# STRUCTURAL ISOMERISM AND CHIRALITY OF *N*-MONOSUBSTITUTED PROTOPORPHYRINS

## Possible relevance to binding of *N*-methyl protoporphyrin by ferrochelatase and to orientation of haem in cytochrome P450

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### 1. Introduction

A modified porphyrin or green pigment which is a powerful inhibitor of ferrochelatase (protohaem ferrolyase, EC 4.99.1.1) has been isolated from the liver of mice treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) [1] and the inhibitory porphyrin has been identified as *N*-methyl protoporphyrin [2–4]. Interaction of the inhibitory porphyrin with the active centre of ferrochelatase is important for inhibition of the enzyme [5,6]. Authentic *N*-methylated meso and protoporphyrin have been synthesized and shown to be powerful inhibitors of the enzyme in vivo and in vitro [2,6,7]. Synthetic *N*-methyl protoporphyrin could be separated by high-performance liquid chromatography (HPLC) into 2 separate fractions possessing different inhibitory activity and possibly representing different structural isomers, where different pyrrole nitrogens had been methylated [2].

Here, we provide evidence that the two HPLC fractions contain in fact different structural isomers of *N*-methyl protoporphyrin. The different inhibitory activity of these two isomeric fractions is now confirmed and extended to their respective zinc chelates. In the isomers with less inhibitory activity, where the propionic acid substituted rings of protoporphyrin are *N*-methylated, the spatial arrangement and distance between the two propionic acid sidechains may be significantly altered. We also wish to report that the naturally occurring *N*-methyl protoporphyrin and the *N*-alkylated protoporphyrins produced in vivo from degradation of the haem of cytochrome P450 during metabolism of unsaturated chemicals are all optically active and appear to possess the same absolute configuration.

### 2. Materials and methods

Male mice were treated with DDC and phenobarbitone-pretreated male rats were exposed to ethylene or given a single injection of 2-allyl-2-isopropylacetamide or of secobarbitone [5-allyl-5(1-methylbutyl)-barbiturate], as in [5,6]. The corresponding *N*-substituted porphyrins or green pigments were extracted from liver homogenates, chromatographed on Sephadex LH-20 [5] and further purified by TLC [6] after methylation of the propionate sidechains by reaction with $BF_3$ in methanol [8].

Authentic *N*-methylated protoporphyrin was prepared from protoporphyrin IX dimethyl ester by reaction with methyl fluorosulphonate at 20°C for 1 day or alternatively with methyl iodide at 100°C in a sealed tube for 1 day. It was first purified by TLC on silica gel plates or on $Al_2O_3$, grade III [9] columns. It was then resolved by HPLC into 2 isomeric fractions ($F_1$ and $F_2$, in order of elution) employing silica columns [either a Hypersil preparative column or a Nucleosil 5 (4.6 × 250 mm) analytical column] developed isocratically with dichloromethane/methanol/$NH_3$, spec. grav. 0.88 (60:40:0.3, by vol.). These 2 fractions were obtained in sufficient amount to allow further characterization by electronic absorption spectroscopy, and also study of the in vitro inhibitory



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activity on ferrochelatase [1] as either the free porphyrins (after hydrolysis of the methyl esters by aqueous 6 M HCl for 16 h) or as their zinc chelates. Chelates for enzymic studies were prepared by reacting the corresponding porphyrins (free carboxylate) with an excess of zinc acetate in methanol and, when conversion was complete, the metal derivative was transferred to chloroform, washed with water and taken to dryness under $N_2$. The inhibitory activity of the free porphyrins and metal derivatives was related to their Soret absorbance in dimethyl sulphoxide/1.0 M Tris–HCl buffer (pH 8.2) (10:1, v/v) and was expressed as inhibitory units/nmol pigment, using the $\epsilon$-values given below for the corresponding methyl esters. In one experiment, $F_1$ and $F_2$ (each comprising 2 different structural isomers of *N*-methyl protoporphyrin, see below) were further resolved into the individual isomers by HPLC on a column (4.6 × 250 mm) of Partisil 10-PAC, as in [3].

### 2.1. *Spectral studies*

To determine the $\epsilon$-value for each of the two isomeric fractions ($F_1$,$F_2$) of synthetic *N*-methyl protoporphyrin, the porphyrin methyl esters were allowed to react with $^{65}ZnCl_2$ (0.44 mCi/mmol) in chloroform/methanol (1:1, v/v), the zinc chelates transferred to chloroform and, after repeatedly washing with water to eliminate the unreacted zinc, they were purified by TLC, dissolved in chloroform/methanol (100:8.3, v/v) and the Soret absorbance (at 430 nm) related to radioactivity and therefore the amount of Zn present. Values of 148 000 and 142 000 were thus obtained for the zinc chelates of $F_1$ and $F_2$, respectively on the basis of a 1:1 (zinc:porphyrin) stoichiometry. The $\epsilon$-values for the metal-free porphyrins ($F_1$ = 125 900 and $F_2$ = 129 700 at 420 nm in chloroform) were then derived by comparison of the Soret absorbance of the porphyrin methyl esters, before and after complete conversion into the zinc chelate.

