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On the Conformation of Bilirubin Dianion

Yu-Ming Pu and David A. Lightner*

Department of Chemistry, University of Nevada
Reno, Nevada 89557-0020 USA

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Abstract: ($\alpha R,\alpha' R$)-Mesobilirubin-XIIIα, a tetrapyrrole dicarboxylic acid with **P**-helicity conformation stabilized by intramolecular hydrogen bonding, exhibits intense bisignate circular dichroism in $CHCl_3$ ($\Delta\epsilon^{436}_{max}$ +246, $\Delta\epsilon^{398}_{max}$ -133) and many other solvents, except in $(CH_3)_2SO$, where the intensity drops 10-fold ($\Delta\epsilon^{428}_{max}$ +18, $\Delta\epsilon^{393}_{max}$ -9). In contrast, the dicarboxylate anion exhibits intense circular dichroism in both $CHCl_3$ ($\Delta\epsilon^{438}_{max}$ +194, $\Delta\epsilon^{394}_{max}$ -130) and $(CH_3)_2SO$ ($\Delta\epsilon^{433}_{max}$ +260, $\Delta\epsilon^{390}_{max}$ -164).

(4*Z*,15*Z*)-Bilirubin-IXα (Fig. 1) the cytotoxic yellow-orange tetrapyrrole pigment of jaundice[1] consists of two dipyrrinone units conjoined by a -CH_2- group. Rotation of the dipyrrinones about the central -CH_2- at C_{10} generates a large array of conformations,[2,3] one of which uniquely places the propionic acid COOH group of one dipyrrinone unit in position for intramolecular hydrogen bonding with the pyrrole and lactam N-H and C=O resides of the other dipyrrinone. Stabilization of this conformation by a network of intramolecular hydrogen bonds (Fig. 1) was first detected in the solid by X-ray crystallography,[4,5] where bilirubin was seen to have folded into either of two ridge-tile shaped enantiomers (Fig. 1). Intramolecular hydrogen bonding has also been detected in solution by NMR,[6,7] where the bilirubin conformers are thought to persist as a pair of interconverting conformational enantiomers[8,9] in solvents which do not strongly perturb the matrix of intramolecular hydrogen bonds. The situation is less clear for solvents or other agents that may disrupt the folded pigment's hydrogen bonding matrix and lead to new conformations with different properties. Of particular interest have been $(CH_3)_2SO$ solvent,[11] in which bilirubin exhibits its greatest solubility,[10] and bases, which convert bilirubin into its mono and dicarboxylate anions. In the former, ^{13}C-NMR relaxation studies (in $(CD_3)_2SO$)) of segmental motion in the propionic acid groups suggest that the COOH residues are tethered to the dipyrrinones via bound solvent molecules.[11] Although it is probably folded,[12] the shape of the pigment is unknown. In the latter, it has been thought that ionization of bilirubin causes breaking of all of the intramolecular hydrogen bonds, leaving the pigment free to adopt new and different conformations.[13] Whether conformation stabilization of the dianion can be through residual intramolecular hydrogen bonding has been an important question in studies of bilirubin protein binding and metabolism, where mono and dianions are thought to be the major species.[1]

