N-Methylprotoporphyrin IX. Identification by NMR of the Nitrogen Alkylated in Each of the Four Isomers

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Abstract: The four isomers of *N*-methylprotoporphyrin IX (as the dimethyl esters) have been obtained by methylation of the parent porphyrin. The isomers have been separated by high-pressure liquid chromatography and have been individually characterized. The nitrogen alkylated in each of the four isomers has been identified by a series of NMR studies. Relaxation time measurements and nuclear Overhauser effects have played a crucial role in differentiating the four isomeric structures. The assignment of a specific structure to each of the four synthetic isomers permits definition of the porphyrin isomer primarily formed in rats treated with 3,5-bis(ethoxycarbonyl)-2,4,6-trimethyl-1,4-dihydropyridine.

The accumulation of an unusual green porphyrin in the liver of rats treated with 2-isopropyl-4-pentenamide was first reported some 3 decades ago.[1] In the intervening years, analogous pigments have been found in rats treated with a variety of olefinic and acetylenic agents, including ethylene[2] and acetylene.[3] Investigations of the structure of the porphyrins obtained with these unsaturated substrates recently culminated in our unambiguous spectroscopic identification of the ethylene-derived pigment as *one* of the four possible isomers of *N*-(2-hydroxyethyl)protoporphyrin IX.[4] It was not possible, however, in the absence of a precedent for differentiation of the possible isomers, to determine which of the four possible nitrogens was alkylated in the isolated isomer.

Hepatic accumulation of a green porphyrin in mice treated with 3,5-bis(ethoxycarbonyl)-2,4,6-trimethyl-1,4-dihydropyridine [3,5-bis(ethoxycarbonyl)-1,4-dihydrocollidine, DDC] has also recently been reported.[5] The structure of this abnormal porphyrin is of particular interest because of the radically different nature of the causal agent and because, unlike that obtained with 2-isopropyl-4-pentenamide, this porphyrin is a potent inhibitor of ferrochelatase.[6] Using high-pressure liquid chromatography, we have resolved the hepatic pigment obtained from rats treated with 3,5-bis(ethoxycarbonyl)-2,4,6-trimethyl-1,4-dihydropyridine into four components, each of which appears to be an isomer of *N*-methylprotoporphyrin IX (isolated as the dimethyl ester).[7] Only one of these isomers, however, was formed in amounts sufficient for detailed structural analysis. Its identification as an isomer of *N*-methylprotoporphyrin IX, first based on mass spectrometric and NMR studies, has been confirmed by direct comparison with authentic samples of the four isomers of this porphyrin synthesized in our laboratory.[7] These four isomers are the first synthetic *N*-alkylprotoporphyrin IX derivatives to be reported. However, although it has been possible to determine which synthetic isomer corresponds to that which predominates biologically, it has not been possible, from the NMR spectra of the four isomers, to convincingly identify the specific nitrogen alkylated in each one.

The precise assignment of structure to the isomers of *N*-methylprotoporphyrin IX and eventually to the isomers of other biologically formed *N*-alkylprotoporphyrin IX derivatives is important, since the regiospecificity of the alkylation process can be used to probe the mechanism and sterochemical requirements of the alkylative interaction. The fact that at least some of the N-alkylated porphyrins reflect alkylation of the prosthetic heme in cytochrome P-450 during catalytic interaction of the enzyme with the administered agent[4b] suggests, for example, that the regiospecificity of the alkylation process can be used to explore the active site of this class of enzymes. We report here a detailed study of the four synthetic *N*-methylprotoporphyrin IX (dimethyl ester) isomers, using primarily nuclear magnetic resonance techniques, which has enabled us to specifically define the site of alkylation in each of the isomers. The complexity of the NMR spectra of the four isomers, coupled with the absence of any previous study of isomeric N-alkylated protoporphyrin IX derivatives, has made this a challenging task. Differentiation of the isomers has, however, been achieved through a combined use of deuterium labeling, proton relaxation time measurements, and nuclear Overhauser effect determinations. The relatively rigid structure of porphyrins makes the use of nuclear Overhauser effects particularly valuable.[12] The four isomeric structures defined here should now serve as models for, and the techniques developed in their assignment should be applicable to, the differentiation of other *N*-alkylprotoporphyrin IX isomers.

Experimental Section

Synthesis and Separation of the Esterified *N*-Methylprotoporphyrin IX Isomers.[7] A solution of methyl fluorosulfonate (1.0 mL, 12 mmol) and 500 mg of the dimethyl ester of protoporphyrin IX (0.84 mmol) in dry CH_2Cl_2 (50 mL) was stirred at 25 °C in the dark for 3 days. *Caution: methyl fluorosulfonate is an exceedingly toxic substance and must be handled with appropriate care!* After washing with water, drying over anhydrous sodium sulfate, and solvent removal (rotary evaporator), the residue obtained was chromatographed on a 4 × 37 cm column of 10% (w/w) H_2O-deactivated Merck silica gel 60 using 20:1 (v/v) $CHCl_3$/methanol as solvent. The unreacted dimethyl ester of protoporphyrin IX (75 mg), a mixture of the isomers of dimethyl esterified *N*-methylprotoporphyrin IX (250 mg), and an isomeric mixture of the dimethyl esters of *N*,*N*-dimethylprotoporphyin IX (50 mg) were eluted, in that order, from the column. The monomethylated isomers were dissolved in hot $CHCl_3$ and were precipitated out of the solution with hexane. The precipitate, dried under vacuum, was subjected to high-pressure liquid

