Helically Shaped Bilirubins: Natural Optical Activity and Chemical Correlation of *M* and *P* Backbone Helicity with Biliverdins

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The natural optical activity of 2,18-bridged helically shaped bilirubins **2** and an unambigous chemical correlation of the *M* and *P* backbone helicity with the corresponding biliverdins **1** is reported. Complete resolution of the four component stereoisomeric mixture of (*M*,1'*S*), (*M*,1'*R*), (*P*,1'*S*) and (*P*,1'*R*) chirality was performed at the biliverdin stage **1**. The optical activity of compounds is discussed in terms of the diagnostic value of CD spectra in the conformational analysis of non-restricted bilirubins.

The metabolic pathway of haem proceeds through bilirubin, a lipophilic linear tetrapyrrol of large conformational mobility.[1-3] During transportation to the liver it is non-covalently bound to serum albumin, by which its solubility becomes substantially increased. Thereby, the chirality of the most flexible biladiene-ac backbone becomes more or less adapted to the handedness of the protein host.[2-6] This enantioselective binding takes place rapidly in aqueous solvents since barrier heights between species of *M* and *P* helicity are even lower than those reported for chloroform.[7] Thus, if bilirubin is added to a solution containing an excess of serum albumin, it becomes instantaneously optically active detectable by a CD spectrum in the absorption region between 500 and 300 nm. However, a detailed knowledge of the bilirubin – serum albumin interactions underlying the spectral phenomenon, that is conformation and ionisation state of the guest and locations of preferential binding sites within the conjugate, could not be assessed until now.

An important step towards a solution of these problems has been put forward by Lightner's group, by a systematic investigation of the mechanism of optical activity of bilirubins and a determination of the chiroptical properties in a defined geometry.[8-10] These studies make use of the covalent binding of the discriminating agent or, even more elegantly, introduce chiral centres into the α- or β-position of the propionic acid side-chains. Using this approach even complete displacements of the $M \rightleftharpoons P$ equilibrium has been achieved. However, the studies reported so far are devoted to the most abundant ridge-tile like conformation. The optical activity of bilirubins in other defined geometries is still lacking. Therefore, the diagnostic value of CD spectra in conformational analysis in general remains, strictly speaking, unclear. The convenient access of 2,18-bridged bilirubins like **2**[11] now offers the opportunity also to study helically shaped members. Apart from geometry the bilirubins **2** differ from those studied hitherto in that interconversion of the biladiene backbone of *M* and *P* helicity can be 'frozen out' at moderately low temperatures even in strong hydrogen bonding solvents. This restricted mobility allows for an isolation of optically pure enantiomers. The chirality centre located at C-1' of the bilirubins **2** will only serve as an auxiliary for complete resolution of the corresponding biliverdin precursors **1**. This is quite different to the α,α'- and β,β'-substituted analogues,[9,10] for which the chirality centres are essential to maintain an overpopulation of still rapidly interconverting ridge-tile like conformers of *M* and *P* helicity.

A further object of this study comprises a chemical correlation of the backbone helicities, *M* and *P*, of bilirubins and biliverdins. This has been attempted by reduction of natural biliverdin-IXα embedded in apomyoglobin,[12] but failed because equilibria between *M* and *P* helical species are too quickly attained. The outstanding kinetic properties inherent to the bridged tetrapyrrols **1** and **2**[11] were encouraging for an unambiguous solution of this problem.

Table 1 Repetitive enrichment of (*M*,1'*S*)-**1a** and (*M*,1'*R*)-**1b** starting from the four component mixture **1** (14 mg)[a]

	(*M*,1'*S*)-**1a**		(*M*,1'*R*)-**1b**	
n	e.e. (%)	yield/mg	e.e. (%)	yield/mg
1	60	6.4	65	5.0
2	80	4.0	90	3.0
3	>95	2.4	>95	1.8

[a] Yields and e.e.s (determined by ^{1}H NMR) for the 2nd (n = 2) and 3rd (n = 3) enrichment cycle refer to the main fractions only.

