Synthetic and Biosynthetic Studies of Porphyrins. Part 11.[1] The Synthesis of *meso* Oxygenated Protoporphyrins[2]

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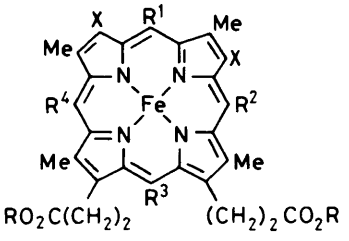
Treatment of protoporphyrin IX dimethyl ester (**12**) with benzoyl peroxide affords a mixture of the four isomeric *meso*-benzoyloxy derivatives (**13**). These have been separated by h.p.l.c. and distinguished from each other by hydrolysis to the corresponding oxophlorins (oxyporphyrins), insertion of iron to form the oxyhaems (**3**), and oxidation by molecular oxygen to the corresponding biliverdins (**7**).

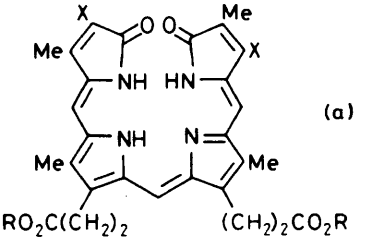
During the course of normal metabolism the red blood pigment haem (**1**) is oxidatively degraded to biliverdin-1Xα (**6a**), which is subsequently reduced to bilirubin (**10a**). The latter is then excreted as its glucuronide, or may be further reduced by intestinal micro-organisms to other bile pigments.[3,4] The oxidative degradation to biliverdin (**6a**) which also occurs in the formation of avian and plant bile pigments, can be divided into three main stages, *meso*-oxidation of the haem, oxidative ring-opening by molecular oxygen, and loss of the iron. The earliest (circumstantial) evidence that the primary intermediate in haem degradation is the α-*meso*-oxygenated haem (**4a**) came from Fischer and Lemberg's[5] chemical oxidations of porphyrin



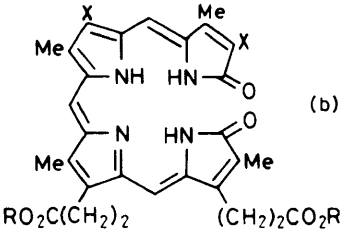
(**1**) R = R^1 = R^2 = R^3 = R^4 = H, X = CH=CH$_2$
(**2**) R = H, X = CH=CH$_2$
(**3**) R = Me, X = CH=CH$_2$
(**4**) R = H, X = Et
(**5**) R = Me, X = Et

a ; R^1 = OH, R^2 = R^3 = R^4 = H
b ; R^2 = OH, R^1 = R^3 = R^4 = H
c ; R^3 = OH, R^1 = R^2 = R^4 = H
d ; R^4 = OH, R^1 = R^2 = R^3 = H

iron complexes with oxygen or hydrogen peroxide in pyridine in presence of a reducing agent such as ascorbic acid, or hydrazine (the latter being necessary to ensure that the iron is in its divalent oxidation state). This work has since been substantiated by several other groups,[6] and has been extensively reviewed elsewhere.[3,4] These *meso*-oxyhaems can be converted into biliverdins by oxidation with molecular oxygen in pyridine, thus closely mimicking the natural process. More recently, we have synthesized α- and β- oxymesohaem esters (**5a**) and (**5b**) *via* the corresponding oxophlorins;[7] the corresponding acids (**4a**) and (**4b**) were converted into mesobiliverdins-IXα and IXβ (glaucobilins) (**9a**) and (**9b**) by oxygenation in pyridine. Tritium labelled α-oxymesohaem (**4a**) was also converted[8] *in vivo* in the rat into tritium labelled mesobilirubin-IXα (**11a**); incorporation of the β-isomer (**4b**) into mesobilirubin-IXβ (**11b**) was very much less efficient, probably because the biliverdin reductase is more specific for the α-isomer.[9] These experiments thus, so far, provide the best direct evidence for the involvement of α-oxyhaem (**4a**) in the primary stage of haem degradation, and ideally it would be very desirable to synthesize labelled α-oxyhaem for direct metabolic experiments. Our earliest attempts to carry out the ring synthesis of α-oxyhaem (**4a**) failed owing to problems encountered in generation of the vinyl groups in the terminal stages of the synthesis, in which the two carbon side-chain cyclised onto the *meso*-oxygen substituent.[10] Shortly afterward, Clezy[11] reported a synthesis of α-oxyhaem (**4a**) from an oxophlorin prepared by condensation of an α-formylpyrroketone and a pyrromethane. However, the product was only obtained in very low yield, and was characterised by conversion into biliverdin-IXα dimethyl ester (**7a**). Thus, there still remains a need for an efficient synthesis of α-oxyhaem (**4a**)



