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SYNTHESIS AND UNUSUAL PROPERTIES OF AN 8,12-BIS-PIVALIC ACID ANALOG OF BILIRUBIN

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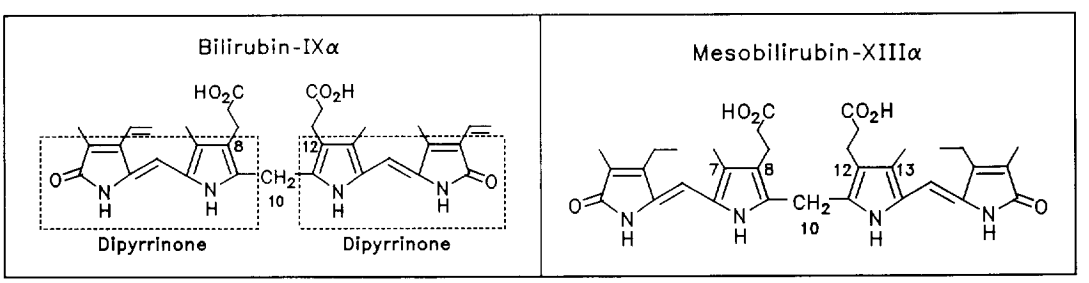
Abstract: A sterically congested analog of bilirubin with propionic acid groups replaced by pivalic acids (**1**) was synthesized from methyl 3-(2,4-dimethyl-5-ethoxycarbonyl-1*H*-pyrrol-3-yl)-2-methylpropionate (**8**). UV-visible and NMR spectroscopic analyses of **1** suggest intramolecular hydrogen-bonding and a preference for a ridge-tile conformation. The activation parameters for $M \rightleftarrows P$ conformational inversion of **1** were determined by dynamic NMR analysis to be $\Delta H^{\ddagger}$ 19.7 ± 1.4 kcal/mole and $\Delta S^{\ddagger}$ + 10.4 ± 4.5 eu. Molecular dynamics computations predict a global energy minimum for a somewhat more open ridge-tile conformation as compared with bilirubin. Circular dichroism of the pigment complex with human serum albumin gives a bisignate Cotton effect: $\Delta\epsilon_{431}^{max} = -51$, $\Delta\epsilon_{382}^{max} = +30$, with the opposite signed order as compared with that found for the parent mesobilirubin-XIIIα and bilirubin.

INTRODUCTION

Complicated, structurally interesting linear tetrapyrroles such as the natural products bilirubin and biliverdin are formed in animal metabolism from normal turnover of hemoglobin and other heme proteins.[1-3] Considerable effort has been devoted to understanding the properties and metabolism of bilirubin, with particular attention being focussed on its unique ability to fold into a conformation where the carboxylic acid groups embrace the opposing dipyrrinones in intramolecular hydrogen bonding.[4-6] This decreases the polarity of the pigment and renders it unexcretable in normal metabolism, except by glucuronidation.[2,3,7] Recently it has become evident that translocation of the pigment's propionic acid groups away from the natural locations at C(8) and C(12) leads to pigments which were more polar than bilirubin and do not require glucuronidation for hepatic excretion.[7,8] However, bilirubin analogs with propionic acid groups at C(8) and C(12), *e.g.*, mesobilirubin-XIIIα, typically exhibit the same unique polarity and excretability properties as bilirubin. These pigments, like bilirubin, tuck their carboxylic acid groups inward, where they are tethered to an opposing dipyrrinone by intramolecular hydrogen bonding shown in Figure 1.



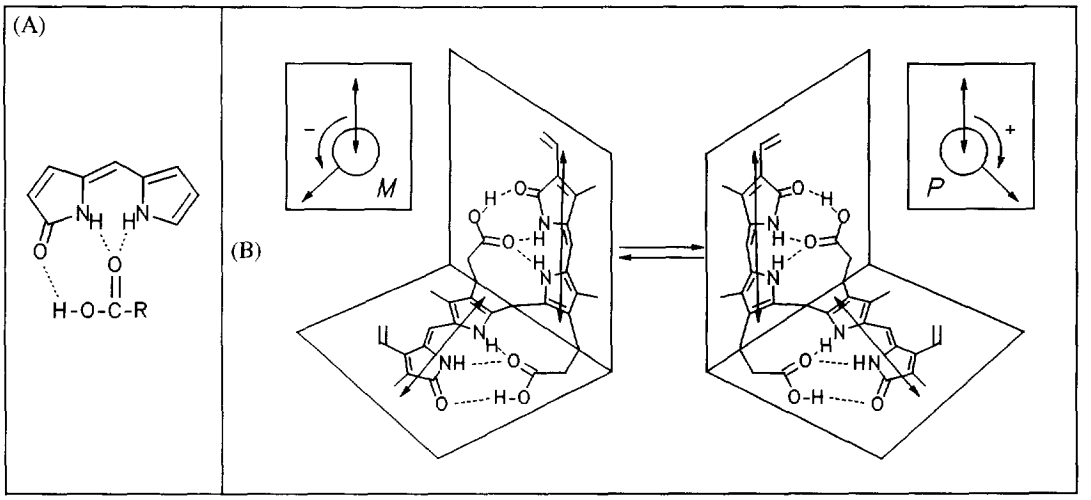
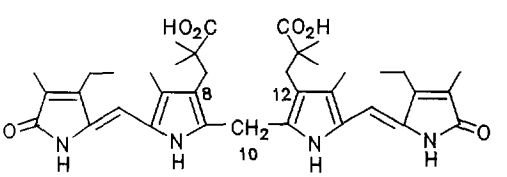


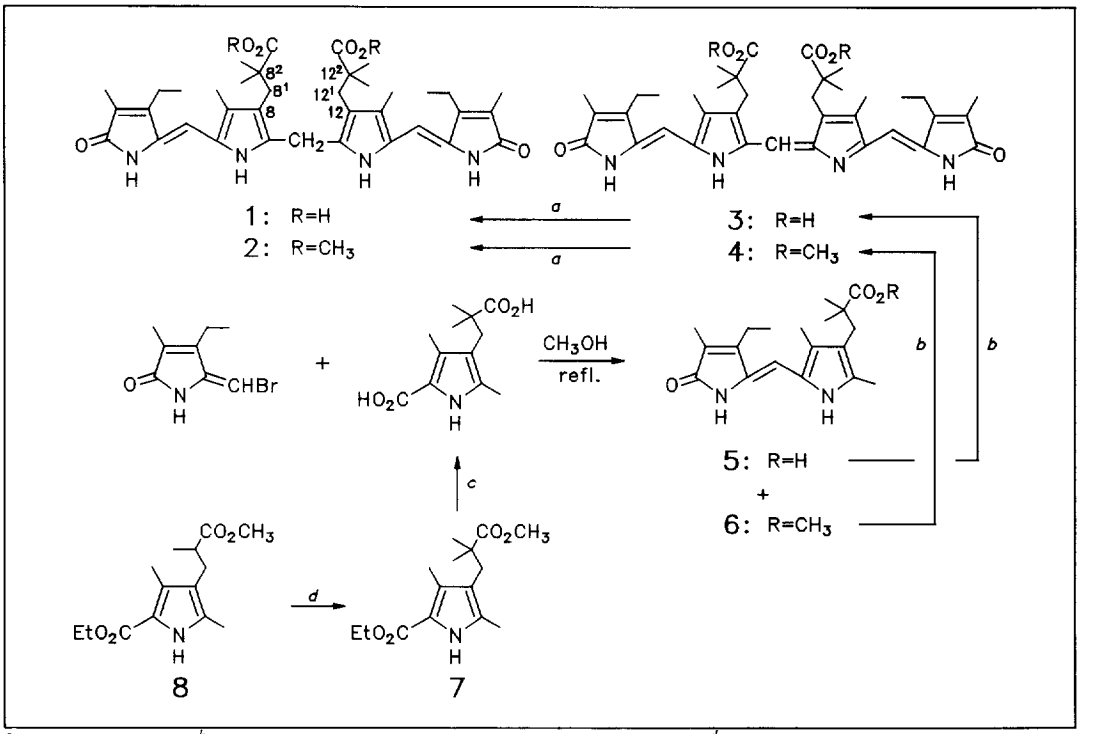
FIGURE 1. (A) Partial structure showing a dipyrrinone fragment hydrogen bonded to a carboxylic acid group. (B) Interconverting intramolecularly hydrogen-bonded enantiomeric conformers of bilirubin-IXα. The double headed arrows represent the dipyrrinone long wavelength electric transition moment vectors (dipoles). The relative helicities (*M*, minus or *P*, plus) of the vectors are shown (inset) for each enantiomer.