Circular dichroism spectra were run on a Jobin-Yvon Dichrograph III instrument using a 1 cm pathlength cell and the spectra were scanned from 350–700 nm.

## 3. Results and discussion

### 3.1. *Differential inhibition of ferrochelatase by different structural isomers of* N*-methyl protoporphyrin*

Synthetic *N*-methyl protoporphyrin dimethyl ester could be resolved by HPLC into two fractions ($F_1$,$F_2$) with retention times of 2.86 and 3.66 min, respectively (using a nucleosil 5 analytical column and a flow rate of 1.5 ml/min).

NMR studies of the 2 fractions showed the expected high field resonances for the *N*-methyl groups, at −4.85 δ for $F_1$ and −5.2 δ for $F_2$; each was a singlet indicating that the *N*-methyl group was likely to be on similarly substituted rings in the two components of each fraction. The vinyl resonances were ~0.2 ppm to higher field in fraction $F_1$, than those in $F_2$, whereas the methyl and methylene resonances of the groups attached to the C and D rings in $F_1$ were slightly to low field of those in fraction $F_2$. These results are consistent with fraction $F_1$ containing the $N_A$ and $N_B$ methylated pigments and fraction $F_2$ the $N_C$ and $N_D$ methylated pigments (the suffix A-C indicating the pyrrole ring which is *N*-methylated in each structural isomer; see fig.1). This was confirmed by further

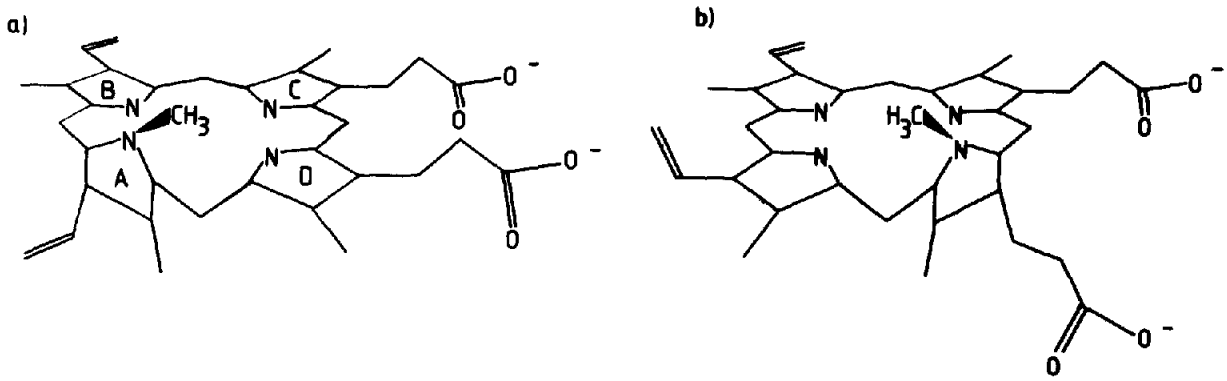


Fig.1. Two structural isomers of *N*-methyl protoporphyrin are illustrated, where a vinyl-substituted ring (a) or a propionic acid-substituted ring (b) is *N*-methylated. Note distortion of the *N*-methylated ring out of the porphyrin plane and, in (b), change in alignment of the 2 propionate sidechains.

studies with europium shift reagent Eu(FOD-$d_9$)$_3$, which complexes with neighbouring ester side chains in porphyrins in a bi-dentate fashion, causing marked low field shifts of these groups and especially of the meso-bridge proton resonance between the groups [10]. Here, the shifts of the two propionate resonances and the γ-meso proton observed on addition of the europium reagent were much less marked for fraction $F_2$ than for fraction $F_1$ owing to the less effective complexing of the reagent with the two propionate residues in $F_2$. This result was attributed to the distortion of the macrocycle by the *N*-methyl group, which is clearly greatest on the pyrrole ring to which it is attached. A detailed description of these results will be published elsewhere [11].