Results and Discussion

Resolution of the two diastereoisomeric pairs of enantiomers of **1**, related by equilibria (1) and (2), was performed by

$$(M, 1'R)\text{-}\mathbf{1b} \rightleftharpoons (P, 1'R)\text{-}\mathbf{1a} \quad (1)$$

$$(P, 1'S)\text{-}\mathbf{1b} \rightleftharpoons (M, 1'S)\text{-}\mathbf{1a} \quad (2)$$

an analogous method to the corresponding methanol adduct[13] by repetitive first-order asymmetric transformation. Repetitive enrichment of *M* helical isomers with (*R*)-(−)-cyclohexylhydroxyacetic acid (CHA) followed by chromatographic separation afforded one enantiomer of each diastereoisomeric pair, *viz.* (*M*,1'*S*)-**1a** and (*M*,1'*R*)-**1b**. The corresponding enantiomers (*P*,1'*R*)-**1a** and (*P*,1'*S*)-**1b** were each obtained from their respective diastereoisomeric counterpart by thermal equilibration (60 h) in benzene and chloroform, respectively, and subsequent TLC at 10 °C (Table 1). The enantiomeric purities achieved (e.e >95%) after the third enrichment cycle were determined by ^{1}H NMR spectroscopy in benzene containing 3 equiv. of (*R*)-(−)-phenylhydroxyacetic acid (PHA). The resonance absorptions due to 10-H are sufficiently resolved to allow for a determination of (i) the ratio of diastereoisomers **a**/**b**, (ii) the helical excess (h.e.) and (iii) the enantiomeric excess (e.e.).†

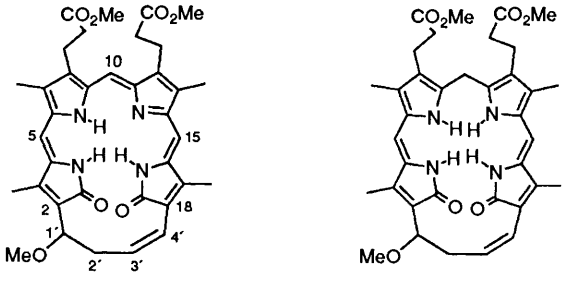
The CD data [band positions, Δε-values and rotational strengths (*R*)] obtained for the four stereoisomers of **1** (Table 2)

† The methods used for resolution and determination of e.e. closely follow those described for the dimethoxy derivative.[13]

Table 2 UV–VIS [ε_{max}/dm^3 mol^{-1} cm^{-1} (λ_{max}/nm)] and CD [$\Delta\varepsilon_{max}$/dm^3 mol^{-1} cm^{-1} (λ_{max}/nm); R/erg cm^3] data[a] of the four stereoisomers of **1**[b] and **2**[c]

Compound	$\Delta\varepsilon(\lambda)$	$R \times 10^{38}$	$\varepsilon(\lambda)$
(*M*,1′*S*)-**1a**	−125.5 (652)	−5.1	
	−243.4 (377)	−7.8	14 400 (657)
(*P*,1′*R*)-**1a**	+126.6 (652)	+5.2	48 100 (377)
	+242.3 (375)	+7.8	
(*M*,1′*R*)-**1b**	−122.9 (652)	−5.2	
	+241.7 (378)	+7.7	14 500 (658)
(*P*,1′*S*)-**1b**	+125.4 (652)	+5.3	49 600 (376)
	−245.3 (378)	−7.8	
(*M*,1′*S*)-**2a**	−196.9 (447)	−5.0	
	+256.3 (387)	+5.8	s21 200 (430)
(*P*,1′*R*)-**2a**	+192.3 (446)	+4.9	55 300 (387)
	−250.2 (386)	−5.7	
(*M*,1′*R*)-**2b**	−215.6 (444)	−5.2	
	+253.5 (384)	+5.9	s22 500 (425)
(*P*,1′*S*)-**2b**	+219.6 (444)	+5.4	53 000 (385)
	−257.7 (385)	−6.0	

[a] For *ca.* 1 × 10^{-5} mol dm^{-3} solutions in methanol; e.e. >95%; errors ± 5%. [b] At 291 K. [c] At 273 K; 1% v/v pyridine added.