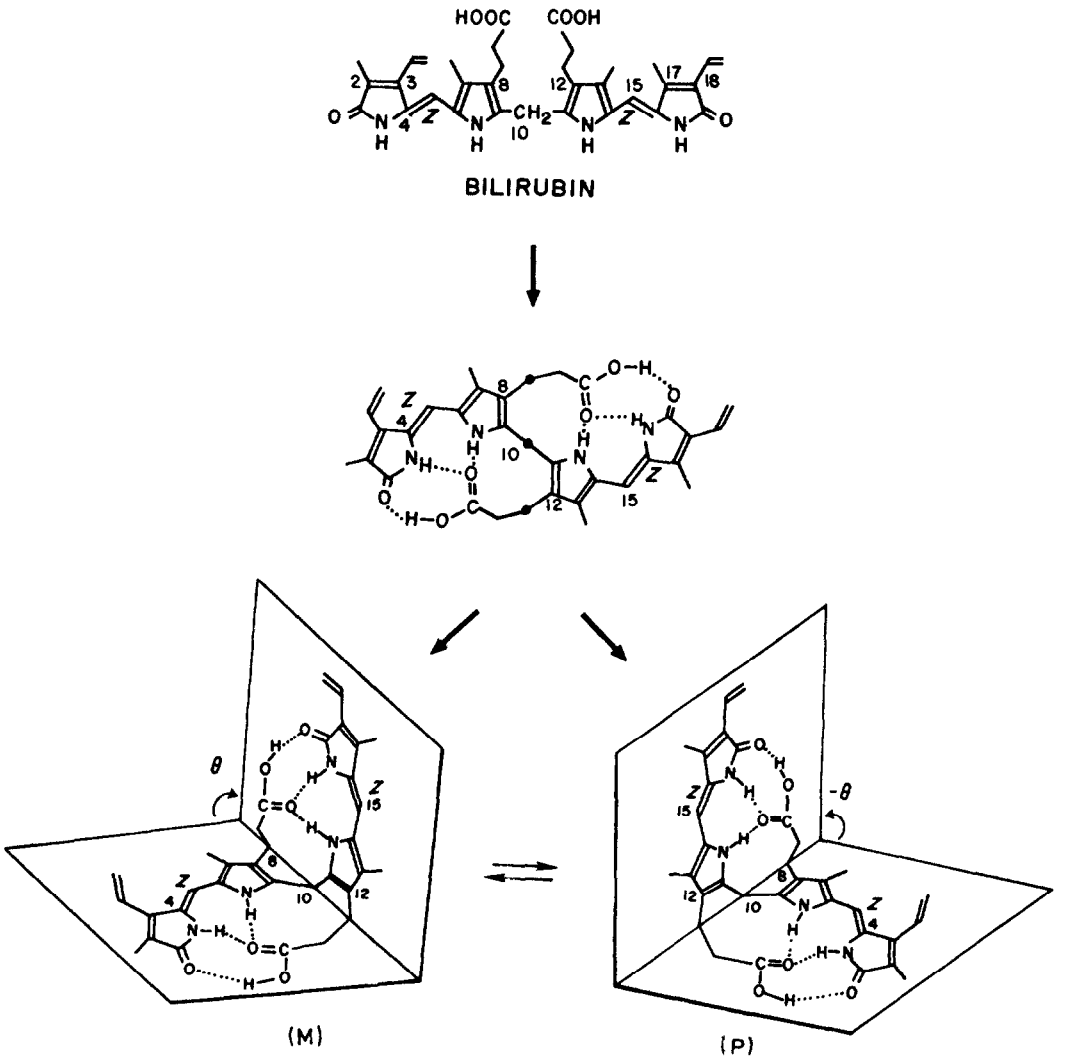
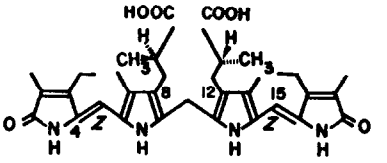


FIGURE 1. (Upper) Linear representation for (4Z,15Z)-bilirubin-IXα showing two dipyrrinone chromophores connected by a -CH_2- group. (Middle) Planar projection of the intramolecularly hydrogen bonded conformation adopted following rotation of the two dipyrrinones about the central -CH_2-. (Lower) Three-dimensional, ridge-tile shaped representations for the intramolecularly hydrogen bonded conformations. There are two enantiomeric ridge-tile conformers, which interconvert by breaking all six hydrogen bonds, rotating the dipyrrinones about C_{10} and remaking the hydrogen bonds. The helicity or chirality of the two enantiomers is designated **M** or **P** according to the helical arrangement made by the electric dipole transition moments oriented along the long axis of each of the two dipyrrinone chromophores of each molecule (reference 16).

