

On the Conformation of Bilirubin Ditaurate

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Summary. The first optically active taurine conjugate of a bilirubin was prepared by reaction of taurine sodium salt with the mixed anhydride formed from reaction of ($\beta S, \beta' S$)-dimethylmesobilirubin-XIII α with isobutyl chloroformate. Analysis of the circular dichroism spectra of the conjugate in water and chloroform indicate a conformational preference for the (M)-helical ridge-tile conformation, thus providing the first spectroscopic evidence on the conformation of ditaurobilirubins.

Keywords. Taurine; Bilirubin; Dipyrinone; CD.

Introduction

Taurine (${}^{-}\text{O}_3\text{SCH}_2\text{CH}_2\text{NH}_3^{+}$), a conditionally essential nutrient important to mammalian development is found in plasma and milk, *inter alia*, and in bile as conjugates of bile acids, *e.g.*, taurocholic acid [1, 2]. It has also been found as a conjugate of the dicarboxylic acid bilirubin (the yellow pigment of jaundice) in the bile of certain fish (yellowtail, red sea bream, and flounder) [3]. It is not found in mammalian bile, however, where the principle bilirubin conjugates excreted into bile are mono and di-conjugates of glucuronic acid [4]. Bilirubin glucuronides are reactive, undergoing acyl migration and facile hydrolysis, and they are not readily available [5]. In contrast, the ditaurate does not undergo either and is far more stable, and it is available commercially. Consequently, it has been used as a surrogate for bilirubin diglucuronide *in vitro* and in animal studies, where it is smoothly excreted by the liver [6, 7]. Although its constitutional structure is known (Fig. 1), little is known of its conformation [8].

In the following, we describe the syntheses of the ditaurate of ($\beta S, \beta' S$)-dimethylmesobilirubin (**1**) and β -methylxanthobilirubic acid (**2**) (Fig. 2) and a spectroscopic study of **1** designed to provide new information on the three-dimensional structure of ditaurobilirubin.

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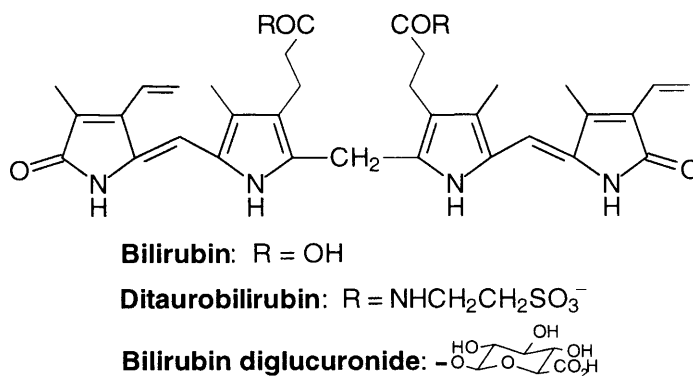


Fig. 1. Linear constitutional structures of bilirubin-IX α and its ditaurate and diglucuronide conjugates

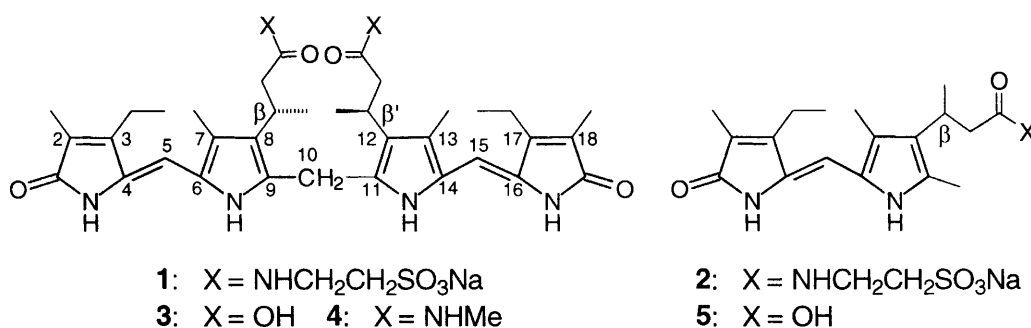


Fig. 2. Ditauro-(β S, β' S)-dimethylmesobilirubin-XIII α disodium salt (**1**), tauro-(β S)-methylxanthobilirubinate sodium salt (**2**), their parent carboxylic acids **3** and **5**, and the bis-*N*-methylamide (**4**) of **3**

