ON THE CONFORMATION OF GLYCOBILIRUBIN

Stefan E. BOIADJIEV[1] and David A. LIGHTNER[2,*]

Chemistry Department, University of Nevada, Reno, Nevada 89557-0020 U.S.A.;
e-mail: [1] stefcho@scs.unr.edu, [2] lightner@scs.unr.edu

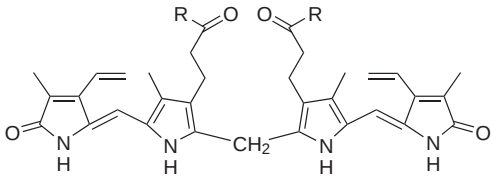
Received December 18, 2002
Accepted March 20, 2003

Dedicated to the memory of Professor Otakar Červinka.

The first optically active glycine conjugate **1** of a bilirubin was prepared in several steps from (*S*)-β-methylxanthobilirubic acid glycine conjugate **8**. The latter was synthesized by reaction of benzyl glycinate tosylate with the mixed anhydride formed in the reaction of (*S*)-β-methylxanthobilirubic acid **6** with isobutyl chloroformate. Spectroscopic analysis of the circular dichroism spectra of **1** in various solvents, including aqueous buffer, indicate a conformational preference for the ***M***-helical ridge-tile conformation, thus providing the first spectroscopic evidence on the conformation of glycobilirubins.

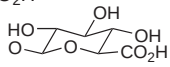
Keywords: Glycine conjugate; Bilirubin; Hydrogen bonding; Pyrroles; CD spectroscopy; NMR; Porphyrins; Oligopyrroles; Amino acids; Conformation analysis.

The essential amino acid glycine ($^{-}O_2CCH_2NH_3^{+}$) is found widely in nature, serving as a flexible link in proteins, recognition sites on enzymes and cell membranes, a component of molecular activity modifying conjugation, *etc.*[1] Together with taurine ($^{-}O_3SCH_2CH_2NH_3^{+}$), it is an important amide-linkage conjugate of bile acids[2–4], and both serve in detoxification mechanisms of xenobiotics *in vivo*[5]. Although taurine conjugates of the natural dicarboxylic acid bilirubin (the yellow pigment of jaundice) have been found in the bile of certain fish (yellowtail, red sea bream and flounder)[6], neither taurine nor glycine conjugates (Fig. 1) of bilirubin seem to be present in mammalian bile, where the principal bilirubin conjugates are mono- and diglucuronides[7]. Bilirubin glucuronides are reactive, undergoing acyl migration and facile hydrolysis, and pure conjugates are not readily available[8]. In contrast, bilirubin ditaurate (taurobilirubin) is very stable and commercially available, thus making it a useful surrogate for bilirubin diglucuronide *in vitro* and in animal studies, where it is smoothly excreted by the liver[9]. The bis-glycine amide of bilirubin (glycobilirubin[10]) is also predicted to be stable, but far less is known of its properties and conformation; few citations appear in the literature, and only one synthesis has been described[10].



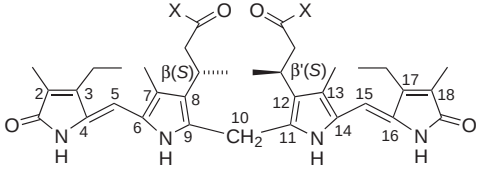
Bilirubin: R = OH

Glycobilirubin: R = $NHCH_2CO_2H$

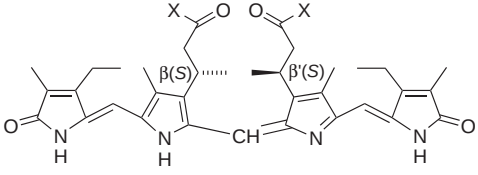
Bilirubin diglucuronide: R = 

Taurobilirubin: R = $NHCH_2CH_2SO_3^-$

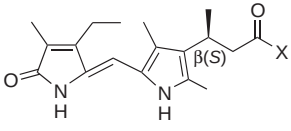
In the following, we describe the syntheses of the bis-glycine amides **1** and **2** of (*S*,*S*)-β,β′-dimethylmesobilirubin **4** [11] and (*S*,*S*)-β,β′-dimethylmesobiliverdin **5** [11], respectively, and the glycine conjugate **3** of (*S*)-β-methylxanthobilirubic acid **6** [12] (Fig. 1). NMR and circular dichroism spectroscopic analyses of **1** provide detailed information on the three-dimensional structure of the glycorubin.



1, X = $NHCH_2CO_2H$
4, X = OH
10, X = $NHCH_3$



2, X = $NHCH_2CO_2H$
5, X = OH
7, X = $NHCH_2CO_2Bn$



3, X = $NHCH_2CO_2H$
6, X = OH
8, X = $NHCH_2CO_2Bn$
9, X = $OCOCH_2CH(CH_3)_2$

FIG. 1

Linear representations of glyco-(*S*,*S*)-β,β′-dimethylmesobilirubin XIIIα **1**, glyco-(*S*,*S*)-β,β′-dimethylmesobiliverdin XIIIα **2** and its dibenzyl ester **7**, the glycine conjugate **3** of (*S*)-β-methylxanthobilirubic acid and its benzyl ester **8**, the parent carboxylic acids **4**, **5** and **6** of **1**, **2** and **3**, respectively, the mixed anhydride **9** of **6** and the bis-*N*-methylamide **10** of **4**