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chromatography (HPLC) on a 9.4 × 250 mm Partisil-10 PAC column using 97:97:6 (v/v) hexane/tetrahydrofuran/methanol as solvent. The leading or trailing edge of each of the four peaks, as appropriate, was collected and was rechromatographed in the same system. Each isomer was obtained free of the others by this procedure. The zinc chloride complex of each of the isomers was prepared by washing a solution of the porphyrin in zinc acetate-saturated $CHCl_3$ with a saturated aqueous NaCl solution three times. Solvent removal from the dried (anhydrous sodium sulfate) solutions yielded the desired zinc chloride complexes in essentially quantitative yield.

Meso-Deuterated *N*-Methylprotoporphyrin IX Isomers. Partially deuterated protoporphyrin IX (dimethyl ester) was obtained by the acid-catalyzed deuteration procedure of Smith, Langry, and De Ropp.[8] Approximately 1 g of *p*-toluenesulfonic acid monohydrate was dissolved in 10 mL of 99% deuterated water. The solvent was then removed under vacuum. The process was repeated twice in order to ensure complete replacement of the acidic proton by deuterium. Deuterated *p*-toluenesulfonic acid (1 g, 5.3 mmol) and dimethyl esterified protoporphyrin IX (200 mg, 0.34 mmol) were dissolved in 25 mL of dry 1,2-dichlorobenzene, and the solution was stirred at 90 °C under argon in the dark for 3 days. The cooled reaction mixture was partitioned between CH_2Cl_2 and 5% aqueous $NaHCO_3$. The organic layer, after drying (anhydrous sodium sulfate) and solvent removal (rotary evaporator, 1 mmHg, <50 °C), yielded a residue which was allowed to stand overnight in 5% H_2SO_4/methanol at 25 °C to reesterify any carboxyl groups hydrolyzed during the acid-catalyzed exchange reaction. The esterified porphyrin extracted with $CHCl_3$, after washing with water, drying, and solvent removal, was purified by chromatography on 10% (w/w) H_2O-deactivated Merck silica gel 60 using 2% (v/v) methanol/CH_2Cl_2. The partially deuterated protoporphyrin IX (dimethyl ester) thus obtained was recrystallized from methanol/$CHCl_3$ (90 mg, 45% overall yield). The procedure already described was used, starting with deuterated protoporphyrin IX, to prepare the four partially deuterated *N*-methylprotoporphyrin IX (dimethyl ester) isomers.

Spectroscopic Studies. Electronic absorption spectra were recorded on a Varian Cary 118 spectrophotometer using $CHCl_3$ solutions. Field-desorption mass spectra were obtained on a modified AEI MS-9 instrument as previously described.[3b,9] NMR studies, performed on a 360-MHz Nicolet NT-360 FT NMR instrument, were carried out with nondegassed (degassing has been shown not to be required[12]) 2 mM solutions of the samples in deuterated $CHCl_3$. The $CHCl_3$ peak at 7.21 ppm was used as an internal reference. Proton relaxation times (T_1 values) were determined by the inversion recovery method with alternating phases to minimize pulse nonidealities. For these measurements, spectra were accumulated over a 2000-Hz spectral width using 16K data points and a list of 11 τ values. A 2-Hz line-broadening function was applied to each FID prior to transformation. Actual T_1 values were computed using a three parameter fit of relative peak heights. Nuclear Overhauser effects (NOE)[10,12,13] were measured using a 4-s gated presaturation pulse and a sweep width of 2000 Hz. Methyl group saturation was achieved with a 20-μs, 42-dB pulse, while a 25-μs, 48-dB pulse was necessary for saturation of the methylene groups. A reference spectrum was obtained at each of the two power levels with the decoupler set at 4.8 ppm. A line-broadening function of 0.5 Hz was applied to all NOE FID signals prior to zero filling the 16K spectra to 32K. The NOE data reported in the text are the difference between the transformed experimental spectrum and an appropriate reference (control) spectrum. NOE signal enhancements of approximately 7–15% were observed.

Results

A mixture of the four possible isomers of the dimethyl ester of *N*-methylprotoporphyrin IX is obtained, as we have briefly reported,[4c,7] when protoporphyrin IX (dimethyl ester) is alkylated with methyl fluorosulfonate. The four isomers, after preliminary purification, can be resolved and individually isolated by HPLC (Figure 1). The formation of approximately equal amounts of the four isomeric structures suggests that there is little intrinsic difference in the reactivity of the four nitrogens in protoporphyrin IX.