Results and Discussion

Synthesis Aspects

A synthesis of ditaurobilirubin, reported in 1958 involved reaction of bilirubin with ethyl chloroformate to form the mixed anhydride, followed by reaction with taurine in dioxane-triethylamine [9]. Other methods of activation of carboxylic acids for reaction with taurine involve acid chloride, *N*-acylsuccinimide, azide, acyl imidazole, and anhydride intermediates [10]. Given *Jirsa's* procedure [9] for preparing ditaurobilirubin, we were inclined toward syntheses involving mixed anhydrides [10h-j] and found an attractive variation using isobutyl chloroformate described in the synthesis of a water-soluble taxol [11]. Using this modification and a change of solvent (to *THF*) with the presence of triethylamine as HCl scavenger, we converted **3** cleanly and quantitatively to its mixed anhydride when the solvents and reagents were oxygen-free and anhydrous, and the medium was basic. Addition of sodium taurate in *DMSO* to the mixed anhydride of **3** afforded the desired ditaurobilirubin **1** in 90% yield, isolated after radial chromatography on silica using a polar mobile phase. In several experiments, **1** crystallized on the chromatography plate (rotor) and could not be moved by the CH₂Cl₂-MeOH eluent – a problem that was

solved by pre-wetting the rotor with a small volume of 10% (*v/v*) MeOH in CH₂Cl₂ before loading the sample. Using the same procedure, β -methylxanthobilirubic acid (**5**) was converted to its taurate derivative **2** in 86% yield.

NMR and Structure

The constitutional structures and absolute configuration (Fig. 2) of ($\beta S, \beta' S$)-dimethylmesobilirubin-XIII α (**3**) [12], its bis-*N*-methylamide (**4**) [13] and β -methylxanthobilirubic acid (**5**) [14] are well established. Consequently, it is not surprising that the ¹³C-NMR chemical shifts in DMSO-*d*₆ of the carbons of the common skeleton are nearly identical (Table 1), especially in comparing **1**, **3** and **4**. The small differences seen, especially in the β -methylpropionic chains, among these three are probably relevant only to changes in the state of ionization and the presence of a sulfonate ion. It is interesting to note that the propionic carbonyl carbon is 1–2 ppm more shielded in **1** and **2** than in **3** and **4** but the α -carbon is more deshielded, and the β -carbons of **1–4** have nearly the same chemical shifts. Similar shieldings of carbonyl and α -carbon are found in taurate derivatives of bile acids, *e.g.*, taurodeoxycholate, $\delta_{C=O} = 172.3$ ppm and $\delta_{\alpha CH_2} = 32.7$ ppm relative to the parent bile acid, deoxycholic acid, $\delta_{C=O} = 174.9$ and $\delta_{\alpha CH_2} = 30.8$ ppm.

Table 1. Comparison of ¹³C NMR assignments ($5 \times 10^{-3} M$) in DMSO-*d*₆ at 25°C; δ in ppm of the sodium salt of ditauro-($\beta S, \beta' S$)-dimethylmesobilirubin (**1**), sodium tauro-(βS)-methylxanthobilirubinate (**2**), ($\beta S, \beta' S$)-dimethylmesobilirubin (**3**) and its bis-*N*-methylamide (**4**)

Position	1	2	3	4
1,19–CONH	171.98	171.92	172.10	171.96
2,18	122.94	122.50	122.97	123.04
2,18–CH ₃	8.10	8.08	8.09	8.12
3,17	147.29	147.20	147.33	147.45
3,17–CH ₂ CH ₃	17.19	17.18	17.18	17.22
3,17–CH ₂ CH ₃	14.83	14.88	14.86	14.84
4,16	128.00	127.19	128.01	127.98
5,15–CH=	97.71	97.65	97.67	97.30
6,14	122.60	121.82	122.48	122.67
7,13	121.43	123.55	121.70	121.02
7,13–CH ₃	10.63	10.03	10.65	10.54
8,12	123.34	121.51	123.21	123.14
β, β' –CH	27.46	27.28	26.88	27.20
β, β' –CH ₃	20.25	20.12	19.84	21.05
α, α' –CH ₂	42.48	42.76	39.41	41.59
α, α' –CO	171.46	170.96	173.68	173.55
CONHCH ₂ CH ₂ SO ₃ Na	35.50	35.42	–	–
CONHCH ₂ CH ₂ SO ₃ Na	50.47	50.64	–	–
CONHCH ₃	–	–	–	25.73
9,11	130.16	128.79	130.20	130.57
9–CH ₃	–	12.03	–	–
10–CH ₂	23.19	–	23.91	21.84