EXPERIMENTAL

Circular dichroism spectra were recorded on a Jasco J-600 spectropolarimeter, and UV-VIS spectra on a Perkin–Elmer Lambda 12 spectrophotometer. All organic solutions for CD and UV-VIS measurements contained 0.12 vol.% of CH_3OH and 1.88 vol.% of $CHCl_3$. NMR spectra were recorded on a 500 MHz Varian Unity Plus spectrometer. Chemical shifts are reported in δ (ppm) and referenced to the CHD_2SOCD_3 signal at 2.49 (^{1}H) and $(CD_3)_2SO$ at 39.50 (^{13}C) or the residual $CHCl_3$ signal at 7.26 (^{1}H) and $CDCl_3$ at 77.00 (^{13}C). Coupling constants (*J*) are given in Hz. A *J*-modulated spin-echo experiment was used to obtain carbon multiplicities. Radial chromatography was carried out on Merck silica gel PF-254 with $CaSO_4$, preparative thin layer grade, using a Chromatotron (Harrison Research Inc., Palo Alto, CA). High-resolution FAB mass spectra were obtained at the Nebraska Center for Mass Spectrometry, University of Nebraska, Lincoln, for samples which were >95% pure by NMR. Commercial reagents and HPLC grade solvents (Aldrich or Fisher) were dried and purified following standard procedures[13]. Isobutyl chloroformate was distilled prior to use, and the reactions were carried out under Ar and light protection. The syntheses of pigments **4** [11], **5** [11], **6** [12] and **10** [14] were reported previously in the literature as noted and were available in our laboratory.

(*S*)-8-[2-(*N*-{[(Benzyloxy)carbonyl]methyl}carbamoyl)-1-methylethyl]-3-ethyl-2,7,9-trimethyl-(10*H*)-dipyrrin-1-one (**8**)

To a suspension of (*S*)-β-methylxanthobilirubic acid **6** [12] (632 mg, 2 mmol) in anhydrous THF (40 ml) and Et_3N (0.84 ml, 6 mmol), isobutyl chloroformate (0.78 ml, 6 mmol) was added under Ar, and the mixture was stirred for 1.5 h. Then it was transferred to a solution of benzyl glycinate tosylate[15] (1.69 g, 5 mmol) in anhydrous DMSO (20 ml) and Et_3N (0.84 ml, 6 mmol). The mixture was stirred for 1.5 h under water aspirator vacuum with occasional heating to 35–40 °C to remove THF solvent. Then more Et_3N (0.84 ml, 6 mmol) was added and stirring was continued at ambient temperature for 4.5 h. The mixture was diluted with $CHCl_3$ (500 ml) and washed with 0.2% aqueous HCl (200 ml) and water (4 × 150 ml). After drying (anhydrous Na_2SO_4), filtration and removal of the solvent under vacuum, the residue was purified by radial chromatography (eluent 2–4% CH_3OH in CH_2Cl_2 v/v) and recrystallization from CH_2Cl_2–CH_3OH to afford 771 mg (83%) of the title ester amide of xanthobilirubic acid. Bright yellow crystals had m.p. 217–218 °C. ^{1}H NMR ($CDCl_3$): 11.21 br s, 1 H (lactam NH); 10.26 br s, 1 H (pyrrole NH); 7.33 m, 5 H (phenyl); 6.13 s, 1 H (5-CH=); 5.90 br t, 1 H, J = 5.0 (amide NH); 5.150, 5.153 AB, 2 H, ^2J = 12.2 (benzyl CH_2); 4.06 A**B**X, 1 H, ^3J = 5.4, ^2J = 18.4 (glycine CH_2); 3.99 **A**BX, 1 H, ^3J = 5.0, ^2J = 18.4 (glycine CH_2); 3.33 m, 1 H (β-CH); 2.57 A**B**X, 1 H, ^3J = 7.8, ^2J = 14.3 (α-CH_2); 2.54 q, 2 H, J = 7.6 (3-C**H**$_2$CH$_3$); 2.53 **A**BX, 1 H, ^3J = 7.4, ^2J = 14.3 (α-CH_2); 2.46 s, 3 H (9-CH_3); 2.19 s, 3 H (7-CH_3); 1.94 s, 3 H (2-CH_3); 1.30 d, 3 H, J = 7.2 (β-CH_3); 1.18 t, 3 H, J = 7.6 (3-CH_2C**H**$_3$). ^{13}C NMR ($CDCl_3$): 174.13 (1-CO), 172.37 (-CH_2**C**OOBn), 169.88 (α-CONH), 148.41 (3), 135.14 (*i*-C), 130.98 (9), 128.60 (*o*-CH), 128.48 (*p*-CH), 128.30 (*m*-CH), 127.36 (4), 123.86 (8), 123.32 (2), 122.54 (6), 122.53 (7), 100.85 (5), 67.13 (O**C**H$_2$C$_6$H$_5$), 43.40 (glycine CH_2), 41.41 (α-CH_2), 27.98 (β-CH), 20.69 (β-CH_3), 17.94 (3-**C**H$_2$CH$_3$), 15.03 (3-CH_2**C**H$_3$), 12.62 (9-CH_3), 10.42 (7-CH_3), 8.54 (2-CH_3). ^{13}C NMR data in $(CD_3)_2SO$ are given in Table I. For $C_{27}H_{33}N_3O_4$ (463.6) calculated: 69.95% C, 7.18% H, 9.06% N; found: 69.73% C, 7.29% H, 8.99% N.