The intramolecular hydrogen bonding depicted in Figure 1 is one of the most interesting and important facets of bilirubin structure.[1,4-6,9,10] Although the two component dipyrrinone units of bilirubinoid molecules may rotate relatively freely and independently about the interconnecting central -CH_2- group, two conformations are uniquely stabilized through an extensive network of intramolecular hydrogen bonds.[10] The two conformations of Fig. 1B are non-superimposable mirror images, which (for bilirubin) are known to interconvert fairly rapidly at room temperature over a barrier of ~20 kcal/mole.[5,6] Our interest in pigment stereochemistry that is stabilized by intramolecular hydrogen bonding between propionic acid and dipyrrinone groups led us to consider: (1) whether a full complement of hydrogen bonds might be retained in a bilirubin analog with both propionic acid groups replaced by more sterically demanding alkanoic acid groups, and (2) how altered hydrogen bonding might affect the conformation of the pigment. In the following, we report on the synthesis, properties and conformational analysis of a new, symmetric analog (**1**) of mesobilirubin-XIIIα with pivalic acid groups replacing the conventional propionic acids at C(8) and C(12). The spectral properties of **1** are compared to those of mesobilirubin-XIIIα and used, along with molecular dynamics simulations in a stereochemical analysis to define the most stable conformations. Variable temperature NMR analysis is used to determine kinetic parameters associated with the $\boldsymbol{M} \rightleftarrows \boldsymbol{P}$ conformational inversion.

$8^2,8^2,12^2,12^2$-Tetramethyl-mesobilirubin-XIIIα (**1**)



Synthetic Scheme



[a] $NaBH_4$, then HCl; [b] *p*-chloranil, HCOOH, CH_2Cl_2; [c] NaOH, then HNO_3; [d] LDA, CH_3I.

RESULTS AND DISCUSSION

Synthesis. Mesobilirubin-XIIIα bis-pivalic acid (**1**) was prepared in five steps as outlined in the Synthetic Scheme. Introduction of the first α-methyl group was achieved early, in the preparation of **8**. This key synthetic intermediate had been prepared in earlier studies following a conventional Fischer pyrrole synthesis by reaction of ethyl acetoacetate with methyl 4-acetyl-2-methyl-5-oxo-hexanoate.[11] The latter was prepared by a Michael addition of pentane-2,4-dione to methyl methacrylate. Introduction of the second α-methyl group was accomplished by alkylation of pyrrole **8**. Reaction of **8** with a 2.5-fold excess of LDA in THF at −40°C followed by treatment with methyl iodide afforded the *gem*-dimethyl pyrrole ester **7** in 93% yield. Saponification of **7** followed by careful acidification in cold aq. $NaNO_3$-HNO_3 afforded the corresponding diacid in quantitative yield. Reaction of the diacid with the bromomethylenepyrrolinone shown, a standard reaction[12] for forming dipyrrinones, gave a mixture of dipyrrinone acid **5** and its methyl ester **6**. Typically, the reaction conditions lead to high yields of dipyrrinone ester, but with the presence of more sterically hindered pivalic acids, only a 22% yield of methyl ester **6** was obtained, along with a 50% yield of acid **5**, which were separated by chromatography. Dipyrrinones **5** and **6** were oxidatively coupled using *p*-chloranil[13] to give the corresponding verdins, **3** and **4**, respectively. Verdin diacid **3** was reduced directly with $NaBH_4$ to give rubin **1**; the verdin diester **4** was converted similarly to the rubin dimethyl ester (**2**).

Polarity from Chromatographic Behavior.

Mesobilirubin-XIIIα bis-pivalic acid **1** has a longer retention time (~ 19.1 minutes) when coinjected with its parent mesobilirubin-XIIIα (~ 11.5 minutes) on reverse phase HPLC, suggesting that **1** is more lipophilic than the parent. However, on silica gel TLC, **1** has a smaller R_f value (0.24) when compared with the mesobilirubin-XIIIα standard (R_f=0.78) using 1.5% CH_3OH in CH_2Cl_2 as eluant, suggesting that it is more polar. It is difficult to reconcile these conflicting results, except to note that **1** is more soluble in chloroform than its parent, and in this sense, it is probably less polar.

TABLE 1. Mesobilirubin Lactam and Pyrrole N-*H* Chemical Shifts[a] in $CDCl_3$ and $(CD_3)_2SO$.[b]

Pigment	$CDCl_3$			$(CD_3)_2SO$		
	Lactam	Pyrrole	CO_2H	Lactam	Pyrrole	CO_2H
Bis-pivalic Rubin (**1**)	10.41	9.06	13.53	9.87	10.02	12.11
Mesobilirubin-XIIIα	10.59	9.15	13.64	9.72	10.27	11.83
Etiobilirubin-IVγ	10.58	10.28	—	9.78	10.28	—
Bis-pivalic Rubin Ester (**2**)	10.69	10.36	—	9.87	10.06	—
Mesobilirubin-XIIIα Dimethyl Ester	10.54	10.27	—	9.74	10.40	—

[a] δ, ppm downfield from $(CH_3)_4Si$; [b] Run as 10^{-2} *M* $(CD_3)_2SO$ and 10^{-3} *M* $CDCl_3$ solutions at 22°C.

NMR Analysis and Intramolecular Hydrogen Bonding.

The ^{1}H-NMR chemical shifts of the pyrrole and lactam N-*H*s have proven to be an excellent way to determine whether the dipyrrinone units of rubins are involved in intramolecular hydrogen bonding.[14,15] Previous studies of bilirubin pigments have shown that the pyrrole N-*H* appears near 9.2 δ in $CDCl_3$ solvent (*e.g.* for mesobilirubin-XIIIα) when the dipyrrinone and carboxylic acid groups are linked through intramolecular hydrogen bonds, as shown in Fig. 1.[14,15] The ^{1}H-NMR data for **1** in $CDCl_3$ reveal a pyrrole N-*H* chemical shift characteristic of intramolecular hydrogen bonding. In fact, the pyrrole N-*H* chemical shifts of **1** and mesobilirubin-XIIIα in $CDCl_3$ solvent are so similar that one is tempted to conclude that the CO_2H groups of **1** are tethered to the dipyrrinones much as they are in bilirubin and mesobilirubin (Fig. 1). In $(CD_3)_2SO$, however, the dipyrrinone N-*H*s become hydrogen bonded to solvent, and thus the distinctions seen in $CDCl_3$ and due to intramolecular hydrogen bonding are lost. As anticipated, the pyrrole (and lactam) N-*H* chemical shifts of **1** and mesobilirubin-XIIIα are very similar in $(CD_3)_2SO$ solvent (Table 1). When mesobilirubin is esterified, however, or when its propionic acid groups are relocated to C(7) and C(13) (as in mesobilirubin-IVα), or when they are replaced by ethyl (as in etiobilirubin-IVγ), the pyrrole hydrogens become more deshielded (10.3 δ) due to dipyrrinone-dipyrrinone intermolecular hydrogen bonding (Table 1).[16,17] This is also seen in the bis-pivalic acid dimethyl ester **2** which like mesobilirubin dimethyl ester and etiobilirubin exhibits the N-*H* chemical shifts in $CDCl_3$ solvent expected for intermolecular dipyrrinone-dipyrrinone hydrogen bonding.[13-15]

UV-Visible Spectral Analysis and Conformation from Exciton Coupling.