(a)



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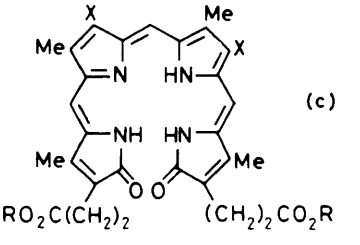

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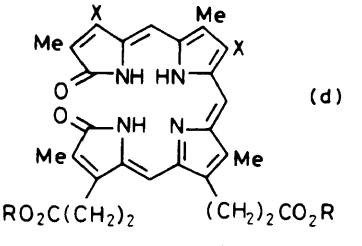


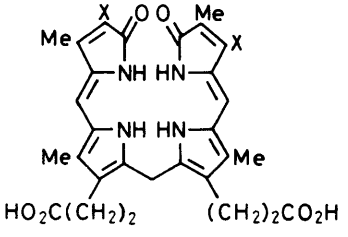
(d)

(**6**) X = CH=CH$_2$, R = H
(**7**) X = CH=CH$_2$, R = Me
(**8**) X = Et, R = H
(**9**) X = Et, R = Me

which will provide sufficient material for metabolic studies in order to provide substantive, rather than circumstantial, evidence for its involvement in haem metabolism. It will also enable further studies to be carried out on the mechanism of the oxidative ring opening process, a topic of increasing interest at the present time.[12–14]

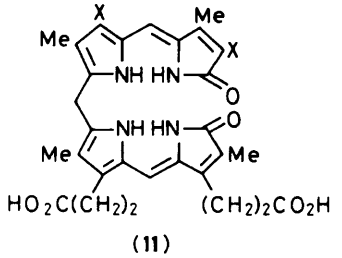
Several years ago, we completed a synthesis of γ-oxyhaem (**3c**)[15] *via* ring synthesis of the corresponding oxophlorin, and originally we had intended to adapt the method used in that work for the ring synthesis of α-oxyhaem (**3a**). However, in the event the very rapid improvements which have occurred in h.p.l.c. during the last few years made it feasible to consider the more direct approach of oxidising haem itself (or a closely related analogue) and separating the isomeric products obtained. A number of methods have been devised for *meso*-oxidation of porphyrins and their metal complexes, *e.g.* hydrogen peroxide oxidation of haem in pyridine solution,[3–6] benzoyl peroxide oxidation[16] and the use of thallium(III) trifluoroacetate.[17] The latter reagent gives excellent yields with many porphyrins and has been utilised for the preparation of *meso*-oxyhaems from several porphrins including deuteroporphyrin-IX and mesoporphyrin IX.[18] However, it also reacts with vinyl groups.[19] We, therefore, selected the benzoyl peroxide oxidation method as this led directly to the *meso*-benzoyloxyporphyrins; the hydrogen peroxide–pyridine method could also have been utilised but the *meso*-hydroxy groups would then have had to be protected and the iron removed in order to facilitate chromatographic separations.

In Bonnett's original studies[16] of the *meso*-benzoyloxylation of octaethylporphyrin, he found that the use of 1 mol equiv. of benzoyl peroxide in 1,2,4-trichlorobenzene at reflux for 1 h gave a 30% yield of the mono *meso*-benzoyloxy octaethylporphyrin and very little if any side-chain benzoyloxylation occurred if the reaction was carried out in presence of air or oxygen. In the present instance, we carried out the reaction of protoporphyrin-IX dimethyl ester (**12**) with benzoyl peroxide under a range of conditions and found (by monitoring of visible spectra and t.l.c.) that the most useful method was to carry out the reaction in chlorobenzene for 10 min at 95 °C with 2.5 mol equiv. of benzoyl peroxide; a mixture of the four isomeric *meso*-benzoyloxyprotoporphyrin dimethyl esters (**13**) was obtained in 20% yield. If longer reaction times were used, the reaction mixture became dark, the yield of the mono-substituted product diminished, and other (poly-substituted?) products were formed. As in Bonnett's earlier studies[16] the presence of oxygen inhibited side-chain attack, and column chromatography readily enabled the mixture of *meso*-monobenzoyloxyprotoporphyrins (**13**) to be separated from unchanged starting material (75%) and a small amount of a *meso*-dibenzoyloxyprotoporphyrin.

