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A STUDY OF PORPHYRIN ANALOGUES—III
SYNTHESES, ENZYME INTERACTIONS AND SELF-AGGREGATION OF NEW MODELS FOR TYPES I, III AND IX PORPHYRINS

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Abstract—Three new porphyrin free bases have been synthesised and their interaction with the mitochondrial enzyme Ferrochelatase has been studied. The model compound for type IX porphyrins is the best substrate for Ferrochelatase so far studied, whereas the model compound for type I porphyrins is the only compound of this type to act as a substrate for this enzyme. The model compound for type III porphyrins is not a substrate, but does act as a competitive inhibitor.

The ^{1}H NMR spectra of the new compounds in their dimethyl diester form differ substantially from the spectra of their zinc(II)bis-pyrrolidine adducts, showing that aggregation is taking place. The results for the *α-meso* and *γ-meso* protons in particular are unusual and indicate that aggregation is taking place anomalously, with electronic effects dominating steric effects.

One of the most stringent tests to which a proposed model for a biological compound can be subjected involves exposing the model compound to an appropriate enzyme system. In the case of proposed model compounds for porphin or porphyrins, an appropriate enzyme is the mammalian liver mitochondrial enzyme, Ferrochelatase (protohaem ferrolyase EC.4.99.1.1).

The majority of naturally occurring porphyrins that act as substrates[1] for Ferrochelatase have the substitution pattern MPPM for the substituents R_{12}, R_{13}, R_{17} and R_{18} respectively where M = CH_3 and P = $(CH_2)_2COOH$. Earlier work has shown that if the MPPM pattern is retained, the rate of uptake of Co^{2+} or Fe^{2+} ions by naturally occurring porphyrins is enhanced as the size or polarity of the R_2, R_3, R_7 and R_8 substituents is reduced. However, this work did not eliminate the possibility that permutations of the M_2P_2 pattern might give porphyrins that still act as substrates for Ferrochelatase.

In order to determine the optimum position for the two carboxylic side chains, and to assess the role of the other substituents, three new porphyrins having no substituents on the A/B rings have been synthesised. The pattern of substitution in rings C and D of the new porphyrins (Fig. 1) is identical to rings C and D of type IX porphyrins (i.e. **8** with MPPM), to rings C and D of type I porphyrins (i.e. **9** with MPMP) and to rings A and D of coproporphyrin III (i.e. **10** with PMMP).

The self aggregation of highly substituted porphyrins has been studied in detail by Abraham *et al.*[2] Our new compounds have a degree of substitution that is intermediate in extent between porphin and the natural porphyrins. We report some effects of the lessened steric hindrance and the paucity of hydrophobic substituents upon the self-aggregation of **8e**, **9e** and **10e**, where e implies the dimethyl diester.

Synthesis

Strategy. Two features of the structures of the new porphyrins help to simplify the synthetic route—namely, the unsubstituted dipyrrolic units comprising the A and B rings, and the presence of a mirror plane between the C and D rings of **8** and **10**. Thus the longer synthetic routes involving tetrapyrrolic intermediates[3] were avoided in favour of the single-step coupling of two dipyrrolic intermediates. The choice of the four intermediate dipyr-

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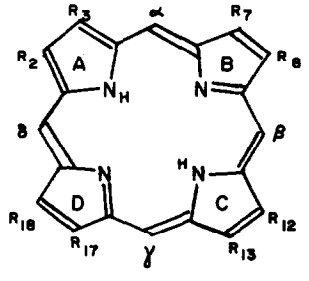


R_2	R_3	R_7	R_8	R_{12}	R_{13}	R_{17}	R_{18}	Code Number	Name
M	V	M	V	M	P	P	M	1	protoporphyrin IX
M	Et	M	Et	M	P	P	M	2	mesoporphyrin IX
M	H	M	H	M	P	P	M	3	deuteroporphyrin IX
M	V	M	V	M	P	M	P	4	protoporphyrin I
M	Et	M	Et	M	P	M	P	5	mesoprophyrin I
M	P	M	P	M	P	M	P	6	coproporphyrin I
M	P	M	P	M	P	P	M	7	coproporphyrin III
H	H	H	H	M	P	P	M	8	new compounds
H	H	H	H	M	P	M	P	9	
H	H	H	H	P	M	M	P	10	
M	P	P	M	M	P	P	M	11	coproporphyrin II
P	M	M	P	M	P	P	M	12	coproporphyrin IV
A	P	A	P	A	P	P	A	13	uroporphyrin III

Fig. 1. Topology, labelling code numbers and names of porphyrins. The suffix e, used in the text, implies that all acid groups have been converted into the corresponding methyl esters (M = CH_3, Et = C_2H_5, V = $-CH{=}CH_2$, P = $(CH_2)_2COOH$ and A = CH_2COOH).

roles was influenced by the extra reduction steps required in the dipyroketone-oxophlorin route,[3] and the reported higher yields in dipyrromethane route[4] compared to its dipyrromethene counterpart.[5]

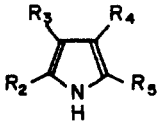
The preparation of highly substituted diesters of 5,5'-dicarboxy-2,2-dipyrromethanes is straightforward due to the ease of preparation of the relevant 2-alkoxy, or 2-aryloxy carbonyl pyrroles by the Knorr or Kleinspehn methods.[6] Our choice for the substituted dipyrromethanes dictated that the eventual C-10 and C-20 porphyrin methine bridges be provided in the form of 5,5'-diformyl-2,2'-dipyrromethane.