Further evidence on intramolecular hydrogen bonding comes from solvent-dependent UV-visible spectra. Over a wide range of solvents with varying polarity and hydrogen bonding ability (benzene, chloroform,

methanol and dimethylsulfoxide), the UV-visible spectra of mesobilirubin-XIIIα change very little, with λ^{max} being near 430 nm and a shoulder with λ^{sh} near 395 nm[13] — corresponding to the two exciton components from electric transition dipole-dipole interaction of the two proximal dipyrrinone chromophores approximately 90° apart (Fig. 1).[10,18,19] Since mesobilirubin-XIIIα is known from NMR studies to adopt the intramolecularly hydrogen bonded conformation of Fig. 1 in $CDCl_3$ solvent and a similar conformation in $(CD_3)_2SO$ solvent,[5,6] it might be argued that a UV-visible exciton couplet with $\lambda^{max} \simeq 430$ nm, $\lambda^{sh} \simeq 395$ nm can be taken as an indicator of a folded (but not necessarily fully hydrogen-bonded) conformation akin to that of Fig. 1. The UV-visible spectral data for bis-pivalic acid **1** (Table 2) are very much like those of mesobilirubin-XIIIα and are consistent with a folded conformation, probably one involving intramolecular hydrogen bonding in $CHCl_3$ and other non-polar solvents.

TABLE 2. Comparison UV-Visible Spectroscopic Data for $8^2,8^2,12^2,12^2$-Tetramethylmesobilirubin-XIIIα (**1**) and Its Dimethyl Ester (**2**).[a]

Solvent	**1**				**2**			
	λ^{max}	ϵ^{max}	λ^{sh}	ϵ^{sh}	λ^{max}	ϵ^{max}	λ^{sh}	ϵ^{sh}
Benzene	451	65,000	432	58,400	386	55,800	402	53,300
$CHCl_3$	436	62,100	—	—	381	61,000	415	34,100
CH_3CN	425	70,000	—	—	375	71,900	—	—
CH_3OH	396	61,600	418[b]	61,500[b]	392	55,200	413[b]	52,600[b]
$(CH_3)_2SO$	392	64,200	420	55,900	391	56,600	412-420	51,100

[a] Run at 1-2 x 10^{-5} *M* concentrations; λ^{max} and λ^{sh} in nm, ϵ^{max} and ϵ^{sh} in M^{-1} cm^{-1}. [b] Max.

The UV-visible spectral solvent dependence of bis-pivalic acid dimethyl ester **2** is different from that of **1** (Table 2) but quite similar to that of mesobilirubin-XIIIα dimethyl ester, which typically exhibits a strong solvent, concentration and temperature dependence due to formation of dimers[20] in non-polar solvents such as benzene and chloroform. This results in spectra with a narrow bandwidth intense absorption at λ^{max} near 380 nm and a weaker shoulder at λ^{sh} near 410 nm.[13] In more polar solvents such as CH_3OH and $(CH_3)_2SO$ the solutions are expected to be largely monomeric, and the UV-visible spectra begin to approach those of the parent acid in these solvents: λ^{max} near 435 nm and λ^{sh} near 400 nm.[13,15a] The UV-visible spectra of **2** are thus consistent with the expectations drawn from earlier studies of mesobilirubin and bilirubin dimethyl esters.[14]

Conformational Analysis from Molecular Dynamics Calculations.

Additional insight may be gained from conformational analysis by molecular dynamics methods using the force field in SYBYL. A conformational energy map (Fig. 2) can be constructed by rotating the dipyrrinone units independently about the 9-10 and 10-11 carbon-carbon single bonds, corresponding to torsion angles ϕ_1 and ϕ_2, respectively. With ϕ_1 and ϕ_2 defined as 0° in the porphyrin-like conformation (Fig. 3), a large array of conformations can be created through rotations about ϕ_1 and ϕ_2, e.g., the linear conformation, with $\phi_1 = \phi_2 = 180°$. Figure 2 shows a mapping of conformational energy *vs* rotation angles, ϕ_1 and

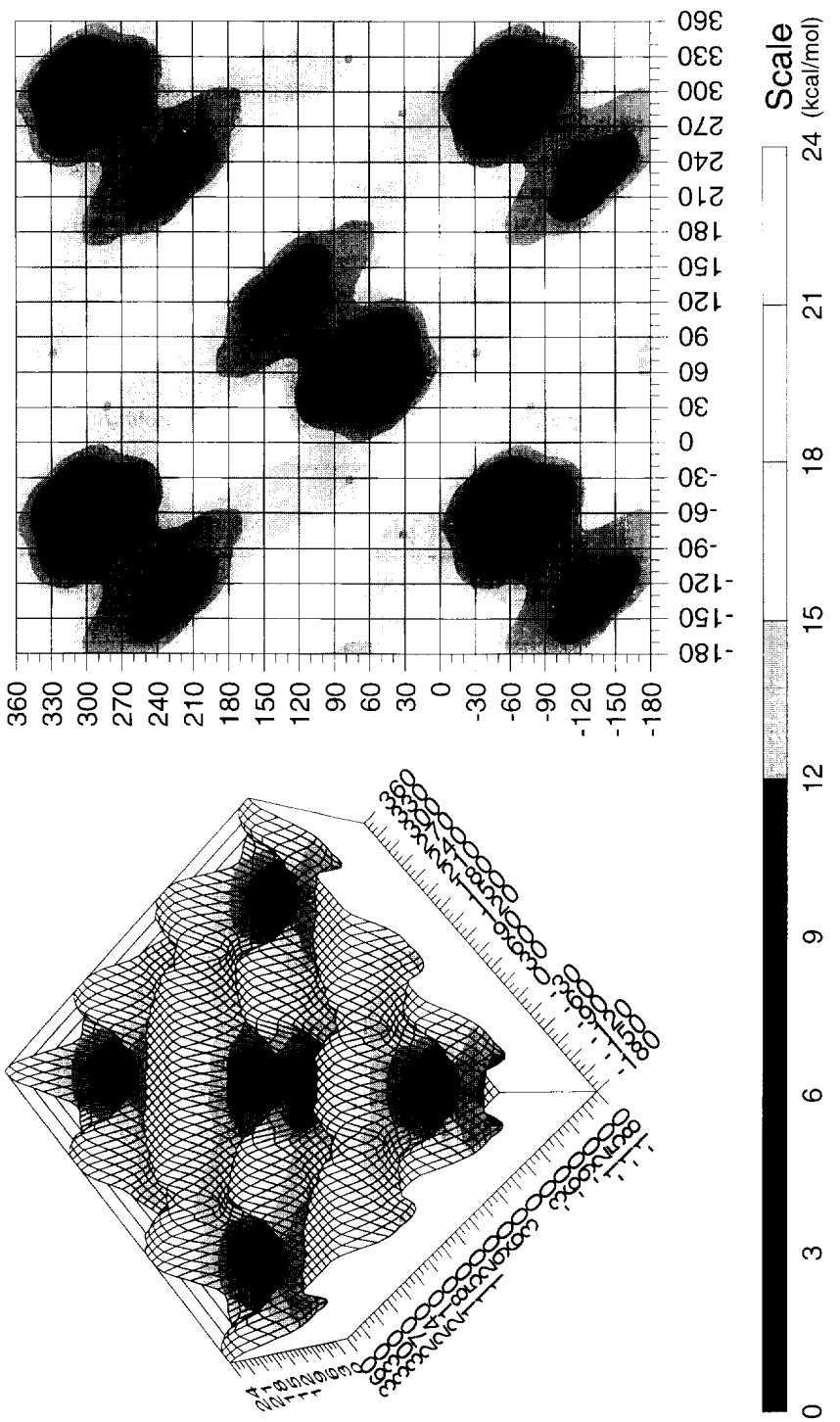


FIGURE 2. Potential energy surface (left) and contour maps (right) for $8^2,8^2,12^2,12^2$-tetramethylmesobilirubin-XIIIα (**1**) conformations generated by rotating the two dipyrrinone groups independently about the C(9)-C(10) and C(10)-C(11) bonds (ϕ_1 and ϕ_2 respectively). The energy scale (below) is in kcal/mol, and isoenergetic global minima (set to 0 kcal/mol) are found near $(\phi_1,\phi_2) \equiv (-60°,-60°)$ and $(-60°,300°)$, $(300°,-60°)$ and $(300°,300°)$, corresponding to the ***M***-chirality enantiomer and near $(\phi_1,\phi_2) \equiv (60°,60°)$ for the ***P***-chirality enantiomer. Local minima are found near $(\phi_1,\phi_2) = (140°,140°)$, $(-140°,-140°)$, $(-140°,220°)$, $(220°,-140°)$ and $(220°,220°)$ and lie 8.3 kcal/mole above the global minima.

