Evidence That Heme d_1 Is a 1,3-Porphyrindione†

C. K. Chang,‡ R. Timkovich,*,§ and W. Wu‡

Department of Chemistry, Michigan State University, East Lansing, Michigan 48823, and Department of Chemistry, University of Alabama, University, Alabama 35486

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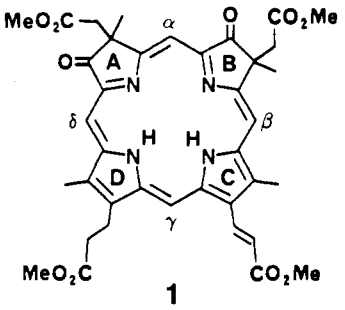
ABSTRACT: Heme d_1 is the noncovalently associated heme prosthetic group of the bacterial nitrite reductase known as cytochrome cd_1. Additional evidence has been obtained in support of a dioxoisobacteriochlorin, or 1,3-porphyrindione, skeleton for this heme. The new data include the natural abundance ^{13}C NMR spectrum of the free base methyl ester derivative of d_1, mass spectrometric determinations of the molecular mass of the free base methyl ester and the Cu and the Zn chelates, visible and ^1H NMR spectral comparisons between d_1 and synthetic porphyrindione model compounds, and the isolation and characterization of several byproducts formed during the purification of the free base methyl ester of d_1. The accumulated evidence strongly supports the following structure for the skeleton of d_1: 1-oxo-2-methyl-2′-acetyl-3-oxo-4-methyl-4′-acetyl-5-methyl-6-acrylyl-7-propionyl-8-methylporphyrin.

Heme d_1 is the active site prosthetic group for nitrite reduction in a dissimilatory nitrite reductase found in many chemoautotrophic bacteria including *Pseudomonas aeruginosa*, *Paracoccus denitrificans*, and *Thiobacillus denitrificans*. A structure was originally proposed for the organic macrocycle skeleton of d_1 on the basis of the visible, mass, infrared, and NMR spectroscopic data on the metal-free, esterified derivative of the heme (Timkovich et al., 1984a,b). A new interpretation of the structure as **1** has been given on the basis of



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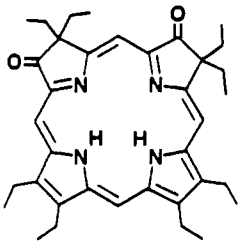

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studies of dioxoisobacteriochlorin model compounds such as **2** (Chang, 1985). To resolve these two interpretations, more experimental data have been obtained on both the isolated d_1 pigment and synthetic model compounds. The purpose of this paper is to present such experimental evidence in favor of the dioxoisobacteriochlorin interpretation.

Some questions of nomenclature for this system should be addressed. Chlorin has been the traditional term for a porphyrin with one reduced pyrrole. We prefer the term partially saturated pyrrole, since substituents on natural systems are being found to contain directly bonded oxygen in different formal oxidation states. Isobacteriochlorin has been the term for a porphyrin with two adjacent (in contrast to diagonally opposite) partially saturated pyrroles. In most model compounds, they have a total of four sp^3-hybridized β-pyrrolic carbon atoms while a chlorin has two. The case of the skeleton of d_1 may be viewed as intermediate. Two adjacent rings are partially saturated, but there are only two sp^3 carbons. The chemical reactivity of this skeleton in model compounds shows very little in common with that of isobacteriochlorin (Chang et al., 1986). For these reasons, we refer to the skeleton of d_1 as a porphyrindione, where it can be considered as a porphyrin quinone. To further specify the position of the oxo groups, the prefix 1,3 has been added (Change & Wu, 1986). *Chemical Abstracts* uses a system based upon porphine-2-propionic acid such that the acrylate should be drawn in the structures to the right of the propionate. This is not a structural revision from other earlier representations but only corresponds to drawing the molecule turned over. We have used the conventional Fischer 1, 2, ..., 8 system for the substituents, and the keto groups as the dominant substituents have been assigned the lowest numbers (1,3 instead of 2,4).

Materials and Methods

The isolation and preparation of the free base methyl ester derivative of d_1 has been described (Timkovich et al., 1984a). The material for the ^{13}C NMR natural abundance spectrum to be reported represented many batches pooled over several years of isolation of this compound. The free base ester is quite stable when stored below 0 °C in the dark as a dry film. The pooled material was subjected to the final reverse-phase chromatography step before use. During this work, it was possible to isolate and further characterize three minor products that were present and had been mentioned only briefly in the original work (Timkovich et al., 1984a). These were initially separated during the preparative reverse-phase chromatography step. Of the three, the most abundant was a blue pigment with an R_f of 0.88 compared to 0.27 for d_1 at this step. Subsequent chromatography in an HPLC[1] system (10-μm silica, 4.6 × 250 mm, eluted with neat chloroform) showed that it was not homogeneous. The main component eluted with a capacity factor k' of 2.37 compared to 0.40 for free base esterified d_1 in the same system and was accumulated to provide the material for further spectroscopic studies that will be described under Results. The next most abundant side

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*Author to whom correspondence should be addressed.

‡Michigan State University.

§University of Alabama.

[1] Abbreviations: ppm, parts per million; NOE, nuclear Overhauser enhancement; Me_4Si, tetramethylsilane; s, d, and t, apparent singlet, doublet, and triplet; FT-IR, Fourier transform infrared; HPLC, high-performance liquid chromatography.

