On the Aggregation of Bilirubins using Vapor Pressure Osmometry

Stefan E. Boiadjiev and David A. Lightner*

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

Received April 17, 2000

Dedicated to the memory of the late Professor Raymond N. Castle.

Bilirubin and its analogs are carboxylic acids that engage in intramolecular hydrogen bonding and are thus thought to be monomeric in solution, although the evidence for the molecularity in solution is indirect. Contrastingly, the dimethyl esters favor intermolecular hydrogen bonding and are thought to be dimeric, yet they, like the bilirubin (acids), exhibit essentially no concentration dependence of their NH nmr chemical shifts upon dilution from 10^{-2} to 10^{-5} (or even 10^{-6}) *M* in chloroform-d. Vapor phase osmometry (vpo) studies of chloroform solutions of eight bilirubins and their dimethyl esters clearly indicate that the former are monomeric, while the latter are dimeric — except when a β-methyl group (but not an α*-methyl) is present in each methyl propionate chain. Bilirubin mono-esters might be monomeric or dimeric in solution. Using vpo to study some seven mono-esters or mono acids, we found that the pigments were monomeric in chloroform.

J. Heterocyclic Chem., **37**, 863 (2000).

Introduction.

Bilirubin (Figure 1) is a tetrapyrrole dicarboxylic acid formed in the normal metabolism of heme proteins [1, 2]. In a healthy adult, it is produced at the rate of ~300 mg per day, principally from the breakdown of red blood cells. Bilirubin is intrinsically unexcretable but is efficiently eliminated by the liver, following uptake and enzymic conversion to water-soluble glucuronides that are promptly secreted into bile. Impaired excretion of the glucuronides occurs in many types of hepatobiliary disease, but retention of native bilirubin is principally observed in newborn babies [1-3]. Accumulation of either native bilirubin or its glucuronides in the body is manifested in jaundice.

Bilirubin is a conformationally mobile bichromophore with characteristics of a molecular propeller. Rotation of its two dipyrrinone chromophores about the central C(10) CH_2 unit generates a large number of conformational isomers, of which the folded conformations, shaped like a ridge-tile have their non-bonded steric interactions minimized [4]. The ridge-tile conformation, which is not rigid, brings the pigment's propionic acid groups into close proximity of the dipyrrinone NH and C = O groups, thus easily engaging a network of six intramolecular hydrogen bonds to make the ridge-tile conformation unusually stable [4]. This inward tucking of the CO_2H groups and tethering to opposing dipyrrinones through intramolecular hydrogen bonding decreases the polarity of the pigment, leaving it unexcretable in normal metabolism (hepatic excretion), except by glucuronidation [1-3]. The ridge-tile conformation is found in crystalline bilirubin and its salts [5,6], and it is the favored conformation in solution [4,7,8]. However, when the propionic acid groups are translocated away from C(8) and C(12), *e.g.*, to C(7) and C(13), the solution properties of the pigment undergo significant changes [9]. Such pigments are less lipophilic than bilirubin and much less soluble in non-polar organic solvents. Analogs with propionic acid groups at C(8) and C(12), such as mesobilirubin-XIIIα (Figure 1B), typically mimic bilirubin's unique lipophilic properties and hepatic excretability

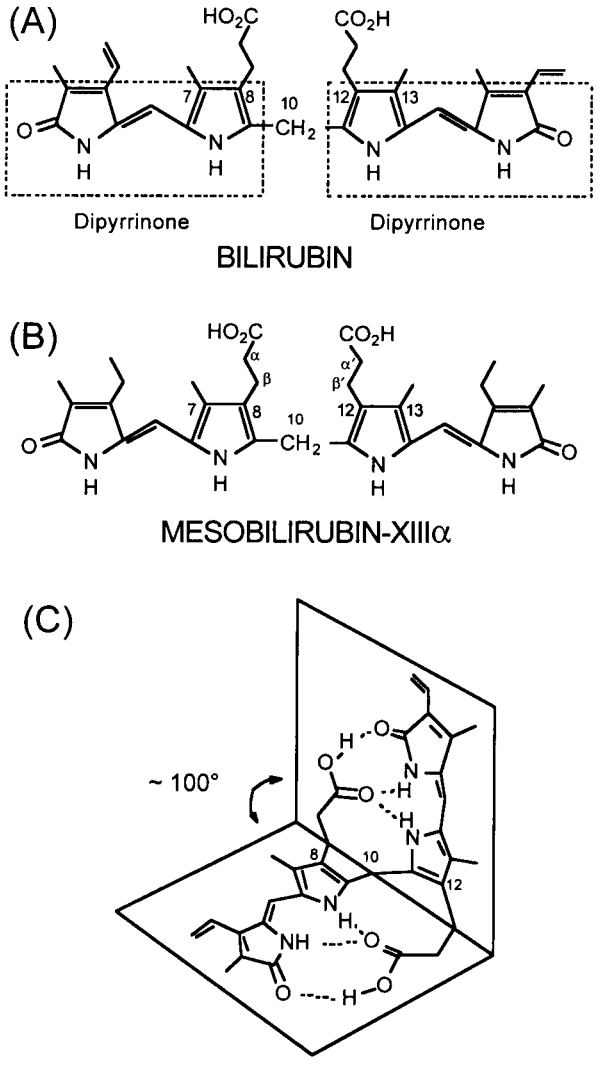


Figure 1. (A) Linear conformation of bilirubin; (B) linear conformation of mesobilirubin-XIIIα; (C) enantiomeric ridge-tile conformation of bilirubin stabilized by intramolecular hydrogen bonding. Mesobilirubin-XIIIα also preferentially adopts a very similar conformation. Hydrogen bonds are indicated by dashed lines.

because they also have their CO_2H groups sequestered by intramolecular hydrogen bonding (Figure 1C).