In the following we present evidence from circular dichroism (CD) and NMR spectroscopy for the persistence of intramolecular hydrogen bonding and the ridge-tile structure in an optically active analog of bilirubin, (+)-($\alpha R,\alpha' R$)-dimethylmesobilirubin-XIIIα.[14]

($\alpha R,\alpha' R$)-Dimethylmesobilirubin-XIIIα:



Experimental: All CD spectra were run on a JASCO J-600 spectropolarimeter, and all NMR spectra were run on a GE QE-300 FT-NMR spectrometer in either $CDCl_3$ (99.9% d_1, Aldrich) or $(CD_3)_2SO$ (99.9% d_6, Aldrich). Mesobilirubin-XIIIα,[15], (+)-($\alpha R,\alpha' R$)-dimethylmesobilirubin-XIIIα[14] and (-)-($\alpha S,\alpha'$S)-dimethylmesobilirubin-XIII[14] (with the latter two having 100% e.e.) were prepared by total synthesis as described previously. The bis-tetramethylammonium and bis-tetra-*n*-butylammonium salts were prepared from the corresponding mesobilirubin (4.65 mg, 7.6-7.8 x 10^{-3} mmole) dissolved in 5.0 mL of CH_2Cl_2 by adding excess methanolic (15.0 μL of 1 *M*, 1.5 x 10^{-2} mmole) tetramethylammonium hydroxide or tetra-*n*-butylammonium hydroxide then evaporating to dryness (roto-vap).

Results and Discussion: The α-methylated mesobilirubins serve as excellent chiral probes of pigment stereochemistry.[14] Thus, when bilirubin or its symmetric analog, mesobilirubin-XIIIα (Fig. 2) is folded and held in the **M**-helicity ridge-tile conformation by intramolecular hydrogen-bonding, the *pro-R* α and α' hydrogens are in a sterically more compressed environment than the *pro-S* with steric crowding coming from the C_7 and C_{13} methyl groups. Consequently, the equilibrium depicted should be driven toward the **P**-helicity conformer when the *pro-R* hydrogens are replaced by CH_3 groups, and toward the **M**-helicity conformer when the *pro-S* hydrogens are replaced by CH_3. When held in the **P**-helicity conformation, the inverse holds: the *pro-S* α and α' hydrogens are more sterically crowded than the *pro-R* by the C_7 and C_{13} methyl groups. However, in the absence of intramolecular hydrogen bonding stabilization of the ridge-tile enantiomeric conformations, the allosteric effect of such methyl subsitution would be lost, and minimal conformational enantioselectivity would be expected.

In full agreement with the predictions of the allosteric model, the *R,R*-enantiomer of α,α'-dimethylmesobilirubin-XIIIα shows an intense bisignate CD (Table 1) that is characteristic of the **P**-helicity conformational enantiomer.[14,16] The $\Delta\epsilon$ values in non-polar solvents such as benzene and $CHCl_3$ are close to the theoretically predicted maximum values,[16] as expected from solvents which do not disrupt the intramolecular hydrogen bonding network. The $\Delta\epsilon$ values decrease; implying a decreased enantioselectivity due to a loosening or disruption of the hydrogen bonding network, in more polar solvents with higher dielectric constants. The most profound effect is found with $(CH_3)_2SO$ solvent, where the $\Delta\epsilon$ values are decreased more than 10-fold from the large values seen in $CHCl_3$ and benzene. This might have been anticipated from the Resonance Raman[17] and NMR[11] studies of bilirubin structure in $(CH_3)_2SO$, which suggest strong

solvent interference with the hydrogen bonds. Although the NMR studies indicate that the CO_2H residues are tied to the nearest pyrrole and lactam groups by bound $(CH_3)_2SO$ molecules, the emerging picture is