Table 2. Comparison of the proton NMR spectral assignments ($2 \times 10^{-3} M$) in $DMSO-d_6$ at $25^\circ C$; δ in ppm of the sodium salt of ditauro- $(\beta S, \beta' S)$ -dimethylmesobilirubin (**1**), sodium tauro- (βS) -methylxanthobilirubinate (**2**), $(\beta S, \beta' S)$ -dimethylmesobilirubin (**3**) and its bis-*N*-methylamide (**4**)

Site	1	2	3	4
α, α' -CONH- or COOH	7.87 ^a	7.71 ^h	11.98	8.30 ^p
21,24-NHCO	9.85	9.79	9.85	10.05
22,23-NH	10.33	10.23	10.10	10.25
-CONHCH ₂ CH ₂ SO ₃ Na	3.30 ^b	3.26 ^b	-	-
-CONHCH ₂ CH ₂ SO ₃ Na	2.52 ^b	2.52 ⁱ	-	-
-CONHCH ₃	-	-	-	2.50 ^q
2,18-CH ₃	1.75	1.76	1.76	1.73
3,17-CH ₂ CH ₃	2.48 ^c	2.48 ^c	2.44 ^m	2.48 ^m
3,17-CH ₂ CH ₃	1.07 ^d	1.07 ^d	1.07 ⁿ	1.06 ⁿ
5,15-CH=	5.94	5.91	5.95	5.92
7,13-CH ₃	2.09	2.06	2.08	2.13
β, β' -CH	3.25 ^b	3.12 ^b	3.17 ^b	3.39 ^b
β, β' -CH ₃	1.04 ^e	1.10 ^j	0.98 ^j	1.16 ^j
α, α' -CH ₂	2.28 ^f	2.19 ^k	2.40 ^o	2.63 ^r
	2.39 ^g	2.32 ^l		
9-CH ₃	-	2.21	-	-
10-CH ₂	3.97	-	3.98	3.90

^a t, $J = 5.3$ Hz; ^b m; ^c q, $J = 7.5$ Hz; ^d t, $J = 7.5$ Hz; ^e d, $J = 7.2$ Hz; ^f ABX, $^3J = 7.7$ Hz, $^2J = 13.8$ Hz; ^g ABX, $^3J = 8.0$ Hz, $^2J = 13.8$ Hz; ^h t, $J = 5.6$ Hz; ⁱ t, $J = 7.7$ Hz; ^j d, $J = 7.1$ Hz; ^k ABX, $^3J = 6.6$ Hz, $^2J = 13.7$ Hz; ^l ABX, $^3J = 8.8$ Hz, $^2J = 13.7$ Hz; ^m q, $J = 7.4$ Hz; ⁿ t, $J = 7.4$ Hz; ^o ABX, $^3J = 8.7, 8.1$ Hz; ^p br.q; ^q d, $J = 4.5$ Hz; ^r ABX, $^3J = 10.8$, overlapped

The ¹H-NMR (Table 2) of **1–4** in $DMSO-d_6$ also exhibit considerable similarity, as might be anticipated. The lactam and pyrrole NH chemical shifts are in the normal region for rubins and dipyrinones in this solvent, and the amide NHs of the taurine (**1** and **2**) and bis-*N*-methylamide (**4**) lie in the expected range. Consistent with the NMR data and interpretations of Navon [15] for bilirubin in $DMSO-d_6$, segmental motion within the β -methylpropionamide chains of **1**, **2**, and **3** is indicated by the similar J_{AX} and J_{BX} coupling constants of diastereotopic α -hydrogens of the $-CH_X(CH_3)-CH_AH_B-C=O$ segment. In addition, NOE measurements indicate that the β (and β')-methyl is near the C(7)/C(13) ring methyl and the C(10)H₂ in **1**. Ideally, one would wish to examine the ¹H-NMR of **1** in $CDCl_3$ solvent, in which the rubin conformation is typically restricted to either of the two enantiomeric ridge-tile conformations [12, 13], but the insolubility of **1** precluded such measurements.