Bilirubin intramolecular hydrogen bonding is apparently driven by the avidity of its dipyrrinones to form hydrogen bonds, which is one of the most interesting and important facets of bilirubin conformation [4-10]. Even simple dipyrrinones such as kryptopyrromethenone and methyl xanthobilirubinate have a strong disposition toward hydrogen bonding and form dipyrrinone to dipyrrinone planar dimers (Figure 2A); whereas, xanthobilirubic acid and its homologs form π-stacked dimers involving acid to dipyrrinone hydrogen bonding (Figure 2B) characteristic of that found in bilirubin (Figure 1C) [4,10]. Dipyrrinone to dipyrrinone intermolecular hydrogen bonding is also thought to be important in bilirubin dimethyl ester, which forms a dimer in nonpolar solvents [11-16]. Our interest in (i) the role of dipyrrinones and carboxylic acid groups in the monomer-dimer equilibrium of bilirubins and (ii) stabilization of pigment stereochemistry through the action of intramolecular hydrogen bonding led us to determine for the first time the state of aggregation of bilirubins and their mono-esters [17] as well as bilirubin analogs with but one propionic acid group [18], or none [19]. Those studies also led us to evaluate the influence of propionic α and β-methyl substituents on the monomer-dimer equilibrium.

In the current study involving vapor pressure osmometry (vpo) measurements and proton nuclear magnetic resonance spectroscopy (^{1}H-nmr) of some eighteen related synthetic rubins and their esters, we present new information on the state of aggregation and the monomer-dimer equilibrium of bilirubins and their di- and monoesters in the hydrogen bond-promoting, nonpolar solvent chloroform. This comprehensive study reports the first systematic investigation of the monomer-dimer equilibrium in bilirubins by vpo.

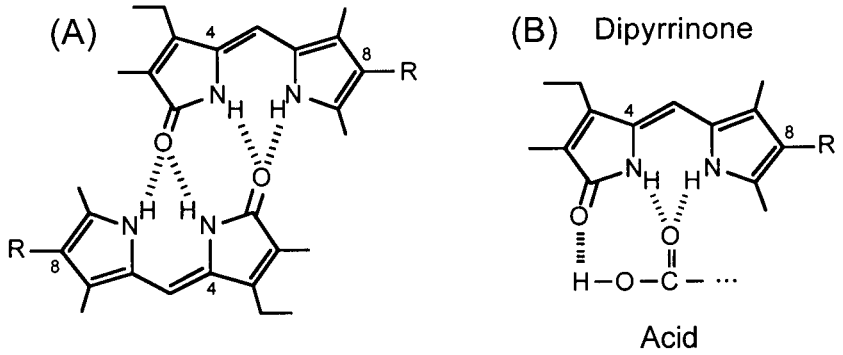


Figure 2. (A) Planar dipyrrinone to dipyrrinone hydrogen bonding found in bilirubin esters, rubins incapable of intramolecular hydrogen bonding and in many 4(Z)-dipyrrinones, such as kryptopyrromethenone (R=CH_2CH_3) and methyl xanthobilirubinate (R=$CH_2CH_2CO_2CH_3$). Hydrogen bonds are shown by hatched lines. (B) Acid to dipyrrinone hydrogen bonding found in bilirubin and its analogs with propionic acids at C(8) and/or C(12), and in 4(Z)-dipyrrinones with alkanoic acids at C(8), such as xanthobilirubic acid (R=$CH_2CH_2CO_2H$).

Results and Discussion.

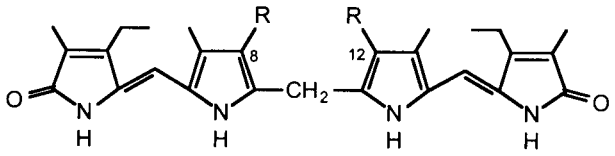
The apparent molecular weights of only a few dipyrrinones and bilirubins have been determined in solution [11]. Yet, such determinations are among the best methods available for detecting and quantitating aggregation phenomena. Vpo measurements by Falk *et al.* [11, 20] of methyl xanthobilirubinate and kryptopyrromethenone in chloroform solution (Figure 2) indicate dimer formation, with K_{dimer} ~1,700 M^{-1} at 37 °C, with experimentally determined molecular weights ~525 and ~500 grams/mole at 0.06-0.1 M concentrations, respectively. More recent studies involving ^{1}H- nmr analyses of the concentration dependence of the NH chemical shifts implicate higher values of K_{dimer} (~25,000 M^{-1} at 25 °C) for these and related dipyrrinones [10c]. Those studies clearly indicated an increased shielding of the pyrrole and lactam NH ^{1}H-nmr signals with dilution, *e.g.*, from 10.22 ppm to 9.54 ppm for the pyrrole NH when methyl xanthobilirubinate in chloroform-d is diluted from ~2 x 10^{-3} *M* to ~9 x 10^{-5} *M* at 22 °C; and from 10.10 ppm to 8.62 ppm for the lactam NH when diluted from ~8 x 10^{-4} *M* to ~7 x 10^{-5} *M* at 60 °C in 1,2-dideuteriotetrachloroethane. The drift to more shielded NH protons with decreasing concentration is large, despite a large K_{dimer}.

Using vpo, Falk *et al.* [11,12a] also determined the molecular weight of bilirubin dimethyl ester (formula weight 612.7 grams/mole) to be 850 ± 20 in chloroform and 1110 ± 30 in tetrahydrofuran (but 595 ± 30 in methanol) at 2 x 10^{-2} to 2 x 10^{-3} moles/kg. This preference for dimers was confirmed independently by Schaffner *et al.* [13] and by Kaplan and Navon [14]. A related tetrapyrrole, etiobilirubin-IVγ (formula weight 586.7 grams/mole) is incapable of intramolecular hydrogen bonding, and was determined to have a molecular weight of 966 ± 25 at concentrations 7 x 10^{-3} to 3 x 10^{-4} moles/kg in chloroform [11,12a]. More recently, Ribó *et al.* [16] showed by vpo that the dimethyl esters of mesobilirubin-IXα and XIIIα exhibited molecular weights corresponding to dimers, ~950 and ~1250, respectively.