ϕ_2. Some conformations are stabilized through intramolecular hydrogen bonding.[10,21,22] In the absence of intramolecular hydrogen bonding, the global energy minimum conformation of **1** is essentially the same as that of mesobilirubin-XIIIα and other bilirubin pigments — a ridge-tile shape.[22,23] In both mesobilirubin-XIIIα and **1** intramolecular hydrogen bonding stabilizes the ridge tile conformation, and the potential energy surface of **1** (Fig. 2) is not very different in appearance from that of mesobilirubin-XIIIα.[10] However, the more sterically demanding pivalic acid chains of **1** force the molecule to adopt a slightly broader set of global minima than those found in mesobilirubin-XIIIα, which has simple propionic acid chains. The global minima of **1** correspond to identical or enantiomeric structures, with each enantiomer being represented by several different points on the surface of Fig. 2. Thus global minima are found for identical ***M***-helicity conformers (Fig. 4) near (ϕ_1,ϕ_2) = (−60°,−60°), (−60°,300°), (300°,-60°) and (300°,300°) and for the isoenergetic ***P***-helicity conformer located near (ϕ_1,ϕ_2) = (60°,60°). Through the action of intramolecular hydrogen bonding, the molecule adopts one basic three-dimensional molecular structure corresponding to the cited minima, a conformation where the two dipyrrinone chromophores are aligned in a ridge-tile conformation (Fig. 4). This conformation is essentially identical to the typical ridge-tile[4a] conformation[10] corresponding to global minima found for mesobilirubin and bilirubin. In **1**, local minima are found near (ϕ_1,ϕ_2) = (−140°,−140°), (−140°,220°), etc. and their enantiomeric counterpart at (140°,140°) and are computed to lie 8.3 kcal/mole above the global minima. These conformations also use the same hydrogen bonding scheme as is seen in mesobilirubin, but the pigment is somewhat stretched and occupies a flatter ridge-tile while still maintaining intramolecular hydrogen bonding.

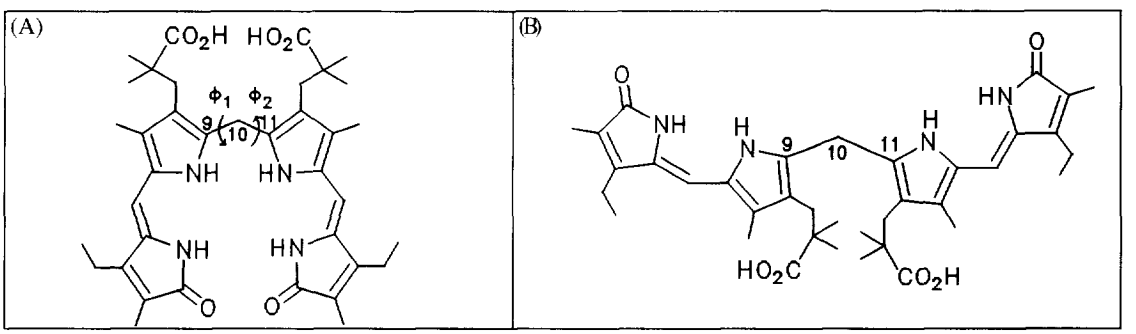


FIGURE 3. $8^2,8^2,12^2,12^2$-Tetramethylmesobilirubin-XIIIα (**1**) in: (A) the porphyrin-like conformation, and (B) the linear conformation. The representations in (A) and (B) may be interconverted by rotating the dipyrrinones about the 9-10 and 10-11 bonds (torsion angles ϕ_1 and ϕ_2). The porphyrin-like conformation (A) corresponds to $\phi_1=\phi_2=0°$, and the linear or extended conformation (B) corresponds to $\phi_1=\phi_2=180°$. There are a multitude of conformations lying between these two extremes, and some are stabilized by intramolecular hydrogen bonds between the pivalic acid CO_2H and the dipyrrinones, *viz.* the global minimum conformation shown in Fig. 4 with $\phi_1=60°$, $\phi_2=60°$ (or its mirror image at $\phi_1=-60°$, $\phi_2=-60°$) and the local minimum conformer at $\phi_1=140°$, $\phi_2=140°$ (or its mirror image at $\phi_1=-140°$, $\phi_2=-140°$).

Conformational Inversion.

M ⇄ ***P*** conformational inversion of bilirubin (Fig. 1) and mesobilirubin-XIIIα has been detected and evaluated by ^{1}H-NMR spectroscopy. Thus, from coalescence of the ABCX pattern of the propionic acid segments (-CH_2-CH_2-CO_2H), the conformational inversion rate at 53°C was determined to be k ≃ 3.1 and 3.9 sec^{-1} for bilirubin and mesobilirubin, respectively.[5d] Arrhenius plots gave $\Delta H^{\ddagger}$ 17.7 ± 0.6 kcal/mole, $\Delta S^{\ddagger}$

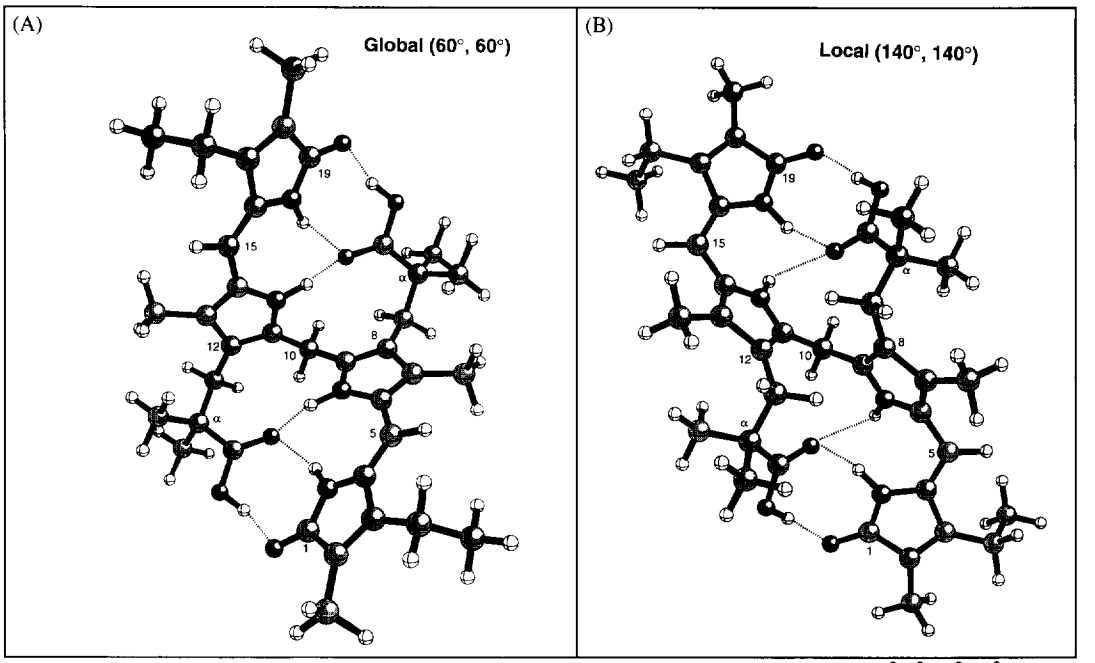


FIGURE 4. (A) Global minimum energy conformation of intramolecularly hydrogen bonded $8^2,8^2,12^2,12^2$-tetramethylmesobilirubin-XIIIα (**1**) at $\phi_1,\phi_2 = 60°,60°$ corresponding to the ***P***-helicity. (B) Local minimum energy ***P***-helicity conformation of **1** at $\phi_1,\phi_2 = 140°,140°$ and lying ~8.3 kcal/mol higher in energy than the global minimum. (Hydrogen bonds between CO_2H and dipyrrinone pyrrole NH and lactam -NH-C=O groups are represented by dashed lines.)