8-{2-[*N*-(Carboxymethyl)carbamoyl]-1-methylethyl}-3-ethyl-2,7,9-trimethyl-(10*H*)-dipyrrin-1-one (**3**)

A mixture of *rac*-**8** (93 mg, 0.2 mmol), CH_3OH (10 ml) and 1 M aqueous NaOH (2 mmol; 2 ml) was heated at reflux for 40 min. After cooling, the mixture was diluted with $CHCl_3$ (100 ml) and H_2O (25 ml), and acidified with 10% aqueous HCl. The organic layer was partially evaporated and purified by radial chromatography (eluent 4–8% CH_3OH in CH_2Cl_2 v/v) to give a bright yellow polar band. After evaporation of the solvent and recrystallization from EtOAc–Et_2O 55 mg (74%) of the title compound was obtained. M.p. 237–240 °C (decomp.). ^{1}H NMR ($CDCl_3$): 13.15 very br s, 1 H (COOH); 9.97 br s, 1 H (lactam NH); 8.44 br s, 1 H (pyrrole NH); 6.15 s, 1 H (5-CH=); 5.80 br t, 1 H, J = 4.3 (amide NH); 4.14 A**B**X, 1 H, ^3J = 5.3, ^2J = 18.5 (glycine CH_2); 3.93 **A**BX, 1 H, ^3J = 3.2, ^2J = 18.5 (glycine CH_2); 3.31 m, 1 H (β-CH); 2.52 q, 2 H, J = 7.7 (3-C**H**$_2$CH$_3$); 2.49 d, 2 H, J = 6.7 (α-CH_2); 2.38 s, 3 H (9-CH_3); 2.14 s, 3 H (7-CH_3); 1.89 s, 3 H (2-CH_3); 1.32 d, 3 H, J = 7.2 (β-CH_3); 1.17 t, 3 H, J = 7.7 (3-CH_2C**H**$_3$). ^{13}C NMR data ($(CD_3)_2SO$) in Table I and ^{1}H NMR data in Table II. For $C_{20}H_{27}N_3O_4$ (373.4) calculated: 64.32% C, 7.29% H, 11.25% N; found: 64.01% C, 7.10% H, 11.09% N.

(*S*,*S*)-8,12-Bis[2-(*N*-{[(benzyloxy)carbonyl]methyl}carbamoyl)-1-methylethyl]-3,17-diethyl-2,7,13,18-tetramethyl-(21*H*,24*H*)-bilin-1,19-dione (**7**)

To a solution of compound **8** (464 mg, 1 mmol) in CH_2Cl_2 (220 ml), *p*-chloranil (615 mg, 2.5 mmol) was added followed by 97% formic acid (11 ml), and the mixture was heated at reflux for 24 h. The volume of the mixture was reduced by distillation to one half, and reflux was continued for 5 h. After cooling, the mixture was chilled at –20 °C for 16 h. The separated solid was removed by filtration and discarded. The blue filtrate was neutralized with saturated aqueous $NaHCO_3$, then washed with 4% aqueous NaOH (2 × 100 ml) and H_2O (4 × 100 ml). After drying (anhydrous Na_2SO_4), filtration and evaporation of the solvent under vacuum, the residue was purified by radial chromatography (gradient CH_2Cl_2:CH_3OH:CH_3CO_2H = 100:2:3 to 100:8:3, v/v/v). The combined pure fractions were washed with 1% aqueous $NaHCO_3$ and H_2O, then dried (anhydrous Na_2SO_4). After filtration, the solvent was evaporated under vacuum and the residue was recrystallized from $CHCl_3$–hexane to afford 377 mg (83%) of dark blue glycomesobiliverdin dibenzyl ester **7**. M.p. 190–192 °C. ^{1}H NMR ($CDCl_3$): 8.23 br s, 2 H (21,24-NHCO); 7.29 m, 6 H (phenyl); 7.22 m, 4 H (phenyl); 7.01 s, 1 H (10-CH=); 6.66 br s, 2 H (amide NH); 5.90 s, 2 H (5,15-CH=); 5.01, 5.05 AB, 4 H, ^2J = 12.3 (benzyl CH_2); 3.89, 3.90 AB, 4 H, ^2J = 14.9 (glycine CH_2); 3.58 m, 2 H (β,β′-CH); 2.70 A**B**X, 2 H, ^3J = 6.1, ^2J = 13.8 (α,α′-CH_2); 2.60 **A**BX, 2 H, ^3J = 9.5, ^2J = 13.8 (α,α′-CH_2); 2.49 q, 4 H, J = 7.7 (3,17-C**H**$_2$CH$_3$); 2.14 s, 6 H (7,13-CH_3); 1.82 s, 6 H (2,18-CH_3); 1.45 d, 6 H, J = 7.2 (β,β′-CH_3); 1.19 t, 6 H, J = 7.7 (3,17-CH_2C**H**$_3$). ^{13}C NMR ($CDCl_3$): 172.33 (1,19-CO), 172.14 (-CH_2**C**OOBn), 169.67 (α,α′-CONH), 150.01 (6,14), 146.70 (3,17), 141.65 (9,11), 140.10 (4,16), 140.05 (8,12), 135.11 (*i*-C), 128.51 (*o*-CH), 128.35 (2,18), 128.33 (*p*-CH), 128.12 (*m*-CH), 126.76 (7,13), 116.06 (10-CH=), 96.17 (5,15-CH=), 67.20 (O**C**H_2Ph), 44.09 (glycine CH_2), 41.22 (α,α′-CH_2), 28.43 (β,β′-CH), 20.87 (β,β′-CH_3), 17.85 (3.17-**C**H_2CH$_3$), 14.42 (3,17-CH_2**C**H$_3$), 10.34 (7,13-CH_3), 8.33 (2,18-CH_3) and ^{13}C NMR ($(CD_3)_2SO$) in Table I. For $C_{53}H_{60}N_6O_8$ (909.1) calculated: 70.02% C, 6.65% H, 9.25% N; found: 70.13% C, 6.55% H, 9.24% N.

(*S*,*S*)-8,12-Bis{2-[*N*-(carboxymethyl)carbamoyl]-1-methylethyl}-3,17-diethyl-2,7,13,18-tetramethyl-(21*H*,24*H*)-biladiene-*ac*-1,19-dione (**1**)

To a solution of diester **7** (182 mg, 0.2 mmol) in deoxygenated THF (30 ml) and CH_3OH (30 ml) was added 0.2 M aqueous NaOH (12 mmol; 60 ml) and the mixture was stirred at 50 °C for 2 h. After cooling, the mixture was diluted with $CHCl_3$–CH_2Cl_2 (1:1, v/v) (150 ml), H_2O (50 ml), and acidified with 10% aqueous HCl (pH <3). The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 50 ml). The combined organic extracts were evaporated under vacuum and residual moisture was removed by coevaporation with C_6H_6.