We found it unusual that the expected concentration dependence [10c] of the pyrrole and lactam NH chemical shifts in the ^{1}H-nmr spectra of bilirubin dimethyl ester or mesobilirubin-XIIIα dimethyl ester [21] in chloroform-d was not detected. Unlike the increased shielding of the NHs observed with increasing dilution of the dipyrrinones kryptopyrromethenone and methyl xanthobilirubinate [10c], we

Table 1

Concentration Dependence of Pyrrole (P) and Lactam (L) NH Chemical Shifts of Bilirubin Analogs in Chloroform-d Solutions at 25 °C [a]



Conc. (*M*)	1 R = CH_2CH_3 L-NH	P-NH	2 R = $CH_2CH_2CO_2CH_3$ L-NH	P-NH	3 R = $CH_2CH_2CH_3$ L-NH	P-NH
$1x10^{-2}$	10.61	10.31	10.58	10.28	10.65	10.31
$1x10^{-3}$	10.61	10.31	10.58	10.28	10.65	10.31
$1x10^{-4}$	10.61	10.31	10.60	10.28	10.65	10.31
$1x10^{-5}$	10.61	10.31	10.59	10.30	10.65	10.31

Conc. (M)	4 R = $CH(CH_3)CH_2CO_2H$ L-NH	P-NH	5 R = $CH(CH_3)CH_2CH_3$ L-NH	P-NH	6 R = $CH(CH_3)CH_2CO_2CH_3$ L-NH	P-NH
$1x10^{-2}$	10.67	9.04	10.77	10.17	9.89 [b]	9.89
$1x10^{-3}$	10.67	9.04	10.77	10.17	9.62	~9.12(br)
$1x10^{-4}$	10.67	9.04	10.77	10.17	9.41	8.60
$1x10^{-5}$	10.67	9.04	10.77	10.16	9.37	8.56

[a] Entries 4-6 have the *S* configuration stereochemistry. [b] $7.5 x10^{-3}$ *M*.

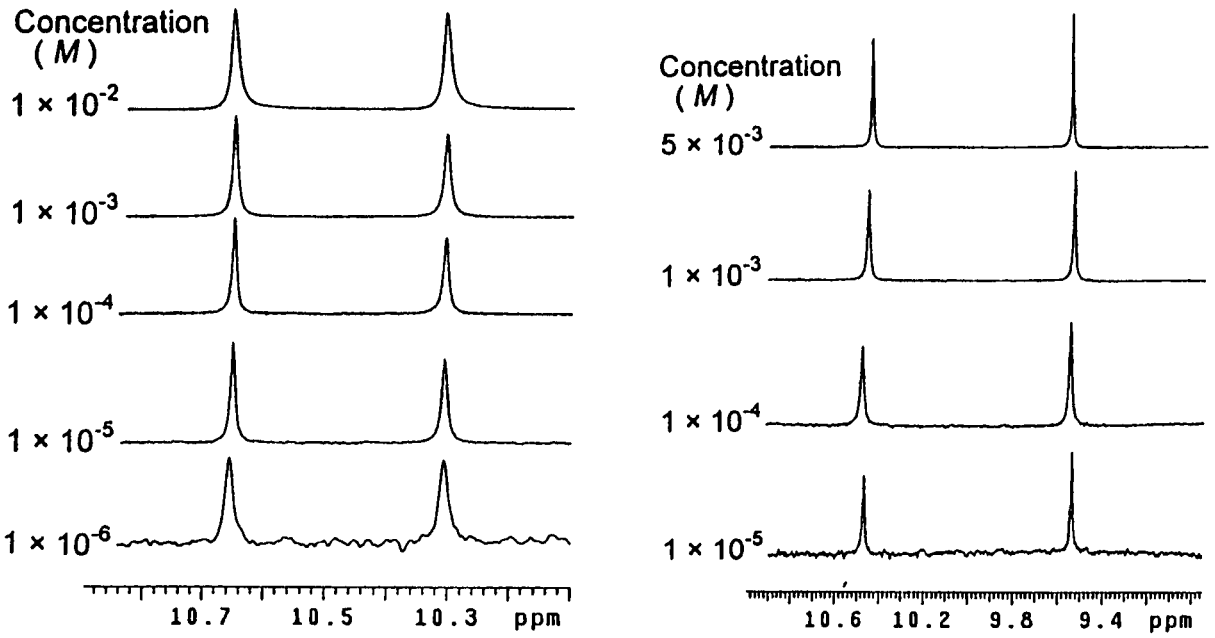


Figure 3. Partial ^{1}H-nmr of 8,12-des-ethyl-8,12-dipropyl-etiobilirubin-IVγ in chloroform-d (left) and etiobilirubin-IVγ in *N,N*-dimethylformamide-d_7 (right) showing the concentration invariance of the lactam (left) and pyrrole (right) NH signals. Molar concentrations are indicated on each spectrum.

could detect no changes in the NH chemical shifts of either etiobilirubin-IVγ (Table 1, entry 1) or mesobilirubin- XIIIα dimethyl ester (entry 2 of Table 1) with increasing dilution, from 1 x 10^{-2} *M* to 1 x 10^{-5} *M* concentrations in chloroform-d at 25 °C. This surprising result, confirmed also in the etiobilirubin analog with C(8) and C(12) propyl groups (Figure 3, left, and Table 1, entry 3) over the concentration range 1 x 10^{-2} *M* to 1 x 10^{-6} *M*, would appear to indicate that the K_{dimer} for these tetrapyrroles is very much greater than that (K_{dimer} ~25,000 M^{-1} at 25 °C) of the component dipyrrinones. Alternatively, the data are consistent with all three tetrapyrroles being monomers in solution.