Each of the four *N*-methylprotoporphyrin IX (dimethyl ester) isomers has been fully characterized by spectroscopic methods. The field-desorption mass spectrum of each isomer, characterized by a molecular ion at *m/e* 604 and a monoprotonated molecular ion at *m/e* 605, is essentially identical with the mass spectrum we have already reported for the mixture of the four isomers.[4c,7]

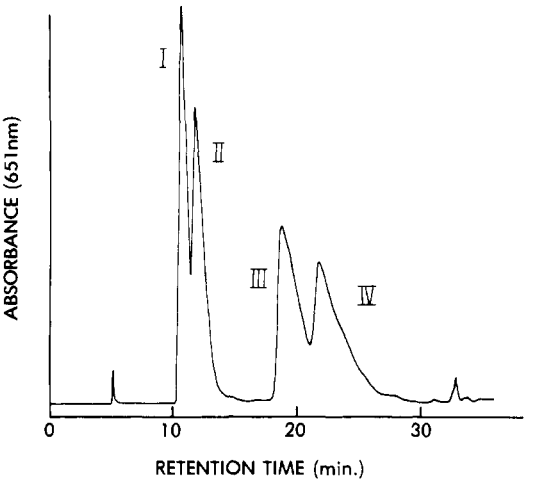

Figure 1. Separation of the four metal-free isomers of synthetic *N*-methylprotoporphyrin IX (dimethyl ester) by HPLC. Conditions are given in the Experimental Section. The isomers are labeled I–IV in order of elution from the column.

The electronic absorption spectra of the isomers, which differ slightly but are in all instances consistent with their formulation as *N*-alkylated protoporphyrin IX derivatives, have also been reported.[4c,7]

Definitive structural evidence is provided by the NMR spectra of the four isomers (Figure 2). Although the NMR spectrum of each isomer has also been obtained in the free base, monoprotonated, and diprotonated forms, the NMR spectra of the zinc complexes are reproduced here because these complexes exhibit sharp, well-resolved NMR signals which are less affected by traces of acids in the NMR solvents. Signals in the following regions of the spectrum of each isomer are due to protons at the following positions: (a) meso (10.1–10.4 ppm), (b) internal vinyl (7.9–8.3 ppm), (c) terminal vinyl (6.0–6.4 ppm), (d) side-chain methylene groups adjacent to the porphyrin ring (4.0–4.4 ppm), (e) ester and ring methyls (3.4–3.8 ppm), (f) side-chain methylene groups adjacent to the carboxyl moieties (2.7–3.3 ppm), and (g) *N*-methyl (−4.4 to −4.6 ppm). In each case the appropriate number of protons are shown to be associated with the signal by integration.

The NMR spectra of the first two isomers eluted from the HPLC column (isomers I and II) are similar in that both exhibit well-resolved multiplets for the two internal vinyl protons (7.9–8.3 ppm) but, conversely, only one multiplet for the four side-chain protons adjacent to the ring and one multiplet for the protons adjacent to the side-chain carboxyl groups (2.7–3.3 ppm). In contrast, isomers III and IV exhibit a single NMR multiplet for the internal vinyl protons but two multiplets for the side-chain protons next to the ring and two multiplets for the four protons next to the carboxyl groups. In sum, in isomers I and II the two vinyl groups are in different magnetic environments but the two propionic acid side chains are not, while the converse is true in the case of isomers III and IV. Earlier studies with simple N-alkylated porphyrins have shown that substituents on the N-alkylated ring appear upfield in the NMR relative to the similar substituents on the nonalkylated pyrrole rings of the porphyrin.[11b] Since crystallographic studies have established that the N-alkylated pyrrole is tilted out of the plane defined by the vicinal rings in the porphyrin,[14] it is probable that this chemical-shift difference reflects the difference in the position of the substituents with respect to the porphyrin ring current. The observation that one of the two internal vinyl protons in isomers I and II is displaced

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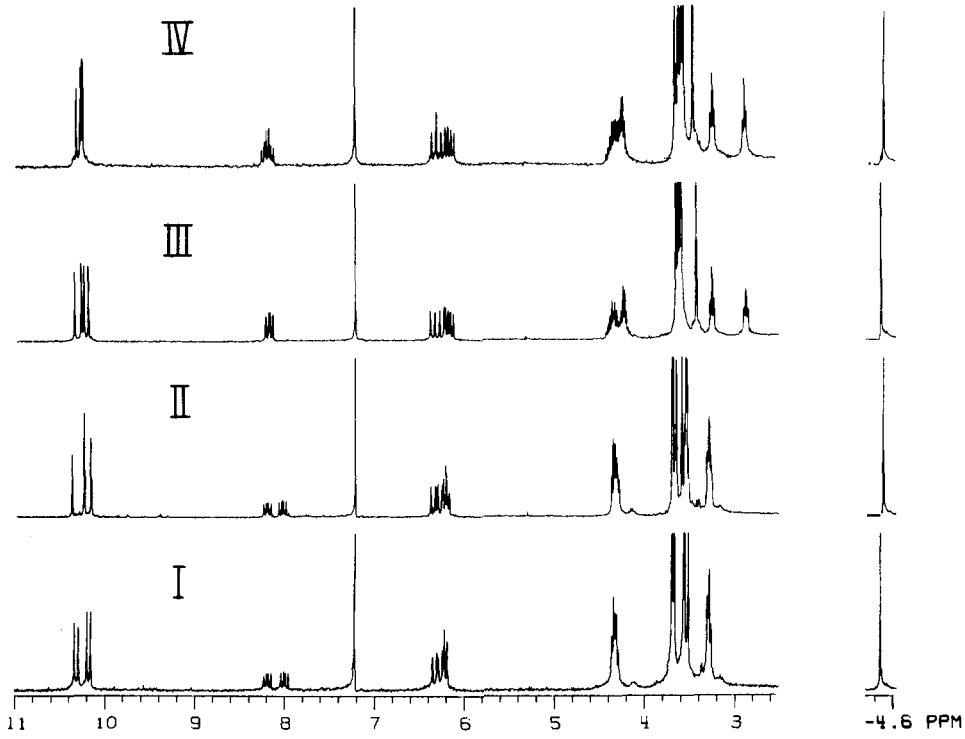

