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Synthesis and Acidity Constants of $^{13}CO_2H$-Labelled Dicarboxylic Acids. pK_as from ^{13}C-NMR

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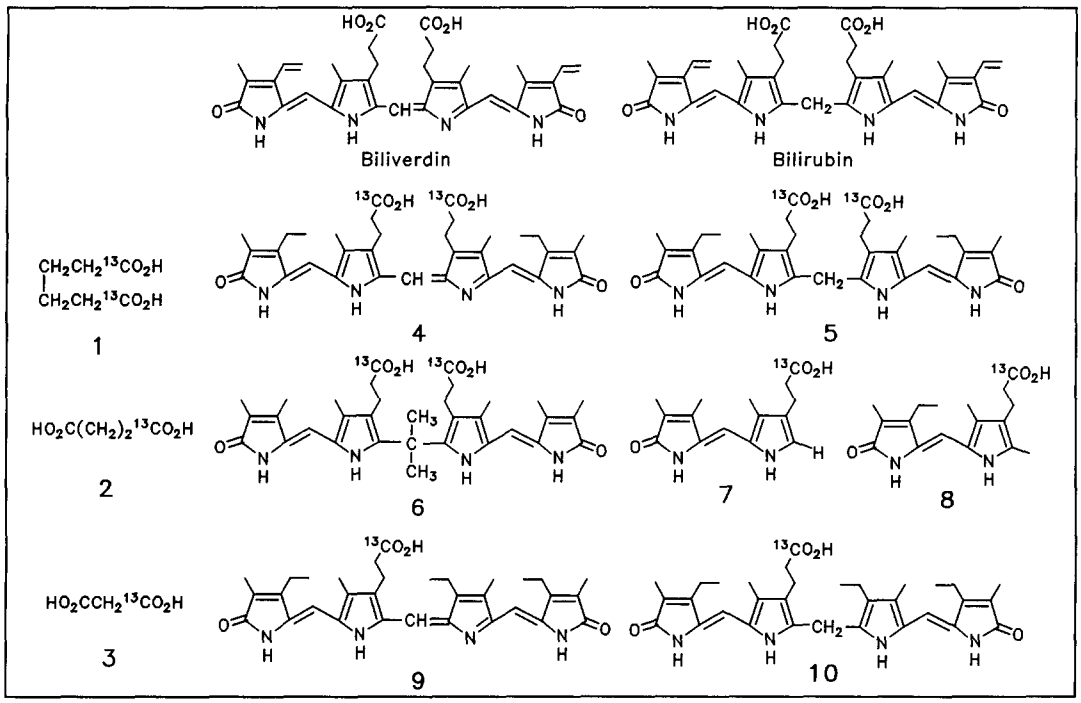
Abstract: Simple dicarboxylic acids: adipic (**1**), succinic (**2**) and malonic (**3**); more complex linear tetrapyrroles with two propionic acid groups: mesobiliverdin-XIIIα (**4**) and mesobilirubin-XIIIα (**5**), and a rubin analog with a *gem*-dimethyl at C_{10} (**6**) were prepared with 99% ^{13}C-enrichment in their CO_2H groups. Their pK_a values were determined from titration curves in water and in H_2O-$(CD_3)_2SO$ solutions: plots of carboxyl carbon ^{13}C-NMR chemical shifts with varying *p*H. Titration curves of diacids **1-3** with known pK_a's in H_2O served as calibration standards for determination of pK_as of tetrapyrrole diacids **4-6**: pK_{a1} = 3.9, pK_{a2} = 5.3 for **4**; pK_{a1} = 4.2, pK_{a2} = 4.9 for **5**, and pK_{a1} = 4.7, pK_{a2} = 5.7 for **6** in H_2O. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Carbon-13 nuclear magnetic resonance spectroscopy (^{13}C-NMR) has been shown to be an excellent diagnostic for studying the acid dissociation equilibria of carboxylic acids.[1,2,3] Over 25 years ago, the carboxylic acid carbon was reported to undergo a large ($\sim$5 ppm) ^{13}C-NMR deshielding upon deprotonation to the carboxylate anion in aqueous solution.[4,5] Yet, from this seminal observation there have been relatively few studies of carboxylic acid deprotonation equilibria using ^{13}C-NMR. The most successful applications involving natural isotopic abundance ^{13}C-NMR were found with water-soluble acids such as acetic, propionic and butyric at high concentration (0.04-0.05 M)[1c] and amino acids.[5,6] Those investigations accurately reproduced carboxylic acid pK_a values determined earlier by emf methods and were found to be especially useful in studying the microscopic deprotonation equilibria of amino acids.[2,7,8] The major factors limiting the application of ^{13}C-NMR in the determination of carboxylic acid pK_a's are: (*i*) the aqueous insolubility of most carboxylic acids, especially at low *p*H and (*ii*) the lack of sensitivity of the method due to the low natural isotopic abundance ($\sim$1.1%) of carbon-13. However, recently it was shown that these two limitations might be overcome in determinations of pK_a values of monocarboxylic acids by using: (*i*) ^{13}C-enriched carboxylic acids[1a,b,3] and (*ii*) dimethylsulfoxide co-solvent.[3] With such modifications, accurate pK_a's of water-insoluble monocarboxylic acids could be determined on concentrations as low as 10^{-4} – 10^{-6} M.

In the following, we describe how ^{13}C-NMR may be used to determine the apparent pK_a's of simple dicarboxylic acids, such as adipic, succinic and malonic acids (**1-3**), and structurally more complicated, water-insoluble diacids, such as analogs (**4-6**) of naturally-occurring bile pigments, biliverdin and bilirubin.[9] Biliverdin and bilirubin are the blue-green and yellow-orange heme degradation products found in animals,

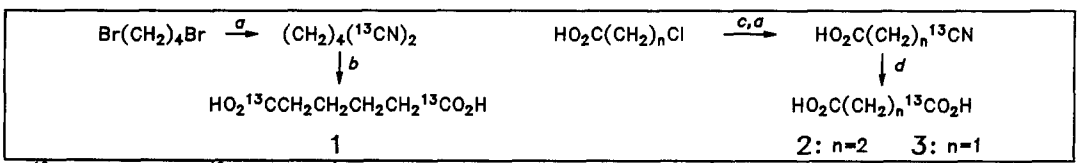
and their acidity and solubility properties are thought to be important in biological transport and metabolism. Yet, the pK_a values of biliverdin are not known, and those of bilirubin (the yellow pigment of jaundice) are controversial. The various acids (**1-10**) used in the pK_a studies were prepared with high ^{13}C enrichment (90-99%) at the carboxyl group to facilitate pK_a determinations in very dilute solutions.



RESULTS AND DISCUSSION

Synthesis. Syntheses of $^{13}CO_2H$-labelled dicarboxylic acids **1-3** were straightforward (Scheme 1). [1,6-$^{13}C_2$]-Adipic acid (**1**) was prepared from 1,4-dibromobutane, first by smooth displacement of bromide with $K^{13}CN$ (90% ^{13}C) in warm $(CH_3)_2SO$ then hydrolysis of the resulting dinitrile in refluxing aqueous KOH. ^{13}C-labelled succinnic (**2**) and malonic (**3**) acids with ^{13}C-enrichment in one CO_2H group were prepared (Scheme 1) from β-chloropropionic acid and chloroacetic acid, respectively — again using $K^{13}CN$ as the source of label.

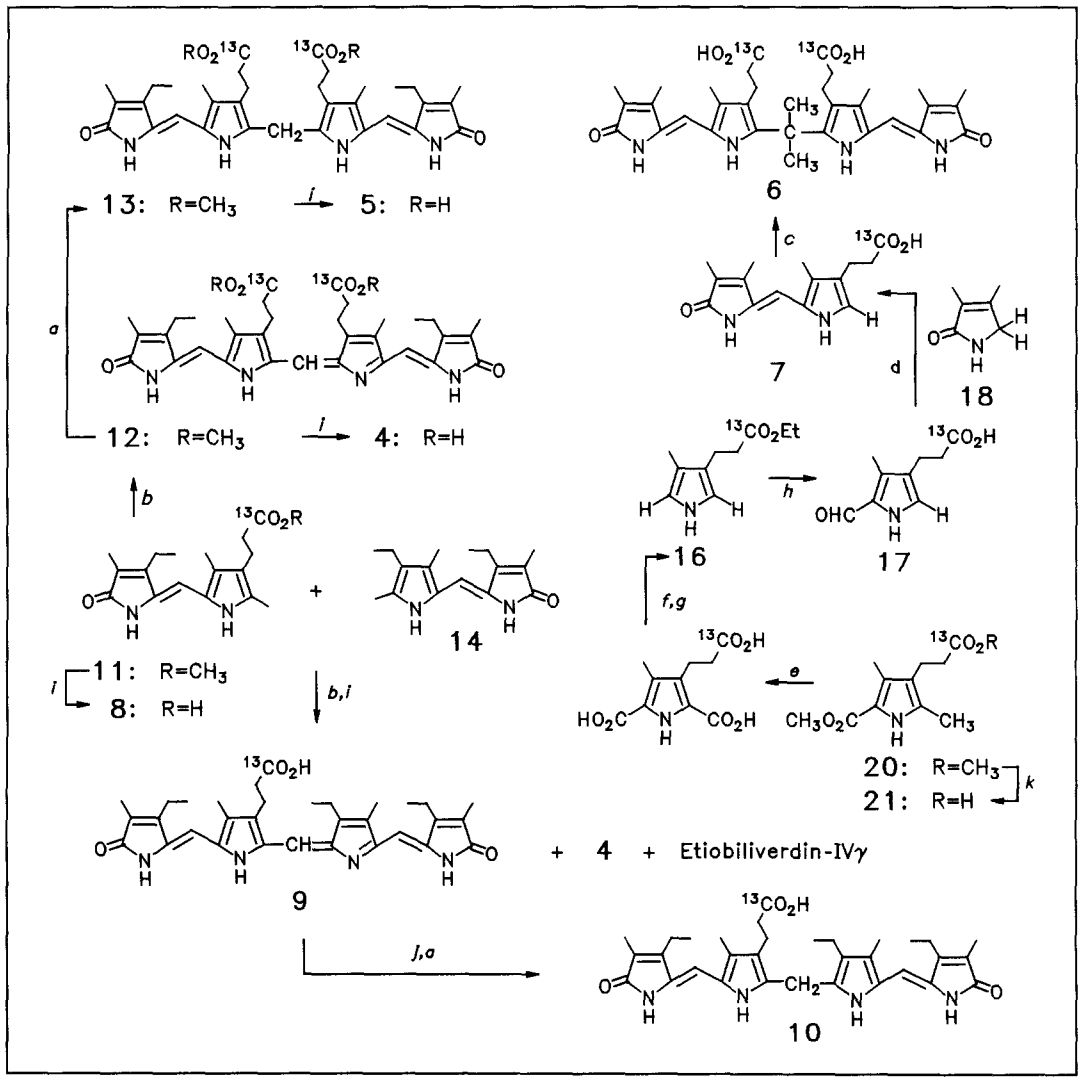
Synthetic Scheme 1



[a] $K^{13}CN/DMSO/\Delta$ or $K^{13}CN/H_2O/\Delta$. [b] Aq. KOH/refl.; then HCl. [c] Aq. Na_2CO_3. [d] Aq. KOH/refl.; then $CaCl_2$; then HCl.