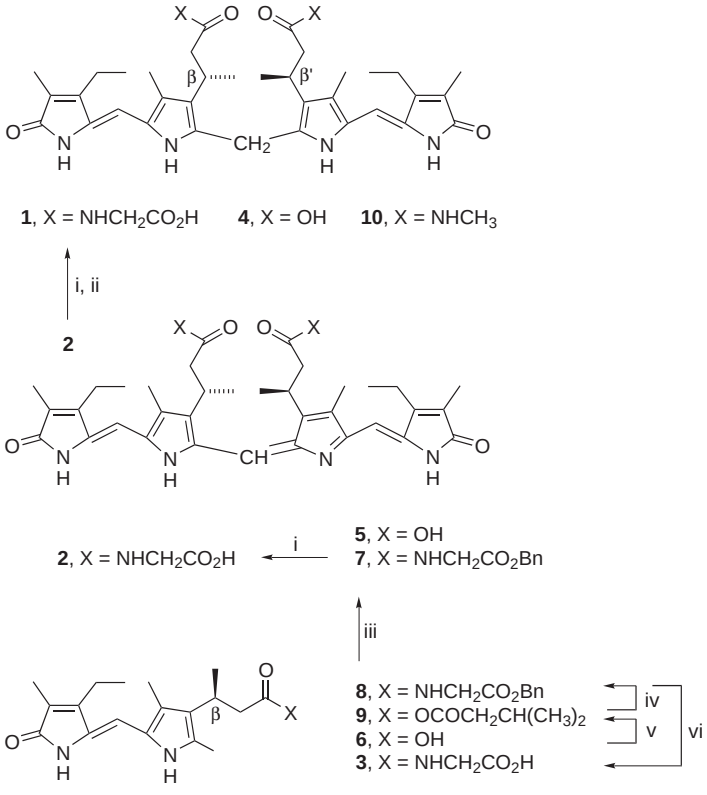
The following steps were performed without delay and under N_2 protection to avoid excessive decomposition (oxidation) of the sensitive, highly acidic glycorubin **1**. The crude verdin diacid was dissolved in anhydrous deoxygenated CH_3OH (60 ml) and sodium borohydride (756 mg, 20 mmol) was added in small portions during 10 min. After stirring for 10 min more, the mixture was diluted with H_2O (100 ml) and of $CHCl_3$–CH_2Cl_2 (1:1, v/v) (100 ml), and acidified with CH_3CO_2H (1 ml) followed by enough 10% aqueous HCl to bring pH ≈3. The product was extracted with $CHCl_3$–CH_2Cl_2 (1:1, v/v) (3 × 50 ml) and the combined organic extracts were evaporated under vacuum. The residue was purified by radial chromatography (eluent 3–8% CH_3OH in CH_2Cl_2 v/v) and the pure fractions were combined and evaporated to dryness. The residue was triturated with a minimum volume of EtOAc and dry Et_2O and the product was separated by filtration to afford 121 mg (83%) of glycorubin **1**. M.p. 206–208 °C (decomp.). NMR data are given in Tables I and II. For $C_{39}H_{50}N_6O_8$ (730.8) calculated: 64.09% C, 6.90% H, 11.50% N; found: 63.75% C, 7.09% H, 11.37% N.

RESULTS AND DISCUSSION

Synthesis

A synthesis of glycobilirubin, reported by Jirsa[10] in 1958, involved reaction of bilirubin in dry dioxane with ethyl chloroformate in dioxane– triethylamine to form the mixed anhydride, followed by the reaction with sodium glycinate in methanol. We modified Jirsa's procedure for preparing glycobilirubin, adopting it to (*S*)-β-methylxanthobilirubic acid **6** [12], whose mixed anhydride was prepared using isobutyl chloroformate as previously described[16]. Using this modification and a change of solvent (to tetrahydrofuran) with added triethylamine as an HCl scavenger, we converted **6** cleanly and quantitatively to its mixed anhydride (**9**, not isolated) when the solvents and reagents were oxygen-free and anhydrous, and the medium was basic. However, addition of sodium glycinate in DMSO–Et_3N [16] proved unsatisfactory due to inhomogeneity of the reaction mixture. Addition of benzyl glycinate tosylate[15] in DMSO to the mixed anhydride **9** afforded ester amide **8** of (*S*)-β-methylxanthobilirubic acid in 83% yield (Scheme 1), isolated after radial chromatography on silica. Benzyl ester **8**

was oxidatively coupled using chloranil[17] to afford the verdin diester **7**, in high hield, from which the target glycomesobilirubin **1** was obtained, following alkaline hydrolysis and reduction. Alkaline hydrolysis of *rac*-**8** gave the amide **3** of β-methylxanthobilirubic acid in 74% yield; alkaline hydrolysis of **7** gave the glycine conjugate **2** of mesobiliverdin **5**.



(i) NaOH; (ii) $NaBH_4$ (83%, steps i + ii); (iii) *p*-chloranil/HCO_2H/CH_2Cl_2, 83%; (iv) $BnOCOCH_2NH_3^+ {}^-OTs$ (83%, steps iv + v); (v) i-BuOCOCl; (vi) NaOH, 74%

SCHEME 1

Constitutional Structure and Conformation

The constitution and absolute configuration (Fig. 1) of (*S*,*S*)-β,β′-dimethylmesobilirubin XIIIα **4**[11] and its bis-*N*-methylamide **10**[14], and β-methylxanthobilirubic acid **6**[12] and (*S*,*S*)-β,β′-dimethylmesobiliverdin XIIIα **5**[18] are well established[19]. Consequently, it is not surprising that the ^{13}C NMR chemical shifts in $(CD_3)_2SO$ of the carbons of the common skeleton of

glycorubin **1**, **4**, the glycine conjugate **3** of **6**, and **10** are nearly identical (Table I). The two glycine conjugates (**1** and **3**) exhibit ^{13}C NMR chemical shifts characteristic of the glycinate moiety, with (again) nearly identical chemical shifts. Similarly, the ^{13}C NMR data for verdin glycine conjugate dibenzyl ester **7** correlates nicely with data for the parent verdin acid **5**, and the ^{13}C NMR chemical shift data of the (*S*)-β-methylxanthobilirubic acid conjugate benzyl ester **8** with that of (*S*)-β-methylxanthobilirubic acid **6** (Table I).