1a [(*P*, 1′*R*) + (*M*, 1′*S*)] **2a**

1b [(*M*, 1′*R*) + (*P*, 1′*S*)] **2b**

(*P*,1′*S*)-**1b** $\xrightarrow[fast]{i}$ (*P*,1′*S*)-**2b** $\underset{slow}{\overset{ii}{\rightleftharpoons}}$ (*M*,1′*S*)-**2a**

(*M*,1′*S*)-**1a** $\xrightarrow[fast]{i}$ (*M*,1′*S*)-**2a** $\underset{slow}{\overset{ii}{\rightleftharpoons}}$ (*P*,1′*S*)-**2b**

Scheme 1 *Reagents and Conditions:* i, $NaBH_4$, MeOH, 283 K; ii, thermal energy, 283 K

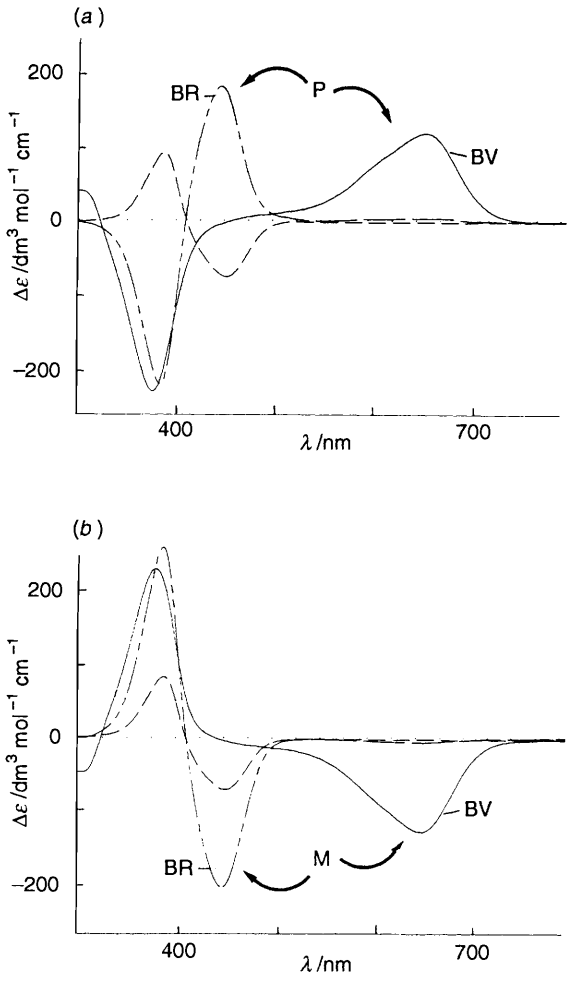


Fig. 1 Intercorrelation of *M* and *P* backbone helicity of a biliverdin (BV) and a bilirubin (BR) *via* sodium borohydride reduction of (*a*) (*P*,1′*S*)-**1b** and (*b*) (*M*,1′*S*)-**1a**. CD spectra refer to the starting biliverdin (*t* = 0, ———), the resulting bilirubins **2b** and **2a**, respectively, after completion of reaction (*t* = 15 min, — — — —) and after completion of equilibration (*t* = 3 h, - - - - - -) (see also footnote ‡ and text).

are close to each other and do not differ substantially from those reported for other helical members[13] including even non-bridged biliverdin peptides.[14,15] These spectral similarities are also found for the corresponding UV–VIS spectra.[11]