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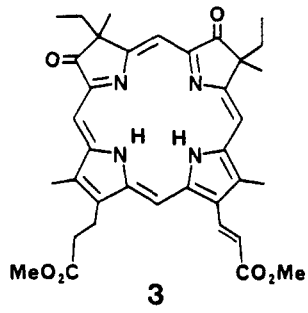
product was a green pigment that eluted with an R_f of 0.35 in the reverse-phase system. Visible spectroscopy, mass spectroscopy, and comparative chromatography revealed that this was the Cu chelate of d_1. Cu and Zn incorporate very rapidly into the center of Fe-free d_1, and it is believed that the product arose by scavenging of trace amounts of Cu by free base d_1 from solvents. A minor side product was another green pigment that will be refered to as the desmesoporphyrin. With an R_f of 0.22 in the reverse-phase step, it was contaminated by tailing of the main d_1 peak. It was purified to homogeneity by HPLC (silica eluted with chloroform–hexane, 85:15; k' was 0.98 compared to 1.48 for d_1). Cu and Zn derivatives of d_1 were prepared by simple addition at room temperature of an excess of the metal acetate salt, dissolved in glacial acetic acid, to chloroform solutions of the free base ester. Changes in the visible spectra were complete in 10–60 min depending on the metal concentration, but the kinetics were not studied in detail.

Compound **2** was synthesized from octaethylporphyrin (Chang, 1980) and the Fe chelate prepared (Chang et al., 1980) as previously described. Compound **3** was synthesized



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(Chang & Wu, 1986b) from mesoporphyrin and the Cu chelate prepared by addition of cuprous acetate.

Proton NMR spectra were obtained at 200 MHz on a Nicolet spectrometer and at 250 MHz on a Bruker spectrometer, usually in deuterated chloroform. For the diamagnetic Fe chelates, the solvent was deuterium oxide–[$^2H_5$]pyridine (1:1) with reduction by the addition of solid dithionite followed by sealing the sample under argon. ^{13}C NMR spectra were obtained on a Bruker (63 MHz for ^{13}C) or on a Varian CFT-20 (20 MHz for ^{13}C) equipped with a custom-made high-sensitivity probe for 5-mm or smaller samples in spherical cells (Wilmad Model 510 microcell). The natural abundance, broad-band proton-decoupled ^{13}C NMR spectra for d_1 to be reported required 1.3 million averaged transients in a 333-h data acquisition. The sample was checked after acquisition by chromatography and visible spectroscopy, and no decomposition was observed. Data collection parameters were optimized to produce the best signal-to-noise ratio for methylene carbons (based upon experiments with model porphyrins), since these were the critical issue between the two interpretations in hand. The relative intensities were therefore distorted from expectations based upon stoichiometry. For example, certain carbons, carbonyl and quaternary, were not observed and others, methylene and meso, appeared strong, even compared to methyl carbons. All chemical shifts are reported vs. Me_4Si, although in some cases residual C^1HCl_3 (7.240 ppm) was used as the actual internal standard. Nuclear Overhauser enhancements were measured as previously described (Timkovich et al., 1984b). Two-dimensional homonuclear J-resolved spectra (Bax & Freeman, 1981) were obtained for the free base **2** and enabled the determination of chemical shifts for the overlapping multiplets. Two-dimensional correlation spectra (NOESY; Wider et al., 1984) were obtained with a published pulse sequence. Fluorescence spectra

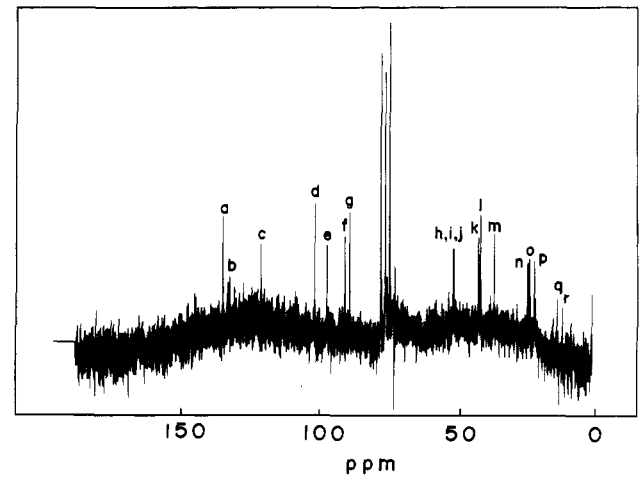


FIGURE 1: Natural abundance, broad-band proton-decoupled ^{13}C NMR of the free base methyl ester of d_1 in deuterated chloroform at 32 °C. Resonances labels correspond to Table I. Not all carbons provide a signal sufficiently strong for detection under these conditions.

were measured on a Perkin-Elmer Model 204 spectrophotometer in terms of relative intensities. Visible spectra were recorded on Perkin-Elmer Lambda 5 or Cary 219 spectrophotometer in standard 1-cm cells. For some compounds involving d_1, approximate extinction coefficients were measured after determining concentrations by the dry mass on a microanalytical balance. Extinction coefficients are more confidently reported for the more abundant model compounds to be discussed. Spectra of the protonated forms of porphyrins were obtained in formic acid as solvent.

Fast atom bombardment spectra were obtained on a Varian MAT CH5 mass spectrometer equipped with an ION Tech FAB gun. Solvents for the FAB matrix were a mixture of thioglycerol, dithiothreitol, and dithioerythritol (2:1:1). Addition of 0.1 M trifluoroacetic acid to the matrix facilitated the ionization of the porphyrinoid pigments during FAB analysis (Musselman et al., 1986). This acidic matrix always produces the monoprotonated [$(M + H)^+$] ions as the most abundant species in the spectra. Some spectra were obtained at the Midwest Center for Mass Spectrometry located at the University of Nebraska in Lincoln, NE.