Pyrroles. For 2-ethoxycarbonyl-4-(2'-methoxycarbonylethyl)-3, 5-dimethylpyrrole (**14**, Fig. 2) and 2-ethoxycarbonyl - 3 - (2'-methoxycarbonylethyl) - 4,5-dimethyl pyrrole (**15**, Fig. 2), the condensation of a β-dione with a 2-amino-3-ketoester by a Kleinspehn synthesis was preferred to a Knorr condensation because the latter usually yields an ester of a 4-carboxypyrrole. Suitable β-diones for **14** are 4-acetyl-5-oxohexanoic acid or its methyl ester. The methyl ester was obtained by a Michael addition[7] of pentan-2,4-dione to methyl acrylate: the method of Gresham *et al.*[8] for the free acid gave yields below 2%. Reaction of ethyl 2-oximinoacetoacetate[9] with the foregoing methyl ester gave **14**. Pyrrole **15** is similar to pyrroles required in the synthesis of **7** and was obtained from methyl-5-methyl-4,6-dioxoheptanoate and diethyl-2-oximinomalonate.[12]

The 5-unsubstituted pyrrole, 2-ethoxycarbonyl-3-(2'-methoxycarbonylethyl)-4-methylpyrrole (**16**, Fig. 2) was obtained by published methods[13,14] *via* trichlorination of the appropriate 5-methylpyrrole (in a proton free solvent with ^{1}H NMR monitoring)[15] followed by hydrolysis, decarboxylation iodination and catalytic hydrogenolysis.[14] This compound and its precursors are related to series of pyrroles prepared by Jackson *et al.*[16] in which t-butyl ester groups occurred at the 2-positions.

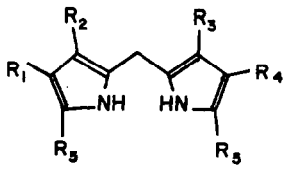
The treatment of the 5-methyl pyrroles **14** and **15** with lead tetraacetate in acetic acid[17] gave 2-carbethoxy-3-(2'-methoxy carbonylethyl) - 4 - methyl - 5 - acetoxymethyl pyrrole (**17**, Fig. 2) and 2-carbethoxy - 3 - methyl - 4 - (2'-methoxycarbonylethyl) - 5 - acetoxymethyl pyrrole (**18**, Fig. 2) respectively.

Dipyrromethanes. The two symmetrically substituted dipyrromethanes (**19** and **20**, Fig. 3) were prepared using a two step synthesis from the 5-acetoxy pyrroles (it was found by us that acetic anhydride gives low yields[18]) instead of bromomethylpyrroles as used by other workers[10] in synthesising precursors to coproporphyrin III. Compound **19** has been prepared before[19] and is the diethyl ester analogue of a previously synthesised



R_2	R_3	R_4	R_5	Code Number
EtO_2C	M	P^M	M	14
EtO_2C	P^M	M	M	15
EtO_2C	P^M	M	H	16
EtO_2C	M	P^M	$AcOCH_2$	17
EtO_2C	P^M	M	$AcOCH_2$	18

Fig. 2. Substituted pyrroles used for syntheses of dipyrromethanes. (M = CH_3, Et = C_2H_5, P^M = $(CH_2)_2COOCH_3$, Ac = $=OC{\cdot}CH_3$).



R_1	R_2	R_3	R_4	R_5	Code Number
M	P^M	P^M	M	EtO_2C	19
P^M	M	M	P^M	EtO_2C	20
P^M	M	P^M	M	EtO_2C	21
H	H	H	H	CHO	22

Fig. 3. Substituted dipyrromethanes used as porphyrin precursors. (abbreviations as for Fig. 2).

dibenzyl-5,5'-dicarboxylate.[20] The unsymmetrically substituted dipyrromethane (**21**, Fig. 3) was obtained by the condensation of **16** with **17** in the presence of toluene-*p*-sulphonic acid. 5,5'-diformyl 2,2'-dipyrromethane (**22**) was obtained in acceptable yield after the modification of published methods.[21,22]

Porphyrins. Compounds **8**, **9** and **10** were obtained by MacDonald-type[4] syntheses following alkaline hydrolysis of the appropriate dipyrromethane tetra–ester (i.e. **19**, **21** or **20** respectively). The low yields based on the tetra–esters (*ca* 10%) complement the findings of Clezy *et al.*[23] concerning the influence of propionate ester side chains.