The transformation of the four diastereoisomers of **1** into the corresponding bilirubins **2** was accomplished by treatment with sodium borohydride in methanol at +10 °C. The course of reduction and the concomitant changes in CD spectra were followed with time. While the biliverdins **1** are optically stable at the conditions employed [half-life ($t_{\frac{1}{2}}$) *ca.* 50 h] the barriers of the bilirubins formed are lower by about 10 kJ mol^{-1},[11] corresponding to $t_{\frac{1}{2}}$ *ca.* 40 min, and therefore slow isomerisation occurs. The results obtained for two isomers (*P*,1′*S*)-**1b** and (*M*,1′*S*)-**1a**, are displayed in Fig. 1. The CD spectra refer to the starting biliverdins [(*P*,1′*S*)-**1b** and (*M*,1′*S*)-**1a**, respectively] the corresponding bilirubin formed after completion of reaction [mainly (*P*,1′*S*)-**2b** and (*M*,1′*S*)-**2a**, respectively]‡ and a 30:70 mixture of **b** and **a** species after completion of equilibration. In Scheme 1 these sequences of events are summarized. Since the species **2a** predominates after equilibration in methanol, sign inversion of the CD takes place if equilibrium is approached from (*P*,1′*S*)-**2b**. Spectra run after equilibration of the two isomers are superimposable in agreement with the requirements of Scheme 1. This comprises the first intercorrelation of the helicities of biliverdins and bilirubins: an *M*-helical biliverdin exhibits a CD which is negative for the long wave length band around λ 650 nm and positive around λ 380 nm.[5,16–18] The bilirubin of the same helicity shows a CD couplet (λ *ca.* 450, 390 nm) in which the sequence of signs is retained. Analogously, for the *P* helicity inverted signs apply. For both bilirubins and biliverdins the correlation outlined confirms the theoretical predictions connecting the CD spectra with helicity.[5,16,17] Simultaneously the assignment of **a** and **b** species of **2** as derived by ^{1}H NMR[11] is corroborated.

For the synthesis of pure stereoisomers of **2** *via* **1** reduction and work-up was performed at ambient temperature and the equilibrated mixtures then subjected to preparative TLC at −10 °C. The bisignate exciton split CD spectra (Fig. 2 and Table 1) show the strict mirror-image relationships of the two enantiomorphic pairs, (*P*,1′*R*)-**2a**/(*M*,1′*S*)-**2a** and (*P*,1′*S*)-**2b**/(*M*,1′*R*)-**2b**. But even CD spectra of diastereoisomers of like helicity are close to each other reflecting the negligible small influence of the chirality centre at C-1′. Therefore the

‡ If equilibrium is approached from the thermodynamically less favoured **2b** isomer the CD spectrum run immediately after completion of reaction must be somewhat smaller than if approached from **2a**.

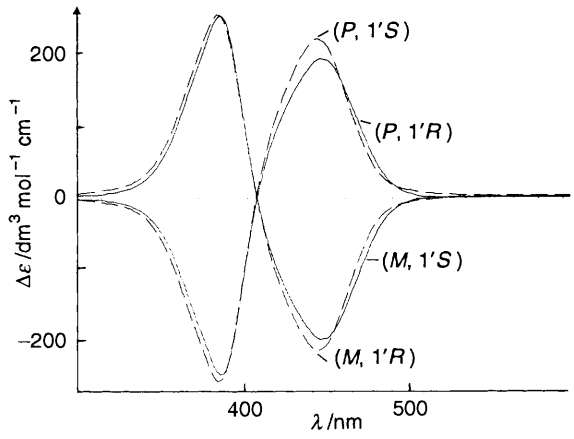


Fig. 2 CD spectra of the two diastereoisomeric pairs of **2** in methanol containing 1% v/v pyridine at 273 K (see also Table 2)