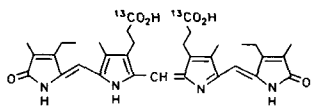
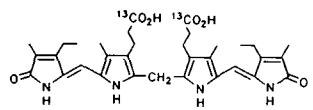
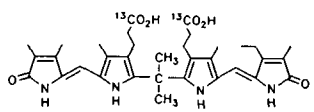
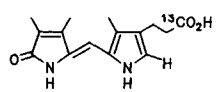
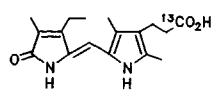
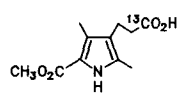
The syntheses of labelled pigments **4-10** were much more complicated but followed conventional methods employed previously in preparations of bile pigments.[10] The key intermediate in the preparation of tetrapyrroles **4**, **5**, **9** and **10** is methyl [8^3-^{13}C]-xanthobilirubinate (**11**) with 99% ^{13}C-enrichment in the carboxyl carbon. It had been prepared earlier from the labelled monopyrrole **20**.[3] As described in Scheme 2, oxidative self-coupling of **11** gave [8^3,12^3-^{13}C$_2$]-mesobiliverdin-XIIIα dimethyl ester (**12**), which was hydrolyzed to give the verdin diacid **4** or reduced with $NaBH_4$ to give [8^3,12^3-^{13}C$_2$]-mesobilirubin-XIIIα dimethyl ester (**13**). The latter was smoothly saponified to give ^{13}C-labelled mesobilirubin-XIIIα (**5**).

Synthetic Scheme 2



[a] $NaBH_4/CH_3OH$; then HCl. [b] *p*-Chloranil/HCO_2H/CH_2Cl_2/refl. [c] $(CH_3)_2C(OCH_3)_2$/TFA. [d] KOH/CH_3OH; then CH_3CO_2H. [e] SO_2Cl_2/THF; then NaOH refl.; then HCl. [f] $HC(OCH_2CH_3)_3$/TFA/EtOH. [g] Δ/distil. [h] Vilsmeier; separate isomers. [i] NaOH/refl.; then HCl. [j] Chromatography. [k] NaOH (aq)/room temp; then HCl (ref. 3).

TABLE 1. Solvent Dependence of ^{13}C-NMR Chemical Shifts[a] of $^{13}CO_2H$-Labelled Dicarboxylic Acids (**1-6**), Dipyrrinone Monocarboxylic Acids (**7** and **8**) and Their Carboxylate Anions.

Compound	H_2O			H_2O-9% vol $(CD_3)_2SO$			H_2O-27% vol $(CD_3)_2SO$			H_2O-64% vol $(CD_3)_2SO$			$(CD_3)_2SO$		
	δ_{CO_2H}	$\delta_{CO_2^-}$	Δ^b	δ_{CO_2H}	$\delta_{CO_2^-}$	Δ^b	δ_{CO_2H}	$\delta_{CO_2^-}$	Δ^b	δ_{CO_2H}	$\delta_{CO_2^-}$	Δ^b	δ_{CO_2H}	$\delta_{CO_2^-}$	Δ^b
$HO_2{}^{13}C(CH_2)_4{}^{13}CO_2H$ **1**:	177.8	182.8	5.0	177.6	182.5	4.9	177.2	181.4	4.2	175.7	179.6	3.9	174.3	177.9	3.6
$HO_2C(CH_2)_2{}^{13}CO_2H$ **2**:	176.2	181.4	5.2	176.0	181.2	5.2	175.7	180.4	4.7	174.9	179.8	4.9	173.9	176.0	2.1
$HO_2CCH_2{}^{13}CO_2H$ **3**:	170.2	176.3	6.1	170.1	176.3	6.2	169.8	175.6	5.8	169.2	174.6	5.4	168.7	172.8	4.1
 **4**:	175.7[c]	180.8	5.1	175.5[c]	180.5	5.0	175.4	180.2	4.8	174.3	178.3	4.0	173.6	176.4	2.8
 **5**:	176.3[c]	181.6	5.3	176.2[c]	181.4	5.2	176.0	181.0	5.0	175.1	179.6	4.5	173.0	176.4	3.4
 **6**:	176.5[c]	181.4	4.9	176.3[c]	181.0	4.7	176.2	180.5	4.3	175.0	178.4	3.4	174.0	175.4	1.4
 **7**:	177.2[c]	181.8	4.6	177.0	181.6	4.6	176.9	180.9	4.0	175.4	178.9	3.5	174.0	175.1	1.1
 **8**:	177.1[c]	181.9	4.8	176.9	181.6	4.7	176.8	180.9	4.1	175.4	178.8	3.4	173.7	175.0	1.3
 **21**:	177.4	181.8	4.4	177.2	181.4	4.2	176.6	180.8	4.2	175.3	178.9	3.6	179.3	174.0	0.7

[a] $(CD_2)_2SO$ was used as an external reference (δ 39.50) in aqueous and $CDCl_3$ solutions at 25°C, with δ reported in ppm downfield from $(CH_3)_4Si$. Only one signal from the acids was observed, that of the labelled carbon. [b] $\delta_{CO_2^-}$ minus δ_{CO_2H}. [c] Extrapolated value.

Preparation of 10,10-dimethylglaucorubin (**6**) was slightly lengthier and required synthesis of [8^3-^{13}C]-3^2-nor-neoxanthobilirubic acid (**7**). ^{13}C-Labelled monopyrrole **20** was converted to opsopyrrole ethyl ester **16** in four steps: (i) trichloromethylation of the 5-methyl group using SO_2Cl_2, (ii) base-catalyzed hydrolysis to a triacid, (iii) selective esterification at the propionic acid carbon using ethyl orthoformate, and (iv) double decarboxylation.[11] A Vilsmeier reaction on **16** afforded aldehyde **17**, which could be condensed with 3,4-dimethylpyrrolinone (**18**)[11] to give **7**. Reaction of **7** with 2,2-dimethoxypropane gave the desired rubin (**6**).

Preparation of rubin and verdin analogs of **4** and **5** with only one propionic acid group was achieved by oxidative cross coupling of **11** with kryptopyrromethenone (**14**)[12] to afford, after saponification, a separable mixture of verdins: etiobiliverdin-IVγ, **9** and **4**, in the ratio 1:2:1.[13] The desired verdin **9** was easily removed by radial chromatography and could be converted to rubin **10** by reduction with $NaBH_4$.

Titration Shift. Table 1 shows that carboxylic acid ^{13}C-NMR chemical shifts (δ_{CO_2H}) of the simple diacids adipic, succinic and malonic acids (**1-3**), as well as the more complex tetrapyrrole diacids (**4-6**) undergo a large deshielding upon deprotonation ($\delta_{CO_2^-}$). This "titration shift" ($\Delta = \delta_{CO_2^-} - \delta_{CO_2H}$) was noted over 25 years ago for monocarboxylic acids[4] and is thought to be approximately equal to the acid's pK_a value.[2] A titration shift has not been reported previously for dicarboxylic acids, but it seems clear from the data of Table 1 that dicarboxylic acids (**1-6**) exhibit large titration shifts in water, and only slightly decreased Δ values in aqueous dimethylsulfoxide. By way of comparison, dipyrrinone monopropionic acids (**7** and **8**) also show large titration shifts in water and in aqueous dimethyl sulfoxide. It is not surprising that solvent change from water to aqueous dimethylsulfoxide scarcely affects the titration shift, because the aqueous dimethylsulfoxide solutions used are mainly water, as measured on a mole fraction basis (Table 2). The titration shift data suggest that ^{13}C-NMR might be used to determine the pK_a values of carboxylic acids in water or aqueous dimethylsulfoxide, as explained in the following.

TABLE 2. Correlation Between Vol % $(CD_3)_2SO$ and Mole % of H_2O in H_2O-$(CD_3)_2SO$ Solutions at 25°C.

Vol % $(CD_3)_2SO$:	3.6	9.0	27	41	50	64	80
Mole % H_2O:	99	97.5	91.4	90	80	69	50

Monocarboxylic Acid pK_a Determination. In water and aqueous dimethylsulfoxide, the apparent deprotonation equilibrium constant (K_a) for $RCO_2H + H_2O \rightleftarrows H_3O^+ + RCO_2^-$ may be expressed as:

$$K_a \simeq \frac{[H_3O^+]\,[RCO_2^-]}{[RCO_2H]} \qquad (1)$$

When $[RCO_2^-] = [RCO_2H]$, pK_a = pH and $\delta_{obs} = 0.5(\delta_{CO_2H} + \delta_{CO_2^-})$.[3] Thus, the p$K_a$ of a monocarboxylic acid can be determined from its ^{13}C-NMR titration curves (plots of δ_{obs} *vs* pH) simply by reading the pH at the defined δ_{obs}. This procedure has been shown to give accurate apparent pK_a values in aqueous solutions, even in aqueous solutions containing low molar concentrations of $(CD_3)_2SO$.[3] (Small amounts of added $(CD_3)_2SO$ are necessary for maintaining the solubility of bilirubin and biliverdin analogs over the entire pH range studied.) As recognized earlier for **21** and other mono-acids,[3] the titration curves typically show

a downward displacement with increasing % $(CD_3)_2SO$.[3] This behavior may also be seen for dipyrrinone **7** in Fig. 1A. Yet, the apparent pK_a values hardly change, *i.e.*, the *p*H (=pK_a) is nearly the same at $\delta_{obs} = 0.5(\delta_{CO_2H} + \delta_{CO_2^-})$ on each curve. Apparently small amounts of dimethylsulfoxide have only a minor effect on pK_a. And as might be expected, dipyrrinone **8** gave titration curves nearly identical to those of **7**.

Where pK_a values must be determined in solutions with added $(CD_3)_2SO$, the data may be extrapolated to 100% water. In an earlier study,[3] we noted that monopyrrole **21** and other arylalkanoic acids gave excellent straight line correlations in plots of pK_a *vs* log vol % $(CD_3)_2SO$ that accurately extrapolated to the true pK_a value determined in water with no $(CD_3)_2SO$. With this calibration standard, we were able to predict the apparent pK_a values of **7** in 100% water (Table 3). As expected, **8** had a nearly identical extrapolated pK_a value of 4.57 in water, and the extrapolated apparent pK_as of **9** and **10** were quite similar: $pK_a \simeq 4.3$ for **9** and 4.5 for **10**.

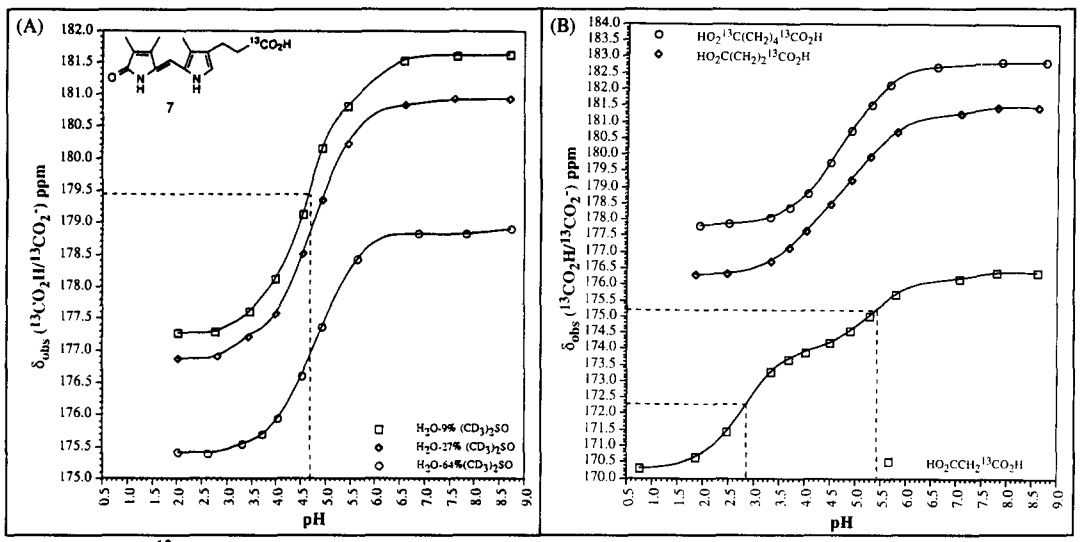
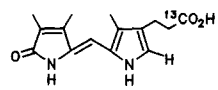
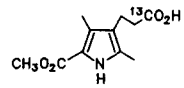


FIGURE 1. (A) ^{13}C-NMR titration curves showing the solvent and *p*H-dependent behavior of δ_{obs} for the carboxyl resonance in dipyrrinone **7**. (B) ^{13}C-NMR titration curves (δ_{obs} *vs* *p*H) for $^{13}CO_2H$-labelled adipic (**1**), succinic (**2**) and malonic (**3**) acids in water. The dashed lines in (A) connect $\delta_{obs} = 0.5\ (\delta_{CO_2H} + \delta_{CO_2^-})$ to the *p*H value corresponding to the pK_a. The dashed lines in (B) connect $\delta_{obs} = \delta_{(CO_2H)_2} + (1/4)\Delta$ and $\delta_{obs} = \delta_{(CO_2H)_2} + (3/4)\Delta$ to their respective correlated *p*H values, which correspond very approximately to pK_{a1} and pK_{a2}, respectively, where $\Delta = \delta_{(CO_2^-)_2} - \delta_{(CO_2H)_2}$.