RESULTS AND DISCUSSION

The interaction with ferrochelatase. The metal ion selected as a substrate was Co^{2+} rather than Fe^{2+} because Co^{2+} has been shown to react at rates comparable to Fe^{2+}, and does not pose the problem of autoxidation to a higher valence state.[1] The initial rate of incorporation of Co^{2+} into a porphyrin, in the presence of sheep-liver mitochondria, was determined by monitoring the decay of band IV of the visible spectrum of free-base porphyrin relative to an adjacent isosbestic point for the reaction

$$Co^{2+} + \text{porphyrin} \rightleftharpoons (\text{porphyrinato})Co(II).$$

The use of an isosbestic point as an internal reference automatically compensates for any overall change in the optical density of the medium due, for instance, to the swelling of the suspended mitrochondria. Each new sample of mitochondria was calibrated against a *bona fide* sample of **3** (Fig. 1).

The respective values of the Michaelis constant (K_m) and maximum rate (V_{max}) for **1**, **2** and **3** are 1.4μM, 0.25 nmole min^{-1} mg protein^{-1}: 1.9μM, 1.0 nmole min^{-1} mg protein^{-1} and 4.0μM, 1.4 nmole min^{-1} mg protein^{-1}. Both these sets of values show a steady increase as the size and hydrophobicity of the substituents on the C and D rings decreases. This trend is highlighted by the behaviour of **8** which has a K_m of 5.0μM and a V_{max} of 2.8 nmole min^{-1} mg protein^{-1}: this artificial porphyrin is therefore the best substrate for Ferrochelatase yet studied, having a V_{max} over ten times larger than that of the natural substrate (i.e. **1**). However, we have found that **8** does not inhibit the interaction between Ferrochelatase and **1**.[24,25] A possible explanation for these observations is that the enzyme has a much greater affinity for **1** than for **8**, and that the much greater reaction rate for **8** is due to the ease of release of the metallated porphyrin from the enzyme-substrate complex.[24] It has been noted that

the presence of organic solvents enhance the rate of interaction of Ferrochelatase with natural, type IX porphyrins.[24,26] We have found no equivalent effect for **8** in the presence of acetone.

In the light of earlier work[1] on **5** and our results for **8**, the effect of the change in substitution pattern from MPPM to MPMP between **8** and **9** was expected to reduce the value of V_{max} of **9** to *ca* 2.3 nmole min^{-1} mg $protein^{-1}$ in accord with the reduction factor of 0.8 between **2** and **5**. In contrast to this expectation, we have found that the V_{max} of **9** is only 0.4 nmole min^{-1} mg $protein^{-1}$. However, *bona fide* samples of **5** (synthesised from fully characterised **4**) and **4** were both found to have values of V_{max} too small to be measured. Thus **9** is the only type I porphyrin yet found that acts as a substrate for Ferrochelatase. The substantial reduction of activity between **8** and **9** shows that the optimum separation of the propionic acid side chains occurs in the type IX porphyrins. The lack of bulky, hydrophobic substituents in **9**, compared to **4** and **5**, presumably permits the unfavourably located acid side chains sufficient freedom of movement to interact with the active site(s) on the enzyme.

In common with **7** and **11**, **10** exhibits zero activity towards Ferrochelatase. Thus, the combined absence of extra acid groups and other bulky substituents from the A and B rings of **10** do not overcome the critically unfavourable disposition of the propionic acid side chains. However, **10** must be able to gain access to the active site(s) on the enzyme because **10** is a competitive inhibitor to **8**.[24,25]

Self-aggregation. The ^{1}H NMR spectra of **8e**, **9e** and **10e** are substantially different from the ^{1}H NMR spectra of their Zn(II)bis-pyrrolidine adducts.[27] All protons exhibit downfield, dis-aggregation shifts upon the addition of the Zn-pyrrolidine moiety (Table 1) which is similar to the behaviour of a range of natural porphyrins.[27,28] It is clear that, in common with the latter, **8e**, **9e** and **10e** are aggregating in solution. Two of our ^{1}H NMR assignments need special justification before any comments can be made about the nature of the aggregates.

It is not possible to distinguish between the ring-Me and ester-Me proton signals by inspection of the ^{1}H NMR spectra. However, the stepwise addition of the lanthanide shift reagent Eu(fod-d_9)$_3$ to solutions *ca.* 0.05M in porphyrin diester gradually shifted the downfield Me signals (at *ca.* 3.64 ppm) to lower applied magnetic field whilst leaving the upfield proton signals virtually unshifted. These observations imply that the shift reagent is not dis-aggregating the porphyrin systems and enable us to eliminate the direct involvement of the ester groups in the aggregation process because the dis-aggregation shifts of the upfield ester Me protons are almost zero (Table 1).