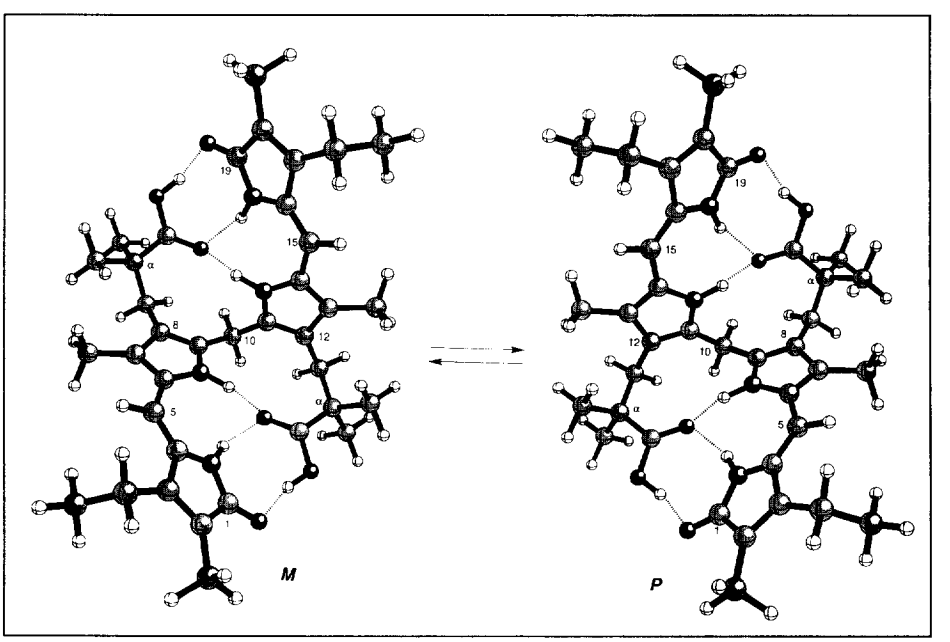


FIGURE 5. ***M*** ⇄ ***P*** conformational inversion of bis-pivalic acid rubin **1**. The *gem*-dimethyls at the α-carbon and the β-CH_2 hydrogens of the propionic acid chain are diastereotopic.

-2.1 ± 1.7 eu for bilirubin and $\Delta H^{\ddagger}$ 17.3 $\pm$ 0.3 kcal/mole, $\Delta S^{\ddagger}$ -3.0 ± 0.9 eu for mesobilirubin.[5d] In bis-pivalic acid rubin **1** the *gem*-dimethyl groups are diastereotopic (Fig. 5), as may be seen by the two different methyl signals appearing in the ^{1}H-NMR spectrum between δ 1.4 and 1.5 in $CDCl_3$ solvent (Fig. 6). The 8^1- and 12^1-methylene hydrogens are also diastereotopic and are seen as doublets at 2.27 and 3.09 ppm in $CDCl_3$ due to their different position relative to the pyrrole ring plane. This pattern collapses in $(CD_3)_2SO$ solvent to two singlets for the $8^2,12^2$-methyls and $8^1,12^1$-methylene protons. The ^{1}H-NMR spectrum in $CDCl_3$ is consistent with ***M*** and ***P*** conformers interconverting slowly on the NMR time scale, but in $(CD_3)_2SO$ the interconversion is relatively rapid.[5a] Upon increasing the temperature of the $CDCl_3$ solution from 273 to 333°K, the methyls undergo coalescence, and from a lineshape analysis, we were able to determine the rate constant ($k \simeq 6.8$ sec^{-1} at 30°C) for the ***M*** $\rightleftarrows$ ***P*** interconversion of **1** (Fig. 5). The corresponding activation perameters were determined to be $\Delta H^{\ddagger}$ 19.7 $\pm$ 1.4 kcal/mole and $\Delta S^{\ddagger}$ + 10.4 $\pm$ 4.5 eu.

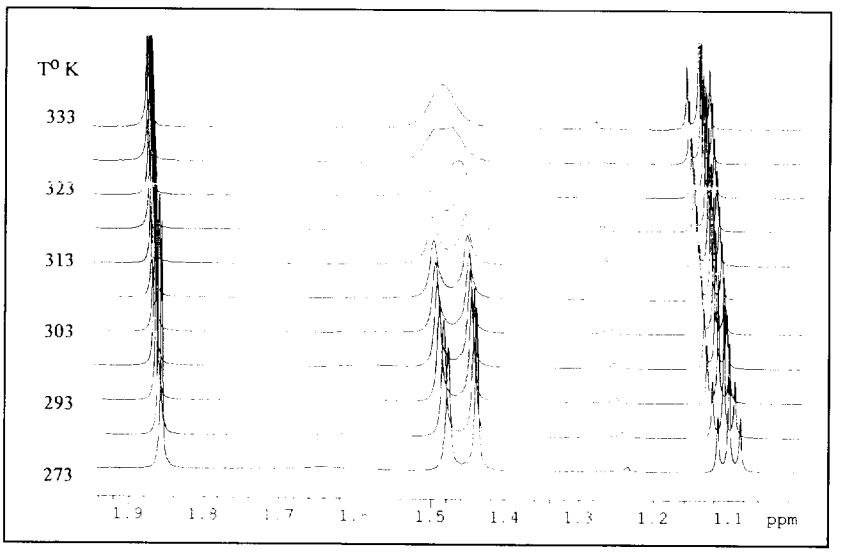


FIGURE 6. Variable temperature partial ^{1}H-NMR spectrum of bis-pivalic acid rubin **1** in $CDCl_3$ showing two signals between δ 1.4-1.5 for the diastereotopic *gem*-dimethyls and their coalescence near 328°K.

Circular Dichroism and Binding to Albumin.

As may be seen in Fig. 7, a solution of **1** in buffered solution with 2 mole equivalents of human serum albumin (HSA) gives a well-defined, intensely bisignate circular dichroism (CD) spectrum for the long wavelength UV-visible transition. When bound to HSA and other species' serum albumin, bilirubin-IXα is known to exhibit optical activity, seen typically as an induced circular dichroism (CD), which is usually intense and bisignate.[24,25] The origin of the optical activity comes from the pigment adopting a chiral conformation selected at the binding site on the protein. The observed bisignate CD comes from exciton coupling of two electric dipole transitions (Fig. 1): one from each of the pigment's twin dipyrrinone chromophores, *viz.* those from the long wavelength UV-visible excitation near 421 nm. The protein is acting as an enantioselective binding agent and constrains the pigment to adopt a chiral conformation, but as reported previously,[24] the presence of at least one propionic acid group at C(8) or C(12) is essential to the enantioselectivity in binding. Thus, bilirubin pigments with both propionic acid groups esterified as methyl esters give only very weak induced CDs, as do analogs with no acid groups, *e.g.*, etiobilirubin-IVγ. As expected, therefore, diester **2**

gave only a negligible CD (Table 3). In contrast, **1**, the bilirubin analog with pivalic acid rather than propionic acid chains at C(8) and C(12), gives an intense bisignate CD. The CD is opposite in sign and magnitude to that observed for bilirubin-IXα, which suggests that **1** adopts a more open ridge-tile conformation in which the relative helical orientation of the dipyrrinone long wavelength electric transition moments is reversed as compared with bilirubin and mesobilirubin. This phenomenon was observed earlier in bilirubin-HSA solutions upon the addition of a drop of chloroform.[26] It is surprising that the bulkier pivalic acid chains do not diminish the enantioselection, for the CD magnitudes observed are nearly the same as those observed for mesobilirubin (Table 3).