TABLE 3. Solvent Dependence of Dipyrrole and Monopyrrole Monopropionic Acid Acidity Constants.[a]

Compound	H_2O[b]	H_2O-9% $(CD_3)_2SO$	H_2O-27% $(CD_3)_2SO$	H_2O-64% $(CD_3)_2SO$
	pK_a	pK_a	pK_a	pK_a
 **7**:	4.59	4.68	4.74	4.82
 **21**:	4.68[c]	4.78	4.82	4.84

[a] Determined from plots of δ_{obs} *vs* *p*H. [b] Extrapolated value; insoluble. [c] Measured in water.

Dicarboxylic Acid pK_a Determination. Determination of the two pK_a values of dicarboxylic acids is somewhat more difficult than determining the single pK_a of monocarboxylic acids. In aqueous solvents, the successive acid deprotonation equilibria of a dibasic acid may be expressed as: (i) $R(CO_2H)_2 + H_2O \rightleftarrows H_3O^+ + {}^-O_2CRCO_2H$ and (ii) $^-O_2CRCO_2H + H_2O \rightleftarrows H_3O^+ + R(CO_2^-)_2$, with

$$(2) \quad K_{a1} \simeq \frac{[H_3O^+]\,[^-O_2CRCO_2H]}{[R(CO_2H)_2]} \qquad \text{and} \qquad K_{a2} \simeq \frac{[H_3O^+]\,[R(CO_2^-)_2]}{[^-O_2CRCO_2H]} \quad (3)$$

As the equilibrium is driven from diacid to mono-anion to dianion (or vice-versa), the observed ^{13}C-NMR chemical shift of the carboxyl carbon (δ_{obs}) varies between $\delta_{(CO_2H)_2}$ and $\delta_{(CO_2^-)_2}$. If pK_{a1} and pK_{a2} differ by more than ~3, a plot of δ_{obs} *vs* p*H* (the titration curve) is expected to have a well-defined slope change near $\delta_{obs} = 0.5(\delta_{(CO_2^-)_2} + \delta_{(CO_2H)_2})$. This is clearly seen (Fig. 1B) for malonic acid (**3**), whose reported[14] pK_{a1} = 2.85 and pK_{a2} = 5.70. However, in most dicarboxylic acids, pK_{a1} and pK_{a2} differ by only ~1 unit (Table 4), and the titration curves do not show the inflection — as in Fig. 1B for adipic (**1**) and succinic (**2**) acids. Added dimethylsulfoxide displaces the titration curves downward (Fig. 2A), but the shape remains unchanged, as seen also for monocarboxylic acids (Fig. 1A). Interestingly and usefully, small quantities of dimethylsulfoxide cause only a minor displacement with essentially no change in slope in the rising part of the curve. Even large quantities of added dimethylsulfoxide cause only minor changes in slope.

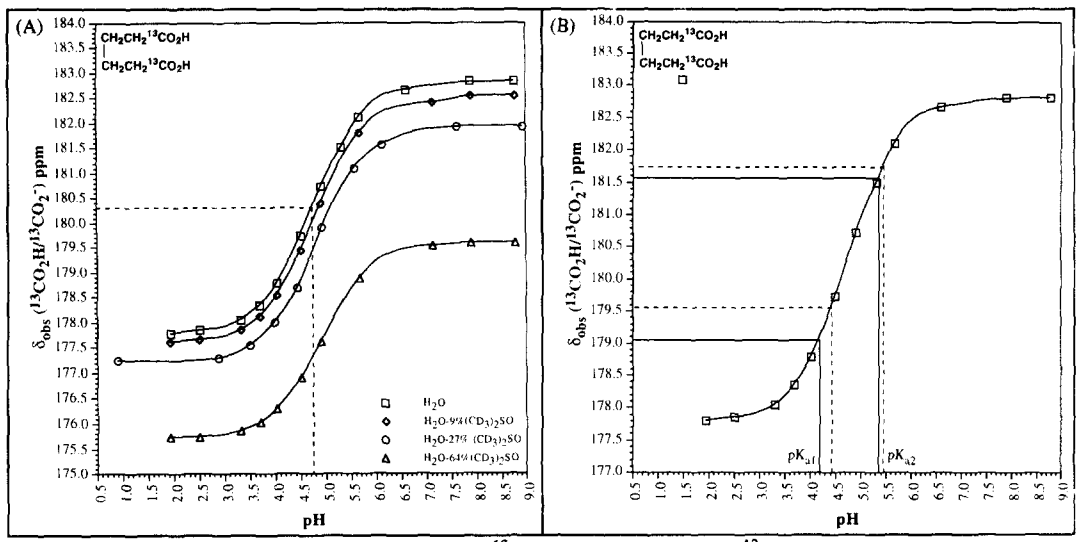


FIGURE 2. (A) Influence of added $(CD_3)_2SO$ on the ^{13}C-NMR titration curves of $^{13}CO_2H$-labelled adipic acid (**1**). The dashed line correlates $\delta_{obs} = (1/2)\Delta$ with *p*H. (B) Titration curve for **1** in H_2O showing (———) the *p*H (pK_a) values predicted by equations 4 and 5, and correlating (- - - -) known pK_a values to δ_{obs} for use in deriving equations 6 and 7.

While the curves of Fig. 2 clearly indicate that pK_{a1} and pK_{a2} of **1** and **2** lie between ~3.5 and 6, determination of the exact values of pK_{a1} and pK_{a2} is difficult and lengthy and involves iterative or extrapolation approaches.[15] Simplistically, however, from the titration curves of Fig. 1B, a crude approximation of the adipic and succinic acid apparent pK_as can be made by assuming that $[R(CO_2H)_2] = [^-O_2CRCO_2H]$ and

hence $pK_{a1} = pH$ (equation 2) when

$$\delta_{obs} = \delta_{(CO_2H)_2} + (1/4)\Delta \tag{4}$$

and that $[R(CO_2^-)_2] = [^-O_2CRCO_2H]$ and thus $pK_{a2} = pH$ (equation 3) when

$$\delta_{obs} = \delta_{(CO_2H)} + (3/4)\Delta \tag{5}$$

where $\Delta = \delta_{(CO_2^-)_2} - \delta_{(CO_2H)_2}$. The pK_as of adipic, succinnic and malonic acids (1-3) approximated by equations 4 and 5 from the data of Fig. 1B are close to the literature values (Table 4), approaching them somewhat better as the chain lengthens (*cf.*, adipic acid), especially for pK_{a2}.

TABLE 4. Comparison of Alkanoic and Alkanedioic Acid pK_a Values.

Number of Carbons	Monocarboxylic Acid	Lit.[a] pK_a	Dicarboxylic Acid	Literature[a]			Approximated[b]	
				pK_{a1}	pK_{a2}	ΔpK_a	pK_{a1}	pK_{a2}
3	Propionic	4.88	Malonic	2.85	5.70	2.85	2.59	5.19
4	Butyric	4.82	Succinic	4.21	5.71	1.50	4.03[c]	5.48[c]
5	Valeric	4.82	Glutaric	4.35	5.40	1.05	—	—
6	Caproic	4.85	Adipic	4.44	5.44	1.00	4.19	5.40
7	Heptanoic	4.84	Pimelic	4.46	5.58	1.12	—	—
8	Caprylic	4.89	Suberic	4.53	5.52	0.99	—	—
9	Nonanoic	4.95	Azeleic	4.56	5.53	0.97	—	—
10	Capric	N/A	Sebacic	4.58	5.54	0.96	—	—

[a] Values in H_2O, from ref. 14. [b] Approximated from the titration curves of Fig. 1B, assuming $pK_{a1} = pH$ for equation 4, and $pK_{a2} = pH$ for equation 5. Values good to no more than 2 significant figures. [c] Further refinement based on adipic acid literature pK_a values and equations 6 and 7 gives $pK_{a1} = 4.30$ and $pK_{a2} = 5.55$.

Potentially better, yet still approximate values may be obtained by calibrating equations 4 and 5 to give the literature pK_a values ($pK_{a1} = 4.44$ and $pK_{a2} = 5.44$) of adipic acid. From a plot of δ_{obs} *vs* pH for adipic acid (Fig. 2B), one finds $\delta_{(CO_2H)_2} = 177.80$ and $\delta_{(CO_2^-)_2} = 182.83$ in water. We find that $\delta_{obs} = 179.57$ at $pK_{a1} = pH = 4.44$; and $\delta_{obs} = 181.74$ at $pK_{a2} = pH = 5.44$. Using these values for δ_{obs}, one obtains the calibrated equations 6 and 7 that relate δ_{obs} to $\delta_{(CO_2H)_2}$. Thus, $pK_{a1} = pH$ when

$$\delta_{obs} = \delta_{(CO_2H)_2} + 0.354\ \Delta \tag{6}$$

and $pK_{a2} = pH$ when

$$\delta_{obs} = \delta_{(CO_2H)_2} + 0.785\ \Delta \tag{7}$$

It is interesting to note that the pK_{a2} values calculated from equations 5 and 7 will be very nearly the same, but the pK_{a1} values calculated from equations 4 and 6 will differ somewhat.

Tetrapyrrole Diacid pK_as. The tetrapyrroles of this work are insoluble in water but soluble in H_2O-$(CD_3)_2SO$ mixtures, in which they exhibit typical titration shifts (Table 1). Typical "titration" curves are obtained for the bilirubin and biliverdin analogs (**4-6**) in aqueous dimethylsulfoxide by plotting their ^{13}C-NMR carboxyl chemical shifts (δ_{obs}) with varying *p*H. No inflections are detected in the rising portions of the dicarboxylic acid curves (Fig. 3A). This means K_{a1} and K_{a2} must differ by less than a factor of 10^3, as is expected for a diacid with two non-interacting, well separated carboxylic acid groups.[15] It may be seen that δ_{obs} achieves constant values at either end of the rising portion of the curve, forming lower and upper plateaus, corresponding to $\delta_{obs} = \delta_{(CO_2H)_2}$ and $\delta_{obs} = \delta_{(CO_2^-)_2}$, respectively. That is, the pigment is entirely in the diacid form at *p*H values at the lower plateau, and it is entirely in the dianion form at *p*H values at the upper plateau of Fig. 3A. No changes in δ_{obs} are seen with further decreases or increases in *p*H, even to *p*H 10.5. This behavior is also seen in the tetrapyrrole monocarboxylic acid analogs **9** and **10**, which exhibit titration curves very similar to those of diacids **4** and **5**. Consequently, the apparent pK_a values for **4-6** must lie at a *p*H in the rising portion of the titration curves of Fig. 3A, *viz.*, between ~3.5 and 5.5. This means that the apparent pK_a values of biliverdin and bilirubin lie in the normal range for aliphatic dicarboxylic acids.