The assignments of the positions of the *N*-methyl substituents in the 2 fractions were also confirmed by further HPLC studies using the method in [3] which separates all 4 structural isomers, in the following order of elution ($N_B$-, $N_A$-, $N_C$- and $N_D$-methyl derivatives). When $F_1$ and $F_2$ were analysed by this HPLC technique, each could be resolved into 2 components with the following retention times (amount recovered in each peak given in parenthesis as a percentage of the total recovery of 417 nm – absorbing material from each sample): $F_1$ 5.7 min (43%), 6.4 min (50%); $F_2$ 11.1 min (48%), 12.7 min (49%). Retention times obtained in individual peaks from unresolved synthetic *N*-methyl protoporphyrin were: 5.8 min, 6.4 min, 11.1 min and 12.8 min, corresponding to the $N_B$-, $N_A$-, $N_C$- and $N_D$-methyl derivatives of protoporphyrin dimethyl ester, respectively [3].

The in vitro inhibitory effect on ferrochelatase of the 2 isomeric fractions and of 2 individual isomers is shown in table 1. Isomers with the *N*-methyl on propionic acid-substituted rings (C,D) were less inhibitory than isomers with the *N*-methyl on vinyl-substituted rings (A,B). This was true both for the mixed isomeric fractions (compare $F_2$ with $F_1$, and see also [2]) and for the purified isomers tested on their own (compare isomer IV with isomer II). The difference in inhibitory activity was small with the free porphyrins (~10% av., 18.5–4.5% in different experiments) but was considerably increased by incorporating a metal in the centre of the tetrapyrrolic system.

A possible interpretation for these findings lies in the spatial arrangement of the two propionic acid sidechains in position 6 and 7, both of which may be important for a porphyrin to be accepted at the active centre of the enzyme [12]. The central *N*-substituent cannot fit into the inner cavity of the porphyrin macrocycle and consequently causes a distortion of the *N*-alkylated ring which is significantly tilted out of the porphyrin plane; this is particularly so in the

Table 1
Inhibitory activity towards ferrochelatase of synthetic *N*-methylprotoporphyrin IX and of its zinc chelate – Comparison between two mixed isomeric fractions ($F_1$ and $F_2$) and between two individual structural isomers (II and IV)

| Pigment tested | Isomer fraction or isomer | *N*-Methylated ring(s)[a] | Inhibitory activity | |
|---|---|---|---|---|
| | | | units/nmol | % of corresponding value in $F_1$ or isomer II |
| Free porphyrin | $F_1$ | A and B | 12.6 | 100 |
| | $F_2$ | C and D | 11.7 | 92.8 |
| | II | A | 10.6 | 100 |
| | IV | D | 9.6 | 90.6 |
| Zinc chelate | $F_1$ | A and B | 12.9 | 100 |
| | $F_2$ | C and D | 2.0 | 15.5 |
| | II | A | 12.8 | 100 |
| | IV | D | 2.1 | 16.4 |

[a] See fig.1.

Results given were obtained with the same preparation of mouse liver mitochondria (as the source of ferrochelatase) employing ≥3 different concentrations of inhibitor [5]. The pure isomers, obtained from unresolved synthetic *N*-methyl protoporhyrin as in [3], were only tested once; all other results were confirmed in ≥2 additional experiments

metal chelate derivatives where the space available in the centre of the porphyrin system is further reduced by the presence of a metal [13–16]. We therefore suggest that when a propionic acid-substituted ring is *N*-methylated, the alignment of the 2 propionic acid chains with respect to each other may be significantly altered (fig.1, cf. b and a): as a consequence, the binding of the modified porphyrin by the ferrochelatase may be impaired, particularly in the metal chelate derivatives, where the distortion of the *N*-methylated ring will be expected to be greater. It must be emphasized that this concept is still hypothetical: more direct evidence is required that the 2 propionic acid side-chains are in fact involved in the binding of the inhibitory porphyrin to the enzyme.

Similar steric factors may be responsible for the less marked inhibition of ferrochelatase observed when the *N*-substituent is increased in size from 1–2 or more carbon units [17]. This interpretation is supported by the observation that in both the non-inhibitory *N*-hydroxyethyl protoporphyrin produced by treatment with ethylene [6] and in the weakly inhibitory *N*-methyl protoporphyrin [18], the loss of inhibitory activity may be largely correlated with the presence of the *N*-alkyl group on propionic acid-substituted rings [19,20].