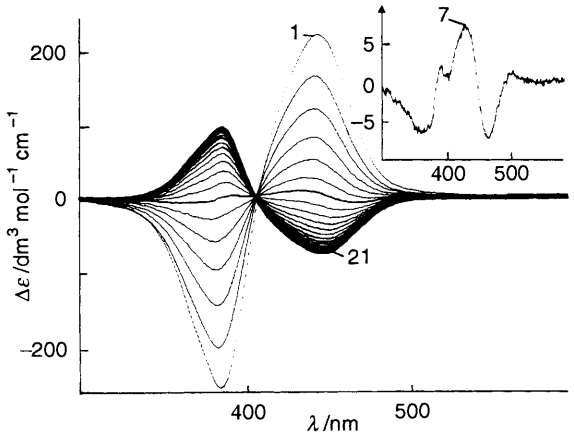


Fig. 3 Thermal equilibration of (*P*,1'*S*)-**2b** (*ca.* 1 × 10^{-5} mol dm^{-3} solutions in methanol containing 1% v/v pyridine, 293 K). CD spectra 1–21 were taken in intervals of *ca.* 3 min. Inset: Spectrum 7 with modified scales for a better identification of the multisignate phenotype.

isodichroic points of diastereoisomers (λ 408 nm) coincide with those of the enantiomers at the base line. The absolute $\Delta\varepsilon$ values of the short and long wave length bands of the bisignate CD spectra are quite different, but considering the rotational strengths *R*, their values approach each other, accounting for the pseudo C_2-symmetry of the stereoisomers of **2**. If the CD spectra of these helical conformers are compared with those reported for the ridge-tile like geometry of β,β'-substituted bilirubins [$\Delta\varepsilon$ (431) ±285/$\Delta\varepsilon$ (386) ±177 (methanol)][10] no striking distinguishing feature becomes evident. This is remarkable if the different phenotypes of UV–VIS absorption spectra characteristic for these geometries are considered.[11] The differences in CD spectra are restricted to the relative band widths of the components of the respective couplet. For the helical conformation the width of the long wavelength band [$\Delta\lambda_{\frac{1}{2}}$(450) 22 nm; $\Delta\lambda_{\frac{1}{2}}$(390) 18 nm] is larger. For the ridge-tile like conformation the inverse holds true.[10] However, additional examples for completely resolved bilirubins of defined geometry would be needed to substantiate the conformational relevance.

If thermal equilibration of (*M*,1'*R*)-**2b** (Fig. 3) or (*P*,1'*S*)-**2b** (not shown) is followed with time, a second feature becomes apparent, which throws additional doubt upon the reliability of CD spectra. Spectrum 7 (inset) refers to an intermediate stage for which the **2a** and **2b** diastereoisomers, being of opposite helicity, have become nearly equipopulated. This multisignate CD, however differs appreciably from the usual bisignate phenotype: clearly, all spectra in Fig. 3 except the initial one reflect difference CD spectra of diastereoisomers of *M* and *P* helicity of varying population. Even if deviations from mirror-image relationships between the CD spectra of (*P*,1'*S*)-**2b** and (*M*,1'*S*)-**2a** isomers are small (Fig. 2), they increasingly come into play as the difference in the population of diastereoisomers approaches zero. Consequently, no stage of vanishing CD exists at all and a residual composite CD spectrum remains instead.

Conclusions

The first, optically fairly stable, optically active bilirubin has been described and the CD measurements performed at temperatures at which interconversion of the backbone helicity is frozen out. Due to this outstanding kinetic property it was possible to make a chemical intercorrelation of backbone helicities of bilirubins and biliverdins. This is in accord with theoretical predictions[5,16–18] and conforms with molecular dynamics calculations performed on ridge-tile shaped (*R*,*R*) and (*S*,*S*) β,β'-disubstituted mesobilirubins.[10]