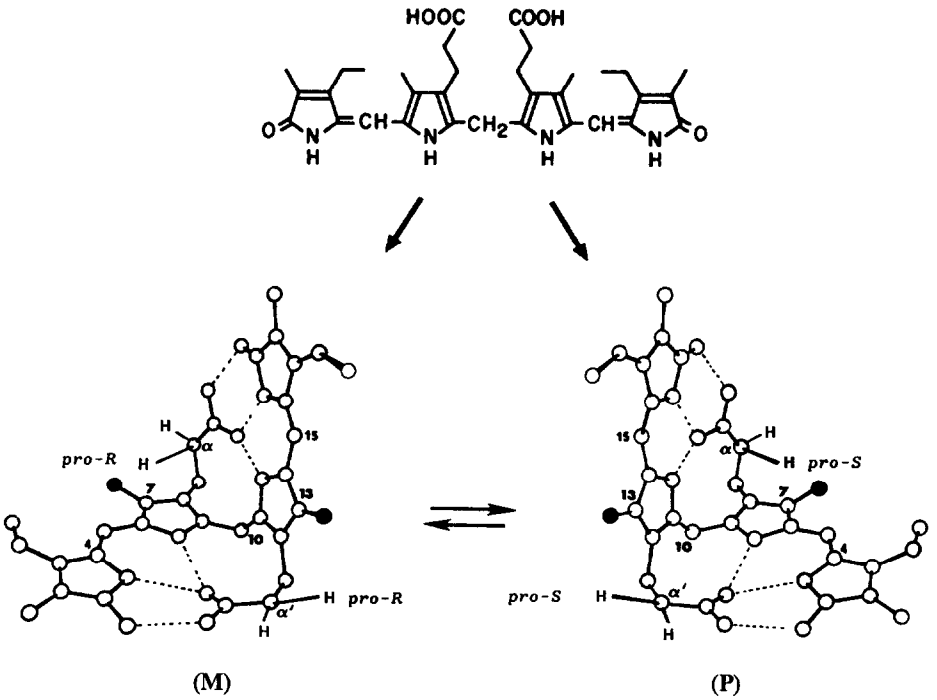


FIGURE 2. (Top) Linear representation for mesobilirubin-XIIIα. (Bottom) Ball and stick conformational representations for the ridge-tile shape intramolecularly hydrogen bonded, interconverting enantiomers. The C_7 and C_{13} methyl groups are represented by the darkened circles. The hydrogens on the α and α′ $-CH_2-$ groups are either *pro-R* or *pro-S*, only one of the set is designated. When the **M**-helicity conformer inverts into the **P**- helicity, steric crowding of the *pro-R* hydrogens is relieved and taken up by the *pro-S* hydrogens.

one of a swollen matrix of hydrogen bonds where the allosteric effect of the α and α′ CH_3 groups is not as profound as in the more compact conformations (Fig. 1). The markedly diminished CD in $(CH_3)_2SO$ may be contrasted with that in pH 7.4 H_2O, where the pigment is undoubtedly ionized and might therefore be expected to possess fewer hydrogen bonds. Here the $\Delta\epsilon$ values (which are the same at pH 9-11) are half as great as those in $CHCl_3$ and an order of magnitude greater than those in $(CH_3)_2SO$. Such data indicate that $(CH_3)_2SO$ is probably a poor organic solvent model for H_2O when one is interested in bilirubin structure in aqueous solution, for they point to a peculiar ability of $(CH_3)_2SO$ to engage in conformation-altering hydrogen bonding with the pigment. No exact correlation of CD with solvent dielectric is apparent, although it might have been thought that solvents with very high dielectric constants would better support CO_2H ionization with a concomitant loss of stereochemistry associated with intramolecular hydrogen

bonding. The data suggest that $HCONHCH_3$, which has a very large dielectric constant, might be a better organic solvent analog for H_2O than is $(CH_3)_2SO$. What is the effect of ionization on the pigment conformation?

TABLE 1. Circular Dichroism and Ultraviolet-Visible Spectral Data for (+)-($\alpha R,\alpha' R$)-Dimethylmesobilirubin-XIIIα and Its Tetramethylammonium Carboxylate Salt[a]