TABLE I

Comparison of ^{13}C NMR chemical shifts (δ, ppm) of 5×10^{-3} M glyco-(*S*,*S*)-β,β′-dimethylmesobilirubin **1**, the glycine conjugate **3** of (*S*)-β-methylxanthobilirubic acid, (*S*,*S*)-β,β′-dimethylmesobilirubin **4**, (*S*,*S*)-β,β′-dimethylmesobiliverdin XIIIα **5** and their benzyl glycinate amides **7** and **8**, respectively, (*S*)-β-methylxanthobilirubic acid **6** and the bis-*N*-methylamide **10** of **4**, in $(CD_3)_2SO$ at 25 °C

Carbon	**1**	**3**	**4**	**5**	**6**	**7**[a]	**8**[b]	**10**
1,19-CONH	171.98	171.71	172.10	172.16	171.99	172.09	171.93	171.96
2,18	123.00	122.54	122.97	127.57	122.67	127.50	122.55	123.04
2,18-CH_3	8.07	8.05	8.09	8.09	8.13	8.08	8.04	8.12
3,17	147.24	147.19	147.33	146.40	147.29	146.37	147.19	147.45
3,17-CH_2CH_3	17.16	17.15	17.18	17.00	17.23	17.00	17.14	17.22
3,17-CH_2CH_3	14.81	14.85	14.86	14.43	14.95	14.43	14.84	14.84
4,16	128.14	127.24	128.01	140.05	127.37	139.94	127.25	127.98
5,15-CH=	97.66	97.54	97.67	95.50	97.56	95.61	97.52	97.30
6,14	122.63	121.78	122.48	149.60	121.83	149.57	121.74	122.67
7,13	121.45	121.54	121.70	126.62	121.65	126.41	121.55	121.02
7,13-CH_3	10.62	9.99	10.65	10.02	10.03	10.13	9.99	10.54
8,12	123.56	123.68	123.21	139.22	122.98	139.27	123.61	123.14
8^1,12^1-CH	27.34	27.17	26.88	27.33	27.14	27.60	27.21	27.20
β,β′-CH_3	20.15	20.01	19.84	20.92	20.55	20.39	19.98	21.05
8^2,12^2-CH_2	41.87	42.13	39.41	41.04	41.04	40.70	40.67	41.59
8^3,12^3-CO	171.23	171.38	173.68	173.09	173.62	169.77	169.89	173.55
$CONHCH_2CO_2H$ (Bn)	40.56	40.53	–	–	–	42.13	42.10	25.73[c]
$CONHCH_2CO_2H$ (Bn)	172.23	171.88	–	–	–	171.52	171.88	–
9,11	129.95	128.62	130.20	141.56	128.81	142.01	128.61	130.57
10	23.49	11.99	23.91	115.99	12.05	115.80	11.99	21.84

[a] $CO_2CH_2C_6H_5$: 65.77, CO_2CH_2-*i*-C_6H_5: 135.84, CO_2CH_2-*o*-C_6H_5: 128.34, CO_2CH_2-*m*-C_6H_5: 127.86, CO_2CH_2-*p*-C_6H_5 127.99; [b] $CO_2CH_2C_6H_5$: 65.73, CO_2CH_2-*i*-C_6H_5: 135.91, CO_2CH_2-*o*-C_6H_5: 128.37, CO_2CH_2-*m*-C_6H_5: 127.90, CO_2CH_2-*p*-C_6H_5: 128.02; [c] $CONHCH_3$.

The ^{1}H NMR spectra (Table II) of **1**, **3**, **4** and **10** in DMSO-d_6 also exhibit considerable similarity, as might be anticipated. The propionamide NHs fall into the narrow range 8.15–8.30 ppm. The diastereotopic glycine CH_2 hydrogens showed geminal coupling in an ABX pattern, coupled also to the NH with slightly different vicinal coupling constants. These were not seen in the quadrupolar-broadened NH resonance appearing as a triplet.

TABLE II

Comparison of the ^{1}H NMR spectral assignments (δ, ppm) of 2 × 10^{-3} M solution of glyco-(*S*,*S*)-β,β′-dimethylmesobilirubin **1**, the glycine conjugate **3** of (*S*)-β-methylxanthobilirubic acid, (*S*,*S*)-β,β′-dimethylmesobilirubin **4** and its bis-*N*-methylamide **10** in $(CD_3)_2SO$ at 25 °C

Proton	**1**	**3**	**4**	**10**
α,α′-CONH- or COOH	8.16[a]	8.15[a]	11.98	8.30[r]
21,24-NHCO	9.74	9.74	9.85	10.05
22,23-NH	10.06	10.16	10.10	10.25
–CONHC**H**$_2$CO$_2$H	3.70[b] 3.78[c]	3.67[j] 3.73[j]	–	–
–CONHCH$_2$CO$_2$**H**	12.46	12.42	–	–
–CONHC**H**$_3$	–	–	–	2.50[s]
2,18-CH$_3$	1.76	1.77	1.76	1.73
3,17-C**H**$_2$CH$_3$	2.48[d]	2.49[k]	2.44[o]	2.48[o]
3,17-CH$_2$C**H**$_3$	1.07[e]	1.07[l]	1.07[p]	1.06[p]
5,15-CH=	5.95	5.91	5.95	5.92
7,13-CH$_3$	2.11	2.07	2.08	2.13
β,β′-CH	3.22[f]	3.14[f]	3.17[f]	3.39[f]
β,β′-CH$_3$	1.06[g]	1.13[g]	0.98[g]	1.16[g]
α,α′-CH$_2$	2.38[h] 2.46[i]	2.27[m] 2.44[n]	2.40[q]	2.63[t]
10	3.97	2.21	3.98	3.90