Figure 2. NMR spectra in deuterated chloroform of the zinc chloride complexed isomers of *N*-methylprotoporphyrin IX (dimethyl ester). Each spectrum is labeled with the number of the porphyrin isomer (legend, Figure 1) from which the zinc complex was prepared. The methyl group signals, truncated here to conserve space, are shown in expanded scale in Figure 4.

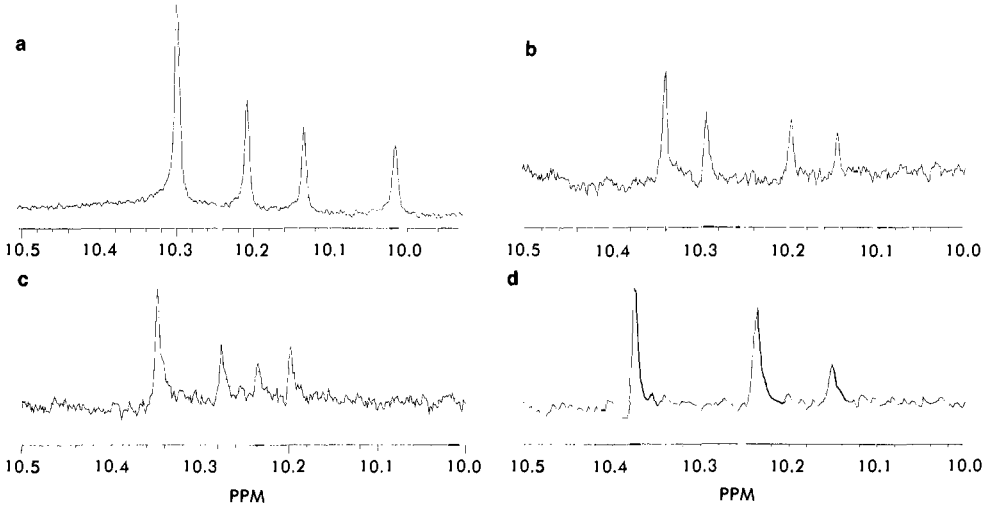

Figure 3. Meso proton region in the NMR spectra of dimethyl esterified, partially deuterated (a) zinc protoporphyrin IX (spectrum taken in the presence of 5 mM perdeuteriopyridine to suppress stacking interactions), (b) zinc-complexed isomer I of *N*-methylprotoporphyrin IX, (c) zinc-complexed isomer II of *N*-methylprotoporphyrin IX, and (d) zinc-complexed isomer III of *N*-methylprotoporphyrin IX. The meso proton signal identities are given in Figure 5.

to higher field, giving rise to two distinct internal vinyl proton multiplets, thus clearly identifies these isomers as the two structures in which the vinyl-substituted rings (rings A and B) are N-methylated.[4a,7] Likewise, the fact that the propionic acid side-chain signals are resolved in isomers III and IV (but not in isomers I and II) indicates that the *N*-methyl group is borne by the propionic acid substituted rings in these structures. It is not possible, however, to determine from the NMR spectra which of the two possible rings is alkylated in each pair of isomers.

To clearly define the pyrrole ring alkylated in each of the isomers, we have found it necessary to specifically assign every methyl group and meso proton signal in the NMR spectrum of each isomer. This has involved (a) synthesis of the four *N*-methylprotoporphyrin IX isomers from protoporphyrin IX with a known (but different) distribution of deuterium at the meso positions, (b) determination of the spin–lattice relaxation times (T_1 values) for all the protons in each of the isomers, and (c) identification of the methyl group signals by measurement of their nuclear Overhauser effect (NOE) with respect to each of the meso protons.

Meso-Deuterated *N*-Methylprotoporphyrin IX (Dimethyl Ester) Isomers. The relative rates of acid-catalyzed deuterium exchange into the four meso positions of the dimethyl ester of protoporphyrin IX have been shown by Smith et al.[8] to decrease in the order $\gamma > \delta > \beta > \alpha$. Heating the dimethyl ester of protoporphyrin IX in 1,2-dichlorobenzene with deuterated *p*-toluenesulfonic acid, as reported,[8] yielded a sample of partially deuterated protoporphyrin IX, the NMR spectrum of which (Figure 3a) established that the greatest degree of deuteration had occurred at the γ, and the least at the α, meso positions. The correspondingly deuterated *N*-methylprotoporphyrin IX isomers were then synthesized by methylation of this partially deuterated protoporphyrin IX sample.