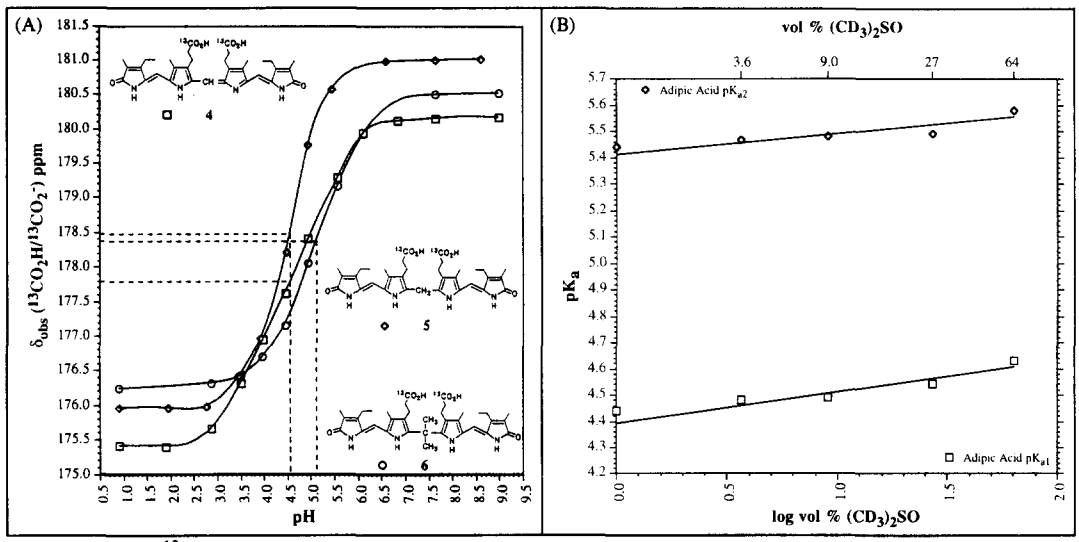
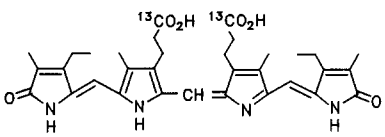
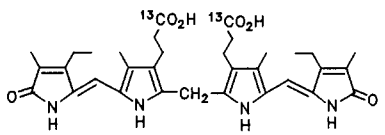
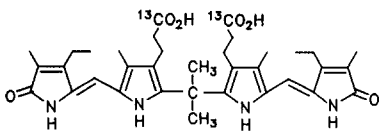


FIGURE 3. (A) ^{13}C-NMR titration curves showing the *p*H-dependent behavior of the carboxyl carbon (δ_{obs}) for mesobiliverdin-XIIIα (**4**) and mesobilirubin-XIIIα (**5**) and its 10,10-dimethyl analog (**6**). The data are taken for 10^{-4}-10^{-6} M solutions in 0.1 M buffers containing 27 (vol/vol) percent $(CD_3)_2SO$ in H_2O at 25°C. The dashed lines connect δ_{obs} = 0.5 Δ to *p*H. (B) Plots of pK_{a1} and pK_{a2} of adipic acid (**1**) *vs* log vol % $(CD_3)_2SO$ extrapolated to 100% H_2O. For ◇, the line is y= 0.079x + 5.411, (r^2=0.726); for □, the line is y= 0.118x + 4.395, (r^2=0.879).

Applying equations 4-7 to the tetrapyrrole dipropionic acids **4**, **5** and **6** gives the apparent pK_a values listed in Table 5 for aqueous solutions containing 27% and 64% by volume $(CD_3)_2SO$. Since these solutions are mainly water (Table 2), it might be expected that the pK_a's, especially those in 27 vol % $(CD_3)_2SO$, would be predictably close to those in water — as observed in earlier studies.[3] In order to extrapolate pK_as from H_2O-$(CD_3)_2SO$ solutions to H_2O, we used two calibration standards: adipic acid (**1**) which might be viewed as two propionic acids strung together, and monopropionic acid pyrrole **21**. The pK_a values of adipic acid in water containing 27 vol % and 64 vol % $(CD_3)_2SO$ are very close to its pK_a values in pure water

(Table 5), as are those of monopropionic acid pyrrole **21**.[3] One might thus assume that the pK_a values of the tetrapyrrole analogs of Table 5 would behave analogously, that the pK_a values in water would be close to the values determined in 27% $(CD_3)_2SO$, in which the mole fraction of $(CD_3)_2SO$ is only 0.086. To support the validity of such extrapolations, we observed that plots of pK_a *vs* log vol % $(CD_3)_2SO$ for adipic acid (**1**) (Fig. 3B) are observed to follow the same type of good straight line behavior as seen for monopropionic acid pyrrole **21**.[3] In adipic acid, the plots accurately predict the literature[14] values for the pK_a values in water. Assuming adipic acid-like slopes obtain for the tetrapyrrole dicarboxylic acids, one can thus extrapolate from the pK_a's determined in $H_2O-(CD_3)_2SO$ mixtures to predict the values in water (Table 5). Alternatively, one might assume a behavior similar to that found earlier in monopropionic acid pyrrole **21**,[3] and from this slope extrapolate to nearly the same sets of pK_a values for tetrapyrroles **4-6** in H_2O (Table 5). The extrapolated pK_a values found by using the adipic acid and the pyrrole standards are in reasonably good agreement. Extrapolations for biliverdin analog **4**, give $pK_{a1} \simeq 3.76$-3.92 and $pK_{a2} \simeq 5.23$-5.28, and for bilirubin analog **5**, $pK_{a1} \simeq 4.11$-4.18 and $pK_{a2} \simeq 4.82$-4.86. The presence of a C(10) *gem*-dimethyl on bilirubin analog **6** raises the values to $pK_{a1} \simeq 4.60$-4.67 and $pK_{a2} \simeq 5.64$-5.69.

TABLE 5. Dicarboxylic Acid Acidity Constants (pK_a) Determined from Titration Curve Plots of ^{13}C-NMR $^{13}CO_2H$ Chemical Shifts (δ_{obs}) *vs* pH.[a]

Compound	H_2O		H_2O-27% $(CD_3)_2SO$		H_2O-64% $(CD_3)_2SO$	
	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}
 **4**	3.92[b,c] 3.87[d] 3.76[c,e]	5.28[b,c] 5.25[d] 5.23[c,e]	3.95[b] 3.58[e]	5.33[b] 5.16[e]	4.09[b] 3.73[e]	5.47[b] 5.34[e]
 **5**	4.18[b,c] 4.13[d] 4.11[c,e]	4.86[b,c] 4.82[d] 4.82[c,e]	4.21[b] 3.99[e]	4.97[b] 4.86[e]	4.35[b] 4.02[e]	4.97[b] 4.92[e]
 **6**	4.67[b,c] 4.67[d] 4.60[c,e]	5.69[b,c] 5.65[d] 5.64[c,e]	4.86[b] 4.50[e]	5.69[b] 5.70[e]	4.78[b] 4.57[e]	5.87[b] 5.80[e]
$HO_2{}^{13}CCH_2CH_2CH_2CH_2{}^{13}CO_2H$ **1**	4.39[b,f] 4.19[e,f]	5.41[b,f] 5.39[e,f]	4.54[b] 4.29[e]	5.49[b] 5.34[e]	4.63[b] 4.38[e]	5.58[b] 5.49[e]

[a] Extrapolated values good to only 2 significant figures. [b] Based on equations 6 and 7. [c] Calibrated to the adipic acid (**1**) slopes of Fig. 3B and adjusted to pK_as in water. [d] Calibrated to the propionic acid pyrrole (**21**) slopes of ref. 3 and adjusted to pK_a in water. [e] Based on equations 4 and 5. [f] Lit. $pK_{a1} = 4.44$, $pK_{a2} = 5.44$ in H_2O (ref. 14).

Biliverdin and Bilirubin. Biliverdin and bilirubin are tetrapyrrole dicarboxylic acids formed in the normal metabolism of heme proteins.[9,16,17,18] The most stable structure of biliverdin is porphyrin-like but helical and shaped like a lock washer (Fig. 4A).[17] In contrast, bilirubin adopts a very different conformation that is shaped like a ridge-tile[19] to minimize nonbonded steric interactions[20] and stabilized by a network of intramolecular hydrogen bonds that link the each propionic acid carboxyl group to an opposing dipyrrinone lactam and pyrrole (Fig. 4B).[19-21] Intramolecular hydrogen bonding is strongly favored in nonpolar organic solvents and thought to persist in polar, aprotic solvents such as dimethylsulfoxide and in polar, protic solvents.[20-22,23] Remarkably, it persists even in the bilirubin dianion (Fig. 4C),[21,24] where the remaining hydrogen bonds are strengthened by increased electrostatic attraction to a negatively charged carboxylate oxygen. Intramolecular hydrogen bonding explains the differences in polarity and transport of biliverdin and bilirubin. Biliverdin is promptly excreted across the liver into bile; whereas, bilirubin is intrinsically unexcretable but is efficiently eliminated by hepatic uptake and enzymic conversion to water-soluble glucuronides that are promptly secreted into bile.[16,18] Biliverdin is polar, insoluble in $CHCl_3$ but soluble in CH_3OH;[25] bilirubin is lipophilic, soluble in $CHCl_3$ but insoluble in CH_3OH. Unlike biliverdin and many other dicarboxylic acids, bilirubin does not extract into aqueous bicarbonate. Yet, the unusual solution and excretion properties of bilirubin are scarcely changed in analogs with vinyl groups reduced to ethyl, and symmetrically arranged, as in mesobilirubin-XIIIα (**5**) and its *gem*-dimethyl analog (**6**). This is because here too the chemical and biological properties are dominated by similar intramolecular hydrogen bonding. Intramolecular hydrogen bonding has been invoked as an explanation for the phenomenally high bilirubin pK_a values (8.1 to $\geq$9.3) recently measured[26,27] — values well above those (6.2-6.5) preferred by most medical researchers,[28] and significantly higher than the pK_as found in typical dicarboxylic acids (Table 4). Yet other measurements find bilirubin pK_a values (4.3-5.6) more like those of aliphatic carboxylic acids (Table 4).[29,30,31] Clearly, bilirubin acidity is controversial. Yet, knowledge of the exact pK_a values is an important consideration in bilirubin metabolism, as they are thought to be an important factor in cellular uptake, in transport across the blood-brain barrier and in gallstone nucleation.[9,16,18,27]

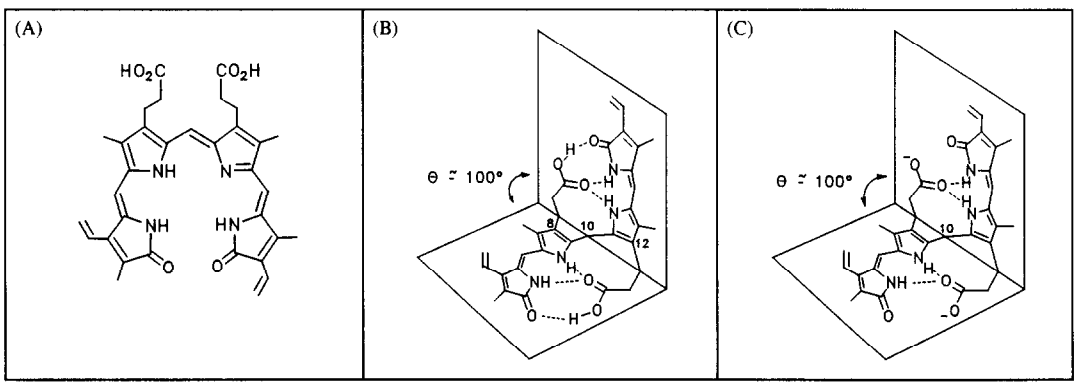


FIGURE 4. The most stable structures of: (A) biliverdin in a porphyrin-like, helical conformation shaped like a lock-washer; (B) bilirubin and (C) bilirubin dianion, shaped like ridge-tiles and intramolecularly hydrogen-bonded.