In a more polar solvent, dimethylformamide-d_7, the lactam NH chemical shift (10.45 ppm) of etiobilirubin-IVγ is

quite close to that found in chloroform-d (Table 1), while the pyrrole NH chemical shift (9.55 ppm) is more shielded by ~0.75 ppm. Yet, there is essentially no variation in chemical shift over a concentration range 5 x 10^{-3} to 1 x 10^{-5} *M* (Figure 3, right). Whether in chloroform-d or dimethylformamide-d_7, if a monomer-dimer equilibrium is taking place, the association constant must be >> 10^6 M^{-1}.

Previous work indicated that bilirubins with propionic acids at C(8) and C(12) preferred an intramolecularly hydrogen-bonded conformation, and it was deduced that their solutions were probably monomeric [8b] because their NH chemical shifts do not vary with changes in concentration. The NH chemical shift data for (β*S*, β'*S*)-dimethylmesobilirubin-XIIIα [22a] (Table 1, entry 4) exemplify and reinforce this deduction. Like entries 1-3 of Table 1, it exhibits no changes in the NH shielding with varying concentration in chloroform-d. However, while the lactam NH chemical shifts are very similar in all four compounds, note that the pyrrole NH is strongly shielded in the rubin diacid, relative to the dimethyl ester or etiobilirubin-IVγ and its homolog. This difference is apparently due to differing conformations. The rubin acid adopts a ridge-tile shape, whereas the ester and etiobilirubin do not. When the carboxylic groups (which are crucial to maintaining an intramolecularly hydrogen-bonded ridge-tile conformation) are replaced with methyls (Table 1, entry 5), the pyrrole NH is again more deshielded and similar to that found in entries 1-3. When the acid is esterified, however, the dimethyl ester (Table 1, entry 6) exhibits strongly shielded dipyrrinone NHs, with chemical shifts that are atypical of either the intramolecularly hydrogen-bonded (Figure 1C) monomeric conformation (Table 1, entry 4) or the intermolecularly hydrogen bonded dimer (Table 1, entries 1-3 and 5). These data seem to suggest that the dimethyl ester of (β*S*,β'*S*)- dimethylmesobilirubin-XIIIα is neither a dimer nor an intramolecularly hydrogen-bonded monomer.

In an attempt to correlate the concentration invariance of the NH chemical shifts in bilirubin analogs in chloroform-d (Table 1) with pigment molecularity, to confirm monomeric species in the rubin acids and dimeric species in the rubin dimethyl esters, and to explore whether mono-esters are dimeric or monomeric, we determined the molecular weights of seventeen linear tetrapyrroles in chloroform solution by vpo. For calibration purposes, we re-measured the molecular weights of kryptopyrromethenone, methyl xanthobilirubinate, bilirubin dimethyl ester and etiobilirubin-IVγ in chloroform (Table 2), finding good agreement with the previous results that showed a strong preference for dimers. Consequently, if K_{dimer} is large for the dipyrrinones (~25,000 M^{-1} at 22 °C) [10c], then K_{dimer} for the tetrapyrroles must be enormous, given the observed concentration independence of the NH chemical shifts over the range 10^{-2} to 10^{-5} M in the latter. In contrast to the concentration-invariance of the NH chemical shifts in the rubins, large changes in NH chemical shifts are seen in the dipyrrinones upon dilution[10c].

Table 2

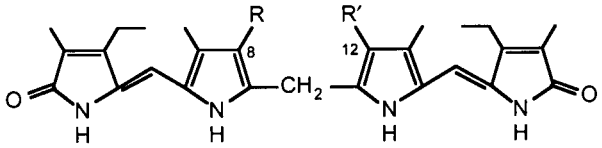
Molecular Weights of Dipyrrinones and Bilirubin Analogs Determined by Vapor Pressure Osmometry in Chloroform [a]

Compound	Formula Weight (grams/mole)	Measured Molecular Weight
Kryptopyrromethenone	258.3	509 [b]
Methyl xanthobilirubinate	316.3	579 [b]
Bilirubin-IXα dimethyl ester	612.7	1173 [c]
Etiobilirubin-IVγ	500.7	800 [d]

[a] Calibrated using benzil: measured molecular weight 207; formula weight 210. Measured molecular weights are ± 5% in this work, for ~1.5-5.6 x 10^{-3} *M* solutions at 45°C. Formula weights and measured molecular weights in grams/mole. [b] Reported previously to be dimeric in chloroform, with K_{dimer} ~1700 M^{-1} in refs. 10c and 11. [c] Previously determined as 850 ± 20 in chloroform, refs. 11 and 12a; as 1100 ± 10% in ref. 13; and that a 16.3 mM solution in chloroform at 30.0 ± 0.1 °C had an activity of 8.5 mM (± 5%) thus indicating a dimer, ref. 14. [d] Previously determined to be 966 ± 25 in ref. 12a.

Table 3

Molecular Weights of Bilirubin Analogs Determined by Vapor Pressure Osmometry in Chloroform [a]



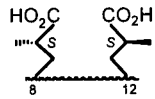
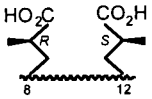
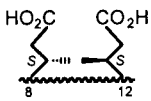
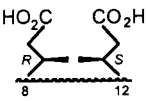
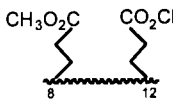
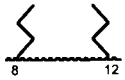
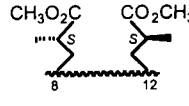
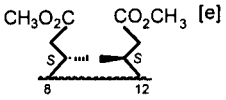
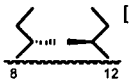
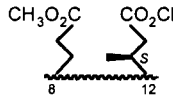
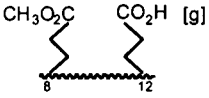
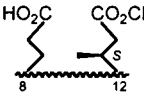
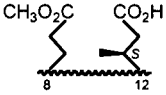
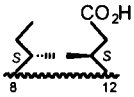
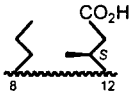
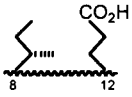
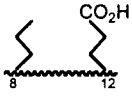
Entry	R	R'	Formula Weight	Measured Molecular Weight
1		[b]	616.7	612
2		[b]	616.7	680
3		[c]	616.7	626
4		[c]	616.7	671
5		[d]	616.7	1139
6			528.7	1027
7		[b]	644.8	1143