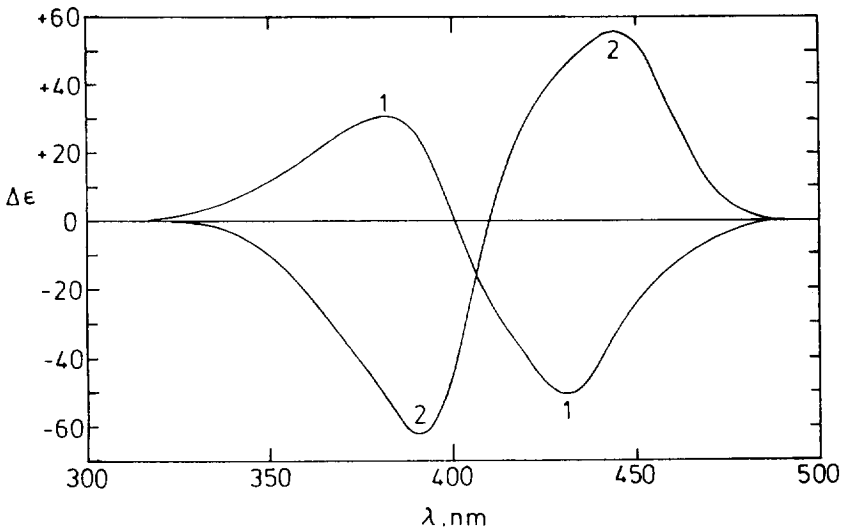


FIGURE 7. Comparison of the circular dichroism (CD) spectra of the pivalic acid rubin **1** (curve 1) on HSA with mesobilirubin-XIIIα on HSA (curve 2) in pH 7.4 phosphate buffer at 22°C. The concentration of pigment in each spectrum is 2 x 10^{-5} *M* and that of HSA is 4 x 10^{-5} *M*.

TABLE 3. Comparison of Circular Dichroism and UV-visible Spectral Data[a] for $8^2,8^2,12^2,12^2$-Tetramethyl-mesobilirubin-XIIIα, Mesobilirubin and Etiobilirubin-IVγ in pH 7.4 Buffered Aqueous Human Serum Albumin Solutions Containing 0.5% Dimethylsulfoxide.[b]

Pigment	Circular Dichroism			UV-Visible
	$\Delta\epsilon_{max}$ (λ_1)	λ at $\Delta\epsilon=0$	$\Delta\epsilon_{max}$ (λ_2)	ϵ_{max} (λ)
$8^2,8^2,12^2,12^2$-Tetramethyl-mesobilirubin-XIIIα (**1**)	−50.5 (431)	400	+30.2 (382)	55,200 (442)
Mesobilirubin-XIIIα	+55.3 (444)	410	−62.0 (391)	46,600 (437) 40,600^{sh} (408)
Etiobilirubin-IVγ	+2.1 (438)	414	-3.5 (388)	22,600^{sh} (432) 37,300 (375)
$8^2,8^2,12^2,12^2$-Tetramethyl-mesobilirubin-XIIIα Dimethyl Ester (**2**)	-0.52 (428)	411	+0.59 (379)	49,500 (375)

[a] $\Delta\epsilon$ and ϵ in L · mole^{-1} · cm^{-1} and λ in nm; [b] For 2 x 10^{-5} *M* pigment solutions run with 2 mole equivalents of human serum albumin, 0.5% $(CH_3)_2SO$ has no effect on the mesobilirubin-HSA CD spectrum.

Metabolism.

As with bilirubin and mesobilirubin-XIIIα, the tetramethyl analog (**1**) was not excreted in the (jaundiced) Gunn rat, which has a congenital deficiency of glucuronsyl transferase. Interestingly, when **1** was administered to a normal Sprague-Dawley rat, it was excreted into bile very slowly and only as its monoglucuronide. This contrasts markedly with the behavior of the parent, mesobilirubin-XIIIα, which is excreted promptly into bile, as mono and diglucuronides. Whether the longer excretion time and the exclusive formation of a monoglucuronide is due to ineffective uptake into the hepatocyte or to a rate decrease at the glucuronidation site is unclear.

CONCLUDING COMMENTS

Intramolecular hydrogen bonding between propionic acid CO_2H and dipyrrinone groups is known to be a dominant, conformation stabilizing force in bilirubin and its analogs.[10] The current study shows that even when the steric bulk of the propionic acid chains is expanded to pivalic acid, intramolecular hydrogen bonding persists in non-polar solvents. And the effect of such hydrogen bonding is to stabilize predominantly a ridge tile conformation and to a lesser extent a flattened ridge-tile shape.

EXPERIMENTAL PART

General Methods. All UV-visible spectra were recorded on a Perkin Elmer model 3840 diode array or Cary 219 spectrophotometer, and all circular dichroism (CD) spectra were recorded on a JASCO J-600 instrument. NMR spectra were obtained on a GE GN-300 or Varian Unity Plus spectrometers operating at 300 and 500 MHz, respectively, in $CDCl_3$ solvent (unless otherwise noted). Chemical shifts were reported in δ ppm referenced to the residual $CHCl_3$ ^1H signal at 7.26 ppm and ^{13}C signal at 77.0 ppm. A J-modulated spin-echo experiment (*A*ttached *P*roton *T*est) was used to assign ^{13}C-NMR spectra. Mass spectra (EI) were measured on Finnigan MAT SSQ 710 instrument. Melting points were taken on a Mel-Temp capillary apparatus and are uncorrected. Radial chromatography was carried out on Merck Silica Gel PF_{254} with gypsum preparative layer grade, using a Chromatotron (Harrison Research, Inc., Palo Alto, CA). HPLC analyses were carried out on a Perkin-Elmer Series 4 high performance liquid chromatograph with an LC-95 UV-visible spectrophotometric detector (set at 410 nm) equipped with a Beckman-Altex ultrashere-IP 5 μm C-18 ODS column (25 x 0.46 cm) and a Beckman ODS precolumn (4.5 x 0.46 cm). The flow rate was 1.0 mL/minute, and the elution solvent was 0.1 *M* di-*n*-octylamine acetate in 3% aqueous methanol (pH 7.7, 31°C). Combustion analyses were carried out by Desert Analytics, Tucson, AZ. Spectral data were obtained in spectral grade solvents (Aldrich or Fisher). Ethyl acetoacetate, pentane-2,4-dione, methyl methacrylate, diisopropyl amine, n-butyllithium in hexane, methyliodide, *p*-chloranil, and sodium borohydride were from Aldrich. Tetrahydrofuran, dichloromethane, chloroform, methanol, hexane, and dimethylsulfoxide were HPLC grade from Fisher. Tetrahydrofuran was dried by distillation from $LiAlH_4$; methanol was distilled from $Mg(OCH_3)_2$; dimethylsulfoxide was freshly distilled from CaH_2 under vacuum.

Methyl 3-(2,4-dimethyl-5-ethoxycarbonyl-1H-pyrrol-3-yl)-2-methylpropionate (**8**) was prepared from ethyl acetoacetate, pentane-2,4-dione, and methyl methacrylate in two steps as described previously.[11]

Methyl 3-(2,4-dimethyl-5-ethoxycarbonyl-1H-pyrrol-3-yl)-2,2-dimethylpropionate (**7**). To a cooled (−40°C) solution of 50 mmol LDA [prepared from 7.1 mL (50.5 mmol) of diisopropylamine and 31.2 mL (50 mmol) of 1.6 M *n*-butyllithium in hexane] in 30 mL of dry THF was added solution of 5.35 g (20 mmol) of **8** in 50 mL of THF. The mixture was stirred for 1 h and a solution of 2.8 mL (45 mmol) of methyliodide in 10 mL of THF was added. The mixture was stirred for 1 h at −35°C and then warmed-up to room temperature over 30 min. The reaction was quenched with water (100 mL) and the product was extracted with Et_2O (4 x 100 mL). The organic extracts were washed with water till neutral (4 x 100 mL), dried ($MgSO_4$), filtered, and the solvent was removed under vacuum. The crude product was purified by radial chromatography on silica gel (10-15% acetone in hexane) and the evaporated pure fractions were recrystallized from minimum volume of CH_2Cl_2 and hexane added in portions to afford 5.24 g (93%) of **7** with mp 110-111°C. It had ^{1}H-NMR δ: 1.14 (6H, s), 1.34 (3H, t, J=7.1 Hz), 2.17 (3H, s), 2.22 (3H, s), 2.65 (2H, s), 3.67 (3H, s), 4.29 (2H, q, J=7.1 Hz), 8.77 (1H, br.s) ppm; ^{13}C-NMR δ: 11.58, 12.26, 14.52, 24.67, 34.02, 44.51, 51.74, 59.60, 117.10, 117.6, 128.2, 131.3, 161.8, 178.5 ppm; MS *m/z* (rel. abund.): 281 (14%), 236 (6%), 180 (100%), 134 (80%) amu.