Precipitation or aqueous insolubility of biliverdin and bilirubin has been a major problem in almost all previous determinations of their pK_a values.[32] Inflections or breaks in the titration curves caused by precipitation have been assumed incorrectly to correspond to two very different pK_{a1} and pK_{a2} values. However,

such inflections or breaks are not observed for solutions where homogeneity is maintained over the entire *p*H range (Figs. 2B and 3A), and unless pK_{a1} and pK_{a2} differ by at least 2-3 *p*K units, none are expected.[15] In the current work, the pK_a values found (Table 5) for biliverdin and bilirubin analogs (**4-6**) are close to those of adipic acid and other long chain dicarboxylic acids (Table 4), and they fall into the normal range of ordinary aliphatic carboxylic acids. They are also close to those estimated for bilirubin about 30 years ago by Overbeek *et al.*,[30] who assumed a dissociation constant of $K_a = 2 \times 10^{-5}$ for a monopyrrole carboxylic acid (a value close to that of **21** in Table 2) and *approximated* the bilirubin K_{a1} as 2 x K_a, and K_{a2} as $K_a/2$: $pK_{a1} \simeq 4.4$ and $pK_{a2} \simeq 5$. And they are similar to the bilirubin values determined under conditions where solubility is maintained; for example, spectrophotometric titrations in dimethylformamide[29] gave $pK_{a1} = 4.3$ and $pK_{a2} = 5.4$, and ^{13}C-NMR titrations in dimethylsulfoxide at high concentration[31] gave an average $pK_a = 4.4$. But they differ from other determinations of bilirubin pK_a found in potentiometric and spectrophotometric titrations: pK_as $\simeq 7.55$,[33] and pK_{a1} and $pK_{a2} \simeq 6.7$ and 7.5;[34] or found in more error-prone experiments by partitioning bilirubin between $CHCl_3$ and aqueous buffers: pK_{a1} and $pK_{a2} \simeq 6.7$ and ≥ 9.3[26a] or 8.1 and 8.4.[26b] The extraordinarily high pK_a values are seldom if ever observed for carboxylic acids[14,15,35,36] and have been rationalized speciously in terms of a weakened carboxylic acid acidity due to strong intramolecular hydrogen bonding.[26,27] Contrary to this belief, intramolecular hydrogen bonding is known to *increase* the acidity of neutral carboxylic acids by stabilizing the resulting carboxylate anion. For example, the lower pK_{a1} (1.92) of the dicarboxylic acid maleic acid relative to that of methyl maleate ($pK_a = 2.94$) and fumaric acid ($pK_{a1} = 3.02$) has been explained in terms of stabilization of the resultant monoanion by intramolecular hydrogen bonding.[35,36] And the unusual acidity of 2,6-dihydroxybenzoic acid ($pK_a \simeq 1.3$), which has an intramolecularly hydrogen-bonded carboxylic acid group, has been attributed to stabilization of the carboxylate anion through intramolecular hydrogen bonding; *cf*, $pK_a = 4.47$ for the isomeric 3,4-dihydroxybenzoic acid, which cannot participate in intramolecular hydrogen bonding either as the acid or carboxylate anion.[35] Carboxylic acid deprotonation is hampered in the second ionization of certain dicarboxylic acids but only when the proton is hydrogen bonded to the proximal, negatively-charged carboxylate anion, as in maleate monoanion ($pK_{a2} = 6.23$, *cf.*, fumarate monoanion $pK_{a2} = 4.38$),[35,36] a situation that does not obtain in bilirubin.

CONCLUDING COMMENTS

Our results indicate that the apparent pK_a values of dicarboxylic acids can be determined by ^{13}C-NMR in water and in aqueous-$(CD_3)_2SO$ solutions. With 99% ^{13}C label in the carboxylic acid groups, the ^{13}C-NMR method described is useful for measuring pK_as in very dilute aqueous solutions (10^{-4} to 10^{-6} M). Where the acid is insoluble, addition of small quantities of $(CD_3)_2SO$ to improve the acid's aqueous solubility has only a small influence on the measured pK_a. The first experimental measurement of biliverdin pK_as gives values $pK_{a1} \simeq 3.9$ and $pK_{a2} \simeq 5.3$, which are similar to those found in ordinary dicarboxylic acids. The pK_a values of bilirubin ($pK_{a1} \simeq 4.2$ and $pK_{a2} \simeq 4.9$) are also similar. We find no evidence for the unusually high pK_a's reported earlier for bilirubin by other workers and attributed to intramolecular hydrogen bonding. Those pK_a values, supported by erroneous assumptions, are unrealistic and probably due, *inter alia*, to the poor solubility of the pigment at $pH \leq 7$ and methodological limitations.

EXPERIMENTAL SECTION

General Procedures. Nuclear magnetic resonance (NMR) spectra were determined in $CDCl_3$ or $(CD_3)_2SO$ on a Varian Unity Plus 500 MHz spectrometer or a General Electric QE-300 300 MHz spectrometer and are reported in δ (ppm) downfield from $(CH_3)_4Si$. High resolution mass spectra were performed by the Midwest Center for Mass Spectrometry with partial support by NSF, Biology Division (Grant No. DIR9017762), Lincoln, NE. *p*H measurements were determined on a model 811 Orion Research microprocessor *p*H/millivolt meter. GC-MS analyses were carried out on a Hewlett-Packard GCMS Model 5890A ion selective detector equipped with a DB-1 (100% dimethylpolysiloxane) column. Melting points were determined on a Thomas-Hoover Uni-Melt capillary apparatus and are uncorrected. All solvents were reagent grade obtained from Fisher. Deuterated chloroform and dimethylsulfoxide were from Cambridge Isotope Laboratories. Labelled potassium cyanide ($K^{13}CN$, 90% and 99% ^{13}C-enriched) were obtained from Cambridge Isotope Laboratories. *p*-Chloranil was obtained from Eastman Kodak and used as received. $NaBH_4$ was from J.T. Baker and formic acid and $CaCl_2$ were from Fisher, all used as received. 2,2-Dimethoxypropane, TFA and SO_2Cl_2 were obtained from Aldrich. Analytical thin layer chromatography (TLC) was carried out on J.T. Baker silica gel IB-F plates (125 μm layer). Flash chromatography was performed on Woelm silica gel F, thin layer chromatography grade. Combustion analyses were carried out by Desert Analytics, Tucson, AZ.

Sample Preparation. NMR samples were prepared in NMR tubes by adding standard aliquots of a stock solution of acid or its tetra-*n*-butylammonium salt to aqueous buffers. The stock solutions were prepared as 6-8 x 10^{-3} M solutions in either H_2O or in $(CD_3)_2SO$. Buffered solutions were 0.1 M acetate (for *p*H ~3.2-6.8) and 0.1 M Tris (for *p*H > ~6.8). At *p*H < 3.2, either 0.1 M acetic acid, or 0.1 M acetic acid-HCl, or 0.2 M HCl were used (non-buffered). Phosphate buffers (0.1 M) were used to compare $\delta_{CO_2H}/\delta_{CO_2^-}$ values derived from 0.1 M Tris buffer. No difference was detected at the same *p*H. For the concentrations of adipic acid used (~10^{-4} M) no difference in $\delta_{CO_2^-}$ could be detected at *p*H 7.4 in 0.1 M and 1.0 M Tris buffer. Ten to eleven simple solutions were prepared in NMR tubes at various *p*H for use in a complete titration curve. Added $(CD_3)_2SO$ did not alter the buffered pH or the UV-visible spectra significantly.

1. Aqueous solutions were prepared by adding 50 μL of a 6-8 x 10^{-3} M stock solution of acid or its salt dissolved in deionized H_2O to 500 μL of buffer.

2. Aqueous dimethylsulfoxide solutions were prepared by adding an aliquot of a 6-8 x 10^{-3} M stock solution of acid or its salt in $(CD_3)_2SO$ to an aliquot of buffer:

Final vol % $(CD_3)_2SO$	μL Aliquot Stock Solution	Vol. Buffer (μL)
3.6	20	530
9.0	50	500
27	50	400 + 100 μL $(CD_3)_2SO$
64	50	200 + 300 μL $(CD_3)_2SO$

Final sample concentrations ranged from ~8 x 10^{-4} M to ~2 x 10^{-5} M, with δ_{obs} for the CO_2H/CO_2^- group being independent of concentration in this range. Compounds with only a limited solubility in the NMR solvent, *i.e.*, at low *p*H, were run at least ten times more dilute in 10 mm NMR tubes using long accumulation times. Turbidity did not interfere with the NMR run, except to require longer data accumulation times to record the ^{13}C chemical shift of the dissolved pigment. The data reported are an average of 2-3 independent determinations with pK_a values reported ±0.05.

NMR measurements of δ_{obs} for CO_2H/CO_2^- were carried out on a Varian Unity Plus 500 MHz spectrometer. The instrument settings and parameters were: frequency 125.706 MHz; spectral width 28,368.8 Hz; acquisition time 2.000 sec; relaxation time 0.000 sec; pulse width 5.0 μsec; decouple ^1H; high power 40; decoupler continuously on; Waltz 16 modulated; double precision acquisition; line broadening 1.8 Hz, number of acquisitions varied depending on sample concentration and % ^{13}C-label; and temperature 25°C. Titration curves for adipic acid run at 37°C gave essentially the same pK_a value. To each sample was added a sealed mp capillary insert filled with 50 μL of $(CD_3)_2SO$ that was used as the lock and external reference to standardize all samples to an independent of environment reference.

[1,6-$^{13}C_2$]-Adipic Acid (1). In a 25-mL Erlenmeyer flask equipped with a magnetic stir bar was placed $K^{13}CN$ (632 mg, 9.56 mmol, 90% ^{13}C-enriched) in $(CH_3)_2SO$ (20 mL) and heated to 75°C while 1,4-dibromobutane (1.03 g, 4.77 mmol) was added. The reaction was allowed to stir at 75°C for 20 h. It was then poured into water (50 mL) and extracted with CH_2Cl_2. The organic phase was washed with water, then saturated aqueous NaCl and dried over anhyd. $MgSO_4$. Filtration and concentration to dryness afforded an orange liquid. This was then placed in a 25 mL round bottomed flask equipped with a magnetic stir bar and reflux condenser with a Teflon sleeve along with ethanol (5 mL) and 40% KOH (10 mL) and heated to reflux for 6 h. The reaction was then cooled in an ice bath, acidified by the addition of conc. HCl and extracted with ethyl acetate. The organic phase was dried over anhyd. $MgSO_4$, filtered, and concentrated to dryness to afford diacid **6** as a pale yellow solid (531 mg, 3.59 mmol, 75%). It had mp 151-152°C (Lit.[37] mp 149-150°C); ^{1}H-NMR 500 MHz ($(CD_3)_2SO$) δ: 1.49 (4H, m, 2 x -$CH_2CH_2{}^{13}CO_2H$), 2.18 (4H, m, 2 x -$CH_2CH_2{}^{13}CO_2H$), 11.98 (2H, brs, 2 x -$^{13}CO_2H$) ppm; ^{13}C-NMR 125 MHz ($(CD_3)_2SO$) δ: 174.32 (C_1/C_6 2 x C=O) ppm (8 scans); ^{13}C-NMR 125 MHz (decoupled) ($(CD_3)_2SO$) δ: 24.02 (d, $^2J_{CC}$=2.6 Hz C_3/C_4 2 x CH_2), 33.38 (d, $^1J_{CC}$=55 Hz, C_2/C_5 2 x CH_2), 174.07 (d, $^1J_{CC}$=55 Hz, C_1/C_6 2 x C=O) ppm.

[1-^{13}C]-Succinic Acid (2). In a 10-mL Erlenmeyer flask equipped with a magnetic stir bar was dissolved chloropropionic acid (587 mg, 5.33 mmol) in water (1 mL). This was heated to 50°C, neutralized by the addition of Na_2CO_3 (293 mg, 2.76 mmol) and cooled to room temperature. To this solution was added a solution of $K^{13}CN$ (353 mg, 5.34 mmol, 90% ^{13}C-enriched) in water (1.2 mL), and the mixture was heated to 80°C for 2 h. To this hot solution was then added NaOH (264 mg, 6.60 mmol), and the reaction was allowed to stir at 80°C for 4 h. A solution of $CaCl_2$ (615 mg, 5.54 mmol) in water (1 mL) was then added to the hot solution with rapid stirring. The resulting white suspension was then cooled in an ice bath and the precipitate was collected by filtration, washed with ice cold water, and dried to give 1-^{13}C-labeled calcium succinate as a white solid. The solid was then taken up in ether (20 mL), then 1 M HCl (15 mL) was added, and the mixture was stirred until the solid was dissolved. The resulting solution was continuously extracted with ether for 14 h, and the ether was concentrated to dryness to give the ^{13}C-labeled succinic acid (**10**) as a white solid (211 mg, 1.77 mmol, 33%). It had mp 183-185°C (Lit.[38] mp 188-190°C); ^{1}H-NMR 500 MHz ($(CD_3)_2SO$) δ: 2.40 (4H, m, 2 x CH_2) ppm; ^{13}C-NMR 125 MHz ($(CD_3)_2SO$) δ: 173.92 (C=O) ppm (8 scans); ^{13}C-NMR 125 MHz (decoupled) ($(CD_3)_2SO$) δ: 29.02 (d, $^1J_{CC}$= 56 Hz and C_2 CH_2 and C_3 CH_2), 173.92 (d, $^1J_{CC}$=56 Hz, C_1 C=O and C_4 C=O) ppm.