We consider that the *γ-meso* proton signals are upfield of the *α-meso* proton signals in **8e**, **9e** and **10e** at all molarities for the following reasons: (i) the *meso* protons of free-base porphin at very low molarity (5×10^{-5} M) have a chemical shift of 10.58 ppm whereas the *γ-meso* proton in **3e** (4×10^{-3} M), **11e** (infinite dilution) and **12e** (infinite dilution) have chemical shifts of 10.10 ppm, 10.10 ppm and 10.12 ppm respectively.[28] (ii) the *γ-meso* proton in **3e** (0.037M) has a chemical shift of 9.93 ppm[29] which compares favourably with our assignment of 9.96 ppm for the chemical shift of the *γ-meso* proton in **8e** at the same molarity. (iii) the ^{1}H NMR spectra of **8e** and **10e** at the same molarity (0.11 M) have unit intensity singlets at 9.40 ppm and 10.00 ppm (**8e**) and at 9.65 ppm and 9.95 ppm (**10e**). The upfield signals can be assigned to the *γ-meso* protons because their environment changes between **8e** and **10e**. The changes in chemical shift are not caused by a pronounced difference in aggregation because the pyrrole-hydrogen signals occur at 9.22 ppm (**8e**) and 9.19 ppm (**10e**) and the *β/δ-meso* proton signals occur at 9.75 ppm (**8e**) and 9.79 ppm (**10e**). (iv) the interaction between **8e** and the shift reagent is similar to that[30] for **12e** or **13e**, and is so specific that the largest lanthanide-induced *meso*-proton shift positively identifies the *γ-meso* proton. Apart from the ester-bearing side-chain, the only non-zero LIS were given by the ring-Me protons (0.02 ppm) and the upfield *meso* proton singlet (0.32 ppm): this latter must therefore be assigned to the *γ-meso* proton.

Table 1. ^{1}H NMR chemical shifts of solutions of new, free-base porphyrin dimethyl diesters (in $CDCl_3$) and the downfield dis-aggregation shifts induced by conversion to the (porphyrinato)Zn(II)bis-pyrrolidine complexes

	PORPHYRIN DIESTER 8e			PORPHYRIN DIESTER 9e			PORPHYRIN DIESTER 10e		
Proton Site	Chemical Shift δ/ppm	Dis-aggregation Downfield Shift Δ/ppm	Shift RATIO	Chemical Shift δ/ppm	Dis-aggregation Downfield Shift Δ/ppm	Shift RATIO	Chemical Shift δ/ppm	Dis-aggregation Downfield Shift Δ/ppm	Shift RATIO
1'-CH_2	4.2	0.24	1.0	4.30	0.14	1.0	4.05	0.40	1.0
2'-CH_2	3.14	0.13	0.54	3.20	0.06	0.43	3.05	0.24	0.60
$COOCH_3$	3.60	0.03	0.13	3.64 3.70	0.03 0.01	0.21 0.07	3.64	0.04	0.10
ring-CH_3	3.42	0.21	0.88	3.50 3.53	0.17 0.14	1.20 1.00	3.20	0.48	1.2
ring-H	9.22	0.18	0.75	9.32 9.37	0.10 0.05	0.71 0.36	9.22	0.23	0.58
α-meso	10.10	0.05	0.21	10.15	0.02	0.14	10.00	0.18	0.45
β/δ-meso	9.88	0.22	0.92	10.02	0.07 0.10	0.50 0.71	9.75	0.34	0.75
γ-meso	9.76	0.27	1.13	9.88	0.17	1.21	9.40	0.69	1.73
NH	-4.3	-	-	-4.06	-	-	-4.52	-	-

The most interesting fact about the dis-aggregation shifts (Δ, downfield, ppm) of **8e**, **9e** and **10e** is that $\Delta\gamma \gg \Delta\alpha$. This disparity is readily understood, in the case of **8e** in particular, if the ester group is directly involved in the aggregation mechanism: but this possibility has already been eliminated. Abraham *et al.*[28] have noted that the chemical shifts of porphin protons at low molarity differ significantly from the corresponding values in the Zn(II)bis-pyrrolidine adduct. In case this difference arises from a particular sensitivity of the chemical shifts of protons in unsubstituted regions of the porphin skeleton to co-ordination and/or chelation, and that we are witnessing a similar effect in the A, B rings of **8e**, **9e** and **10e**, we have studied the concentration dependence of the proton chemical shifts of the free base of **8e**.[31] These results (Table 2) show that, although the disparity is reduced, we still have the situation of $\Delta\gamma > \Delta\alpha$. This is opposite to the behaviour of, for example, **1e**[29] and is contrary to what one would expect from the following simple considerations.