Results and Discussion

The prefered interpretation of the data now in hand on natural d_1 and the porphyrindione model compounds leads to the assignment of **1** as the correct structure. The data and interpretation may be given as follows.

The ^{13}C NMR spectra of porphyrins are more straightforward to assign than the ^1H NMR spectra, although they are also more difficult to obtain on trace samples. The relative contribution of the aromatic ring current to the final chemical shift is much less for ^{13}C, and so there is less ambiguity about bond types or neighboring groups. The natural abundance ^{13}C NMR spectrum of the free base methyl ester derivative of d_1 is shown in Figure 1. Detectable resonances are tentatively assigned in Table I and contrasted to the spectra of **2** (given in Figure 2) and **3**. The original proposed structure postulated hydroxymethyl groups saturating β-pyrrolic carbons while **1** proposes acetate esters. The expected ^{13}C chemical shift is very different for the methylene carbon of these two moieties. Methyl groups on saturated β-pyrrolic carbons in model porphyrins are usually very close to 23 ppm (Smith & Unsworth, 1975; Janson & Katz, 1979). The incremental shift for replacing an H with –COOR is about +20 ppm, predicting 43 ppm for $-CH_2COOR$. The incremental shift for replace-

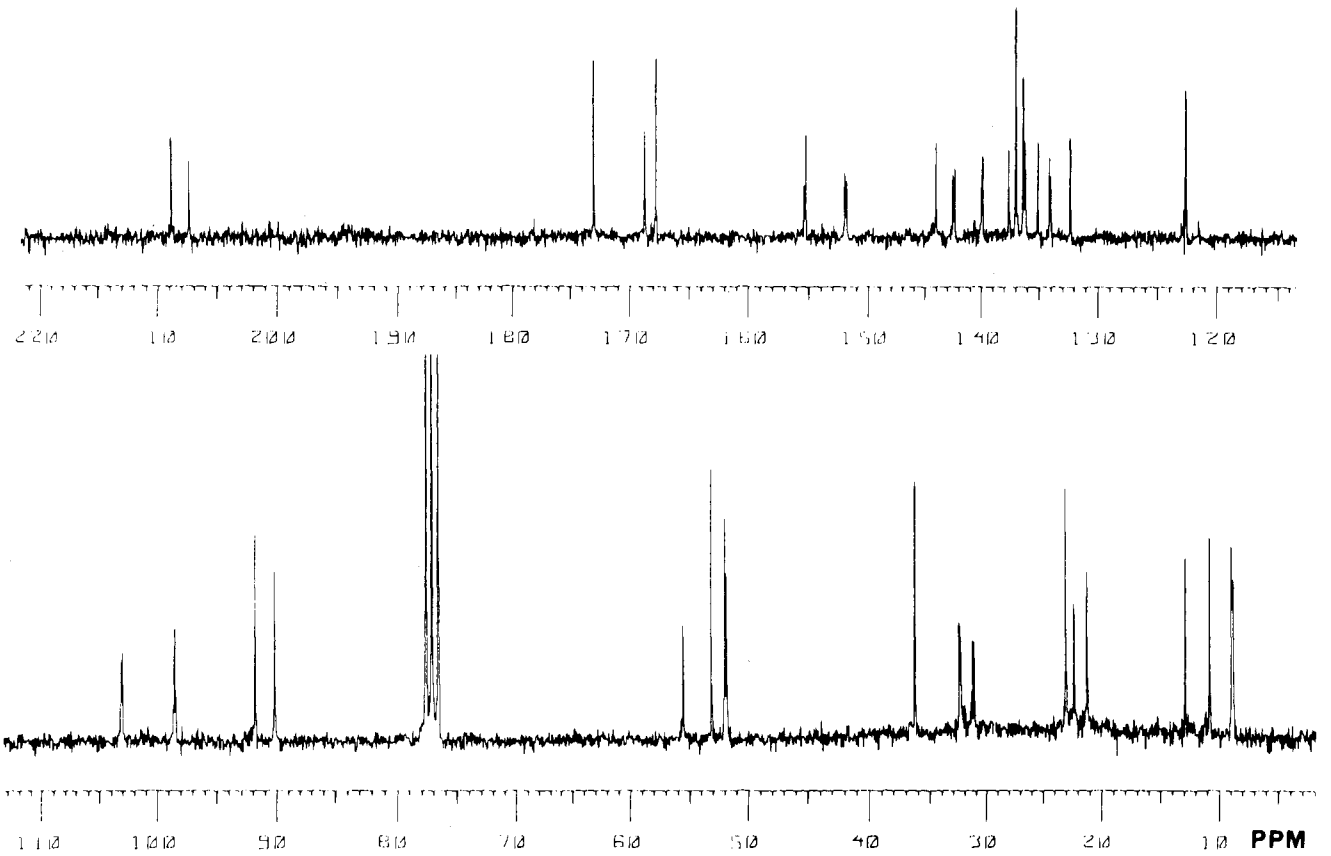


FIGURE 2: Natural abundance, broad-band proton-decoupled ^{13}C NMR of compound **2** at 30 °C. The spectrum is shown folded with the same scale. Peak assignments are given in Table I.