[1-^{13}C]-Propanedioic Acid (3). In a 10-mL Erlenmeyer flask equipped with a magnetic stir bar was dissolved chloroacetic acid (505 mg, 5.34 mmol) in water (1 mL). The solution was heated to 50°C, neutralized by the addition of Na_2CO_3 (291 mg, 2.75 mmol) and cooled to room temperature. To this solution was then added a solution of $K^{13}CN$ (353 mg, 5.34 mmol, 90% ^{13}C-enriched) in water (1.2 mL), and the reaction was heated to 80°C for 2 h. To the resulting hot solution was added NaOH (258 mg, 6.45 mmol), and the solution was stirred at 80°C for 4 h. A solution of $CaCl_2$ (612 mg, 5.51 mmol) in water (1 mL) was then added to the hot solution with rapid stirring. The resulting white suspension was then cooled in an ice bath; and the precipitate was collected by filtration, washed with ice cold water, and dried to give mono-^{13}C-labeled calcium malonate as a white solid. The solid was then taken up in ether (20 mL), 1 M HCl (15 mL) was added; and the mixture was stirred until the solid was dissolved. The resulting solution was then continuously extracted with ether for 14 h; and after evaporating the ether to dryness, mono-^{13}C-labeled malonic acid was collected as a white solid (262 mg, 2.49 mmol, 47%). It had mp 131-133°C (Lit.[39] mp 130°C); ^{1}H-NMR 500 MHz ($(CD_3)_2SO$) δ: 3.22 (2H, d, $^2J_{CH}$=6.7 Hz, C_2 CH_2) ppm; ^{13}C-NMR 125 MHz ($(CD_3)_2SO$) δ: 42.15 (d, $^1J_{CC}$=56 Hz, C_2 CH_2), 168.10 (d, $^1J_{CC}$=56 Hz, C_1 C=O and C_3 C=O) ppm.

[8^3,12^3-^{13}C]-Mesobiliverdin-XIIIα Dimethyl Ester (12). In a 2-L round bottomed flask equipped with a magnetic stirrer and reflux condenser was placed methyl [8^3-^{13}C]-xanthobilirubinate (**11**)[3] (2.15 g, 6.78 mmol) dissolved in hot CH_2Cl_2 (50 mL). To this solution was added *p*-chloranil (4.19 g, 17.0 mmol) dissolved in hot CH_2Cl_2 (750 mL). This green solution was then allowed to stir at reflux for 10 min when 98% formic acid (60 mL) was added in a single portion and allowed to reflux for 20 h. This was then cooled to room temperature and concentrated to ~100 mL to afford a blue-green suspension. This was then cooled

to -20°C and resulting solid filtered off and washed with cold CH_2Cl_2. The filtrate was then washed sequentially with 5% aq. $NaHCO_3$ (3 x 100 mL), 1 M NaOH (5 x 100 mL), water (3 x 100 mL), and saturated aq. NaCl (2 x 100 mL). The resulting solution was then dried over anhyd. Na_2SO_4, filtered, then concentrated to dryness to afford a blue solid. The solid was dissolved in a minimum amount of CH_2Cl_2 and deposited on a silica gel aspirator flash column (3.5 x 6.5 cm diameter), pre-eluted with CH_2Cl_2, and eluted with CH_2Cl_2:CH_3OH (10:1) which gave the verdin as a blue band which was collected and concentrated to dryness to afford the verdin as a blue solid (1.92 g, 3.11 mmol, 92%). It had mp 234-236°C (Lit.[40] 246-247°C); ^{1}H-NMR ($CDCl_3$) δ: 1.21 (6H, t, $^3J_{HH}$=7.6 Hz, 2 x -CH_2CH_3), 1.82 (6H, s, C_7/C_{13} 2 x CH_3), 2.09 (6H, s, C_2/C_{18} 2 x CH_3), 2.25 (4H, q, $^3J_{HH}$=7.5 Hz, 2 x -CH_2CH_3), 2.45 (4H, dt, $^3J_{HH}$= 7.5 Hz and $^2J_{CH}$=8.5 Hz, 2 x -$CH_2CH_2{}^{13}CO_2CH_3$), 2.92 (4H, dt, $^3J_{HH}$=7.5 Hz and $^3J_{CH}$=2.8 Hz, 2 x -$CH_2CH_2{}^{13}CO_2CH_3$), 3.67 (6H, d, $^3J_{CH}$=3.8 Hz, 2 x -OCH_3), 5.93 (2H, s, C_5/C_{15} 2 x CH), 6.75 (1H, s, C_{10} CH), 8.14 (1H, brs, NH pyrrole), 10.32 (2H, brs, 2 x NH lactam) ppm; ^{13}C-NMR ($CDCl_3$) δ: 173.11 ($C_8{}^3/C_{12}{}^3$ C=O) ppm (8 scans).

[8^3,12^3-^{13}C]-Mesobilirubin-XIIIα Dimethyl Ester (13). In a 500-mL Erlenmeyer flask was placed verdin dimethyl ester **12** (532 mg, 0.862 mmol) in tetrahydrofuran (100 mL). This was then placed in a sonicator and swept with N_2 where $NaBH_4$ (1.50 g, 39.7 mmol) and CH_3OH (150 mL) were added, each in 1/3 portions over a 20 min period. Upon complete addition the sonication was continued for an additional 2 h. The resulting yellow suspension was cooled to 4°C and acidified to *p*H 8 by the addition of 10% HCl and extracted with CH_2Cl_2 (200 mL). The extract was then washed with water (2 x 200 mL), saturated aq. NaCl (1 x 200 mL), dried with anhyd. Na_2SO_4, filtered, and concentrated to dryness to afford the rubin as a yellow-green solid. This was then dissolved in a minimum amount of CH_2Cl_2 and deposited on a silica gel aspirator flash column (2.5 x 4.5 cm diameter), pre-eluted with CH_2Cl_2, and eluted with CH_2Cl_2:ethanol (50:1) to removed an orange band which was collected and concentrated to dryness to afford the rubin as a bright yellow solid (432 mg, 0.689 mmol, 81%). It had mp 232-234°C (Lit.[41] 234-236°C); ^{1}H-NMR ($CDCl_3$) δ: 1.00 (6H, t, $^3J_{HH}$=7.4 Hz, 2 x -CH_2CH_3), 1.48 (6H, s, C_7/C_{13} 2 x CH_3), 2.10 (6H, s, C_2/C_{18} 2 x CH_3), 2.32 (4H, q, $^3J_{HH}$=7.4 Hz, 2 x -CH_2CH_3), 2.48 (4H, dt, $^3J_{HH}$=7.0 Hz and $^2J_{CH}$=8.5 Hz, 2 x -$CH_2CH_2{}^{13}CO_2CH_3$), 2.87 (4H, dt, $^3J_{HH}$=7.0 Hz and $^3J_{CH}$=2.8 Hz, 2 x -$CH_2CH_2{}^{13}CO_2CH_3$), 3.68 (6H, d, $^3J_{CH}$=3.8 Hz, 2 x -OCH_3), 4.14 (2H, s, C_{10} CH_2), 5.91 (2H, s, C_5/C_{15} CH), 10.27 (2H, brs, 2 x NH pyrrole), 10.56 (2H, brs, 2 x NH lactam) ppm; ^{13}C-NMR ($CDCl_3$) δ: 173.65 ($C_8{}^3/C_{12}{}^3$ C=O) ppm (8 scans).

[8^3,12^3-$^{13}C_2$]-Mesobiliverdin-XIIIα (4). In a 500-mL round bottomed flask equipped with a magnetic stir bar and reflux condenser was placed verdin dimethyl ester **12** (153 mg, 0.247 mmol) in CH_3OH (200 mL). To this blue solution was added ascorbic acid (42.4 mg), disodium EDTA (10 mg), and 1 M NaOH (75 mL) and heated to 45°C for 20 h. To this blue solution was added acetic acid (30 mL) and *p*H 2.8 glycine hydrochloride buffer (350 mL) and extracted with $CHCl_3$ until the aqueous layer was a clear yellow. The organic extract was then washed with water (2 x 100 mL), saturated aq. NaCl (1 x 100 mL), dried with anhyd. Na_2SO_4, and concentrated to dryness. This was then taken up in $CHCl_3$:CH_3OH (10:1) and deposited on a silica gel aspirator flash column (3 x 4.5 cm diameter), pre-eluted with $CHCl_3$, and eluted with $CHCl_3$:CH_3OH (10:1) to remove some yellow and green impurities, then with $CHCl_3$:CH_3OH (5:1) to removed the verdin as a blue band which was collected and concentrated to dryness to afford a blue solid (71.4 mg, 0.121 mmol, 49%). It had mp 194-196°C (dec) (Lit.[42,43] none reported); ^{1}H-NMR ($CDCl_3$) δ: 1.11 (6H, t, $^3J_{HH}$=7.7 Hz, 2 x -CH_2CH_3), 1.69 (6H, s, C_7/C_{13} 2 x CH_3), 2.04 (6H, s, C_2/C_{18} 2 x CH_3), 2.41-2.85 (12H, m, 2 x -$CH_2CH_2{}^{13}CO_2H$ and 2 x -CH_2CH_3), 5.96 (2H, s, C_5/C_{15} 2 x CH), 6.95 (1H, s, C_{10}CH), 9.87 (1H, brs, NH pyrrole), 12.15 (2H, brs, 2 x NH lactam) ppm; ^{13}C-NMR ($CDCl_3$) δ: 173.62 ($C_8{}^3/C_{12}{}^3$ C=O) ppm (32 scans); ^{13}C-NMR 125 MHz (decoupled) ($(CD_3)_2SO$) δ: 8.11 ($C_3{}^2/C_{17}{}^2$ 2 x CH_3), 9.15 ($C_7{}^1/C_{13}{}^1$ 2 x CH_3), 14.42 ($C_2{}^1/C_{18}{}^1$ 2 x CH_3), 16.97 ($C_3{}^1/C_{17}{}^1$ CH_2), 19.31 ($C_8{}^1/C_{12}{}^1$ CH_2), 35.18 (d, $^1J_{CC}$=54 Hz, $C_8{}^2/C_{12}{}^2$ CH_2), 95.78 (C_5/C_{15} CH), 115.97 (C_{10} CH), 127.36 (C_7/C_{13}), 127.62 (C_6/C_{14}), 137.90 (d, $^3J_{CC}$=2.4 Hz, C_8/C_{12}), 139.90 (C_2/C_{18}), 139.97 (C_4/C_{16}), 146.27 (C_9/C_{11}), 149.33 (C_3/C_{17}), 172.27 (C_1/C_{19} C=O), 173.61 (d, $^1J_{CC}$=54 Hz, $C_8{}^3/C_{12}{}^3$ C=O) ppm.