[a] br t, *J* = 5.8; [b] **A**BX, ^3J = 5.6, ^2J = 17.6; [c] A**B**X, ^3J = 6.0, ^2J = 17.6; [d] q, *J* = 7.5; [e] t, *J* = 7.5; [f] m; [g] d, *J* = 7.1; [h] **A**BX, ^3J = 7.4, ^2J = 13.8; [i] A**B**X, ^3J = 7.9, ^2J = 13.8; [j] ABX, ^3J = 5.8, ^2J = 17.5; [k] q, *J* = 7.6; [l] t, *J* = 7.6; [m] **AB**X, ^3J = 6.1, ^2J = 13.9; [n] A**B**X, ^3J = 9.3, ^2J = 13.9; [o] q, *J* = 7.4; [p] t, *J* = 7.4; [q] **AB**X, ^3J = 8.7, 8.1 overlapped; [r] br q; [s] d, *J* = 4.5; [t] **AB**X, ^3J = 10.8, overlapped.

Conformation by Circular Dichroism Spectroscopy

The optical activity of rubins **1**, **4**, and **10** enables one to measure their circular dichroism (CD) spectra and extract information on their conformation. The most stable conformation of bilirubin and mesobilirubin XIIIα is shaped like a ridge-tile or half-opened book and is stabilized by a network of intramolecular hydrogen bonds formed when the carboxylic acid groups embrace the opposing dipyrrinones[11,19–21]. There are two such ridge-tiles, equi-energetic and interconverting in solution over barriers of ≈20 kcal/mol [20–23] (Fig. 2). Earlier, we showed that β and β′ methyl groups of the parent rubin **4** can act through nonbonded steric interactions to displace the equilibrium toward either the ***M***- or the ***P***-helical conformer, which results in the observation of bisignate CD curves for the long wavelength transition[11]. Since bilirubins, with their two dipyrrinone chromophores may be viewed as molecular excitons, exciton coupling theory[24] can be used to predict the ***M***- or ***P***-helicity of the intramolecularly hydrogen-bonded ridge-tile from the signed order of the bisignate CD couplet[20,25] (Fig. 3). In **4**, the ***M***-helical ridge-tile conformation is present exclusively in nonpolar solvents, as confirmed by the intense negative exciton chirality bisignate CD [11]. With *ent*-**4**, a mirror image positive chirality bisignate CD confirms the ***P***-helical ridge-tile[11]. Thus CD spectroscopy of (*S*,*S*)-β,β′-dimethylrubins can be used in conformational analysis and in confirming intramolecular hydrogen bonding[11,20].

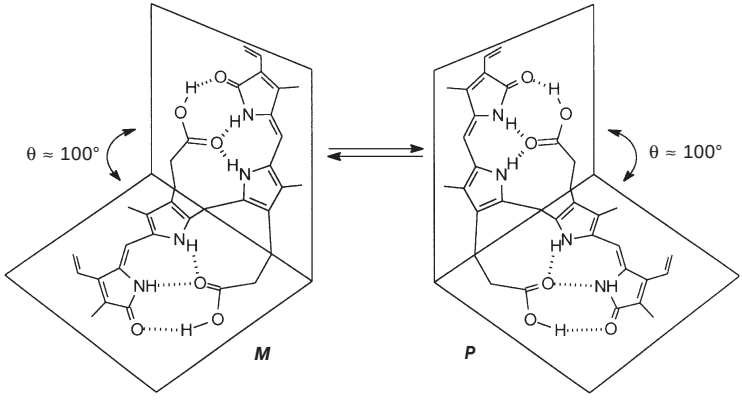


FIG. 2
Bilirubin 3-D conformational structures shaped like ridge-tiles of left- (***M***) and right-handed (***P***) chirality, are isoenergetic, non-superimposable mirror images (enantiomers). Dashed lines are hydrogen bonds

Consistent with what has been observed previously in the CD spectra of the bis-*N*-methylamide **10** [14] of (*S*,*S*)-β,β′-dimethylmesobilirubin XIIIα **4**, we observe intense bisignate negative chirality CD for glycorubin **1** in nonpolar as well as polar organic solvents (Fig. 3), thus confirming the ***M***-helical ridge-tile conformation as well as intramolecular hydrogen bonding between dipyrrinones and the propionamide groups. Stabilized ridge-tile conformations are preserved not only from hydrogen bonding between the propionic carboxylic acid group and a dipyrrinone (Fig. 2), as found in **4**, but also from hydrogen bonding between propionamide groups and dipyrrinones, as shown previously for **10** [14]. Since glycorubin **1** is a propionamide as is **10**, it is clear that the glycine CO_2H group does not interfere excessively with the matrix of conformation-stabilizing intramolecular hydrogen bonds between its amide linkage and an opposing dipyrrinone that preserves the predominant ***M***-helical ridge-tile conformation (Fig. 4).