Table I: ^{13}C NMR Chemical Shifts[a]

assignment	compound **2**	hydrogenated **3**[b]	compound **3**	d_1 (**1**)
pyrrole C	132.25, 136.59, 137.19, 137.31, 138.86, 142.48, 144.98, 145.30, 146.63	131.55, (131.98, 132.03),[c] (132.24, 132.32), 135.69, 136.74, 136.92, 137.10, 139.52, (144.16, 144.24), (147.45, 147.59)	132.22, (133.87, 133.93), 134.90, (136.07, 136.19), 136.84, 137.46, 139.72, (142.19, 142.34), (151.66, 151.78), (155.07, 155.19)	134.7, 137.1 (b, a)[d]
C—C═O	160.33, 165.57	(161.72, 161.87), 167.23	167.57, 168.57	
C═O	210.12, 210.21	209.15, 209.29	207.25, 208.71	
quaternary C	58.47, 61.20	53.11, 55.73	53.03, 55.42	
meso C	90.58, 91.11, 96.13, 98.57	91.31, 96.86, 99.01, 99.11	90.03, 91.67, 98.33, (102.88, 102.97)	90.2, 91.9 (g, f), 98.5, 102.9 (e, d)
unsaturated pyrrole				
$-CH_2CH_3$	18.94, 19.14, 19.23, 19.38			
$-CH_2CH_3$	17.74, 17.93, 18.05			
$-CH_3$		10.71, 11.06	10.72, 12.81	11.2, 13.2 (r, q)
acrylate				
—C═*C*—			122.48	122.9 (c)
—*C*═C—$COOCH_3$			143.83	
$-CH_2CH_2COOCH_3$		36.19	36.24	36.6 (m)
$-CH_2CH_2COOCH_3$		21.18, 21.33	21.16	21.5 (p)
esters				
$-COOCH_3$		173.12	172.83	
$-COOCH_3$		51.62	51.73, 51.91	51.8, 51.9 (j, i), 52.1 (h)
saturated pyrrole				
$-CH_2CH_3$	30.94, 31.44	(31.24, 31.29), (31.97, 32.03)	(31.09, 31.18), (32.26, 32.34)	
$-CH_2CH_3$	8.59	8.86	8.73, 8.85	
$-CH_3$		22.51, 23.00	22.29, 23.01	23.4, 24.1 (o, n)
$-CH_2COOCH_3$				41.7, 42.5 (l, k)

[a] Referenced against Me_4Si (0 ppm) or the center of C^2HCl_3 (77.00 ppm). [b] The skeleton of **3** with two propionates instead of one propionate and one acrylate. [c] Pairs in parentheses indicate bifurcation due to diastereomers. [d] Letters of peaks in Figure 1.

ment with –OH is +48 ppm, predicting 71 ppm for $-CH_2OH$. The spectrum of d_1 has no evidence of any features near 71 ppm while two distinct resonances are evident at 43 ppm corresponding to the two acetate esters of **1**. Other aspects of the assignments of Table I follow from analogy to known models (Smith & Unsworth, 1975; Janson & Katz, 1979). It has been pointed out that the meso carbons are sensitive to the level of saturation of the porphyrin core as well as to precise substituents (Janson & Katz, 1979). The highly similar pattern of the individual meso carbon resonances between **1**

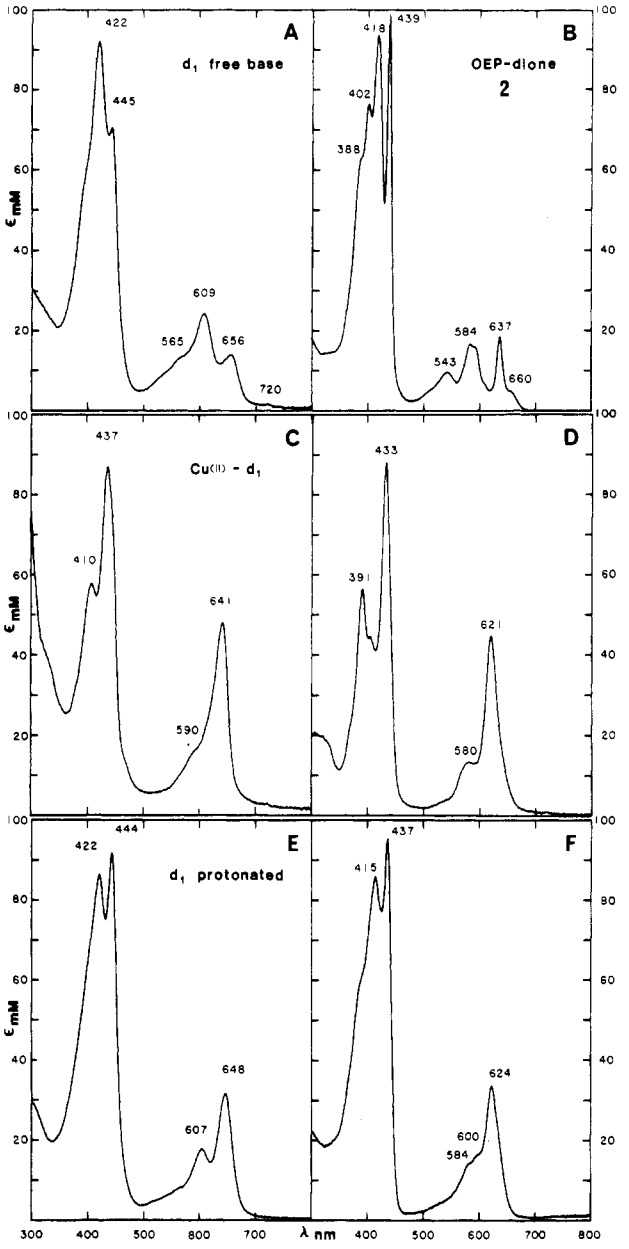


FIGURE 3: Visible spectra of free base forms of d_1 (A) and **2** (B), of the Cu chelates of d_1 (C) and **2** (D) in methylene chloride, and of the protonated forms of d_1 (E) and **2** (F) in formic acid.