Solvent	Dielectric Const.	ACID					DICARBOXYLATE ANION				
		CD			UV		CD			UV	
		$\Delta\epsilon_{max}(\lambda_1)$	λ_2 at $\Delta\epsilon=0$	$\Delta\epsilon_{max}(\lambda_3)$	ϵ_{max}	λ(nm)	$\Delta\epsilon_{max}(\lambda_1)$	λ_2 at $\Delta\epsilon=0$	$\Delta\epsilon_{max}(\lambda_3)$	ϵ_{max}	λ(nm)
C_6H_6	2	+252(436)	408	-135(392)	52,000	435	+196(434)	407	-120(391)	57,500	436
$CHCl_3$	5	+246(436)	408	-133(393)	56,000	433	+194(438)	412	-130(394)	55,000	435
CH_3CN	36	+214(429)	403	-121(386)	55,000	425	+237(426)	399	-137(383)	57,000	425
CH_3CH_2OH	24	+171(432)	404	-109(387)	58,000	429	+220(432)	406	-147(389)	54,000	432
$HCON(CH_3)_2$	37	+ 83(431)	405	- 59(387)	48,000	426	+262(432)	405	-160(389)	50,000	424
$(CH_3)_2SO$	49	+ 18(4283)	406	- 9(393)	48,000	425	+260(433)	406	-164(390)	55,000	431
H_2O	80	+117(425)[c]	399[c]	- 84(379)[c]	45,000	418[c]	+126(424)	400	- 88(379)	45,000	418
$HCONHCH_3$	160	+136(428)	402	- 90(385)	45,000	424	+141(428)	402	- 91(384)	48,500	422

[a] Run on 2 x 10^{-5} *M* solutions at 20°C; [b] From Gordon, A.J.; Ford, R.A. *The Chemist's Companion*, Wiley, NY; (1972) pp 4-8; [c] In pH 7.4 phosphate buffer.

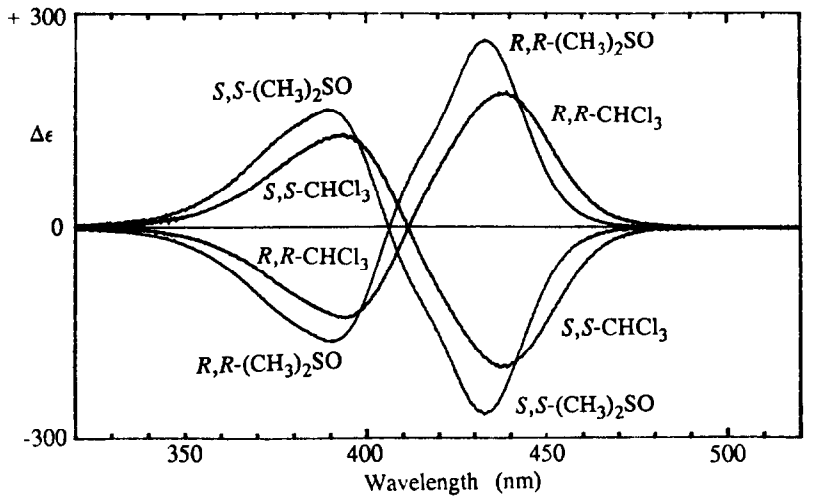


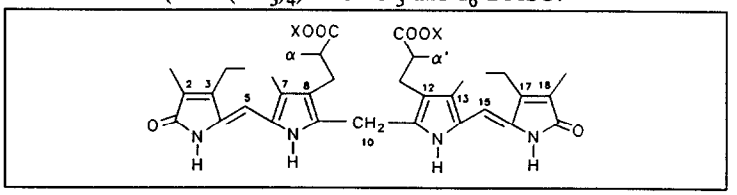
FIGURE 3. Circular dichroism spectra of the *bis*-tetra-*n*-butylammonium salts of (+)-($\alpha R,\alpha R'$) and (-)-($\alpha S,\alpha' S$)-dimethylmesobilirubin-XIIIα in $CDCl_3$ and $(CH_3)_2SO$ solvents at 22°C. The sample concentrations are 2x 10^{-5} *M*.