In the nonpolar, aprotic solvent $CHCl_3$, glycorubin **1**, (*S*,*S*)-β,β′-dimethylmesobilirubin XIIIα **4** and its bis-*N*-methylamide **10** all show high-intensity bisignate CD curves (Fig. 3), with a sign order indicative of the expected ***M***-helicity and Cotton effect magnitudes much larger than those seen when no intramolecular hydrogen bonding is possible[26]. As expected from earlier studies of **4** [11] and **10** [14], the CD intensities of **1** are diminished somewhat

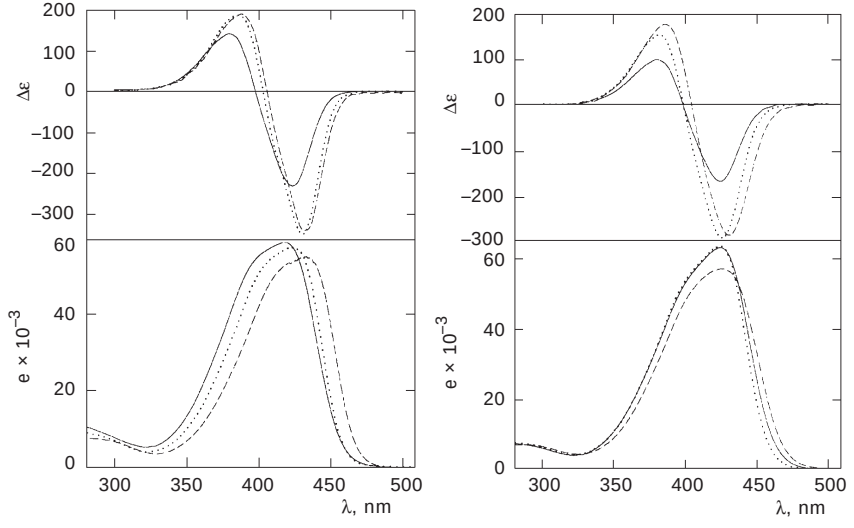


FIG. 3
Circular dichroism (upper) and UV-VIS spectra (lower) of **1** (—), amide **10** (····) and their parent diacid **4** (- - -) in chloroform (right) and methanol (left)

for spectra run in a polar protic solvent (CH_3OH) (Fig. 3). The somewhat larger drop in CD magnitude found for **1** than for **4** and **10** suggests slightly weaker intramolecular hydrogen bonding in **1** than in **4** or **10**. The data indicate the same, mainly ***M***-helical molecular geometry in both $CHCl_3$ and CH_3OH, but with differing ratios of ***M***:***P*** conformations (see Fig. 2). Although molecular mechanics calculations of the pigments indicate an ***M***-helical global energy minimum (see Fig. 3), apparently the glycine carboxylic acid destabilizes this conformation to some extent. With somewhat weaker intramolecular hydrogen bonding, **1** is more sensitive to an increased solvent polarity. In contrast, the bisignate CD magnitudes of the dimethyl ester of **1** are approximately 25% larger ($CHCl_3$: $\Delta\varepsilon_{424}^{max}$ = –297, $\Delta\varepsilon_{381}^{max}$ = +163; CH_3OH: $\Delta\varepsilon_{423}^{max}$ = –229; $\Delta\varepsilon_{380}^{max}$ = +126).

Table III summarizes the CD spectral data of **1** in solvents of a wide range of polarity, from $CHCl_3$ (Fig. 3) to DMSO and water, and compares the data to those of parent acid **4** [11] and *N*-methylamide **10** [14]. In modestly polar (diethyl ether) to polar aprotic (CH_3CN) solvents, intense negative-chirality bisignate Cotton effects persist, consistent with the intramolecular hydrogen-bonded model (Fig. 4). The data from DMSO have typically represented a special case of solvent insertion into the hydrogen bonding network[11,14,20]. In water, a somewhat weaker but still large negative chirality bisignate CD is found for **1**, again as predicted from the stereochemistry of the β,β′ stereocenters of the propionamide groups and considerations of nonbonded steric interactions in the ***M***-helical intramolecularly hydrogen-bonded ridge-tile diglycinate conformation (Fig. 4). In pH 7.4 buffer,

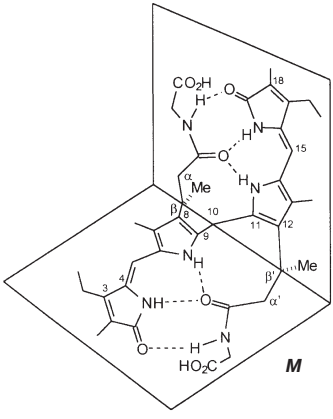


FIG. 4
Most stable 3-D conformational structure of **1**. Dashed lines are hydrogen bonds

the CD intensities of **1** drop to approximately one-half of the values seen in distilled water, indicating that the unionized glycine CO_2H may participate in intramolecular hydrogen bonding but the carboxylate anion does not. Taken collectively and comparatively, the CD data point to a strong preference for the intramolecularly hydrogen-bonded ridge-tile conformation of glycorubin **1** (Fig. 4) in nonpolar solvents and the high probability of the same conformation in water and other polar solvents.

TABLE III

Comparison of circular dichroism and UV-VIS spectral data from 2×10^{-5} M solutions of glyco-(*S*,*S*)-β,β′-dimethylmesobilirubin **1**, (*S*,*S*)-β,β′-dimethylmesobilirubin **4** and its bis-*N*-methylamide **10** at 22 °C[a]

Pigment	Solvent	CD			UV	
		$\Delta\varepsilon_1^{max}(\lambda_1)$	λ at Δε = 0	$\Delta\varepsilon_2^{max}(\lambda_2)$	ε^{max}	λ^{max}
1[b]	$CHCl_3$	–231.4(424)	398	+141.2(380)	58900	419
4		–337.3(434)	407	+186.2(389)	55500	431
10		–347.7(431)	403	+187.2(386)	57600	428
1	Et_2O	–239.5(421)	394	+122.9(378)	62000	417
4		–365.2(429)	402	+182.6(387)	56400	429
10		–371.2(424)	397	+167.7(381)	59200	424
1	CH_3CN	–196.2(416)	392	+123.4(374)	66100	418
4		–315.1(429)	403	+181.4(384)	56100	424
10		–318.3(421)	395	+164.9(378)	58100	417
1	CH_3OH	–167.0(425)	399	+99.1(380)	63100	425
4		–285.4(431)	405	+177.1(386)	56600	425
10		–290.2(426)	399	+153.9(382)	64800	424
1	$(CH_3)_2SO$	+27.0(429)	397	–16.3(382)	57200	424
4		+23.0(425)	385	–5.8(369)	55900	425
10		–178.9(421)	394	+83.4(379)	60300	421
1[b]	H_2O	–89.0(422)	398	+57.8(379)	50800	417
	H_2O[c]	–35.8(422)	398	+32.5(373)	51100	416
4[b]	H_2O[c]	–150.4(423)	398	+95.2(379)	44500	416