Table 3 (continued)

Entry	R	R'	Formula Weight	Measured Molecular Weight
8		[e]	644.8	664
9		[f]	556.7	1115
10			630.8	1030
11		[g]	602.7	578
12		[h]	616.7	628
13		[h]	616.7	624
14		[h]	586.7	606
15		[h]	572.7	600
16		[h]	572.7	565
17			558.7	574

[a] Measured molecular weights ± 5% for ~1.5-6.5 M solutions at 45°C. Formula weights and measured molecular weights in grams/mole. Calibrated using benzil: measured molecular weight 207; formula weight 210.2. [b] Reference 22b; [c] Reference 22a; [d] Reference 21; [e] Reference 24; [f] Reference 19a; [g] Reference 16a; [h] Reference 27.

The molecular weight of bilirubin in a nonpolar solvent has not been reported due to its limited high end solubility (~1 m*M*) in chloroform, the nonpolar solvent in which it is most soluble. Because of this limit, we could not achieve sufficient concentrations of bilirubin in chloroform to satisfactorily determine its molecular weight. In order to improve the solubility of bilirubin while preserving its ability to engage in intramolecular hydrogen bonding, we examined synthetic rubins that are somewhat more soluble in chloroform, namely analogs of mesobilirubin-XIIIα (Figure 1) with methyls at the α or β positions in the propionic acid chains [22]. These bilirubin analogs had been synthesized earlier in order to understand bilirubin stereochemistry through chiroptical measurements, and had confirmed the ridge-tile intramolecularly hydrogen-bonded conformations [22]. Vpo measurements of these compounds clearly indicate that the bilirubin pigments are monomeric in chloroform (Table 3, entries 1-4), and they provide an understanding for the observation that the NH chemical shifts remain invariant with changes in pigment concentration (Table 1). Interestingly, while α or β (*S, S*) and (*R, R*)-dimethyl mesobilirubins are known to participate in more effective hydrogen bonding than the *R, S*-isomers, the vpo data for the latter (Table 3, entries 2 and 4) indicate only slightly elevated molecular weights (670-680 grams/mole) over the (*S, S*)-isomers (entries 1 and 3) and thus only a very slight tendency to form dimers.

The data confirm the notion that the presence of two propionic acid groups, appropriately located on the bilirubin skeleton is sufficient to preserve an intramolecularly hydrogen-bonded ridge-tile conformation, thereby thwarting intermolecular hydrogen bonding and preventing the formation of dimers in nonpolar solvents. Absent such intramolecular hydrogen bonding to carboxylic acids, the dipyrrinones latch on to other dipyrrinones via intermolecular hydrogen bonding of the type shown in Figure 2A. Even though such concatenations might lead to polymeric arrays [23], bilirubin dimethyl ester is found to be only dimeric in chloroform (Table 2), as is its close analog, mesobilirubin-XIIIα dimethyl ester [16] (Table 3, entry 5). Although propionate ester groups acting independently are insufficient to sequester the dipyrrinones through intramolecular hydrogen bonding, the pigment's conformation seems to be more amenable to dimer as opposed to polymer formation [16]. Consistent with this behavior, when the propionic esters are replaced with propyl groups, the pigment is also dimeric (Table 3, entry 6), as was found with etiobilirubin-IVγ [11, 12a].

The presence of α-methyl groups on the propionic acid chain has been shown to play an important role in directing and preserving the ridge-tile stereochemistry in a rubin diacid [22b]; however, the methyls do not interfere with the formation of a dimer by the dimethyl ester (Table 3, entry 7). Surprisingly perhaps, but consistent with earlier circular dichroism (cd) studies [24], when methyl groups are located at the β-position of each propionate ester chain, as in the dimethyl ester of (β*S*, β'*S*)-dimethylmesobilirubin-XIIIα, the pigment is clearly a monomer in chloroform (Table 3, entry 8). This unanticipated and interesting result in dimethyl esters suggests that while α-methyls have little influence, β-methyls can act in gear-like fashion to maintain a ridge-tile conformation and guide the carbomethoxy carbonyl groups into position for intramolecular hydrogen

bonding with the dipyrrinone NHs. The two β-methyls apparently act in concert with the carbomethoxy groups to dictate a conformation that discourages dimer formation, because when the carbomethoxy groups are replaced by methyls, as in the rubin analog with *sec-* butyl groups (entry 9, Table 3), the pigment is dimeric in chloroform. Thus, although the presence of β-methyls may become an important factor in preserving a ridge-tile shape in bilirubin analogs when carboxylic acid esters are also present, absent such polar termini, the β-methyls do not alone prevent intermolecular hydrogen bonding. The balance between dimer formation *vs* monomer preference must be delicate, as with only one β-methyl, mesobili rubin-XIIIα dimethyl ester tends strongly toward dimeric (entry 10, Table 3).

The shape of the bilirubin dimethyl ester dimer was reflected on earlier [7a,14-16]. Using molecular mechanics calculations, we investigated the role of intermolecular and intramolecular hydrogen bonding in forming energy-minimized dimers. The results of such calculations indicate a strong preference for a dimeric structure in which dipyrrinone to dipyrrinone intermolecular hydrogen bonds predominate. The component rubin esters adopt a porphyrin-like helical conformation rather than a ridge-tile and lie on intersecting planes (Figure 4). Such a dimer is consistent with that proposed by Kaplan and Navon [7a, 14] based on ^{1}H-nmr studies in chloroform-d, and it is somewhat similar to the dimeric structure proposed recently by Ribó *et al.* [16] for the mesobilirubin cyclic ester (with propane-1,3-diol).