Jansen and Katz have deduced[29] that rings A and B lie above rings C and D (i.e. a face-to-face but head-to-tail aggregation) in free-base **1e** and Abraham *et al.*[27] have made similar observations. In common with **1e**, **8e** has electronically dissimilar pyrrole rings (A, B compared to the slightly electron-rich C, D) and, in an aggregate, will experience a less sterically crowded environment than that found in naturally occurring porphyrins. Therefore, **8e** (and **9e** and **10e**) can be expected to form a face-to-face, head-to-tail dimer, as the first stage in the aggregation process, with some displacement from exact superposition to ease steric hindrance (Fig. 4). The expected direction of such a displacement would be to slide the CD substituents of one porphyrin away from the AB rings of the other porphyrin in the dimer. However, this would give rise to $\Delta\alpha > \Delta\gamma$.

It would appear therefore that, in **8e**, a displacement in a sense opposite to that just described is taking place (Fig. 4), which implies that electronic rather than steric effects are playing a dominant role in the aggregation process, in order to obtain $\Delta\gamma/\Delta\alpha$.

The fact that we cannot use the chemical shifts of the Zn(II)bis-pyrrolidine adducts as monomer shifts prevents us from making a detailed study of monomer-dimer equilibria or dimer geometry. However, using an equilibrium constant of $3.6\,dm^3\,mole^{-1}$, and taking the chemical shifts at 0.18 M (extrapolated) as initial values for the dimer chemical shifts, an iterative solution of Abraham's equations[2] gave consistent values for the dimer and monomer chemical shifts after five iterations: these values gave excellent agreement with our experimental results for **8e**. Most of the monomer shifts so calculated were *ca.* 0.15 ppm downfield from the values observed in the Zn(II)bis-pyrrolidine adducts in accord with other work.[28]

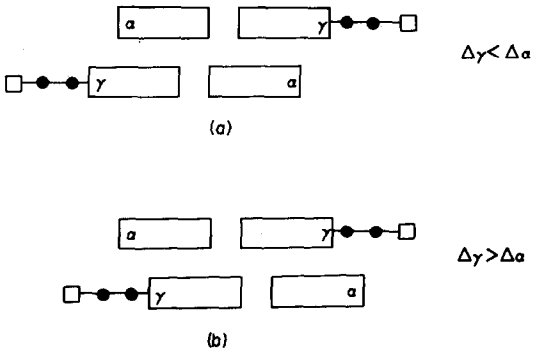


Fig. 4. Schematic representations of relative positions of porphyrin sub-units in dimeric aggregates of **8e**. (a) Steric effects more important than electronic effects: (b) Electronic effects more important than steric effects. The motif ⊸●⊸□ represents the side chains $(CH_2)_2COOCH_3$ which are present on both the C and D rings.

CONCLUSIONS

We have found that the artificial, new porphyrin, **8**, is the best substrate yet found for the enzyme Ferrochelatase. This confirms the importance of the MPPM substitution pattern on the CD rings, and shows that the substituents found on the AB rings of natural type IX porphyrins are not essential for interaction with the enzyme. The importance of the AB ring substituents is exemplified by our observation that the natural substrate, **1**, is not competitively inhibited in its interaction with Ferrochelatase by **8**. In the absence of AB ring substituents, the CD ring substitution patterns MPMP and PMMP yield new porphyrins (**9** and **10**) that act as a poor enzyme substrate, or a competitive inhibitor to **8**, respectively. These results have drawn attention to an error in earlier work[1] on natural type I porphyrins.

The new porphyrin diesters, **8e**, **9e** and **10e** aggregate in

Table 2. ^{1}H NMR chemical shifts at two disparate molarities (in $CDCl_3$) of the new porphyrin dimethyl diester, **8e**. The values were obtained by *interpolation* of the experimental curve

PROTON CHEMICAL SHIFTS/ppm			Downfield Dilution	RATIO
Proton Site	0.11M	0.02M	Shifts/ppm	
1'-CH_2	4.17	4.43	0.26	1.0
2'-CH_2	3.17	3.34	0.17	0.65
$COOCH_3$	3.64	3.68	0.04	0.15
ring-CH_3	3.42	3.64	0.22	0.85
ring-H	9.18	9.42	0.24	0.92
α-meso	9.96	10.22	0.26	1.0
β/δ-meso	9.79	10.14	0.36	1.38
γ-meso	9.65	10.07	0.42	1.62
NH	-4.42	-3.86	0.56	2.15

solution. In common with porphin, and to a lesser extent **3e**,[28] the Zn(II)bis-pyrrolidine adducts yield proton chemical shifts that are at variance with the free-base values, and therefore cannot be used as monomer shifts in the study of a possible monomer-dimer equilibrium. Concentration dependent NMR studies of the free-base of **8e** indicate that the aggregation mechanism is dominated by electronic rather than steric effects, due to the lack of substituents on the AB rings, giving rise to the unusual circumstance of a larger aggregation shift for the *γ-meso* proton than for the *α-meso* proton.