[8^3,12^3-$^{13}C_2$]-Mesobilirubin-XIIIα (5). In a 100-mL round bottomed flask equipped with a magnetic stir bar and reflux condenser was placed rubin dimethyl ester **13** (432 mg, 0.698 mmol) in THF (20 mL) and CH_3OH (30 mL). To this was added 1 M NaOH (5.00 mL) and heated to reflux for 4 h. This was then cooled in an ice bath and 1 M HCl (6.00 mL) was added dropwise to give a green suspension which was washed with ice cold CH_3OH to give the rubin as a bright yellow solid. The mother liquor was concentrated to dryness, taken up in CH_3OH and cooled in an ice bath to afford more of the rubin which was collected by filtration, washed with ice cold CH_3OH, combined with the previously collected rubin, and dried (323 mg, 0.548 mmol, 78%). It had mp 308-311°C (Lit.[41] 312-315°C); ^{1}H-NMR ($CDCl_3$) δ: 1.11 (6H, t, $^3J_{HH}$=7.5 Hz, 2 x -CH_2CH_3), 1.86 (6H, s, C_7/C_{13} 2 x CH_3), 2.16 (6H, s, C_2/C_{18} 2 x CH_3), 2.49 (4H, q, $^3J_{HH}$=7.5 Hz, 2 x -CH_2CH_3), 2.56 (1H, dddd, $^3J_{HH}$=2.6 Hz, $^3J_{HH}$=4.7 Hz, $^2J_{HH}$=15 Hz, $^3J_{CH}$=3.7 Hz, -CH_XHCH_2-$^{13}CO_2H$), 2.79 (1H, dddd, $^3J_{HH}$=2.8 Hz, $^3J_{HH}$=4.7 Hz, $^2J_{HH}$=19 Hz, $^2J_{CH}$=6.7 Hz, -$CH_2CH_2{}^{13}CO_2H$), 2.86 (1H, dddd, $^3J_{HH}$=13 Hz, $^3J_{HH}$=2.6 Hz, $^2J_{HH}$=19 Hz, $^2J_{CH}$=6.7 Hz, -$CH_2CH H_B{}^{13}CO_2H$), 2.99 (1H, dddd, $^3J_{HH}$=13 Hz, $^3J_{HH}$=2.8 Hz, $^2J_{HH}$=15 Hz, $^3J_{CH}$=3.7 Hz, -$CH H_ACH_2{}^{13}CO_2H$), 4.08 (2H, s, C_{10} CH_2), 6.05 (2H, s, C_5/C_{15} CH), 9.14 (2H, brs, 2 x NH pyrrole), 10.59 (2H, brs, 2 x NH lactam), 13.64 (2H, brs, 2 x -$^{13}CO_2H$) ppm; ^{13}C-NMR ($CDCl_3$) δ: 179.49 ($C_8{}^3$/ $C_{12}{}^3$ C=O) ppm (32 scans); ^{13}C-NMR 125 MHz (decoupled) ($(CD_3)_2SO$)) δ: 8.06 ($C_3{}^2$/$C_{17}{}^2$ 2 x CH_3), 9.14 ($C_7{}^1$/$C_{13}{}^1$ 2 x CH_3), 14.81 ($C_2{}^1$/$C_{18}{}^1$ 2 x CH_3), 17.15 ($C_3{}^1$/$C_{17}{}^1$ CH_2), 19.25 (C^1/$C_{12}{}^1$ CH_2), 23.31 (C_{10} CH_2), 34.39 (d, $^1J_{CC}$=54 Hz, $C_8{}^2$/$C_{12}{}^2$ CH_2), 97.66 (C_5/C_{15} CH), 119.15 (d, C_8/C_{12} $^3J_{CC}$=2.3 Hz), 121.93 (C_7/C_{13}), 122.41 (C_6/C_{14}), 122.89 (C_2/C_{18}), 127.78 (C_4/C_{16}), 130.37 (C_9/C_{11}), 147.16 (C_3/C_{17}), 171.88 (C_1/C_{19} C=O), 173.94 (d, $^1J_{CC}$=54 Hz, $C_8{}^3$/$C_{12}{}^3$ C=O) ppm.

Ethyl 4-Methyl-1*H*-pyrrole-3-[1-^{13}C]propanoate (16). In a 250-mL 3-necked round bottomed flask equipped with a magnetic stir bar, thermometer, drying tube, and addition funnel was dissolved 2-methoxycarbonyl-3-5-dimethyl-1*H*-pyrrole-4-[3-^{13}C]propionic acid methyl ester (**15**) (4.89 g, 20.4 mmol) in dry tetrahydrofuran (45 mL). This brown solution was then cooled to -15°C when freshly distilled SO_2Cl_2 (8.26 g, 61.2 mmol) was added dropwise over a 1 h period. This was then allowed to warm to 3°C and stirred for 4 h when water (15 mL) was added and the reaction was allowed to stir at room temperature for 18 h. The excess tetrahydrofuran was removed *in vacuo* to give a tan precipitate which was collected by filtration and washed with ice cold water. This solid was then suspended in water (20 mL) and NaOH (4.02 g, 101 mmol) and heated to reflux for 3.5 h. This brown solution was cooled in an ice bath and to it was added concentrated HCl until acidic by *p*H paper. The resulting brown suspension was cooled in an ice bath and solid collected by filtration and dried to afford the triacid as a light-brown solid (4.45 g, 18.4 mmol, 90%). This was then used directly in the next step.

The triacid (4.45 g, 18.4 mmol) was suspended in absolute ethanol (25 mL) in a 100-mL round bottomed flask equipped with a magnetic stir bar. To this red-brown suspension was added TFA (2.40 mL, 3.55 g, 31.2 mmol) and allowed to stir at room temperature for 26 h. To this red solution was added triethyl orthoformate (2.80 mL) and allowed to stir at room temperature for 24 h when the solvent was evaporated to give a red solid. This was then doubly decarboxylated by distillation in a Kugelrohr apparatus at 180°C under a vacuum of 1.2 mm Hg to afford the pyrrole as a clear tan liquid (2.06 g, 11.3 mmol, 61%).[11] It had ^{1}H-NMR ($CDCl_3$) δ: 1.26 (3H, t, ^{3}J=7.1 Hz, -OCH_2CH_3), 2.05 (3H, s, C_3 CH_3), 2.57 (2H, dt, $^3J_{HH}$=7.7 Hz and $^2J_{CH}$=7.2 Hz, -$CH_2CH_2{}^{13}CO_2Et$), 2.75 (2H, dt, $^3J_{HH}$=7.2 Hz and $^3J_{CH}$=3.6 Hz, -$CH_2CH_2{}^{13}CO_2Et$), 4.13 (2H, dq, $^3J_{HH}$=7.1 Hz and $^3J_{CH}$=3.3 Hz, -OCH_2CH_3), 6.53 (2H, d, $^3J_{HH}$=2.5 Hz, C_2/C_5 CH), 7.79 (1H, brs, NH) ppm; ^{13}C-NMR ($CDCl_3$) δ: 173.56 ($C_4{}^3$ C=O) ppm (32 scans).

2-Formyl-3-methyl-1*H*-pyrrole-4-[1-^{13}C]propanoic Acid (17). In a 100-mL round bottomed flask with magnetic stir bar was placed 4-methyl-1*H*-pyrrole-3-[1-^{13}C]propanoic acid ethyl ester (**16**) (2.06 g, 11.3 mmol) in dry ether (40 mL) and dimethylformamide (1.03 mL, 14.1 mmol) and cooled in an ice bath to 4°C. Phosphorous oxychloride (2.05 g, 13.4 mmol) was added dropwise over a 5 min period then allowed to stir at 4°C for 1 h. This was then warmed to room temperature and allowed to stir another 20 h. This dark brown oily suspension was then concentrated to dryness leaving a dark brown oil. To this was first added water (25 mL) then a solution NaOH (3.28 g, 82.0 mmol) in water (10 mL). This was then heated to reflux for 30 min, cooled in an ice bath, and brown insoluble material filtered off and washed with cold water. The

tan filtrate was then acidified by the addition of conc. HCl to give a tan precipitate which was collected by filtration, washed with ice cold water, and dried leaving a brown solid. This mixture of 2 and 5-formyl isomers was separated by crystallization from methanol giving the 5-formyl isomer as red-tan crystals (603 mg, 3.31 mmol, 30%).[11,44] It had mp 152-153°C (Lit.[44] 155°C); ^{1}H-NMR ($(CD_3)_2SO$) δ: 2.21 (3H, s, C_4 CH_3), 2.43 (2H, dt, ^3J=7.2 Hz and $^2J_{CH}$=6.9 Hz, -$CH_2CH_2{}^{13}CO_2H$), 2.56 (2H, m, -$CH_2CH_2{}^{13}CO_2H$), 6.92 (1H, d, $^3J_{HH}$=3.0 Hz, C_2 CH), 9.54 (1H, s, -CHO), 11.55 (1H, s, NH), 12.09 (1H, s, -$^{13}CO_2H$) ppm; ^{13}C-NMR $(CD_3)_2SO$ δ: 174.02 (s) ppm (8 scans).

[8^3-^{13}C]-3^2-Nor-neoxanthobilirubic Acid (7). In a 50-mL round bottomed flask equipped with a magnetic stir bar was placed 2-formyl-3-methyl-1*H*-pyrrole-4-[1-^{13}C]propanoic acid (**17**) (595 mg, 3.27 mmol) and 3,4-dimethylpyrrolin-2-one[11] (**18**) (363 mg, 3.30 mmol) in CH_3OH (3 mL). To this was added a solution of NaOH (1.61 g, 40.3 mmol) in water (8 mL) and allowed to stir at room temperature for 23 h. The resulting suspension was diluted with water (20 mL) and acidified with acetic acid (3 mL) and allowed to stir in an ice bath for 2 h. The solid was collected by filtration, washed with ice cold water, and dried *in vacuo* to give the dipyrrinone as a yellow-green solid (567 mg, 2.06 mmol, 63%). It had mp 244-246°C (Lit.[11] non-labeled 245-247°C); ^{1}H-NMR ($(CD_3)_2SO$) δ: 1.76 (3H, s, C_7 CH_3), 2.03 (3H, s, C_3 CH_3), 2.05 (3H, s, C_2 CH_3), 2.40 (2H, dt, $^3J_{HH}$=7.3 Hz and $^2J_{HH}$=6.8 Hz, -$CH_2CH_2{}^{13}CO_2H$), 2.55 (2H, m, -$CH_2CH_2{}^{13}CO_2H$), 5.94 (1H, s, C_5 CH), 6.73 (1H, s, C_9 CH), 9.71 (1H, brs, NH pyrrole), 10.49 (1H, brs, NH lactam), 12.05 (1H, brs, $^{13}CO_2H$) ppm; ^{13}C-NMR $(CD_3)_2SO$ δ: 174.01 ($C_8{}^3$ C=O) ppm (8 scans); ^{13}C-NMR 125 MHz (decoupled) ($(CD_3)_2SO$) δ: 8.30 ($C_7{}^1$ CH_3), 9.01 ($C_3{}^1$ CH_3), 9.55 ($C_2{}^1$ CH_3), 20.36 ($C_8{}^1$ CH_2), 38.89 (d, $^1J_{CC}$ =55 Hz, $C_8{}^2$ CH_2), 97.69 (C_5 CH), 119.25 (C_9 CH), 121.01 (C_7), 122.26 (d, $^3J_{CC}$=3.4 Hz, C_8), 123.72 (C_6), 124.20 (C_2), 130.06 (C_4), 141.57 (C_3), 173.43 (C_1 C=O), 173.94 (d, $^1J_{CC}$=55 Hz, $C_8{}^3$ C=O) ppm.