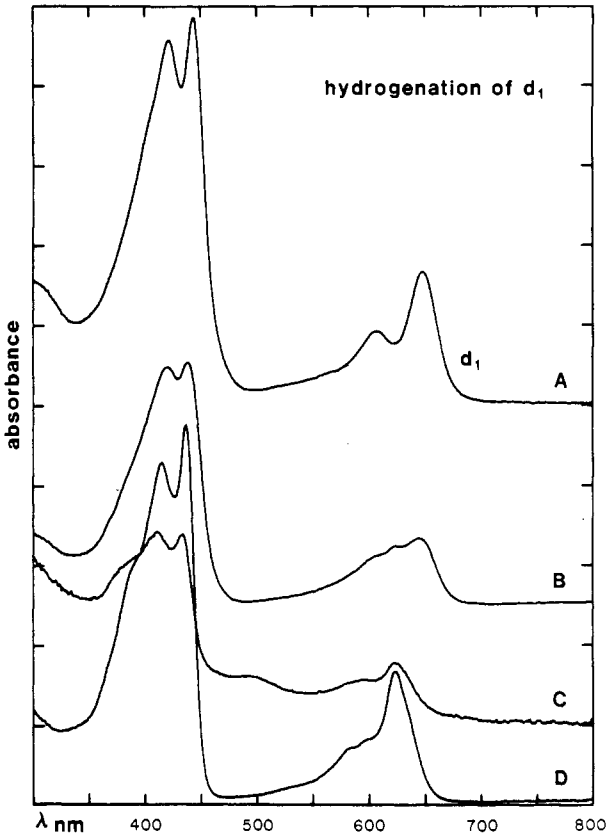


FIGURE 4: Hydrogenation of d_1 in formic acid with PtO_2: (A) before the addition of catalyst; (B) 30 s after catalyst addition; (C) 2 min after catalyst addition; (D) spectrum of compound **2** for comparison. The hydrogenation reaction is not selective, and curves B and C show general decreased absorption and noise because the porphyrin core is undergoing attack.

and **3** is evidence for the common core.

Structure **1** has an integral mass of 712. The original proposed structure had a parent mass of 714, and the observation of the most intense ion peak at 712 was rationalized in terms of an M − 2 ion resulting from loss of the inner pyrrolic protons, an event observed in other porphyrins (Murphy et al., 1979). One might think of the differences in predicted masses as arising from replacing two −OH groups by two =O in **1**. New fast atom bombardment mass spectra were obtained in a matrix that consistently gives $(M + H)^+$ ions for porphyrins (Musselman et al., 1986). Mass observations for the protic methyl ester [$(M + H)^+ = 713$], the [$^2H_3$]methyl ester (725, 726, and 727 corresponding to two ^1H, one ^1H and one ^2H, or two ^2H as inner pyrrolic NH's), the copper chelate of the protic methyl ester (773), and the copper chelate of the protic ethyl ester (829) are only consistent with structure **1**.

The visible absorption spectrum of the free base methyl ester of d_1 is not similar to the free base spectrum of **2** (see Figure 3A,B), although, as pointed out previously, it also does not look like a typical chlorin (Chang, 1985). However, in the free bases, symmetry (or its absence) exerts a strong effect on oscillator strengths and hence band intensities. The band structure of porphyrins simplifies in the protonated or metalloforms. The correspondence between d_1 and **2** becomes more apparent when these forms are compared as shown in Figure 3C–F. The spectra of the Zn chelates (not shown) are similar to the Cu spectra, except that band maxima are shifted to 653, 604 (sh), 454, and 425 nm (sh) for the d_1 methyl ester and to 630, 587, 443, and 421 nm for **2**. A consistent feature of these comparisons is the ca. 20-nm red shift of the highest wavelength band of d_1. The proposed structure **1** does contain the extra conjugation of the acrylate, which could account for the red shift. As shown in Figure 4, when this olefinic substituent is hydrogenated by treatment with PtO_2, the band shifts to a wavelength coincident with **2**. As shown in Figure 5, the visible spectrum for the model compound **3** is indeed sensitive to the presence of the double bond. The correspondence of the visible spectrum of the free base **3** to the free base of d_1 is major evidence that they share a common core.

The pK_3 values (basicity of the pyrrole nitrogens) of these porphyrindiones have been determined in 2.5% sodium dodecyl sulfate to be 1.8 for **2** and 1.7 for d_1 and **3**. In contrast, ordinary porphyrins and chlorins all have pK_3's greater than 4.0 (Falk, 1964). The extremely weak basicity of d_1, therefore, is another diagnostic characteristic indicating that **1** is the correct structure.

As is the usual case for fluorescent porphyrin compounds (Gouterman, 1978), the emission maxima upon excitation into the Soret band simply reflected the wavelength of the longest visible band and did not provide additional evidence.