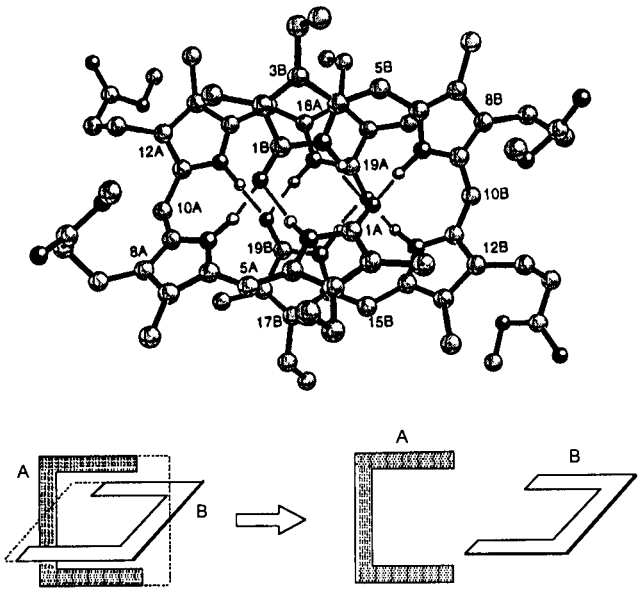


Figure 4. (upper) Ball and Stick representation of the energy-minimized, intermolecularly hydrogen-bonded dimeric conformation of mesobilirubin-XIIIα dimethyl ester. Each dipyrrinone of an **A** molecule is hydrogen bonded to each dipyrrinone in a **B** molecule, with hydrogen bonds shown by hatched lines. (lower) Schematic showing the two esters (**A** and **B**) oriented in orthogonal planes for dimer formation.

There have been no reports of vpo measurements of bilirubin monomethyl esters and thus no information is available on their molecularity in solution. Previous studies involving ^{1}H-nmr and cd spectroscopy [18,19,25] showed that a single propionic acid group was sufficient to maintain a ridge-tile conformation through intramolecular hydrogen bonding with an opposing dipyrrinone, leaving the remaining dipyrrinone free to engage in intermolecular hydrogen bonding to another dipyrrinone. Our vpo results show clearly that mesobilirubin-XIIIα monomethyl ester (Table 3, entry 11) and the corresponding monoesters with a single β-methyl group in the propionic ester (entry 12) or acid (entry 13) chain are monomeric. Thus, when one of the two propionic acids at C(8) and C(12) of the rubin is esterified, irrespective of whether the propionic acid or ester chain has a β-methyl substituent, the remaining propionic acid suffices to stabilize a ridge-tile conformation and prevent intermolecular hydrogen bonding in the "free" dipyrrinone. Whether that dipyrrinone is engaged in intramolecular hydrogen bonding to the ester may be unclear, but it is apparently unnecessary for preventing dimer formation because when the ester group is replaced by a hydrocarbon group (Table 3, entries 14-16), dimers are not observed. The data indicate that by folding into and maintaining a ridge-tile conformation the formation of intermolecular hydrogen bonds in the tetrapyrrole is prevented, even if one dipyrrinone is free to form them.

EXPERIMENTAL

General Procedures.

Nuclear magnetic resonance (nmr) spectra were obtained on a Varian Unity Plus spectrometer operating at 500 MHz (for ^{1}H-nmr) in chloroform-d solvent (unless otherwise noted), and chemical shifts were reported in δ ppm referenced to the residual chloroform ^{1}H signal at 7.26 ppm and $CDCl_3$ ^{13}C at 77.00 ppm. J-modulated spin-echo experiments were used to obtain and assign ^{13}C-nmr spectra. Radial chromatography was carried out on Merck silica gel PF_{254} with calcium sulfate binder preparative layer grade, using a Chromatotron (Harrison Research, Inc., Palo Alto, CA) with 1, 2, or 4 mm rotors. Melting points were determined on a Mel-Temp capillary apparatus and are uncorrected. Combustion analyses were carried out by Desert Analytics, Tucson, AZ. Vapor pressure osmometry measurements were performed on an OSMOMAT 070-SA instrument (Gonotec GmbH, Germany) in chloroform (Allied Signal, Burdick & Jackson, hplc grade with amylene preservative, treated with activated basic alumina just before use) at 45 °C.

Commercial reagents were used as received from Aldrich or Acros and high performance liquid chromatography grade solvents (Fisher) were dried and distilled prior to use according to standard procedures [26].

3, 17-Dimethyl-8, 12-bis(1-propyl)-2, 7, 13, 18-tetramethyl-(21*H*, 24*H*)-bilin- 1,19-dione.

A mixture of 681 mg (2.5 mmoles) of 4-ethyl-9-(1-propyl)-3, 8, 10-trimethyl-2-oxo-1,11-dihydrodipyrrinone [18a], 1.54 g (6.25 mmoles) of *p*-chloranil, 550 ml of dichloromethane, and 25 ml of formic acid was heated at reflux for 24 hours. The mixture volume

was reduced by distillation to one half and reflux was continued for 6 hours. Then the mixture was chilled overnight at -20 °C, and the solid that separated was removed by filtration and washed with cold dichloromethane. The blue cold filtrate was neutralized carefully with 5% aqueous sodium bicarbonate, and the organic layer was washed with 2 (100 ml of 4% sodium hydroxide, followed by 4 (100 ml of water. The organic layer was dried (anhydrous sodium sulfate) and filtered, and the solvent was removed under vacuum. The residue was purified by radial chromatography on silica gel (eluent 2-4% methanol in dichloromethane) to afford 538 mg (82%) of bright blue 8,12-des-ethyl-8,12-di-*n*-propyl-etiobiliverdin-IVγ. It had mp 256-258 °C; ^{1}H-nmr: δ 0.96 (6H, t, J = 7.4 Hz), 1.22 (6H, t, J = 7.6 Hz), 1.57 (4H, m), 1.83 (6H, s), 2.07 (6H, s), 2.52 (4H, q, J = 7.6 Hz), 2.55 (4H, t, J = 7.4 Hz), 5.95 (2H, s), 6.63 (1H, s), 8.17 (2H, br.s), 10.54 (1H, br.s) ppm; ^{13}C-nmr: δ 8.3, 9.6, 13.9, 14.5, 17.9, 24.5, 26.5, 96.4, 114.1, 127.6, 128.2, 139.6, 139.8, 141.5, 146.7, 149.8, 172.4 ppm.