Anal. Calcd. for $C_{15}H_{23}NO_4$ (281): C, 64.03; H, 8.24; N, 4.98.
Found: C, 64.18; H, 8.28; N, 4.88.

3-(5-Carboxy-2,4-dimethyl-1H-pyrrol-3-yl)-2,2-dimethylpropionic acid. A mixture of 2.24 g (8 mmol) of **7**, 3.20 g (80 mmol) of NaOH, 8 mL of water, and 18 mL of ethanol was heated at reflux for 5 h. The ethanol was removed under vacuum, an additional 5 mL of water was added, and the mixture was cooled to −10°C. The free diacid was precipitated by slow addition of solution 40% aq. $NaNO_3$-conc. HNO_3 (5:1 by vol) with vigorous stirring until an acidic *p*H was obtained. The precipitate was filtered, washed with icy water (2 x 10 mL), and dried overnight in vacuum over P_2O_5. The yield is quantitative and the product was converted into dipyrrinones **5** and **6** without further characterization.

3-(3-Ethyl-2,7,9-trimethyl-1-oxo-1,10-dihydrodipyrrin-8-yl)-2,2-dimethylpropionic acid (**5**) and ***Methyl 3-(3-ethyl-2,7,9-trimethyl-1-oxo-1,10-dihydrodipyrrin-8-yl)-2,2-dimethylpropionate*** (**6**). The foregoing free diacid (8 mmol) was mixed with 1.73 g (8 mmol) of 5-bromomethylene-4-ethyl-3-methyl-2-oxo-1*H*-pyrrole[ref] and 40 mL of methanol, and the mixture was heated at reflux for 6 h. After chilling overnight at −20°C, the resulting precipitate was filtered and washed with cold methanol. The crude product was separated by radial chromatography on silica gel (1.5-5% CH_3OH in CH_2Cl_2) — the less polar fractions after evaporation and recrystallization from chloroform-methanol afforded 0.61 g (22%) of methyl ester **6**, and the more polar fractions after evaporation gave 1.33 g (50%) of pure by ^{1}H-NMR acid **5**. It had mp 294-296° (decomp.); ^{1}H-NMR δ: 1.10 (3H, t, J=7.6 Hz), 1.31 (6H, s), 1.91 (3H, s), 1.92 (3H, s), 2.23 (3H, s), 2.43 (2H, q, J=7.6 Hz), 2.64 (2H, s), 5.81 (1H, s), 8.71 (1H, br.s), 10.22 (1H, br.s), 13.29 (1H, very br.s); ^{1}H-NMR $(CD_3)_2SO$ δ: 1.03 (6H, s), 1.07 (3H, t, J=7.3 Hz), 1.77 (3H, s), 1.99 (3H, s), 2.15 (3H, s), 2.47 (2H, q, J=7.3 Hz), 2.53 (2H, s), 5.92 (1H, s), 9.78 (1H, s), 10.34 (1H, s), 12.10 (1H, s) ppm; ^{13}C-NMR $(CD_3)_2SO$ δ: 8.11, 10.18, 12.01, 14.90, 17.20, 24.69, 34.13, 43.52, 97.75, 116.3, 121.8, 122.6, 123.6, 127.2, 131.1, 147.2, 171.9, 179.0 ppm.

The methyl ester **6** had mp 238-239°C; ^{1}H-NMR δ: 1.17 (6H, s), 1.18 (3H, t, J=7.5 Hz), 1.94 (3H, s), 2.10 (3H, s), 2.38 (3H, s), 2.55 (2H, q, J=7.5 Hz), 2.68 (2H, s), 3.69 (3H, s), 6.14 (1H, s), 10.24 (1H,

br.s), 11.04 (1H, br.s) ppm; ^{13}C-NMR δ: 8.54, 10.52, 12.62, 15.06, 17.95, 24.73, 34.32, 44.68, 51.81, 101.3, 116.8, 122.3, 122.6, 125.9, 127.0, 133.2, 148.3, 174.1, 178.8 ppm.

Anal. Calcd. for $C_{20}H_{28}N_2O_3 \cdot 1/2\ CH_3OH$ (360): C, 68.30; H, 8.39; N, 7.77.
Found: C, 68.75; H, 8.12; N, 7.76.

3,17-Diethyl-8,12-bis-(2-carboxy-2,2-dimethylethyl)-2, 7, 13, 18-tetramethyl-(21H,24H)-bilin-1,19-dione (**3**). A mixture of 495 mg (1.5 mmol) dipyrrinone **5**, 924 mg (3.75 mmol) of *p*-chloranil, 330 mL of CH_2Cl_2, and 17 mL of formic acid was heated at reflux for 24 h. Then 160 mL of solvent was removed by distillation, and refluxing was continued for additional 6 h. The reaction mixture was kept overnight at −20°C, filtered, and washed with cold CH_2Cl_2. The filtrate was washed twice with water (150 mL), dried (anhydr. Na_2SO_4), filtered, and concentrated under vacuum to ~15 mL. The resulting precipitate was filtered, and the filtrate was purified by radial chromatography on silica gel (5% CH_3OH in CH_2Cl_2, 100 mL, and 8-10% CH_3OH in CH_2Cl_2, 250 mL) collecting only the bright blue polar band. After removing the solvent and drying under vacuum (P_2O_5), 261 mg (54%) of verdin-diacid **3** was obtained. It had mp 258-261°C; ^{1}H-NMR ($(CD_3)_2SO$) δ: 1.11 (6H, t, J=7.2 Hz), 1.11 (12H, s), 1.69 (6H, s), 2.03 (6H, s), 2.48 (4H, q, J=7.2 Hz), 2.81 (4H, s), 5.96 (2H, s), 6.82 (1H, s), 9.85 (2H, s), 12.27 (4H, very br.s) ppm; ^{13}C-NMR $(CD_3)_2SO$ δ: 8.17, 10.28, 14.51, 17.04, 25.13, 33.80, 43.19, 95.85, 117.8, 127.7, 129.0, 135.2, 140.0, 141.4, 146.3, 149.3, 172.4, 178.6 ppm.

3,17-Diethyl-8,12-bis-(2,2-dimethyl-2-methoxycarbonylethyl)-2,7,13,18-tetramethyl-(21H,24H)-bilin-1,19-dione (**4**). A mixture of 495 mg (1.5 mmol) dipyrrinone **6**, 924 mg (3.75 mmol) of *p*-chloranil, 330 mL of CH_2Cl_2, and 17 mL of formic acid was heated at reflux for 24 h. Then 160 mL of solvent was removed by distillation, and refluxing was continued for an additional 6 h. The reaction mixture was kept overnight at −20°C, and the resulting solid was filtered and washed with cold CH_2Cl_2. The cold filtrate was carefully neutralized with sat. aq. $NaHCO_3$, washed with 1 M NaOH (2 x 100 mL), water until neutral (4 x 200 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under vacuum. The crude product was purified by radial chromatography on silica gel (1.5-2.5% CH_3OH in CH_2Cl_2) to afford 296 mg (59%) of pure verdin-dimethyl ester **4**. It had mp 208-210°C; ^{1}H-NMR δ: 1.21 (6H, t, J=7.5 Hz), 1.22 (12H, s), 1.82 (6H, s), 2.06 (6H, s), 2.50 (4H, q, J=7.5 Hz), 2.86 (4H, s), 3.64 (6H, s), 5.95 (2H, s), 6.64 (1H, s), 8.37 (2H, br.s) ppm; ^{13}C-NMR δ: 8.31, 10.54, 14.45, 17.82, 25.25, 34.49, 44.26, 52.01, 96.51, 116.3, 128.2, 129.7, 135.1, 139.6, 142.5, 146.9, 149.7, 172.6, 178.0 ppm.