The tetramethylammonium and tetra-*n*-butylammonium salts of (+)-($\alpha R,\alpha' R$)-dimethylmesobilirubin-XIIIα exhibit intense bisignate CD spectra in benzene and $CHCl_3$ (Fig. 3 and Table 1), indicating that intramolecular hydrogen bonding remains a powerful force in the enantioselection of the **P**-helicity conformer. This is perhaps not too surprising a result for these solvents, and it indicates that even when the number of hydrogen bonds is reduced by 1/3, the residual hydrogen bonding is still sufficient for stabilizing the ridge-tile structures, and possibly even strengthened by conversion of COOH to COO^-. Unlike the

diacid, however, the $\Delta\epsilon$ values of the dianion do not decrease as the solvent dielectric constant increases from CH_3CN to CH_3CH_2OH to $HCON(CH_3)_2$ to $(CH_3)_2SO$ solvent. The last is especially striking because the $\Delta\epsilon$ values are the largest observed, and the data would suggest that the pigment dianion adopts the same **P**-helicity conformation as the pigment diacid in $CHCl_3$, where intramolecular hydrogen bonding is extensive. Apparently whether the carboxylate-tetraalkylammonium ion pair is tight (as in benzene, $CHCl_3$) or loose or solvent-separated has little bearing on the enantioselection and hence the intramolecular hydrogen bonding between the carboxylate anion and the dipyrrinone N-H and C=O residues.

The major influence of solvent is seen with H_2O and $HCONHCH_3$, the solvents of highest dielectric constant. It is not surprising and even reassuring (of the model) that in H_2O the tetramethylammonium salt gives essentially the same CD as the pigment acid in pH 7.4 aqueous buffer. The CD data for the two species are also the same in $HCONHCH_3$ for reasons that are less clear. Yet one would conclude here that the conformations are much the same and that intramolecular hydrogen bonding plays a major role in the stabilization of such conformations.

Table 2. Proton NMR Spectra[a] of (±)-α,α′-Dimethylmesobilirubin-XIIIα and Its Bis-Tetramethylammonium Salt ($X=N(CH_3)_4$) in $CDCl_3$ and d_6-DMSO.

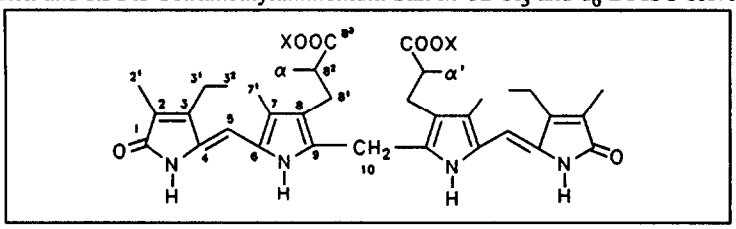


	Racemic Acid (X=H)		Racemic Salt ($X=NMe_4$)		Mesobilirubin-XIIIα Salt	
Hydrogen	$CDCl_3$	d_6-DMSO	$CDCl_3$	d_6-DMSO	$CDCl_3$	d_6-DMSO
COOH (s)	13.68	11.95	—	—	—	—
lactam NH (s)	10.55	9.85	12.35	12.19	12 09	12.36
pyrrole NH (s)	9.09	10.30	12.03	12.04	11 95	12.06
5,15- =CH (s)	6.05	5.95	5.94	5.80	5.87	5 79
10- CH_2 (s)	4.04	3.95	3.81	3.70	3 87	3.69
2,18- CH_3 (s)	1.86	1.77	1.76	1.67	1.73	1.67
7,13- CH_3 (s)	2.15	2.00	2.10	2 00	2 03	1 99
3,17- CH_2-CH_3 (t)	1.12	1.08	1.09	1.01	1.04	1 04
3,17- CH_2-CH_3 (q)	2.48	2.50	2.46	2.42	2.42	2.43
α,α′-CH_3 (d)	1.45	0.89	1.18	1.04	—	—
8,12-CH_2(m)	2.42	2.05	2.35	2.16	2.31	2.11
$^+N(CH_3)_4$	—	—	3.12	3.00	3.10	3.00

[a] Values in δ, ppm downfield from $(CH_3)_4Si$ for 10^{-2} *M* pigment solutions.

Further evidence for the presence of intramolecular hydrogen bonding may be found in the NMR spectra of (±)-α,α′-dimethylmesobilirubin-XIIIα and its dianion (Tables 2 and 3). In the ^{1}H-NMR, one

Table 3. Carbon-13 NMR Spectra[a] of the Racemic Diastereomer of α,α′-Dimethylmesobilirubin-XIIIα and Its Bis-Tetramethylammonium Salt in $CDCl_3$ and d_6-DMSO solvents at 22°C.