Anal. Calcd. for $C_{33}H_{42}N_4O_2$ (526.7): C, 75.25; H, 8.03; N, 10.62. Found: C, 75.46; H, 8.30; N, 10.64.

3,17-Diethyl-8,12-bis(1-propyl)-2,7,13,18-tetramethyl-(10*H*, 21*H*,23*H*,24*H*)-bilin-1,19-dione.

To a cooled (0 °C) solution of 132 mg (0.25 mmole) of the verdin above in 50 ml of dry deoxygenated tetrahydrofuran under nitrogen was added 946 mg (25 mmoles) of sodium borohydride followed by slow addition of 25 ml of dry methanol. The temperature was slowly raised to ambient and after 45 minutes stirring, water/ice (150 ml) was added followed by careful acidification with 10% hydrochloric acid. The product was extracted with chloroform (4 x 75 ml), which was washed with water (4 x 100 ml), dried (anhydrous sodium sulfate), filtered and evaporated under vacuum. The residue, after radial chromatography purification (eluent 1.5-3.0% methanol in dichloromethane) and recrystallization from chloroform-methanol, gave 106 mg (80%) of bright yellow 8,12-des-ethyl-8,12-di-*n*-propyl-etiobilirubin-IVγ (Table 3, entry 6). It had mp 269-272 °C (dec.); ^{1}H-nmr: 0.97 (6H, t, J = 7.3 Hz), 1.00 (6H, t, J = 7.6 Hz), 1.47 (6H, s), 1.51 (4H, m), 2.09 (6H, s), 2.33 (4H, q, J = 7.6 Hz), 2.50 (4H, t, J = 7.7 Hz), 4.10 (2H, s), 5.93 (2H, s), 10.31 (2H, s), 10.66 (2H, s) ppm; ^{13}C-nmr: δ 7.8, 9.8, 14.1, 14.8, 17.8, 22.8, 24.5, 26.7, 100.3, 120.7, 122.9, 123.3, 123.6, 128.6, 131.1, 146.8, 174.2 ppm.

Anal. Calcd. for $C_{33}H_{44}N_4O_2$ (528.7): C, 74.96; H, 8.39; N, 10.60. Found: C, 74.77; H, 8.13; N, 10.45.

3,17-Diethyl-8-(2-methoxycarbonylethyl)-12-(2-methoxycarbonyl-1-methylethyl)-2,7,13,18-tetramethyl-(10*H*,21*H*,23*H*,24*H*)-bilin-1,19-dione.

A solution of 77 mg (0.125 mmoles) of 8-(2-carboxyethyl)-3, 17-diethyl-12-(2-methoxycarbonyl-1-methylethyl)-2,7,13,18-tetramethyl-(10*H*,21*H*,23*H*,24*H*)-bilin-1,19-dione [27] in 25 ml of dry deoxygenated chloroform was treated with a slight excess of freshly prepared ethereal diazomethane, and the reaction was followed by thin layer chromatography. After stirring for 15 minutes, the solvent was evaporated under vacuum, and the residue was purified by radial chromatography on silica gel (eluent 1-3% methanol in dichloromethane). Recrystallization from methanol/ether afforded 70 mg (89%) of dimethyl ester (entry 10, Table 3). It had mp 273-275 °C (dec.); ^{1}H-nmr: δ 1.00 (3H, t, J = 7.6 Hz), 1.01 (3H, t, J = 7.6 Hz), 1.34 (3H, d, J = 7.1 Hz), 1.44 (3H, s), 1.46 (3H, s), 2.10 (3H, s), 2.18 (3H, s), 2.33 (4H, q, J = 7.6 Hz), 2.52 (2H, m), 2.69, 2.70 (2H, AB, ^{2}J = 15.1 Hz), 2.90 (2H, m), 3.52 (1H, m), 3.58 (3H, s), 3.69 (3H, s), 4.11, 4.20 (2H, AB, ^{2}J = 15.9 Hz), 5.90 (2H, s), 10.20 (2H, br.s) 10.56 (2H, br.s) ppm; ^{13}C-nmr: δ 7.6(7), 7.7(2), 9.7, 11.0, 14.7, 17.8, 20.0, 20.7, 22.7, 28.0, 35.4, 41.5, 51.4, 51.6, 99.8, 100.0, 118.7, 121.8, 122.7, 122.8, 123.5(3), 123.5(4), 123.9, 124.2, 128.9, 129.0, 130.4, 131.0, 147.0, 173.2, 173.8, 174.1 ppm.

Anal. Calcd. for $C_{36}H_{46}N_4O_6$ (630.8): C, 68.55; H, 7.35; N, 8.88. Found: C, 68.74; H, 7.54; N 8.86.

Acknowledgment.

We thank the U.S. National Institutes of Health (HD 17779) for generous support of this work. Prof. S. E. Boiadjiev is on leave from the Institute of Organic Chemistry, Bulgarian Academy of Sciences. We thank Prof. T. W. Bell for use of the vapor pressure osmometer.

REFERENCES AND NOTES

[1] A. F. McDonagh, Bile Pigments: Bilatrienes and 5, 15-Biladienes, in The Porphyrins, Vol **VI**, D. Dolphin, ed, Academic Press, New York, 1979, Chapter 6, p 293.