Analysis of Conformation by Circular Dichroism Spectroscopy

The optical activity of **1–4** enables one to measure their circular dichroism (CD) spectra and extract information on conformation [12, 13, 16, 17]. The most stable conformation of bilirubin and mesobilirubin-XIII α 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 dipyrinones

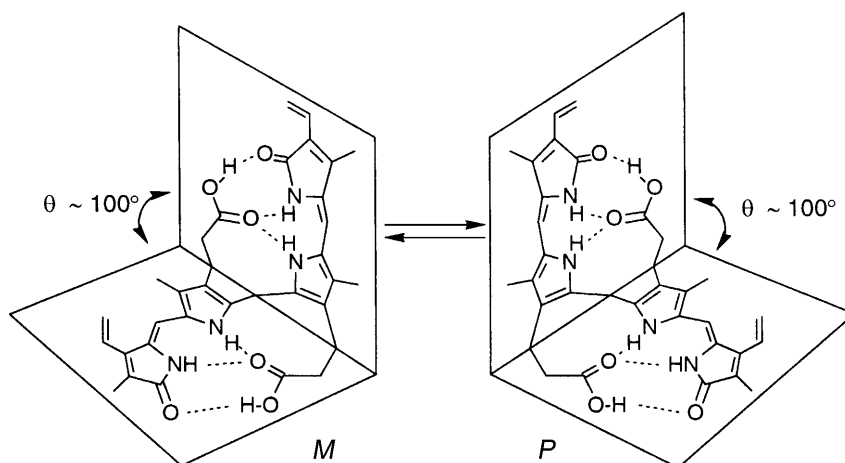


Fig. 3. Bilirubin 3-D conformational structures shaped like ridge-tiles of left (*M*) and right (*P*) handed chirality, are isoenergetic, non-superimposable mirror images (enantiomers); dashed lines are hydrogen bonds

[12, 16, 17]. There are two such ridge-tiles, equi-energetic and interconverting in solution over barriers of ~ 80 kJ/mol [16] (Fig. 3). Earlier, we showed that β and β' methyl groups (**3**) 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 [12]. Since bilirubins, with their two dipyrrole chromophores may be viewed as molecular excitons, exciton coupling theory [18] may be used to predict the (*M*) or (*P*)-helicity of the intramolecularly hydrogen-bonded ridge-tile (Fig. 3) from the signed order of the bisignate CD couplet [19]. In **3**, the (*M*)-helical ridge-tile conformation is confirmed by the intense negative chirality bisignate CD [12]. With *ent*-**3** a mirror image positive chirality bisignate CD confirms the (*P*)-helical ridge tile [12]. Thus CD spectroscopy of $\beta S, \beta' S$ -dimethylrubins can be used in conformational analysis and in confirming intramolecular hydrogen bonding [16].

In the bis-*N*-methylamide **4**, negative chirality intense bisignate CD is also observed, confirming the (*M*)-helical ridge-tile conformation as well as intramolecular hydrogen bonding between dipyrroles and the propionamide groups [13]. Stabilized ridge-tile conformations thus results not only from propionic carboxylic acid to dipyrrole hydrogen bonding but from hydrogen bonding between propionamides and dipyrroles as well. Since taurorubin **1** is a propionamide in the same sense as **4**, we determined its CD spectra for use in conformational analysis (Fig. 4).

Table 3 summarizes the CD spectra of **1** in solvents of a wide range of polarity and compares the data to those of **3** [12] and **4** [13]. In *pH* 7.4 0.1 *M* phosphate buffer, a large negative chirality bisignate CD is found for **1**, 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 ditaurate conformation (Fig. 4). Comparison of the CD with that of **3** in the same buffer (**4** is insoluble), shows that the CD intensity is lower in **1**, suggesting that intramolecular hydrogen bonding is weakened and