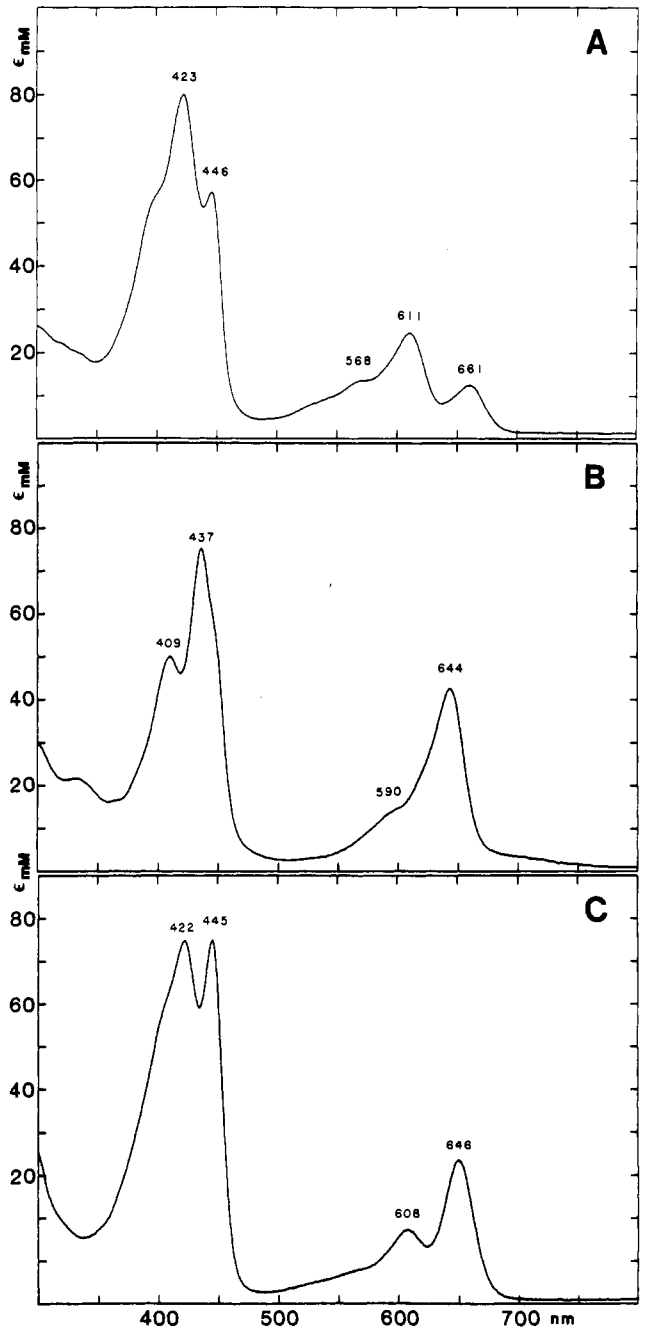


FIGURE 5: Visible spectra of various forms of model compound **3** (A) as the free base in chloroform, (B) as the Cu chelate in chloroform, and (C) as the protonated form in formic acid. These spectra of **3** should be carefully compared to those given for d_1 in Figure 3A,C,E.

It had been hoped that the minor products now isolated during the purification of d_1 would provide some structural evidence. This hope has only been partially realized. The visible spectrum of the freshly isolated blue pigment is shown in Figure 6. This spectrum rapidly degraded under a variety of conditions (in alcohol, chloroform, or ether solutions or upon storage as a dry film at −25 °C under inert atmosphere) and acquired a greenish hue. NMR revealed a very heterogeneous mixture that included some peaks ascribable to native d_1, even though these compounds had been cleanly resolved by HPLC. Only a 713 mass peak, equivalent to $(M + H)^+$ for native d_1, was detected in the mass spectrum of the blue pigment. Rechromatography by HPLC produced multiple chromatographic peaks, even though the material had been previously isolated as a single well resolved peak. Among these peaks were features at the retention times for native d_1 and the

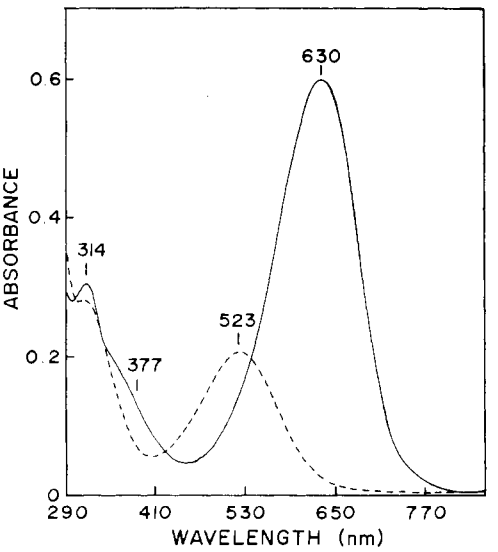


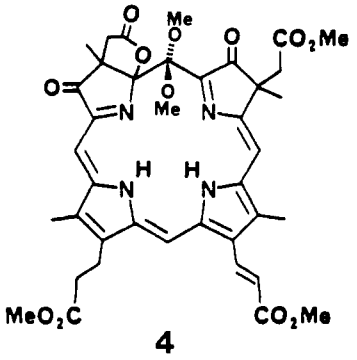
FIGURE 6: Visible spectra of blue pigment isolated during d_1 purification in neat methanol (solid curve) and after the addition of 1% HCl (dashed curve).

desmeso compound (vide infra). The lability of the blue pigment has precluded further characterization. It does appear that it is formed from native d_1 by a reaction that is at least partially reversible. The blue color is reminiscent of oxophlorins or open-chain tetrapyrroles derived from phlorins (Cavaleiro & Smith, 1973; Bonnett et al., 1969) that involve attack of oxygen at a meso carbon.