Two factors strongly limit the diagnostic value of CD spectroscopy in the conformational analysis of bilirubins. One of these simply arises from the apparent poor sensitivity of CD spectra towards even large conformational changes—as distinct from UV–VIS spectra.[11] The second factor is due to an occasional imperfectness of mirror-image relationships of eventual coexisting diastereoisomers of comparable population. No matter whether the optical activity of any bilirubin considered is due to enantioselective binding to a chiral co-solute, or intramolecular in origin, diastereoisomeric species occur if chiral discrimination between *M* and *P* helicities is incomplete. This may be of importance for conjugates of bilirubins with proteins and other chiral compounds for which the extent of chiral discrimination has not been assessed. Other potential ambiguities upon the CD of bilirubins have been raised recently.[17]

Experimental

General.—M.p.s were determined with a Kofler-Reichert hot-stage apparatus. ^{1}H NMR spectra (250 MHz) were run with a Bruker AC250 AF instrument at 297 K in $CDCl_3$, [$^2H_6$]-benzene (both chromatographed on alumina prior to use) or [$^2H_5$]pyridine for *ca.* 10^{-2} mol dm^{-3} solutions. Molecular masses have been determined by fast atom bombardment (3-nitrophenylmethanol or glycerol, Xe) and field desorption mass spectrometry using a Finnigan MAT 900 or 8230 instrument. UV–VIS spectra and CD spectra for solutions in methanol (Uvasol, Merck) were measured with a Perkin Elmer Lambda 7 spectrometer and a CD6 circulardichrograph (I.S.A. Jobin-Yvon), respectively, using thermostatted (±1 °C) quartz cuvettes (1 cm path length). For measurements of the bilirubins **2**, 1% v/v pyridine was added. TLC was performed on Kieselgel 60 precoated plates (0.25 mm, Merck). The optically active acids CHA and PHA (both Fluka) both showed satisfactory optical rotation and were used as purchased. For syntheses methanol (p.A., Riedel de Haën), DMSO (purum, Loba), sodium borohydride and DDQ (both zur Synthese, Merck) were used.

The bridged bilirubins **2** are particularly sensitive towards oxygen and light. Therefore the precautions delineated in ref. 11 were taken into account. All measurements reported here refer to freshly prepared solutions of freshly prepared compounds.

Resolution and Separation of the Four Stereoisomers of (4Z,9Z,15Z,3'Z)-2,18-(1'-*methoxybut*-3'-*en*-1',4'-*diyl*)-8,12-*bis*-(2''-*methoxycarbonylethyl*)-3,7,13,17-*tetramethyl*-1,19-*dioxo*-1,19,21,24-*tetrahydrobilin* (**1**).—The stereoisomers obtained as described below were identical with the racemates in their ^{1}H

NMR, UV–VIS and mass spectra,[11] and can be stored at −30 °C for several months without isomerisation. E.e.s were determined by ^{1}H NMR spectroscopy in [$^2H_6$]benzene after addition of 3 equiv. PHA, the latter being superior to CHA for this purpose. Under these conditions the resonance absorptions due to 10-H of (*M*,1′*S*)-**1a** (7.83 ppm), (*M*,1′*R*)-**1b** (7.79 ppm), (*P*,1′*R*)-**1a**, (7.75 ppm) and (*P*,1′*S*)-**1b** (7.72 ppm) are well resolved and can easily be integrated. Assignment of individual peaks follows on from similar considerations to those given for the corresponding methoxy adduct.[13]

Stereoisomers (M,1′S)-**1a** *and* (M,1′R)-**1b**.—*The biliverdin **1**[11] (14 mg, 0.022 mmol) was dissolved in dichloromethane (5 cm^3) and added to a solution of CHA (11 mg, 0.07 mmol) in methanol (1 cm^3) and the solvents were evaporated under reduced pressure at 5 °C. The residue was dissolved in benzene (0.7 cm^3) and stored at room temp. to allow for equilibration (60 h). This solution was then subjected to TLC at 10 °C [toluene–ethyl acetate–propan-2-ol 85:12:3 v/v; R_f (**1a**) 0.25, (**1b**) 0.40] thereby also stripping off the chiral discriminator. The two separated silica gel adsorbed fractions were each extracted with dichloromethane–methanol (5:1 v/v) at 10 °C and the solutions filtered through a membrane filter (0.2 μm, recycled cellulose, Schleicher & Schüll) and evaporated to dryness (10 °C). This afforded **1a** (6.4 mg) and **1b** (5.0 mg) of configurations and e.e.s as compiled in Table 1 (*n* = 1). These two fractions were then twice subjected to the enrichment procedure described above, disregarding the respective minor fractions (Table 1).