EXPERIMENTAL

Porphyrin dimethyl diesters. **1e** was prepared by the method of Grinstein[3] from human blood. **2e** and **3e** were obtained from a commercial source (Koch–Light Laboratories, Colnbrook, Bucks) whereas **4e** was a gift from Professor K. M. Smith. **5e** was synthesised in 63% yield from **4e** (14 mg) by scaling down the literature method for the conversion of **1e** to **2e**. The syntheses and characterisations of **8e**, **9e** and **10e** and their dipyrromethane precursors are described below: full details of the syntheses of the required pyrroles are given elsewhere.[24]

M.ps were measured on a Gallenkamp Hot Stage Microscopic Apparatus or an Electrothermal Capillary Melting Point Apparatus and are uncorrected. Column chromatography was performed using neutral alumina (Woelm, Brockmann Grade III), and tlc was carried out on Woelm pre-coated sheets of silica gel F(254/366). Electronic spectra ($CHCl_3$ + 0.5% trifluoroacetic acid) were taken on a Perkin Elmer 407 or a Pye Unicam SP1700 spectrophotometer: holmium oxide was used as calibrant. ^1H NMR spectra were recorded using Perkin Elmer R12 (60 MHz), Jeol (100 MHz) and Perkin–Elmer R34 (220 MHz) spectrometers. The chemical shifts in the concentration dependence studies were evaluated at 60 MHz using a frequency counter. Mass spectra (50μA and 70 eV) were determined with AEI MS12, AEI MS902 or Varian MATCH 7 instruments. IR spectra were measured on a Perkin–Elmer 257 grating spectrophotometer using polystyrene film as calibrant.

5,5′ - Di(ethoxycarbonyl) - 3,3′ - di(3″ - methoxycarbonylethyl) - 4,4′ - dimethyl - 2,2′ - dipyrromethane (**19**).[19] Compound **17** (1.6 g, 5 mmole), was refluxed with MeOH (25 cm^3) containing conc HCl (1.5 ml) for 4 hr. The soln was concentrated to about half the original volume and set aside to furnish colourless prisms (1.16 g, 95%), m.p. (MeOH) 134.5–136° (lit.[19] 131.5–132.5); $\bar{\nu}_{max}$ 1655, 1240, 1670, 1315, 3300, 1742, 1735 cm^{-1}; τ ($CDCl_3$) 0.90 br·(2H, s, NH), 5.63 (4H, q, J = 7.3 Hz, $C\underline{H}_2{\cdot}CH_3$), 6.01 (2H, s, py·$C\underline{H}_2$·py), 6.32 (6H, s, $CO_2{\cdot}C\underline{H}_3$), 7.1–7.6 (8H, m, $C\underline{H}_2{\cdot}C\underline{H}_2$), 7.71 (6H, s, py·$C\underline{H}_3$), 8.68 (6H, t, J = 7.3 Hz, $CH_2{\cdot}C\underline{H}_3$); *m/e* (rel. int.%) 490 (90), 462 (33), 445 (29), 444 (64), 417 (79), 403 (37), 371 (44), 357 (52), 252 (61), 210 (32), 166 (20), 146 (25), 46 (25), 45(100), 31 (49), 29(42), 27(99), 26(100).

5,5′ - Di(ethoxycarbonyl) - 4,4′ - di(2″ - methoxycarbonylethyl) - 3,3′ - dimethyl - 2,2′ - dipyrromethane (**20**). This compound was prepared as described for **19** from **18**, in 96% yield: m.p. (MeOH) 134.5–138°: $\bar{\nu}_{max}$ 1652, 1732, 1698, 1277, 3375 cm^{-1}: τ ($CDCl_3$) 0.95 br·(2H, s, N$\underline{H}$), 5.74 (4H, q, J = 7.2 Hz, $C\underline{H}_2{\cdot}CH_3$), 6.16 (2H, s, py·$C\underline{H}_2$·py), 6.33 (6H, s, $CO_2{\cdot}C\underline{H}_3$), 6.7–7.7 (8H, m, $C\underline{H}_2{\cdot}C\underline{H}_2$), 8.03 (6H, s, py·$C\underline{H}_3$), 8.70 (6H, t, J = 7.2 Hz, $CH_2{\cdot}C\underline{H}_3$): *m/e* (rel. int.%) 490(100), 459(20), 418(15), 417(21), 416(18), 401(19), 371(15), 343(19), 329(34), 252(30), 252(30), 251(71). (Found: C, 61.08; H, 7.10; N, 5.80. $C_{25}H_{34}N_2O_8$. Requires: C, 61.21; H, 6.99; N, 5.71%). Compound **20** (1.52 g) was also isolated during the conversion of **15** (5.06 g, 20 mmole) to **18** using lead tetraacetate, AcOH and Ac_2O).[24]