The compound referred to as the desmesoporphyrin has been difficult to interpret. Experimentation has been hampered by very limited quantities. It was purified to apparent homogeneity by HPLC and was stable when stored dried. This green pigment showed a visible spectrum whose general profile was highly similar to free base d_1 methyl ester with the exceptions that the maxima were shifted to 662, 614, 446, and 426 nm and the 446 maxima was now comparable in intensity to the 426-nm band. High-resolution FAB mass spectra on this compound gave an exact mass of 759.2672 ± 0.0024 (four trials). This represents an extra 46 mass units added to the core $(M + H)^+$ ion of native free base d_1 methyl ester. The best fit to the observed mass corresponded to the addition of three oxygen atoms and the loss of two hydrogen atoms from d_1 (calcd 759.2650, 2.9 ppm deviation from observed). The next best corresponded to the addition of two O and one CH_3 and the loss of one H (calcd 759.2968, 39 ppm deviation). The only other remotely possible fit corresponded to the addition of one O and two CH_3 (calcd 759.3286, 81 ppm deviation). Insufficient material was available for infrared spectra or nuclear Overhauser enhancement (NOE) studies. NMR data (20 °C, in chloroform) showed chemical shifts of 9.17, 9.14, and 8.01 (1 H each, s, meso H), 8.78 and 6.83 (1 H each, d, J = 17.2 Hz, acrylate —CH=CH—), 4.78 and 3.71 (1 H each, d, J = 17.6 Hz, uncertain assignment), 3.91 and 2.97 (2 H each, t, J = 7.5 Hz, propionate $-CH_2CH_2-$), 3.78 (uncertain area because of overlap, acetate $-CH_2-$), 3.96, 3.62, 3.20, 3.18, and 3.15 (15–18 H, s, ring and ester $-CH_3$), 2.54 (3 H, s, uncertain assignment), and 1.90 and 1.68 (3 H each, s, ring CH_3).

We are unable to interpret completely these data in terms of a single structure. Partial interpretations can be made with varying degrees of confidence. The HPLC chromatograms discussed previously suggest that the compound is related to the uncharacterized, labile blue pigment and thus may not represent a single chemical transformation on d_1. Most of the

NMR spectrum is readily assigned by comparison to the spectrum of d_1. In particular, it appears that the unsaturated pyrroles have not been modified. A striking feature of the NMR is the observation of only three meso protons. Additional spectra were obtained in $C^2H_2Cl_2$ to ensure that the fourth meso was not under the residual C^1HCl_3 resonance. It is known that the meso position in relatively saturated porphyrins is susceptible to attack by nucleophilic oxygen (Cavaleiro & Smith, 1973; Bonnett et al., 1969), for example, leading to oxophlorins, and that, in turn, certain keto oxophlorins show a preference for the enol tautomeric form (Inhoffen & Gossauer, 1969). Therefore, replacement of a meso proton by hydroxy or methoxy seems plausible. The spin-coupled doublets at 4.78 and 3.71 ppm have a coupling constant (17.6 Hz) typical of geminal coupling. These could arise from an internal lactonization reaction to produce a structure such as **4**. In this rigid lactone ring, the two methylene protons



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experience very different ring current effects and electrostatic shielding effects from the keto oxygen. These could account for the chemical shift inequivalence and the appearance of an essentially first-order subspectrum. There are precedents for the cyclization in the existence of derivatives of sirohydrochlorin (Battersby et al., 1977; Battersby & McDonald, 1978) and for the NMR interpretation for the spirolactone form of *Escherichia coli* terminal oxidase heme *d* (Timkovich et al., 1985). It has also been shown that dicyanocobyrinate (vitamin B_{12} core), which possesses analogous pyrroline acetate groups, undergoes similar cyclization with the *C*-acetate group with concommitant oxidation at the adjacent meso position to form the xanthocorrinoids (Gossauer et al., 1977). The three-proton singlet at 2.54 ppm is at a shift possible for a methoxy group. This could be from a ketal OCH_3 at the meso position. For porphyrin quinones like **1**–**3**, one may write possible, although not necessarily precedented, mechanisms for addition/replacement reactions at meso, carbonyl, and even NH positions. It has not been possible for us to do this for the original chlorin structures first proposed for d_1 (Timkovich et al., 1984a). So the desmeso compound, while not clear evidence in favor of **1**, does appear to be evidence against the original proposal.

Table II compares ^1H chemical shift data for **2**, its Fe chelate, **3**, and **1** and limited data obtained on a diamagnetic Fe^{2+} form of heme d_1. The NOE and lanthanide shift data previously reported for the free base methyl ester of d_1 (Timkovich et al., 1984a) are fully compatible with structure **1** and have been used to make the assignments in Table II for d_1. For example, an enhancement on two meso protons at 8.26 and 8.41 ppm had been observed upon irradiation of the two overlapping saturated pyrrole methyl groups at 1.76 and 1.78 ppm. This was originally interpreted as arising from two meso protons flanking a single saturated pyrrole. Inspection of **1** indicates it could and does arise from each meso proton next to the two partially saturated pyrroles. The assignments of

Table II: ^1H NMR Chemical Shifts in ppm from Me_4Si

assignment	**2**	Fe^{2+}–**2**	**3**	d_1 (**1**)	heme d_1
meso γ	9.415	9.871	9.40	9.439	9.285
meso δ	9.277	9.719	9.13	9.214	9.034
meso α	8.621	8.134	8.40, 8.39[a]	8.415	8.573
meso β	8.438	9.094	8.26	8.261	8.385
$-CH_2CH_3$	3.839	3.731			
(unsaturated)	3.823	3.699			
	3.778				
	3.715				
$-CH_2CH_3$	2.602	2.660			
(saturated)	2.582	2.545			
$-CH_2CH_3$	1.745	1.851			
(unsaturated)	1.723	1.831			
	1.700	1.809			
	1.676	1.652			
$-CH_2CH_3$	0.532	0.507			
(saturated)	0.405	0.359			
4-CH_2CH_3			2.53		
2-CH_2CH_3			2.57		
4-CH_2CH_3			0.48, 0.50[a]		
2-CH_2CH_3			0.63, 0.64[a]		
2,4-CH_2COOR				3.751, 3.778, 3.694	
4′-CH_3			1.81, 1.83[a]	1.763	
2′-CH_3			1.90, 1.92[a]	1.783	
8-CH_3			3.24	3.244	
7-CH_2CH_2COOR			4.07	4.074	
7-CH_2CH_2COOR			3.07	3.051	
6-CH=CHCOOR			6.91	6.901	6.714
6-CH=CHCOOR			8.96	8.967	8.767
5-CH_3			3.32	3.314	
7-CH_3 (ester)			3.65	3.615	
6-CH_3 (ester)			4.01	3.996	
2,4-CH_3 (ester)				3.178, 3.131	
NH	−0.05		0.97	0.92	