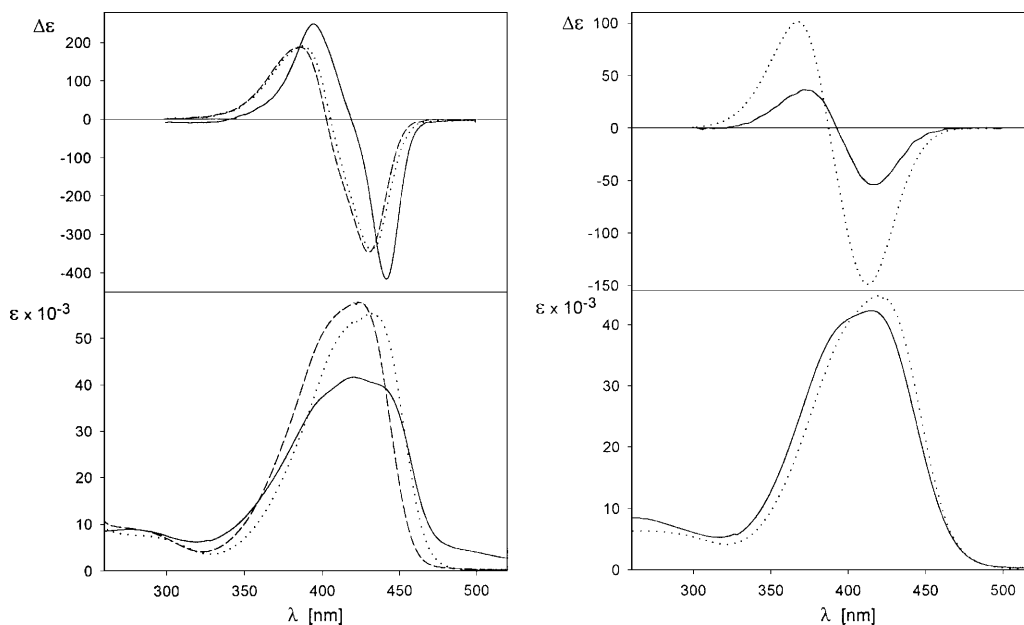


Fig. 4. Circular dichroism (upper) and UV-visible spectra (lower) of **1** (—), **4** (- - -) and their parent diacid **3** (· · · ·) in CHCl_3 (left) and in $\text{pH } 7.4$ phosphate buffer (right)

Table 3. Circular dichroism and ultraviolet-visible spectral data from $2 \times 10^{-5} M$ solutions of ditauro- $(\beta S, \beta' S)$ -dimethylmesobilirubin (**1**), $(\beta S, \beta' S)$ -dimethylmesobilirubin (**3**) and its bis- N -methylamide (**4**) at 22°C^a

Pigment	Solvent	CD			UV	
		$\Delta\epsilon_1^{\max}(\lambda_1)$	λ at $\Delta\epsilon=0$	$\Delta\epsilon_2^{\max}(\lambda_2)$	ϵ^{\max}	λ^{\max}
1	H_2O	- 54.7 (420)	394	+ 36.8 (375)	42 300	413
3^b	$\text{pH } 7.4$	- 150.4 (423)	398	+ 95.2 (379)	44 500	416
1	CH_3OH	- 56.9 (421)	397	+ 32.1 (381)	50 700	424
3		- 285.4 (431)	405	+ 177.1 (386)	56 600	425
4		- 290.2 (426)	399	+ 153.9 (382)	64 800	424
1	$\text{HCON}(\text{CH}_3)_2$	- 27.5 (415)	394	+ 10.7 (377)	52 300	419
3		- 246.1 (429)	404	+ 164.8 (386)	53 100	421
4		- 316.9 (427)	399	+ 165.1 (382)	58 400	421
1	$(\text{CH}_3)_2\text{SO}$	+ 3.6 (426)	-	+ 3.4 (381)	48 600	425
3		+ 23.0 (425)	385	- 5.8 (369)	55 900	425
4		- 178.9 (421)	394	+ 83.4 (379)	60 300	421
1	CH_2Cl_2	- 318.4 (440)	417	+ 208.6 (393)	38 400	424
3		- 319.2 (433)	407	+ 179.9 (389)	54 800	430
4		- 339.6 (427)	400	+ 182.3 (383)	58 100	424
1	CHCl_3	- 420.1 (442)	418	+ 245.7 (395)	38 700	421
3		- 337.3 (434)	407	+ 186.2 (389)	55 500	431
4		- 347.7 (431)	403	+ 187.2 (386)	57 600	428

^a All solutions of **1** contained 2% v/v of CH_3OH ; solutions of **3** and **4** contained 2% v/v of CHCl_3 ; ^b 0.1 M phosphate buffer $\text{pH } 7.40$

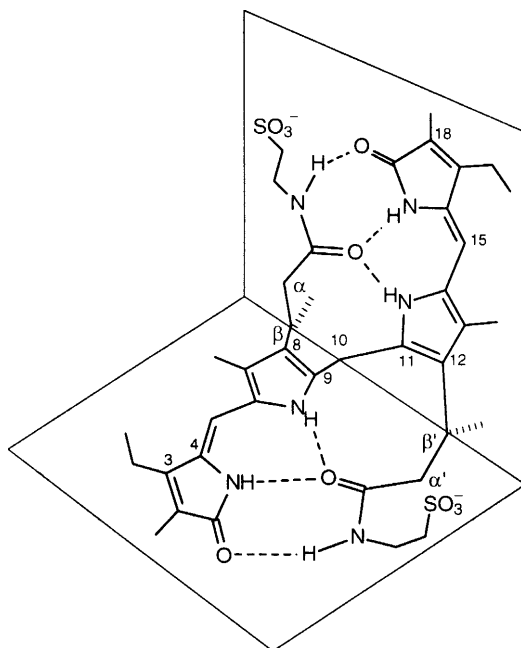


Fig. 5. Most stable 3-D conformational structure of **1**. Dashed lines are hydrogen bonds