5,5′ - Di(ethoxycarbonyl) - 3,4′ - di(2″ - methoxycarbonylethyl) - 3′,4 - dimethyl - 2,2′ - dipyrromethane (**21**).[13] Compound **17** (311 mg, 1 mmole) and the 5-unsubstituted pyrrole, **16** (239 mg, 1 mmole) were heated, under N_2 at 40°, in MeOH (6 cm^3) containing toluene-*p*-sulphonic acid trihydrate (12 mg), monitoring by tlc (chloroform). After digestion of the starting materials (19 hr) sat $NaHCO_3$aq (1 ml) was added dropwise. No solid product was observed, and the mixture was diluted with water (2 ml) and extracted sequentially with CH_2Cl_2 (3 × 10 ml) and ether (3 × 10 ml). The combined extracts were dried and evaporated to produce the required dipyrromethane (350 mg, 71%), m.p. 96–98°; $\bar{\nu}_{max}$ 1650, 1697, 1740, 3350, 1270, 1290, 1320, 1180 cm^{-1}; τ ($CDCl_3$) 0.29 br· and 0.48 br· (2H, s, N$\underline{H}$), 5.75 (4H, q, J = 7.0 Hz, $C\underline{H}_2{\cdot}CH_3$), 6.07 (2H, s, py·$C\underline{H}_2$·py), 6.33 (6H, s, $CO_2{\cdot}C\underline{H}_3$), 6.8–7.9 (m) and 7.74 (s) (11H, $C\underline{H}_2{\cdot}C\underline{H}_2$ and py·$C\underline{H}_3$), 7.99 (3H, s, py·$C\underline{H}_3$), 8.71 (6H, t, J = 7.0 Hz, $CH_2{\cdot}C\underline{H}_3$). (Found: C, 61.24; H, 6.86; N, 5.68, $C_{25}H_{34}N_2O_8$; Requires: C, 61.21; H, 6.99; N, 5.71%).

5,5′ - Diformyl - 2,2′ - dipyrromethane (**22**). Although a literature method[21] was adopted, we experienced considerable difficulty with low yields, and with the instability of **22** (despite storage in the dark at −10°). Although the latter problem was not overcome, our overall yield was doubled by using the crude, green 2,2′-dipyrroketone intermediate instead of the purified compound. The final product was typically obtained in 11.6% yield from 2-formylpyrrole (m.p. 226–230°: lit. 229–231°[21]).

12,18 - Dimethyl - 13,17 - di(2′ - methoxycarbonylethyl)porphin (**8e**). Compound **19** (0.6 g, 1.22 mmole) was heated under reflux, under N_2, in a soln of 10% NaOHaq (7.5 ml) and EtOH (7.5 ml) for 5 hr. The alcohols were then removed under vacuum and the aqueous soln carefully acidified (pH 3) at 0° using SO_2 gas. The ppt was collected by filtration, washed with water, vacuum dried in the dark and then used immediately. To a soln of this crude material (300 mg, 0.74 mmole) and **22** (150 mg, 0.74 mmole) in glacial AcOH (300 cm^3) was added, in the dark, a soln of 56% HI (3 ml) in glacial AcOH (120 ml). The reaction was allowed to proceed, in the dark, for 5 hr with gentle stirring: a soln of anhyd NaOAc (8.4 g) in glacial AcOH (120 ml) was then added, and the mixture aerated in the dark overnight. After removal of the AcOH at the pump, the black residue was refluxed (2 hr) with 5% 9M H_2SO_4 in MeOH (300 ml), and then set aside overnight in the dark. Chloroform (300 ml) was added and the soln washed sequentially with 1M Na_2CO_3aq (3 × 100 ml) and water (2 × 50 ml), then dried and evaporated. This crude product was twice purified by column chromatography (alumina) using CH_2Cl_2 and collecting the red fractions. Purity was checked by tlc (silica gel) using $CHCl_3$, and **8e** was finally recrystallised from MeOH–$CHCl_3$ as purple, metallic crystals (45 mg, 12%), m.p. 214–215°: ^1H NMR ($CDCl_3$), τ (for 10 mg sample in 0.5392 g of solvent) 3.60, 3.42 (2 × $CO_2C\underline{H}_3$ and 12, 18-$C\underline{H}_3$); 9.76, 10.1 (5, 15-H); 9.88 (10, 20-H); 9.22 (pyrrole-H); 3.14, br·t·(2′-$C\underline{H}_2$); 4.20, br·t·(1′-$C\underline{H}_2$); −4.30, br·s·(NH): *m/e* (rel. int. %) 510(100), 498(6), 437(37): UV-vis, phyllotype ($CHCl_3$) λ_{max} nm (ϵ_M) 398 (1.68×10^5), 495 (1.40×10^4), 527 (0.51×10^4), 565 (0.54×10^4), 618 (0.22×10^4): UV-vis ($CHCl_3$ + 0.5% TFA) λ_{max} nm (ϵ_M) 405 (3.17×10^5), 549 (1.45×10^4), 590 (0.34×10^4). (Found: C, 70.68; H, 5.81; N, 11.04. $C_{30}H_{30}N_4O_4$; Requires: C, 70.57; H, 5.92; N, 10.97%).