Table I. Chemical Shift and Relaxation Time (T_1) Values for the Meso and Methyl Protons in the Four *N*-Methylprotoporphyrin IX (Dimethyl Ester) Isomers

group	isomer[a] I	II	III	IV
1-Me	3.659 (0.71)	3.548 (0.66)	3.630 (0.72)	3.663 (0.67)
3-Me	3.517 (0.65)	3.646 (0.71)	3.650 (0.66)	3.620 (0.98)[c]
5-Me	3.554 (0.67)	3.526 (0.65)	3.429 (0.65)	3.566 (0.64)
8-Me	3.546 (0.67)	3.582 (0.64)	3.587 (0.80)	3.469 (0.64)
6-MeO	3.686 (1.36)	3.682 (1.32)[b]	3.592 (1.14)	3.619 (0.98)[c]
7-MeO	3.686 (1.36)	3.693 (1.33)[b]	3.613 (1.30)	3.548 (1.24)
N-Me	−4.492	−4.530	−4.497	−4.535
α-meso	10.338 (1.16)	10.373 (1.12)	10.344 (1.19)	10.315 (1.07)
β-meso	10.291 (1.10)	10.233 (1.05)[c]	10.272 (1.12)	10.265 (1.10)
λ-meso	10.142 (0.65)	10.150 (0.66)	10.232 (0.70)	10.247 (0.90)[c]
δ-meso	10.193 (1.04)	10.236 (1.05)[c]	10.193 (1.08)	10.245 (0.90)[c]

[a] Chemical shifts in parts per million (T_1 values in parentheses in seconds). [b] These two methoxy groups could not be specifically assigned. [c] Relaxation time is an average for two superimposed signals.

Unreacted protoporphyrin IX (dimethyl ester) recovered from the methylation reaction was shown by NMR analysis to have retained the original label distribution (Figure 3a). An alteration in the deuterium label pattern due to exchange during the methylation reaction was, thus, explicitly excluded. The meso proton regions in the NMR spectra of three of the resulting *N*-methylprotoporphyrin IX (dimethyl ester) isomers are reproduced in Figure 3. The α meso proton (least deuterated position) and the γ meso proton (most deuterated position) can be readily identified in the NMR spectrum of each of the isomers. Due to the approximately equal incorporation of label into the β and δ meso positions (Figure 3a), these two proton signals can not be unambiguously differentiated in the NMR spectra of the N-methylated isomers. These positions are differentiated, however, by the NOE experiments described later in this report.

Spin–Lattice Relaxation Times. The relaxation times for all the meso and methyl protons were measured and are presented, in the form of T_1 values, in Table I. Two particularly useful structural inferences can be drawn from an analysis of the relaxation data. The first derives from the observation that one of the four meso protons in each of the isomers relaxes more rapidly (T_1 = 0.6–0.7 s) than the other three (T_1 = 1.0–1.2 s). This difference is not as clearly defined in isomer IV, although it is definitely present, because the signal due to the rapidly relaxed meso proton is essentially superimposed on that of one of the more slowly relaxed meso protons. The observed relaxation time thus represents an averaging of the two individual relaxation times. In view of previous studies with a number of porphyrins which have established that the γ meso proton relaxes with exceptional rapidity, probably through interaction with the flanking propionic acid side chains,[12] the meso proton with a low T_1 value in each of the present isomers can be identified with confidence as that at the γ position. This assignment coincides with, and consequently confirms, that which was made on the basis of the degree of deuterium present at the various positions in the partially deuterated structures.

The proton relaxation results, in addition to confirming the γ meso proton assignment, also allow identification of the signals due to the side-chain methyl ester groups. Because the methyl ester groups are not closely held to other protons which can aid their relaxation, they are expected, and have been shown,[12] to exhibit longer relaxation times than the methyl groups attached to the porphyrin ring. Of the six methyl group signals in isomer I, for example (Table I), four are found to have T_1 values of approximately 0.7 s, while two have T_1 values of 1.36 s. The latter two can thus be attributed to the side-chain methyl ester protons. The methoxy signals in the other isomers are clearly distinguished from the ring methyl signals by similar clear-cut differences in relaxation time, with the exception that one of the methoxy signals in isomer IV is superimposed on that of a ring methyl group. In consequence, the observed relaxation time represents an averaging of the two individual methyl group relaxation times. All of the methoxy signal assignments derived from differences in relaxation time are confirmed, in the following section, by the observation that irradiation of the assigned methoxy protons causes, as expected, no NOE enhancement of meso proton signals.

Nuclear Overhauser Effects (NOE). If relaxation of a proton depends significantly on through-space dipole coupling with a second set of protons, its NMR signal intensity may be enhanced when the set of protons which assist its relaxation are saturated by appropriate irradiation in the NMR spectrometer.[10] This phenomenon, termed the nuclear Overhauser effect, is highly dependent on the distance between the protons.[10] NOE enhancement of a meso proton signal on irradiation of the methyl group directly adjacent to the affected meso position has recently been reported for protoporphyrin IX and other porphyrins.[12,13] It is therefore theoretically possible by NOE studies to identify the meso position to which each of the ring methyl signals is vicinally related and, consequently, to specifically assign the methyl signals in the NMR spectrum. This can be accomplished by irradiating each methyl signal in turn and determining which meso proton exhibits an NOE enhancement. If the meso proton signals have been identified, the only methyls which can not be differentiated without ambiguity by this method are those at positions 1 and 8 of the porphyrin, since both of these methyls flank the *same* meso position.