### 3.2. *Chirality of* N-*monosubstituted protoporphyrins*

In protoporphyrin the 2 faces of the porphyrin plane are not superimposable because of the asymmetric arrangement of the vinyl and propionate side chains. Two antipodes are therefore possible for each *N*-methyl protoporphyrin, depending upon whether the *N*-methyl group lies above or below the porphyrin plane. Preliminary observations have suggested that the *N*-methyl protoporphyrin isolated from the liver after treatment with DDC is optically active [21,22]. In contrast, synthetic *N*-methyl protoporphyrin is optically inactive, as it is a racemic mixture of the 2 enantiomorphs. We now report that the DDC pigment and the green pigments isolated from the liver of animals treated with ethylene, AIA and secobarbitone, all exhibit a circular dichroism spectrum (CD) with a prominent band of positive ellipticity in the 430–445 nm region. The CD spectrum of the ethylene pigment (fig.2) shows that the main band is at 430 nm, as was also the case with the AIA and secobarbitone pigments. The general shapes of the CD spectra from the 2 fractions of the DDC pigment were also very similar, but whereas the main band in the spectrum

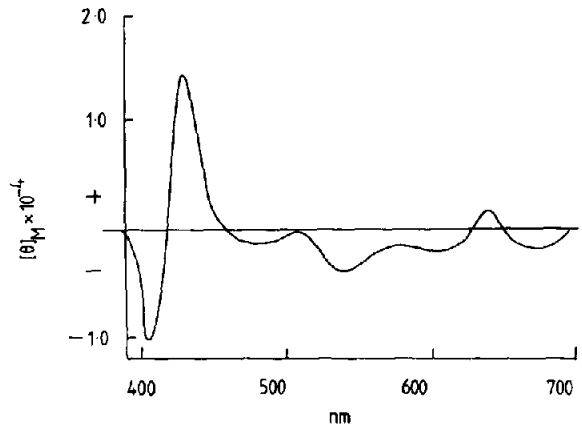


Fig.2. Circular dichroism spectrum of the natural ethylene green pigment. The ellipticity values have been calculated on the basis that $\epsilon_{max}$ at 420 nm is 125 000.

of $F_2$ was also at 430 nm that of $F_1$ was at 444 nm (fig.3). In [23] the pigment derived from propyne is the $N_A$-acetonylprotoporphyrin (on the basis of NMR studies) and not only is the CD spectrum of this material very similar to those shown in fig.2,3, but the main band of positive ellipticity is at 445 nm like that of our fraction $F_1$. In [19] NMR evidence that the ethylene-derived green pigment is substituted on either ring C or D is reported. Thus the CD spectra of these *N*-alkylated protoporphyrin derivatives appear to provide a means of distinguishing between substitution on rings A and B *vs* rings C and D. We attribute the longer wavelength shift of the main band in $N_A$ and $N_B$ alkylated pigment spectra to the greater dis-

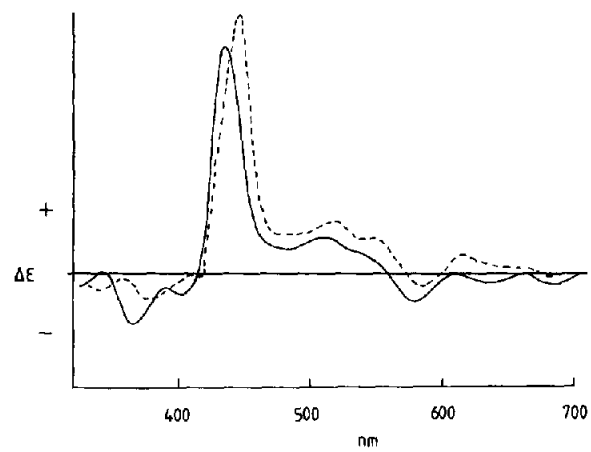


Fig.3. Qualitative circular dichroism spectra of the 2 fractions (– – –, $F_1$; ——, $F_2$) of the DDC green pigment separated by HPLC.

tortion of these rings from planarity, as it is these rings which bear the conjugated vinyl groups.

These findings indicate that alkylation of the tetrapyrrolic macrocycle is stereospecific in biological systems and takes place preferentially, if not exclusively, from one side of the porphyrin plane. They also suggest that the various biological pigments listed above and that isolated from liver after treatment with propyne [23] may all possess the same absolute configuration, although we cannot yet specify which of the 2 possible configurations actually occurs.

In the green pigments originating from the haem of cytochrome P450 during metabolism of unsaturated drugs the attack of the tetrapyrrolic system must take place from the oxygen binding side of the porphyrin plane, since the drug is first monooxygenated (by an active oxygen species generated on the iron of haem) and transfer of the resulting electrophilic species across the porphyrin plane is not probable. It seems therefore likely that the *N*-alkyl substituents of the various green pigments considered above (those arising from unsaturated drugs as well as the DDC pigment) all lie on the same side of the porphyrin plane, the side corresponding to the oxygen ligand in cytochrome P450. By establishing the absolute configuration of one such pigment (by X-ray crystallographic analysis) it should be possible to derive the orientation of the haem plane in cytochrome P450 with respect to each of its 2 axial ligands, oxygen and the mercaptide anion.

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