[a] All solutions contained 2% v/v of $CHCl_3$ and those of **1** in organic solvents – additional 0.1% v/v of CH_3OH; [b] solutions contained 2% v/v of $(CH_3)_2SO$; [c] 0.05 M phosphate buffer pH 7.40

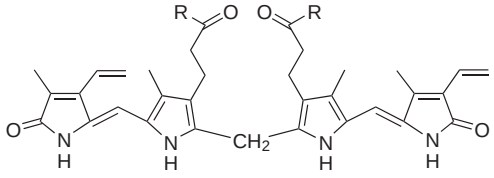
We thank the U.S. National Institutes of Health (HD-17779) for support of this work. S. E. Boiadjiev is on leave from the Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia.

REFERENCES

1. Hall J. C.: *J. Parenteral Enteral Nutr.* **1998**, *22*, 393.
2. Hofman A. F., Mysels K. J.: *J. Lipid Res.* **1992**, *33*, 617.
3. Hofmann A. F., Palmer K. R., Yoon Y. B., Hagey L. R., Gurantz D., Huijghebaert S., Converse J. L., Cecchetti S., Michelotti E.: *Falk Symp. Adv. Glucuronide Conjugation* **1985**, 245.
4. Hay D. W., Carey M. C.: *Hepatology* **1990**, *12*, 6S.
5. Benzi G.: *Ann. Med.* **1968**, *12*, 66.
6. Idle J. R., Millburn P., Williams R. T.: *Xenobiotica* **1978**, *8*, 253.
7. Chowdury J. R., Wolkoff A. W., Chowdury N. R., Arias I. M. in: *The Metabolic and Molecular Bases of Inherited Disease* (C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, Eds), Vol. 2, p. 3063. McGraw–Hill, New York 2001.
8. McDonagh A. F. in: *The Porphyrins* (D. Dolphin, Ed.), Vol. 6, p. 293. Academic Press, New York 1979.
9. a) Kajihara T., Tazuma S., Yamashita G., Kajiyama G.: *J. Gastroenterol. Hepatol.* **1999**, *14*, 578; b) Jansen P. L. M., van Klinken J. W., van Gelder M., Ottenhoff R., Elferink R. P. J. O.: *Am. J. Physiol.* **1993**, *265*, G445.
10. Jirsa M., Večerek B.: *Hoppe-Seyler's Z. Physiol. Chem.* **1958**, *311*, 87.
11. Boiadjiev S. E., Person R. V., Puzicha G., Knobler C., Maverick E., Trueblood K. N., Lightner D. A.: *J. Am. Chem. Soc.* **1992**, *114*, 10123.
12. Boiadjiev S. E., Anstine D. T., Lightner D. A.: *J. Am. Chem. Soc.* **1995**, *117*, 8727.
13. Armarego W. L. F., Perrin D. D.: *Purification of Laboratory Chemicals*, 4th ed. Butterworth–Heinemann, Oxford (U.K.) 1996.
14. Boiadjiev S. E., Person R. V., Lightner D. A.: *Tetrahedron: Asymmetry* **1993**, *4*, 491.
15. Ono N., Katayama H, Nisyiyama S., Ogawa T.: *J. Heterocycl. Chem.* **1994**, *31*, 707.
16. Boiadjiev S. E., Lightner D. A.: *Monatsh. Chem.* **2003**, *134*, 51.
17. Shrout D. P., Puzicha G., Lightner D. A.: *Synthesis* **1992**, 328.
18. Boiadjiev S. E., Anstine D. T., Lightner D. A.: *Tetrahedron: Asymmetry* **1995**, *6*, 901.
19. Boiadjiev S. E., Lightner D. A.: *Tetrahedron: Asymmetry* **1999**, *10*, 607.
20. Person R. V., Peterson B. R., Lightner D. A.: *J. Am. Chem. Soc.* **1994**, *116*, 42.
21. a) Boiadjiev S. E., Lightner D. A.: *J. Am. Chem. Soc.* **2000**, *122*, 11328; b) Boiadjiev S. E., Lightner D. A.: *Tetrahedron: Asymmetry* **1999**, *10*, 2535.
22. Manitto P., Monti D.: *J. Chem. Soc., Chem. Commun.* **1976**, 122.
23. Kaplan D., Navon D.: *Isr. J. Chem.* **1983**, *23*, 177.
24. Harada N., Nakanishi K.: *Circular Dichroic Spectroscopy – Exciton Coupling in Organic Stereochemistry*. University Science Books, Mill Valley (CA) 1983.
25. Lightner D. A., Gawronski J. K., Wijekoon W. M. D.: *J. Am. Chem. Soc.* **1987**, *109*, 6354.
26. Boiadjiev S. E., Pfeiffer W. P., Lightner D. A.: *Tetrahedron* **1997**, *53*, 14547.

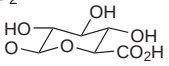
Boiadjiev S. E., Lightner D. A: *Collect. Czech. Chem. Commun.* **2003**, *68*, 1007.

The structure at the top of page 1008 should be bilirubin-IXα:



Bilirubin: R = OH

Glycobilirubin: R = $NHCH_2CO_2H$

Bilirubin diglucuronide: R = 

Taurobilirubin: R = $NHCH_2CH_2SO_3^-$