[a] Mixture of diastereomers.

the four meso protons in **2** and its Fe chelate were independently obtained by observation of appropriate NOE's between these protons and the ethyl substituents on saturated and unsaturated pyrroles. Irradiation of the overlapping multiplets for methylene protons on unsaturated pyrroles at 3.8 ppm gave a 24% NOE to the 9.41 ppm meso proton, 14% to 9.28, and 6% to 8.44 ppm. Irradiation of methylene protons on saturated pyrroles at 2.6 ppm gave a 16% NOE to 8.62 ppm and an 11% NOE to 8.44 ppm. Irradiation of the 9.41 ppm meso gave a 2% NOE in the region of unsaturated methylene protons. This same pattern was also observed for the analogous resonances in the Fe chelate, and in addition, weak NOE's were also seen between 9.09 ppm β meso and methylene protons on both saturated and unsaturated pyrroles and the 9.72 ppm δ meso and an unsaturated pyrrole methylene. The assignments of the meso protons in **3** were similarly obtained by NOE measurements. The strongest NOE's (those greater than 10%) were also observed as appropriate off-diagonal peaks in two-dimensional NOESY NMR spectra. Therefore, the meso assignments in Table II do not rest solely on chemical shift correspondence, although such correspondence is readily apparent in the table.

One of the main reasons the original interpretation of the d_1 data went astray was the observation of d_1 meso resonances as grouped into two distinct pairs. This is almost universally observed for chlorin model compounds with the upfield pair representing the two meso protons adjacent to the sole saturated pyrrole. In most isobacteriochlorins, the usual pattern is one meso proton downfield (the one between the unsaturated pyrroles), a pair at an intermediate shift (the two between a saturated and an unsaturated pyrrole), and one relatively

Table III: Meso Proton Chemical Shift Differences between Hemes and Free Bases[a]

meso	d_1	compound **2**	protoporphyrin IX
α	0.16	0.51	0.18
δ	−0.18	0.44	0.06
γ	−0.15	0.46	0.49
β	0.12	0.66	0.26

[a] Free base shifts were in chloroform at 20 °C. Heme shifts were in 1:1 deuterium oxide–[$^2H_5$]pyridine at 20 °C. Data for protoporphyrin IX represent measurements in this study made for the sake of similar conditions.

upfield (the one between the two saturated pyrroles). In 1,3- or 2,4-porphyrindiones, the deshielding effects of the carbonyl oxygens have distorted the usual isobacteriochlorin pattern into an apparent chlorin pattern. The **3** carbonyl oxygen has deshielded α from its furthest upfield normal position to a shift comparable to β, while the **1** carbonyl has deshielded δ to a range comparable to γ.

Part of the evidence for the incorrect proposed structure for d_1 was the appearance of presumed OH modes in the Fourier-transform infrared spectrum of d_1 taken as dried films on a NaCl surface. These were recurrent, albeit weak, features on admittedly noisy spectra (Timkovich et al., 1984a) that were not present on blanks. Additional experiments on recovering the sample and rechromatographing it by HPLC indicated extensive degradation, especially after exposure to the FT-IR laser system. Therefore, it is likely that the observed peaks arose from unidentified decomposition products. Quinones do have accessible semiquinone and hydroquinone oxidation states, and the desmeso compound has indicated the feasibility of oxygen addition.

The NMR spectrum of **3** has clarified the question of the chemical shift for the NH resonances of d_1. In **3**, they appear at 0.97 ppm, which is unusually downfield for the inner NH protons of a porphyrin, but presumably reflects the decreased ring current and electron-withdrawing effects in an acryloporphyrindione. Features at 0.92 ppm in the spectrum of the protic methyl ester of d_1 had been dismissed as residual impurities.

An important question about the proposed structure **1** for d_1 is whether the porphyrindione is the native form for heme d_1 or whether it arises from a diol by a pinacol–pinacolone rearrangement during its purification. There are several lines of evidence against the possibility of an in vitro rearrangement. It has been pointed out that the conditions for the isolation of the free base methyl ester are not as harsh as is necessary for diol rearrangement in model compounds (Chang, 1985). The chiral **2** and **4** β-pyrrolic carbons give rise to diastereomers in porphyrindiones synthesized by rearrangement, but there is no evidence for diastereomeric resonances in d_1. Similarity of the meso proton chemical shifts in the free base methyl ester and a diamagnetic Fe^{2+} form of heme d_1 in a freshly prepared crude extract (Timkovich et al., 1984a) argues against subsequent chemical transformation. This argument was stringently tested by comparing the differences in meso proton shifts between free base and diamagnetic iron complexes of model compounds. As shown in Table III, the shifts produced on going from a metalloporphyrin to the free base are generally small and especially small for d_1, and they do not grossly distort the profile of resonances. Although it is unlikely that rearrangement occurs during isolation of d_1, it should be mentioned that this transformation may play a critical role in the natural biosynthesis of d_1.

Registry No. **1** (Fe complex), 105253-57-4; nitrite reductase, 9080-03-9.

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