[2] J. R. Chowdhury, A. W. Wolkoff, N. R. Chowdhury and I. M. Arias, Hereditary Jaundice and Disorders of Bilirubin Metabolism, in The Metabolic and Molecular Bases of Inherited Disease, Vol **II**, C. R. Scriver, A. L. Beaudet, W. S. Sly and D. Valle, eds, McGraw-Hill Inc., New York, 1995, p 2161.

[3a] A. F. McDonagh, D. A. Lightner, *Pediatrics*, **75**, 443 (1985); [b] A. F. McDonagh, D. A. Lightner, *Seminars in Liver Disease*, **8**, 272 (1988).

[4] R. V. Person, B. R. Peterson and D. A. Lightner, *J. Am. Chem. Soc.*, **116**, 42 (1994).

[5a] R. Bonnett, J. E. Davies, M. B. Hursthouse and G. M. Sheldrick, *Proc. R. Soc. London*, **B202**, 249 (1978); [b] G. LeBas, A. Allegret, Y. Mauguen, C. DeRango and M. Bailly, *Acta Crystallogr., Sect. B*, **B36**, 3007 (1980); [c] W. Becker and W. S. Sheldrick, *Acta Crystallogr., Sect. B*, **B34**, 1298 (1978).

[6] A. Mugnoli, P. Manitto and D. Monti, *Acta Crystallogr., Sect. C*, **C38**, 1287 (1983).

[7a] D. Kaplan and G. Navon, *Israel J. Chem.*, **23**, 177 (1983); [b] D. Kaplan and G. Navon, *Org. Magn. Reson.*, 198 (1983); [c] D. Kaplan and G. Navon, *Biochem. J.*, **201**, 605 (1982).

[8a] D. Nogales and D. A. Lightner, *J. Biol. Chem.*, **270**, 73 (1995); [b] T. Dörner, B. Knipp and D. A. Lightner, *Tetrahedron*, **53**, 2697 (1997).

[9] F. R. Trull, R. W. Franklin and D. A. Lightner, *J. Heterocyclic Chem.*, **24**, 1573 (1987).

[10a] S. E. Boiadjiev, D. T. Anstine and D. A. Lightner, *J. Am. Chem. Soc.*, **117**, 8727 (1995); [b] S. E. Boiadjiev, D. T. Anstine, E. Maverick and D. A. Lightner, *Tetrahedron: Asymmetry*, **6**, 2253 (1995); [c] D. F. Nogales, J-S. Ma and D. A. Lightner, *Tetrahedron*, **49**, 2361 (1993).

[11] For leading references, see H. Falk, The Chemistry of Linear Oligopyrroles and Bile Pigments, Springer Verlag, New York and Vienna, 1989.

[12a] H. Falk, T. Schlederer and P. Wolschann, *Monatsh. Chem.*, **112**, 199 (1981); [b] H. Falk and N. Müller, *Monatsh. Chem.*, **113**, 111 (1982).

[13] A. R. Holzwarth, E. Langer, H. Lehner and K. Schaffner, *Photochem. Photobiol.*, **32**, 17 (1980).

[14] D. Kaplan and G. Navon, *Org. Magn. Reson.*, **17**, 79 (1981).

[15] D. A. Lightner and F. R. Trull, *Spectroscopy Lett.*, **16**, 785 (1983).

[16] J. Crusats, A. Delgado, J-A. Farrera, R. Rubires and J. M. Ribó, *Monatsh. Chem.*, **129**, 741 (1998).

[17] D. A. Lightner and J-S. Ma, *Spectroscopy Lett.*, **17**, 317 (1984).

[18a] S. E. Boiadjiev and D. A. Lightner, *Tetrahedron: Asymmetry*, **8**, 3603 (1997); [b] D. L. Holmes and D. A. Lightner, *J. Heterocyclic Chem.*, **32**, 113 (1995); [c] S. E. Boiadjiev and D. A. Lightner, *Tetrahedron: Asymmetry*, **10**, 4321 (1999).

[19a] S. E. Boiadjiev, W. P. Pfeiffer and D. A. Lightner, *Tetrahedron*, **53**, 14547 (1997); [b] S. E. Boiadjiev and D. A. Lightner, *J. Heterocyclic Chem.*, **36**, 969 (1999).

[20] H. Falk, K. Grubmayr, G. Höllbacher, O. Hofer, A. Leodolter, F. Neufingerl and J. M. Ribó, *Monatsh. Chem.*, **108**, 1113 (1977).

[21] D. P. Shrout, G. Puzicha, D. A. Lightner, *Synthesis*, 328-332 (1992).

[22a] S. E. Boiadjiev, R. V. Person, G. Puzicha, C. Knobler, E. Maverick, K. N. Trueblood and D. A. Lightner, *J. Am. Chem. Soc.*, **114**, 10123 (1992); [b] G. Puzicha, Y-M. Pu and D. A. Lightner, *J. Am. Chem. Soc.*, **113**, 3583 (1991).

[23a] M. Bernett, A. K. Tipton, M. T. Huggins, J. H. Reeder and D. A. Lightner, *Monatsh. Chem.* **131**, 239-249 (2000); [b] M. T. Huggins, A. K. Tipton and D. A. Lightner, *Monatsh. Chem.*, (2000) in press.

[24] S. E. Boiadjiev, D. T. Anstine and D. A. Lightner, *Tetrahedron: Asymmetry*, **5**, 1945 (1994).

[25] M-H. Zhang and D. A. Lightner, *Tetrahedron Lett.*, **28**, 4033 (1987).

[26] W. L. F. Armarego and D. D. Perrin, Purification of Laboratory Chemicals, 4th Ed. Butterworth-Heinemann, Oxford, England, 1997.

[27] S. E. Boiadjiev and D. A. Lightner, *Tetrahedron: Asymmetry*, **10**, 2535 (1999).