Anal. Calcd. for $C_{39}H_{50}N_4O_6$ (671): C, 69.82; H, 7.51; N, 8.35.
Found: C, 69.84; H, 7.50; N, 8.22.

3,17-Diethyl-8,12-bis-(2,2-dimethyl-2-methoxycarbonylethyl)-2,7,13,18-tetramethyl-(10H,21H,23H,24H)-bilin-1,19-dione (α,α,α′,α′-Tetramethylmesobilirubin-XIIIα dimethyl ester, 2). To a N_2-saturated solution of 67 mg (0.1 mmol) of **4** in 15 mL of dry THF was added 380 mg (10 mmol) of sodium borohydride, followed by dropwise addition of 10 mL of dry methanol. After 30 min stirring, a new portion of 190 mg (5 mmol) of $NaBH_4$ and 20 mL of CH_3OH were added. After 1 h stirring, the mixture was diluted with 100 mL of deoxygenated water and slowly acidified at 0°C with 10% HCl. The pigment was extracted with $CHCl_3$ (2 x 80 mL), washed with water (2 x 100 mL), dried over anhydr. Na_2SO_4 and filtered. The solvent was evaporated under vacuum, and the resulting crude green-brown product was purified by radial chromatog-

raphy on silica gel (4% CH_3OH in CH_2Cl_2). The bright yellow major band was removed. After evaporation, the solid was recrystallized from a minimum volume of $CHCl_3$ and 2 mL of CH_3OH to afford 31 mg (46%) of pure rubin-dimethyl ester **2** with mp 238-240°C (decomp.); ^{1}H-NMR δ: 0.99 (6H, t, J=7.4 Hz), 1.17 (12H, s), 1.44 (6H, s), 2.04 (6H, s), 2.31 (4H, q, J=7.4 Hz), 2.85 (4H, s), 3.70 (6H, s), 4.10 (2H, s), 5.91 (2H, s), 10.36 (2H, br.s), 10.69 (2H, br.s), ppm; ^{1}H-NMR $(CD_3)_2SO$ δ: 1.05 (6H, t, J=7.4 Hz), 1.06 (12H, s), 1.75 (6H, s), 1.95 (6H, s), 2.48 (4H, q, J=7.4 Hz), 2.50 (4H, s), 3.57 (6H, s), 3.85 (2H, s), 5.93 (2H, s), 9.87 (2H, s), 10.06 (2H, s) ppm; ^{13}C-NMR δ: 7.82, 10.73, 14.73, 17.76, 23.93, 24.84, 34.63, 44.48, 51.79, 100.29, 116.30, 123.43, 123.82, 124.10, 128.71, 132.34, 146.97, 174.24, 178.78 ppm; ^{13}C-NMR $(CD_3)_2SO$ δ: 8.11, 10.17, 14.90, 17.18, 24.24, 24.72, 34.02, 43.85, 51.66, 97.84, 116.5, 122.6, 123.1, 123.2, 128.2, 131.6, 147.37, 172.1, 177.5 ppm.

Anal. Calcd. for $C_{39}H_{52}N_4O_6$ (673): C, 69.61; H, 7.79; N, 8.33.
Found: C, 69.75; H, 7.72; N, 8.24.

3,17-Diethyl-8,12-bis-(2-carboxy-2,2-dimethylethyl)-2,7,13,18-tetramethyl-(10H,21H,23H,24H)-bilin-1,19-dione (α,α,α',α'-Tetramethylmesobilirubin-XIIIα, 1). A homogenous solution of verdin-diacid **3** (247 mg, 0.38 mmol) in 3 mL of dry, N_2 saturated dimethylsulfoxide was diluted with 95 mL of dry methanol. While bubbling N_2 through the solution, 1.44 g (38 mmol) of sodium borohydride was added in small portions over 20 min. After 45 min stirring, more 1.44 g (38 mmol) of $NaBH_4$ was slowly added. After 1.5 h, the green-blue mixture was diluted with 100 mL of water and carefully acidified at 0°C with 10% aq. HCl. More water was added (200 mL) and the pigment was extracted with $CHCl_3$ (3 x 100 mL). The organic extracts were washed with water (3 x 100 mL), dried over anhydr. Na_2SO_4 and filtered. The solvent was removed under vacuum, and the resulting crude product was purified by radial chromatography on silica gel (3.5-5.5% CH_3OH in CH_2Cl_2). The collected bright yellow fraction was isolated, evaporated and recrystallized from a minimum volume of $CHCl_3$ and 10 mL of CH_3OH to yield 133 mg (54%) of pure rubin diacid **1** with mp 241-244°C (decomp.); ^{1}H-NMR δ: 1.12 (6H, t, J=7.5 Hz), 1.45 (6H, s), 1.49 (6H, s), 1.86 (6H, s), 2.15 (6H, s), 2.27 (2H, d, ^{2}J=14.8 Hz), 2.48 (4H, q, J=7.5 Hz), 3.09 (2H, d, ^{2}J=14.8 Hz), 4.14 (2H, s), 6.09 (2H, s), 9.06 (2H, br.s), 10.41 (2H, br.s), 13.53 (2H, very br.s) ppm; ^{1}H-NMR $(CD_3)_2SO$ δ: 1.03 (12H, s), 1.06 (6H, t, J=7.4 Hz), 1.75 (6H, s), 1.99 (6H, s), 2.48 (4H, q, J=7.4 Hz), 2.51 (4H, s), 3.92 (2H, s), 5.94 (2H, s), 9.87 (2H, s), 10.02 (2H, s), 12.11 (2H, s) ppm; ^{13}C-NMR δ: 7.92, 12.21, 14.94, 17.89, 23.60, 25.80, 30.44, 34.21, 44.86, 101.08, 119.31, 123.45, 123.85, 123.89, 129.27, 134.10, 148.15, 174.92, 183.35 ppm; ^{13}C-NMR $(CD_3)_2SO$ δ: 8.10, 10.35, 14.88, 17.18, 24.48, 24.74, 33.87, 43.48, 97.92, 116.9, 122.5, 123.1, 123.3, 128.0, 131.8, 147.2, 172.1, 179.1 ppm.

Anal. Calcd. for $C_{37}H_{48}N_4O_6 \cdot CH_3OH$ (677): C, 67.43; H, 7.74; N, 8.28.
Found: C, 67.68; H, 7.27; N, 8.30.

Molecular mechanics calculations and molecular modelling was carried out on an Evans and Sutherland ESV-10 workstation using version 5.41 of SYBYL (Tripos Assoc., St. Louis, MO.) The dipyrrinone units of **1** were rotated independently about the central -CH_2- at C_{10} (torsion angles ϕ_1 and ϕ_2) through 10° increments from 0° to 360°. (The $\phi_1 = 0°$, $\phi_2 = 0°$ conformer has a porphyrin shape.) In this procedure, the two torsion angles were held fixed at each increment while the remainder of the molecule was relaxes to its minimum energy conformation using molecular mechanics. This was followed by a molecular dynamics cooling curve consisting of the following temperatures and times: 100 fs at 20°K, 100 fs at 10°K, 100 fs at

5°K, 200 fs at 2°K, 200 fs at 1°K, 200 fs at 0.5°K, 300 fs at 0.1°K. This was followed by molecular mechanics minimization, which gave the lowest energy conformations for each set of ϕ values. The conformational energy map of Fig. 2 was created using WingZ (Informix), and the ball and stick drawings of Figs. 4 and 5 were from atomic coordinates of the molecular dynamics structures using Müller and Falk's "Ball and Stick" program (Cherwell Scientific, Oxford, U.K.) for the Macintosh.

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