The methyl group regions in the NMR spectra of the four zinc-complexed *N*-methylprotoporphyrin IX (dimethyl ester) isomers are reproduced in Figure 4. The signals due to the side-chain methylene protons adjacent to the aromatic ring, one multiplet in the case of isomers I and II and two multiplets in the case of isomers III and IV, appear downfield of the methyl signals (see Figure 2). The signal intensity of the four meso protons in each of the porphyrin isomers has been measured as the methylene and methyl proton signals for the given isomer have been irradiated in sequential order from low to high field. The results of these NOE experiments are given in Figure 5. Each of the tracings in the figure corresponds to the *difference* in the meso signal intensities between the spectrum in which a specific methylene or methyl signal is being irradiated and the same spectrum in which the irradiation is at a position where no protons are found. The presence of a peak thus indicates a signal enhancement. Note should be taken that the bottom two tracings in the case of isomers III and IV correspond to irradiation of the two distinct side-chain methylene signals in these isomers, whereas only the bottom tracing in the case of isomers I and II is due to methylene proton irradiation.

Irradiation of the methylene protons at 4.32 ppm in isomer I gives an NOE enhancement of the γ meso proton (Figure 5a, lowest trace), as expected, since the side chains bracket the proton at this meso position. Irradiation of the protons identified by relaxation experiments as those of the methyl ester moieties (Figure 5a, second trace from bottom) causes no meso proton signal enhancement, again as expected from the distance which separates the methoxy groups from the meso protons. Saturation of the first methyl group upfield from the methoxy group signal (labeled 1 in Figure 4, lowest spectrum), however, results in enhancement of the δ meso proton signal. That the meso proton

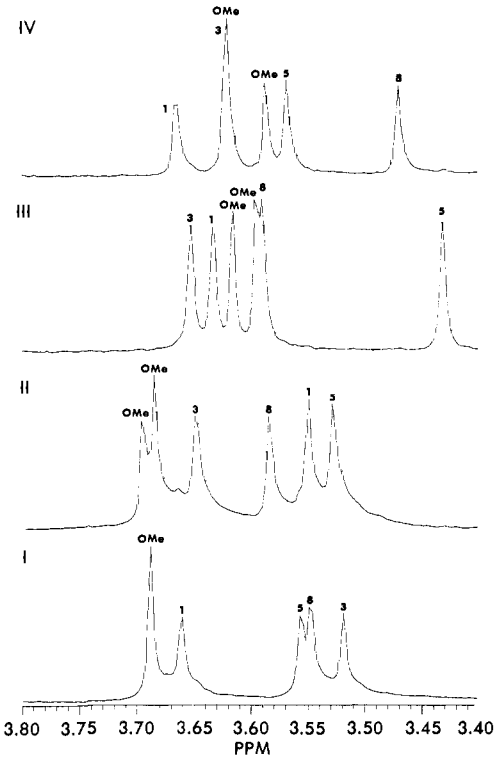

Figure 4. Methyl proton region of the NMR spectra of the four zinc-complexed *N*-methylprotoporphyrin IX (dimethyl ester) isomers. The isomer number (legend, Figure 1) is given with each spectrum. The identity of each of the methyl signals is given.