[8^3,12^3-$^{13}C_2$]-10,10-Dimethylglaucorubin (6). In a 5-mL Erlenmeyer flask equipped with a magnetic stir bar was placed dipyrrinone **7** (316 mg, 1.47 mmol), 2,2-dimethoxypropane (125 mg, 1.20 mmol), and ice cold trifluoroacetic acid (2.00 mL) and allowed to stir for 5 min. The reaction was then quenched by pouring into ice cold water (30 mL). The resulting precipitate was collected by filtration and washed with ice cold water. The precipitate was then washed with CH_2Cl_2 (20 mL) to dissolve formed rubin. The remaining solid, starting dipyrrinone, was placed back in a 5 mL Erlenmeyer flask and to it was added 2,2-dimethoxypropane (110 mg) and ice cold trifluoroacetic acid (2.00 mL) and allowed to stir for 5 min. The reaction was quenched by pouring into ice water (30 mL), the precipitate was collected and treated as above. This procedure was repeated 2 more times. The combined CH_2Cl_2 washings were then washed with water, saturated aq. NaCl and concentrated to dryness to afford an orange solid. This was then dissolved in a minimum amount of CH_2Cl_2 and deposited on a silica gel aspirator flash column (3 x 4 cm diameter), pre-eluted with CH_2Cl_2, and eluted with CH_2Cl_2:CH_3OH (100:1) to remove a yellow band which was collected and concentrated to dryness to afford the rubin and a bright yellow orange solid (135 mg, 0.229 mmol, 40%). It had mp 220-222°C (dec) (Lit.[11] 210°C (dec)); ^{1}H-NMR ($CDCl_3$) δ: 1.85 (6H, s, C_7/C_{13} 2 x CH_3), 2.06 (6H, s, C_3/C_{17} 2 x CH_3), 2.07 (6H, s, C_2/C_{18} 2 x CH_3), 2.16 (6H, s, C_{10} 2 x CH_3), 2.56 (1H, dddd, $^3J_{BX}$= 2.9 Hz, $^3J_{BA}$=12 Hz, $^2J_{BC}$=18 Hz, $^2J_{CH}$=3.7 Hz, -$CH_XHCH_2{}^{13}CO_2H$), 2.78 (1H, ddd, -$CH_2CHH_B{}^{13}CO_2H$, $^3J_{BX}$=2.9 Hz, $^3J_{BA}$=12 Hz, $^2J_{BC}$=18 Hz, $^3J_{CX}$=3.7 Hz), 2.91 (1H, dddd, $^3J_{CA}$=3.2 Hz, $^3J_{CX}$=3.7 Hz, $^2J_{CB}$=18 Hz, $^2J_{CH}$=3.7 Hz, -$CH_2CH_CH{}^{13}CO_2H$), 3.49 (1H, dddd, $^3J_{AB}$=12 Hz, $^3J_{AC}$=3.2 Hz, $^2J_{AX}$=15 Hz, $^3J_{CH}$=2.8 Hz, -$CHH_ACH_2{}^{13}CO_2H$), 6.02 (2H, s, C_5/C_{15} CH), 8.91 (2H, brs, 2 x NH pyrrole), 11.08 (2H, brs, 2 x NH lactam), 13.96 (2H, brs, 2 x CO_2H) ppm; ^{13}C-NMR ($(CD_3)_2SO$) δ: 173.96 ($C_8{}^3/C_{12}{}^3$ C=O) ppm (8 scans) ppm; ^{13}C-NMR 125 MHz (decoupled) ($(CD_3)_2SO$) δ: 8.35 ($C_7{}^1/C_{13}{}^1$ CH_3), 9.30 ($C_3{}^1/C_{17}{}^1$ CH_3), 9.57 ($C_2{}^1/C_{18}{}^1$ CH_3), 19.76 ($C_8{}^1/C_{12}{}^1$ CH_2), 29.05 ($C_{10}{}^1$ 2 x CH_3), 34.25 (d, $^1J_{CC}$=54 Hz, $C_8{}^2/C_{12}{}^2$ CH_2), 36.53 (C_{10}), 98.18 (C_5/C_{15} CH), 118.45 (d, $^3J_{CC}$=4.8 Hz, C_8/C_{12}), 121.66 (C_7/C_{13}), 123.09 (C_6/C_{14}), 124.14 (C_2/C_{18}), 129.89 (C_4/C_{16}), 138.54 (C_9/C_{11}), 141.51 (C_3/C_{17}), 172.34 (C_1/C_{19} C=O), 173.96 (d, $^1J_{CC}$=54 Hz, $C_8{}^3/C_{12}{}^3$ C=O) ppm. HR-MS calcd. for $^{13}C_2C_{31}H_{40}N_4O_6$: 590.3015, found: 590.3008.

[8^3-^{13}C]-12-Despropionic acid-12-ethyl-mesobiliverdin-XIIIα (9). To a solution of methyl [8^3-^{13}C]-xanthobilirubinate (**11**) (396 mg, 1.25 mmol) and kryptopyrromethenone (**19**)[12] (310 mg, 1.26 mmol) dis-

solved in hot CH_2Cl_2 (270 mL) was added *p*-chloranil (1.16 g, 4.72 mmol) followed by 88% formic acid (29 mL). The reaction immediately turned green and was heated to reflux for 40 h. The emerald green solution was concentrated to ~100 mL then placed at -20°C to precipitate out the reduced *p*-chloranil, which was removed via filtration and washed with ice cold CH_2Cl_2. This was then cautiously washed with 5% $NaHCO_3$ (3 x 100 mL), 1 M NaOH (3 x 100 mL), water (1 x 200 mL), saturated aq. NaCl (1 x 200 mL), dried over anhyd. Na_2SO_4, filtered, and concentrated to dryness to afford a blue solid. This was then used without further purification for the next step.

To the mixed verdins, dissolved in hot CH_3OH (140 mL), was added 2 M NaOH (125 mL) and heated to reflux for 17 h. The reaction was cooled, then poured into acetic acid (75 mL) and *p*H 2.8 glycine HCl buffer (500 mL) and extracted with $CHCl_3$ until the aqueous layer was clear yellow. The blue organic extract was then washed with water (5 x 100 mL), 1 M NaOH (until clear) to remove the sodium salt of **4** (which is then acidified with concentrated HCl to recover **4**), water (2 x 100 mL), 1 M HCl (2 x 100 mL) saturated aq. NaCl, dried over anhyd. Na_2SO_4, filtered, and concentrated to dryness to afford a blue solid. This is then dissolved in a minimum amount of $CHCl_3$ and placed on a silica gel aspirator flash column (4 x 6.5 cm diameter), pre-eluted with $CHCl_3$, and eluted with $CHCl_3$:CH_3OH (50:1) to remove etiobiliverdin-IVγ, which was concentrated to dryness to afford a blue solid, then with $CHCl_3$:CH_3OH (10:1) to remove **9** which was concentrated to dryness to afford a blue solid (148 mg, 0.272 mmol, 22%). It had mp 138-139°C (Lit.[13] un-labeled 138-140°C); ^{1}H-NMR ($CDCl_3$) δ: 1.06 (3H, t, $^3J_{HH}$=7.5 Hz, C_{17} -CH_2CH_3), 1.21 (6H, 2 x t, $^3J_{HH}$= 7.5 Hz, C_3 -CH_2CH_3 and $^3J_{HH}$=7.7 Hz, C_{17} -CH_2CH_3), 1.81 (6H, s, C_{13}/C_7 2 x CH_3), 1.97 (3H, s, C_{18} CH_3), 2.05 (3H, s, C_2 CH_3), 2.16-2.62 (10H, m, $C_{12}/C_{17}/C_3$ -CH_2CH_3 and -$CH_2CH_2{}^{13}CO_2H$), 5.89 (1H, s, C_{15} CH), 6.01 (1H, s, C_5 CH), 6.69 (1H, s, C_{10} CH), 9.38 (1H, brs, NH pyrrole), 9.75 (2H, brs, 2 x NH lactam) ppm; ^{13}C-NMR ($CDCl_3$) δ: 176.57 ($C_8{}^3$ C=O), ppm (8 scans); ^{13}C-NMR 125 MHz (decoupled) (($CD_3)_2SO$) δ: 8.11 ($C_{12}{}^2/C_{17}{}^2$ 2 x CH_3), 9.02 ($C_3{}^2$ CH_3), 9.14 ($C_7{}^1$ CH_3), 14.42 ($C_{12}{}^1/C_{18}{}^1$ 2 x CH_3), 16.10 ($C_2{}^1$ CH_3), 16.98 ($C_{12}{}^1/C_{17}{}^1$ 2 x CH_2), 17.10 ($C_3{}^1$ CH_2), 19.32 ($C_8{}^1$ CH_2), 35.29 (d, $^1J_{CC}$=55 Hz, $C_8{}^2$ CH_2), 95.81 (C_5/C_{15} CH), 115.57 (C_{10} CH), 126.71 (C_{12}), 127.20 (C_8), 127.55 (C_{14}), 127.61 (C_6), 137.70 (C_{18}), 139.68 (C_2), 139.81 (C_{13}), 149.89 (C_7), 140.00 (C_{16}), 141.31 (C_4), 146.26 (C_9/C_{11}), 148.91 (C_{17}), 149.78 (C_3), 172.25 (C_{19} C=O), 172.28 (C_1 C=O), 173.63 (d, $^1J_{CC}$=55 Hz, $C_8{}^3$ C=O) ppm.

[8^3-^{13}C]-12-Despropionic acid-12-ethyl-mesobilirubin-XIIIα (10). To a sonicating solution of verdin **9** (47.6 mg, 0.088 mmol) dissolved in cold N_2 saturated CH_3OH (20 mL) was added $NaBH_4$ (650 mg, 17.2 mmol) in a single portion. The reaction turned yellow and sonication was allowed to continue for 15 min. The reaction was then quenched by pouring into an N_2 saturated solution of water (20 mL) and acetic acid (1 mL, 17.5 mmol) and allowed to sonicate under N_2 for 5 min. This yellow suspension was cooled in an ice bath and precipitate collected by centrifugation and washed with water. The resulting pellet was suspended in water (5 mL), collected by filtration, and dried *in vacuo* to afford the rubin as a yellow solid (34.0 mg, 0.062 mmol, 71%). It had mp 267-269°C (Lit.[13] non-labeled mp 268-269°C after blackening from 230-255°C); ^{1}H-NMR ($CDCl_3$) δ: 1.12 (6H, t, $^3J_{HH}$=7.5 Hz, C_3/C_{17} 2 x CH_2CH_3), 1.17 (3H, t, $^3J_{HH}$=7.5 Hz, C_{12} CH_2CH_3), 1.87 (6H, s, C_7/C_{12} 2 x CH_3), 2.10 (3H, s, C_{18} CH_3), 2.14 (3H, s, C_2 CH_3), 2.42-2.80 (10H, m, $C_3/C_{12}/C_{17}$ 3 x -CH_2CH_3 and -$CH_2CH_2{}^{13}CO_2H$), 3.99 (2H, s, C_{10} CH_2), 5.92 (1H, s, C_{15} CH), 6.10 (1H, s, C_5 CH), 7.12 (1H, brs, NH pyrrole), 7.65 (1H, brs, NH lactam), 8.96 (1H, brs, NH pyrrole), 10.49 (1H, brs, NH lactam), 13.87 (1H, brs, -$^{13}CO_2H$) ppm; ^{13}C-NMR ($CDCl_3$) δ: 179.14 (s) ppm (32 scans); ^{13}C-NMR 125 MHz (decoupled) (($CD_3)_2SO$) δ: 8.06 ($C_{12}{}^2/C_{17}{}^2$ 2 x CH_3), 9.11 ($C_3{}^2$ CH_3), 9.16 ($C_{13}{}^1$ CH_3), 14.80 ($C_2{}^1/C_{18}{}^1$ CH_3), 15.09 ($C_7{}^1$ CH_3), 16.78 ($C_{12}{}^1$ CH_2), 17.14 ($C_{17}{}^1$ CH_2), 17.17 ($C_3{}^1$ CH_2), 19.33 ($C_8{}^1$ CH_2), 23.40 (C_{10} CH_2), 34.59 (d, $^1J_{CC}$=55 Hz, $C_8{}^2$ CH_2), 97.70 (C_{15} CH), 97.77 (C_5 CH), 119.25 (C_{12}), 121.86 (d, $^3J_{CC}$=3.2 Hz, C_8), 121.89 (C_{13}), 122.22 (C_7), 122.29 (C_{14}), 122.53 (C_6), 122.75 (C_{18}), 122.87 (C_2), 127.61 (C_{16}), 127.65 (C_4), 129.72 (C_{11}), 130.60 (C_9), 147.13 (C_{17}), 147.21 (C_3), 171.89 (C_{19} C=O), 171.93 (C_1 C=O), 174.04 (d, $^1J_{CC}$=55 Hz, $C_7{}^3$ C=O) ppm.

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