Biophys.* **170**, 375 (1975).