12,17 - Dimethyl - 13,18 - di(2′ - methoxycarbonylethyl)porphin (**9e**). Compound **9e** was prepared from **22** (75 mg) and the hydrolysate of **21** (150 mg, 0.37 mmole) in a method analogous to **8e**, in 11% yield, as a purple 'metallic' solid. m.p. 167–169°: ^1H NMR ($CDCl_3$), τ (for 8.8 mg sample in 0.6058 g of solvent) 3.50, 3.53, 3.64, 3.70 (2 × CO_2CH_3 and 12,17-CH_3): 9.88, 10.02, 10.15, (5, 15-H, 10, 20-H); 9.32, 9.37 (pyrrole-H); 3.2, m·(2′-$C\underline{H}_2$); 4.3, m·(1′-$C\underline{H}_2$); −4.06, br·s·(NH). The following mass spectrum was recorded on a Varian MATCH 7 mass spectrometer using a direct insertion probe at *ca* 230°, 70 eV and 300μA. *m/e* (rel. int.%) 510 (100), 479 (8), 451 (15), 438 (23), 437 (66), 378 (10), 377 (13), 365 (10), 364 (28), 363 (27). UV-vis, phyllo-type ($CHCl_3$) λ_{max} nm (ϵ_M) 398 (1.83×10^5), 495 (1.47×10^4), 528 (0.54×10^4), 566 (0.58×10^4), 619 (0.22×10^4): UV-vis ($CHCl_3$ + 0.5% TFA) λ_{max} nm (ϵ_M) 404 (3.36×10^5), 549 (1.52×10^4), 590 (0.35×10^4). (Found: C, 70.51; H, 5.96; N, 10.88. $C_{30}H_{30}N_4O_4$; Requires: C, 70.57; H, 5.92; N, 10.97%).

12,18 - Di(2′-methoxycarbonylethyl) - 13,17 - dimethylporphin (**10e**). Compound **10e** was prepared as above from **22** and the hydrolysate of **19** in 10.2% yield in the form of a purple 'metallic' solid. m.p. 226–227° ^1H NMR ($CDCl_3$), τ (for 22.3 mg sample in 0.5582 g of solvent) 3.20, 3.64 (2 × $COOCH_3$ and 13, 17-CH_3); 9.40, 10.00 (5, 15-H); 9.75 (10, 20-H); 9.22 (pyrrole-H); 3.05, br·t·(2′-$C\underline{H}_2$); 4.05, br·t·(1′-$C\underline{H}_2$); −4.52, br·s·(NH). *m/e* (rel. int.%) 510 (100), 479 (6), 461 (6) 438 (22), 437 (63), 364 (16), 363

(14): UV-vis, phyllotype ($CHCl_3$) λ_{max} nm (ϵ_M) 398 (1.83×10^5), 495 (1.45×10^4), 527 (0.54×10^4), 565 (0.57×10^4), 618 (0.22×10^4). UV-vis ($CHCl_3$ + 0.5% TFA) λ_{max} nm (ϵ_M) 405 (3.37×10^5), 549 (1.51×10^4), 590 (0.36×10^4). (Found: C, 70.48; H, 5.88; N, 11.05. $C_{30}H_{30}N_4O_4$; Requires: C, 70.57; H, 5.92; N, 10.97%).

Preparation of mitochondria. Liver from freshly slaughtered sheep was weighed and homogenised in an MSE Atomix blender for 2 min at full speed in 2 volumes of buffer soln (containing 0.25M sucrose, 5 mM Tris-HCl and 1 mM ethyleneglycol-di(aminoethyl)tetraacetic acid adjusted to pH 7.4). The homogenate was centrifuged in an MSE 18 centrifuge at 2500G for 7 min and the supernatant collected and spun at 9000G for 10 min. The pellets from the supernatant were resuspended in fresh buffer soln, using a Potter–Elvehjem homogeniser, to give a medium with a protein concentration of 60–80 mg protein ml^{-1}.

Enzyme interactions. The full details of the method and of the construction of a purpose-built double-beam spectrophotometer are given elsewhere.[32] Porphyrin free acids were prepared[3] by overnight hydrolysis of the dimethyl diesters in 25% (w/v) HCl and the precipitation at their isoelectric points with KOH aq. Approximately 1 mM solns of the porphyrins were then prepared by dissolving them in a minimum of base and dilution with a soln of 2% Tween 80 in 40 mM Tris-HCl and the pH adjusted to 8.2 with conc HCl (**10** was insoluble in the buffer and was consequently dissolved in a minimum of base and diluted with 2% aqueous Tween 80). Accurate concentrations of porphyrins were then determinad by spectrophotometry.

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[31]We have studied **8e** in detail because it is much easier to synthesise than **9e** or **10e**: it is also the most interesting biochemically and has an unambiguous γ-*meso* proton assignment.
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