with it, a less well-defined preference for the (*M*)-helical ridge-tile conformation. Similarly, in methanol and in dimethylformamide **1** again exhibited a strong negative chirality bisignate CD, but the CD spectra of **3** and **4** were more intense. In contrast, in nonpolar methylene chloride and chloroform solvents, the CD intensities of **1–3** were comparably very high. The data from *DMSO* have typically represented a special case of solvent insertion into the hydrogen bonding network [12, 17, 20]. Taken collectively and comparatively, the CD data point to a *strong preference* for the intramolecularly hydrogen-bonded ridge-tile conformation of ditaurorubin **1** (Fig. 5) in nonpolar solvents and the high probability of the same conformation in water and other polar solvents.

Experimental

Circular dichroism spectra were recorded on a Jasco J-600 spectropolarimeter, and UV-visible spectra on a Perkin Elmer Lambda 12 spectrophotometer. All solutions for CD and UV-vis measurements of **1** contained 2% (by volume) of CH₃OH and those of **3** and **4** contained 2% of CHCl₃. NMR spectra were recorded on Varian Unity Plus spectrometer operating at a proton frequency of 500 MHz. Chemical shifts are reported in δ (ppm) and referenced to the CHD₂SOCD₃ signal at 2.49 ppm (¹H) and (CD₃)₂SO at 39.50 ppm (¹³C). A *J*-modulated spin-echo experiment was used to obtain carbon multiplicities. Radial chromatography was carried out on Merck silica gel PF-254 with CaSO₄ 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 [21]. Isobutyl chloroformate was distilled prior to use, and the reactions were carried out under Ar and light protection. Sodium taurate was synthesized as previously described [10i].

Disodium bis-tauro-(βS,β'S)-dimethylmesobilirubinate-XIIIα (1, C₃₉H₅₂N₆O₁₀S₂Na₂)

To a suspension of 62 mg (0.1 mmol) of diacid **3** [12] in 4 cm³ of anhydrous THF and 84 mm³ (0.6 mmol) of Et₃N was added isobutyl chloroformate (77 mm³, 0.59 mmol), and the mixture was stirred for 1.5 h. Then it was transferred to a solution of 59 mg (0.4 mmol) of sodium taurate (prepared according to *Nuzzo et al.* [10i]) in 2 cm³ of anhydrous DMSO. The mixture was stirred for 1.5 h under aspirator vacuum with occasional heating to 35–40°C to remove THF. Then Et₃N (84 mm³, 0.6 mmol) was added, and stirring was continued at ambient temperature for 24 h. A solution of 50 mg (0.6 mmol) of NaHCO₃ in 0.3 cm³ of H₂O was added, and the mixture was kept under vacuum for 1 h. The residue was diluted with 1 cm³ of CH₃OH and 5 cm³ of CH₂Cl₂ and purified by radial chromatography (gradient 7–20% v/v CH₃OH in CH₂Cl₂). The most polar bright yellow fractions were combined, and the solid obtained after evaporation was triturated with 1 cm³ of CH₃OH and 5 cm³ of anhydrous Et₂O. The precipitate was collected by filtration and dried under vacuum at 50°C to afford **1**. Yield 79 mg (90%); mp > 260°C (dec); ¹³C and ¹H NMR in Tables 1 and 2; HRMS (FAB, 3-NBA): calcd. for bis-sodium salt: C₃₉H₅₂N₆O₁₀S₂Na₃ (M + Na)⁺ 897.2879; found: 897.2912, Δ = 3.2 mDa, error – 3.6 ppm.

Sodium tauro-β-methylxanthobilirubinate (2, C₂₀H₂₈N₃O₅SNa)

Following the same procedure as above for **1**, and using the same equivalent ratios, 0.2 mmol of β-methylxanthobilirubic acid [14] afforded **2**. Yield 77 mg (86%); mp > 260° (dec); HRMS (FAB, 3-NBA): calcd. for C₂₀H₂₈N₃O₅SNa₂ (M + Na)⁺ 468.1545; found 468.1539, Δ = 0.6 mDa, error 1.2 ppm. ¹³C and ¹H NMR in Tables 1 and 2.

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Bilirubin Ditaurate

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