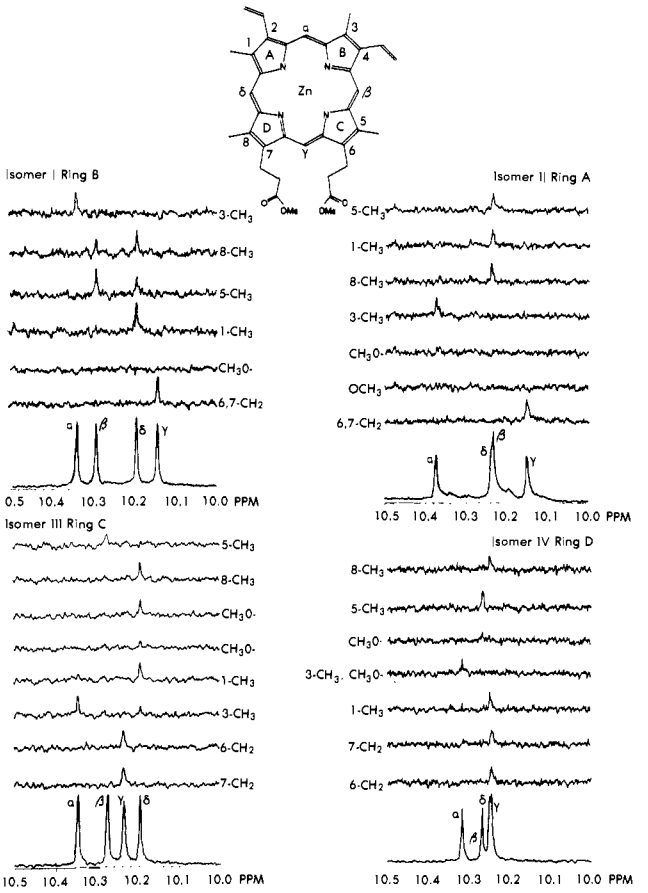

Figure 5. Nuclear Overhauser effect (NOE) enhancement of the meso proton signals in each isomer of zinc-complexed *N*-methylprotoporphyrin IX (dimethyl ester) due to sequential irradiation first of the low-field side-chain methylene protons (Figure 2) and then of each of the methyl groups shown in the corresponding spectrum in Figure 4. The results of these NOE experiments, going from low to high field, are shown from the bottom to the top in the panel which corresponds to each isomer. Each NOE result is given as the difference between the meso proton region in the specifically irradiated sample and the meso proton region of a control spectrum. The presence of a peak thus indicates a signal enhancement: (a) isomer I, (b) isomer II, (c) isomer III, and (d) isomer IV. The structure of zinc protoporphyrin IX (dimethyl ester) is given at the top. The ring methylated as well as the identity of the meso protons and the group irradiated in each NOE experiment are given for each isomer.

involved in this NOE enhancement *is* at the δ position, one of the meso positions not unambiguously defined by the deuteration experiments, is evident from the fact that irradiation of the methyl signal labeled as 8 in Figure 4 causes an NOE enhancement of *the same* meso proton signal. Only the δ meso position is adjacent to two methyl groups. It has been possible in each isomer to identify the δ meso position because it is the only one which exhibits an NOE signal enhancement on irradiation of two different methyl groups. Since the γ and α positions were assigned in the deuteration experiments, localization of the γ meso signal, by exclusion, also results in assignment of the β-meso proton signal. In the case of isomer I, the methyl group at position 8 has almost the same chemical shift as that at position 5 (Figure 4). Specific saturation of these methyl group signals has therefore not been technically possible. Saturation of the peak due to the methyl at position 8 is accompanied by partial saturation of the signal due to the methyl at position 5 and vice versa. In consequence, irradiation of the 8-methyl not only results in a strong NOE enhancement of the δ meso signal but also in a weaker enhancement of the β meso signal (due to partial saturation of the 5-methyl protons). The converse is found on irradiation of the 5-methyl protons (Figure 5a). Finally, irradiation of the highest-field methyl group (Figure 4) results in NOE enhancement of the α meso proton signal, clearly identifying the methyl signal in question as that of the methyl group at position 3 in the porphyrin. The methyl group assignments derived from these NOE experiments, indicated in Figure 4, are unequivocal except for the differentiation of the methyls at positions 1 and 8. The assignment of these two methyl signals as indicated in Figure 4, is based on a chemical-shift correlation yet to be discussed. Similar analysis of the NOE data obtained for the other three isomers of *N*-methylprotoporphyrin IX (dimethyl ester) (Figure 5b–d) leads to the complete meso proton assignments given in Figure 3b–d and to the methyl group assignments indicated in Figure 4.

Isomer Assignment. The chemical-shift values for all the methyl and meso protons in the NMR spectra of each of the four zinc-complexed *N*-methylprotoporphyrin IX (dimethyl ester) isomers are summarized in Table I. All the signals have been unambiguously assigned except for the differentiation of the 1- and 8-methyls. Comparison of the chemical-shift values for a given methyl in the four isomers reveals that the methyl appears at much higher field in one of the isomers than it does in the other three isomers. The 3- and 5-methyl groups, for example, appear at unusually high field in isomers I and III, respectively. In view of the already discussed general observation that the substituents on an N-alkylated ring appear at higher field than those on equivalently substituted but nonalkylated rings,[11b] isomer I (with the upfield-shifted 3-methyl group) must be the structure in which pyrrole ring B is alkylated (Figure 5) and isomer III (with the upfield-shifted 5-methyl group) must be that in which pyrrole ring C is N-methylated.

The ambiguity concerning which methyl signal is due to the 1-methyl and which to the 8-methyl complicates the analogous identification of the other two isomer as either the A- or D-ring alkylated structures. However, as already described, an analysis of their NMR spectra has established that isomers I and II are N-methylated on vinyl-substituted pyrrole rings (rings A and B) and isomers III and IV on propionic acid substituted rings (rings C and D). Specific identification of isomer I as that in which pyrrole ring B is N-methylated thus directly establishes that pyrrole ring A is methylated in isomer II. The demonstration that ring C is methylated in isomer III similarly established that ring D is alkylated in isomer IV.

Table II. Upfield Shift of Ring Methyl Groups When Present on the *N*-Alkylated Ring or on the Ring Opposite to It

group	ref chem shift value,[a] ppm	upfield shift when on alkylated ring,[b] ppm	upfield shift when on opposite ring,[c] ppm
1-Me	3.661 (0.004)	0.113	0.031
3-Me	3.648 (0.004)	0.130	0.028
5-Me	3.560 (0.012)	0.131	0.024
8-Me	3.585 (0.005)	0.116	0.039

[a] The chemical-shift values for the given methyl when on neither the alkylated ring nor on the ring opposite to it (Table I) have been averaged. The difference between the two averaged values is given in parentheses. [b] The difference between the chemical-shift value for the given methyl when on the alkylated ring (taken from Table I) and the average (reference) value calculated in the first column of this table. [c] The difference between the chemical-shift value for the given methyl when on the ring opposite to that which is alkylated and the reference value in the first column.