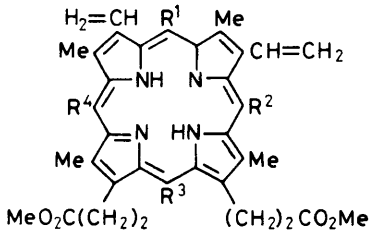


(**10**) (a) X = CH = CH_2

(b) X = Et



(**11**)



(**12**) $R^1 = R^2 = R^3 = R^4 = H$

(**13**) a ; R^1 = OBz, $R^2 = R^3 = R^4 = H$

b ; R^2 = OBz, $R^1 = R^3 = R^4 = H$

c ; R^3 = OBz, $R^1 = R^2 = R^4 = H$

d ; R^4 = OBz, $R^1 = R^2 = R^3 = H$

The separation of the four isomeric benzoyloxy derivatives (**13**) was then investigated by h.p.l.c. using a wide range of adsorption columns and solvents. Partial separations were achieved in many cases but the best conditions found were the use of a mixture of acetone–dichloromethane (1:99, v/v) as eluant, and Hypersil-5 as adsorbent. This enabled all four isomers and a residual trace of protoporphyrin-IX dimethyl ester to be resolved on analytical and semi-preparative h.p.l.c. columns; an illustration of the type of separation achieved is given in our preliminary communication.[2] Subsequently, it was found that more careful open-column chromatography of the original mixture on alumina enabled separation into two fractions each containing a pair of isomers. Each pair of isomers could then be separated by preparative h.p.l.c. and using this method somewhat larger quantities of material could be injected onto the h.p.l.c. column without losing resolution, thus substantially reducing the number of repeat injections required. In this way, 5—10 mg samples of each of the four isomeric *meso*-benzoyloxyprotoporphyrin esters (**13**) were obtained; their purity was shown by h.p.l.c. to be greater than 99%.

The identity of each of the four isomers was originally established by detailed analysis of their n.m.r. spectra taking into account the effects of the *meso*-substituent; this caused a slight shift to highfield of the resonances of the neighbouring peripheral substituents by comparisons with the resonances of unperturbed peripheral substituents. The assignments made in this manner were unequivocally confirmed by conversion of each of the benzoyloxyprotoporphyrins into the corresponding *meso*-oxyhaems by hydrolysis on a basic alumina column followed by insertion of iron using acidified iron(II) sulphate; each of the *meso*-oxyhaems was then oxidised in pyridine solution by molecular oxygen. The resulting verdohaemochromes were then converted with methanolic acid into the corresponding biliverdins each of which was identified individually by their m.p.'s, spectral characteristics and h.p.l.c. As in Clezy's and our own earlier work[11,15] we also found that the intermediate oxophlorins (oxyprotoporphyrins) were very unstable and underwent rapid degradation in solution, especially if exposed to light and air, the colour changing from blue to green and finally to brown.

A mixture of the four *meso*-benzoyloxyprotoporphyrin esters (**13**) was also converted in the same manner into a mixture of the four biliverdin-IX ester isomers (**7**). This mixture was used to evaluate the methods for separating the four isomers by h.p.l.c. One of the original t.l.c. methods for separating them was developed by Bonnett[20] and utilised silica gel with 3% acetone in chloroform as eluant; the retention times decreased in the order $\beta < \alpha < \gamma < \delta$ and the same order of elution was observed in the more recent h.p.l.c. separation described by Rasmussen *et al.*[21] using a Zorbax Sil-850 column. In our own work, we utilised a Hypersil-5 column using acetone–methanol–1,2-dichloromethane (1.2:1.2:97.6, v/v) as eluant and a typical