Isomer (*P*,1′*S*)-**1b**. A solution of (*M*,1′*S*)-**1a** (1.5 mg) in benzene was allowed to equilibrate (20 °C, 60 h). After TLC as described above and evaporation of the solvent *in vacuo*, unconverted (*M*,1′*S*)-**1a** (0.7 mg, 47%) and the target isomer (*P*,1′*S*)-**1b** (0.6 mg, 40%) were obtained.

Isomer (*P*,1′*R*)-**1a**. A solution of (*M*,1′*R*)-**1b** (1.5 mg) in chloroform was allowed to equilibrate (20 °C, 60 h). After TLC as described above and evaporation of the solvent *in vacuo*, unconverted (*M*,1′*R*)-**1b** (0.2 mg, 13%) and the target isomer (*P*,1′*R*)-**1a** (1.0 mg, 67%) were obtained.

Syntheses of the Four Stereoisomers of (4Z,15Z,3′Z)-2,18-(1′-*methoxybut*-3′-*en*-1′,4′-*diyl*)-8,12-*bis*(2″-*methoxycarbonylethyl*)-3,7,13,17-*tetramethyl*-1,19-*dioxo*-1,10,19,21,23,24-*hexahydrobilin* (**2**).—*The stereoisomers obtained as described below were identical with the racemates in their ^{1}H NMR, UV–VIS, and mass spectra,[11] and can be stored at −70 °C for several months without isomerisation.

(M,1′S)-**2a** *and* (P,1′S)-**2b**. Sodium borohydride reduction was performed as described[11] starting from (*M*,1′*S*)-**1a** (2 mg). After equilibration of the product in pyridine (20 °C, 0.5 h) the solvent was evaporated (5 °C) and the residue subjected to TLC (−10 °C) [toluene–ethyl acetate–propan-2-ol-pyridine 70:19:10:1; R_f (**2a**) 0.55, (**2b**) 0.65]. The individual zones (each ca. 0.7 mg, overall yield 70%) were then extracted with methanol containing 1% pyridine and filtered. These solutions were immediately used for recordings of UV–VIS and CD spectra.

(M,1′R)-**2b** *and* (P,1′R)-**2a**. The corresponding enantiomorphic counterparts to those described above were obtained analogously starting from (*M*,1′*R*)-**1b** (1.8 mg).

Re-oxidation of Bilirubins **2** *to Biliverdins* **1**.—An equilibrated solution of (*M*,1′*R*)-**2b** and (*P*,1′*R*)-**2a** (1 mg) in DMSO (2 cm^3) was subjected to oxidation with DDQ (2 mg) in the usual way.[2] After work-up and TLC (see above) the isolated biliverdins (*M*,1′*R*)-**1b** and (*P*,1′*R*)-**1a** (each *ca.* 0.3 mg, overall yield 60%) showed UV–VIS and CD spectra superimposable with those of the biliverdins obtained immediately after resolution.

Acknowledgements

Financial support by the *Hochschuljubiläumsstiftung der Stadt Wien* and by the *Fonds zur Förderung der wissenschaftlichen Forschung* (Nos. P3574, P5767 and P8202C) is gratefully acknowledged.

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Paper 3/02434B
Received 28*th April* 1993
Accepted 29*th June* 1993

* Exact yields were determined spectrophotometrically.