The methyl groups adjacent to the vinyl moieties in protoporphyrin IX appear at lower field than the methyl groups vicinal to the propionic acid side chains.[12] It is therefore to be expected that the 1-methyl, except perhaps in the isomer in which ring A is N-alkylated, will appear at lower field than the 8-methyl. The signal assignments for these two methyls in Table I reflect this argument. The observation that this assignment results in a chemical shift for the 1-methyl in one isomer upfield of its chemical shift in the three other isomers and that the isomer in which this occurs is that which has just been independently identified as the A-ring structure substantiates both the methyl group and structure assignments. The identity of isomer IV is likewise confirmed by a similar correlation of the chemical-shift data for the 8-methyl group (Table I). The further observation has been made that irradiation of the signal attributed to the internal 2-vinyl proton in isomer II (A-ring methylated) results in sharpening of the 1-methyl peak, suggesting that the methyl and the vinyl protons are slightly coupled and, consequently, that both groups are on the same ring. A similar observation has been made with protoporphyrin IX.[12]

Crystallographic studies of several metal-complexed N-alkylated porphyrins have established that, at least in the crystal lattice, the N-alkylated ring is sharply tilted out of the porphyrin plane and the ring trans to it in the structure is also tilted if to a lesser degree.[14] The analysis of the NMR data outlined in Table II convincingly argues that this differential tilting of the N-alkylated ring and of the ring opposite to it in the structure also occurs in solution. The chemical shifts of a given methyl in the two isomers in which it is neither on the alkylated ring nor on the ring opposite to that which is alkylated are virtually identical (Table II). These two chemical-shift values in the case of the 1-methyl differ by 0.012 ppm and in the case of the other three methyls by only 0.004 ppm. If the average of these two values for each of the methyls is taken as the reference position of the methyl in N-alkylated structures, the shift from this reference value can be calculated for the given methyl when it is on the N-alkylated ring or on the ring trans to that which is alkylated. Strikingly similar chemical shifts are thus obtained for the four ring methyl groups, a shift of 0.113–0.131 ppm resulting from the presence of the methyls on the N-alkylated ring and a shift of 0.028–0.039 ppm resulting from their presence on the ring opposite to that which is alkylated. The consistency of the shifts observed for the four methyls and the quantitatively similar difference in the shifts due to their presence on the N-alkylated ring or on the ring opposite to it strongly support the concept that the alkylated ring is tilted to a greater degree than the ring opposite to it in each of the zinc-complexed *N*-methylprotoporphyrin IX (dimethyl ester) isomers.

Conclusions

Although symmetrically substituted N-alkylated porphyrins with no reactive peripheral substituents have been extensively studied,[11b,15] no N-alkylated derivatives of protoporphyrin IX, an asymmetrically substituted porphyrin with two reactive vinyl substituents, had been synthesized or characterized prior to this study. This neglect, albeit understandable from a synthetic and analytical point of view, nevertheless excluded from study the most important porphyrin in mammalian physiology. Our recent unambiguous identification of *N*-methylprotoporphyrin IX as the hepatic pigment formed in rats treated with 3,5-bis(ethoxycarbonyl)-2,4,6-trimethyl-1,4-dihydropyridine,[7] and of *N*-(2-hydroxyethyl)protoporphyrin IX as the porphyrin into which the prosthetic heme of cytochrome P-450 is converted during attempted metabolism of ethylene,[2,4] has rendered this lack of information particularly acute. The present specific characterization of the four isomers of *N*-methylprotoporphyrin IX (dimethyl ester), which remedies this hiatus in our knowledge of N-alkylated porphyrins, provides both models and techniques for the differentiation of other N-alkylated protoporphyrin IX isomers.

The identification of the nitrogen alkylated in each of the synthetic *N*-methylprotoporphyrin IX isomers directly establishes that, in the biological formation of these porphyrin isomers in rats treated with 3,5-bis(ethoxycarbonyl)-2,4,6-trimethyl-1,4-dihydropyridine, the nitrogen in pyrrole ring A of protoporphyrin IX is preferentially alkylated. This follows from the fact that isomer II accounts for approximately 80–90% of the abnormal porphyrin isolated from the livers of treated rats.[7] Methylation of the nitrogen in pyrrole ring C (isomer III) is also significant, whereas methylation of the other two pyrrole rings (isomers I and IV) occurs but is negligible. Pyrrole ring B, however, is not the universally preferred site of alkylation, since the *N*-(2-hydroxyethyl)protoporphyrin IX isomer *exclusively* formed in ethylene-treated rats is alkylated on either ring C or D.[2,4] Determination of whether ring C or D is involved awaits scrutiny of the ethylene adduct by the techniques described here. A clear interpretation of the regiochemical differences in the biological alkylation of protoporphyrin IX brought about by ethylene, 3,5-bis(ethoxycarbonyl)-2,4,6-trimethyl-1,4-dihydropyridine, and other agents requires further exploration of the alkylation processes. In the case of ethylene, the alkylation regiochemistry must reflect steric and electronic properties of the cytochrome P-450 catalytic site at the time of prosthetic heme alkylation. The regiospecificity of alkylation in the case of other agents whose mechanism is less well defined also must bear the steric and electronic imprint of the formative process.

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