	Racemic Acid (X=H)		Racemic Salt (X=NMe_4)		Mesobilirubin-XIIIα Salt	
Carbon	$CDCl_3$	d_6-DMSO	$CDCl_3$	d_6-DMSO	$CDCl_3$	d_6-DMSO
1	174.91	172.40	173.90	172.93	173.38	173.01
2	124.14	123.41	124.15	123.57	123.42	123.76
2^1	7.95	8.54	8.94	9.31	8.98	9.29
3	148.42	147.70	147.92	147.25	147.13	147.14
3^1	17.86	17.20	18.54	18.39	18.47	18.38
3^2	14.89	15.31	15.84	16.18	15.57	18.38
4	128.27	128.24	128.25	128.50	127.71	128.62
5	100.63	98.23	99.41	98.76	99.54	98.68
6	123.91	123.36	123.66	123.24	123.27	123.33
7	119.25	118.84	121.42	121.06	122.03	121.19
7^1	10.27	9.94	10.73	10.69	10.49	10.71
8	123.23	122.40	123.35	122.07	123.13	121.83
8^1	28.06	28.18	30.31	32.39	30.28	30.60
8^2	39.17	40.40	45.17	45.32	41.04	40.81
8^3	182.35	177.86	183.10	180.57	179.45	177.61
9	133.16	131.20	132.50	132.63	133.30	132.52
10	22.17	24.18	21.90	22.64	22.71	22.86
α	19.68	17.63	20.20	20.65	—	—
$^+N(CH_3)_4$	—	—	56.08	55.30	56.01	55.80

[a] Values in δ, ppm downfield from $(CH_3)_4Si$ for 10^{-2} *M* pigment solutions.

should take note of a general shielding influence of $(CD_3)_2SO$ by as much as 0.1 to 0.2 ppm on most C-H's in both the diacid and dianion, as well as the dianion of mesobilirubin-XIIIα. The N-H signals of the diacid are especially sensitive to the change in solvent, reflecting a change from intramolecularly hydrogen bonded pigment in $CDCl_3$ to pigment hydrogen bonded to solvent in $(CD_3)_2SO$.[6,12,15] In contradistinction, the more deshielded N*H* signals of the dianions are fairly insensitive to the solvent change, consistent with the picture wherein intramolecular hydrogen bonding to these hydrogens by the COO^- groups persists in both $CDCl_3$ and $(CD_3)_2SO$. The overall strong deshielding on the N*H*'s may be attributed either to a strengthening of the hydrogen bonds[6] or to field effects of the anion. Unique to the acid is the very large shielding (0.56 ppm) of the α,α′ CH_3 groups upon going from $CDCl_3$ to $(CD_3)_2SO$, again apparently reflecting a change from intramolecular hydrogen bonding in $CDCl_3$ to intense hydrogen bonding with $(CD)_2SO$ solvent. The fact that there is no similarly large shielding and only small N-*H* chemical shift

differences with solvent change for the dianion provides supporting evidence for the same folded, intramolecularly hydrogen bonded conformation in both solvents — one similar to the ridge-tile conformation of the diacid in $CHCl_3$. In the ^{13}C-NMR there are again some general effects of solvent change, both shielding and deshielding. We point to C_{10} as a useful monitor of altered conformation. In the diacid, the change from $CDCl_3$ to $(CD_3)_2SO$ leads to a deshielding of C_{10} by $\sim$2 ppm; whereas, no such strong deshielding is seen in the dianion spectra, and importantly, the chemical shift of this carbon is essentially the same for the dianions (in either solvent) as for the diacid in $CDCl_3$, where intramolecular hydrogen bonding is known to prevail.

Comparison of the CD and NMR data for α,α'-dimethylmesobilirubin-XIIIα and its tetramethylammonium dicarboxylate anion predicts that the latter adopts the bilirubin ridge-tile conformation[4] held in place by a network of intramolecular hydrogen bonds — in $CHCl_3$ solvent and in $(CH_3)_2SO$ solvent.

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