separation has been shown in our preliminary communication.[2] The order of elution of the biliverdin isomers was the same in this system as in the separations of Bonnett[17] and Rasmussen *et al.*,[18] and was confirmed by using naturally derived biliverdin-IXα and synthetic biliverdin-IXγ[15] dimethyl esters (**7a**) and (**7c**). Finally, the identities of the four individual biliverdin-IX dimethyl esters (**7a**—**d**) obtained from the four isomeric benzoyloxyprotoporphyrin-IX dimethyl esters (**13a**–**d**) were confirmed by comparisons of retention times on h.p.l.c. and by 'spiking' the mixture of biliverdins with each separated isomer in turn. Thus the order of elution of the *meso*-benzoyloxyprotoporphyrin esters in acetone–1,2-dichloroethane (1:99, v/v) on Hypersil-5 was shown to be γ, δ, β, α, the latter having the longest retention time. Work is now in progress on the preparation of tritium labelled *meso*-oxyhaems for related metabolic studies in a variety of *in vivo* like systems.

Experimental

M.p.s were determined on a microscope hot-stage apparatus and are uncorrected. N.m.r. spectra were obtained using Varian T60 (60 MHz), Perkin-Elmer R32 (90 MHz) and Bruker WM 360 (360 MHz) spectrometers. Mass spectra were determined on a Varian CH5D instrument with a Varian 620i computer data system, and also on a Finnigan 4000 instrument. T.l.c. plates were coated with Silica gel G and columns were of Merck alumina, neutral, Brockmann Grade III unless otherwise stated. Reactions were monitored, whenever possible, by t.l.c. on silica gel, and by u.v.-visible spectroscopy. H.p.l.c. separations were carried out on appropriate columns (packed in these laboratories) using a Waters 6000 pump and a Cecil 200 u.v. detector (usually set *ca.* 400 nm for porphyrins, *i.e.* on the Soret band).

Protoporphyrin-IX Dimethyl Ester (**12**)—.(*a*) *From haematoporphyrin*. A solution of haematoporphyrin (3.0 g, 5 mmol) in chlorobenzene (1500 ml) was treated with toluene-*p*-sulphonic acid (7.5 g, 45 mmol) and heated in N_2 at 140 °C for 3 h. The cooled solution was shaken with aqueous ammonium hydroxide (2%; 750 ml), glacial acetic acid (300 ml) was added, and the organic layer separated. The aqueous phase was extracted with ethyl acetate (2 × 250 ml) and the combined organic extracts dried (Na_2SO_4) and evaporated to dryness. The dark red residue was set aside in the dark overnight in 5% (v/v) sulphuric acid in methanol (1000 ml). The solution was then poured into ice–cold water (1200 ml), neutralised with aqueous ammonium hydroxide, and extracted with chloroform (3 × 200 ml). The organic extracts were washed with water, dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was chromatographed on grade IV alumina, eluting with chloroform. The porphyrinic eluates were evaporated to dryness, and the residue crystallised from chloroform–methanol to give protoporphyrin-IX as purple plates (1.83 g, 3.1 mmol, 62%), m.p. 221—222 °C (lit.,[22] m.p. 222—223 °C).

(*b*) *From haemin*. Haemin (2.5 g, 4 mmol) was dissolved in pyridine (10 ml), the solution made up to 150 ml with methanol and ferrous sulphate (2.0 g, 10 mmol) added. Dry hydrogen chloride gas was passed rapidly through the solution until the visible absorption bands due to haemin (501 and 625 nm) were replaced by the two bands of the protoporphyrin dication (556 and 598 nm). The mixture was cooled to 20 °C, water (50 ml) was added, and the reaction mixture extracted with chloroform (3 × 60 ml). The organic phase was washed with water (2 × 100 ml), dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was chromatographed, and the porphyrin obtained after evaporation of the appropriate eluates recrystallised to give protoporphyrin-IX dimethyl ester as purple plates (1.75 g, 2.96 mm, 74%), m.p. 222 °C, $\lambda_{max.}$($CHCl_3$) 405, 505, 540, 579, and 632 nm; δ (360 MHz; $CDCl_3$) 10.02, 10.02, 9.90, and 9.89 (4 H, 4 s, 4 × =CH), 8.18 (2 H, m, 2 × C*H*=CH_2), 6.32 (2 H, qd, 2 × CH=C*H*$_2$), 6.15 (2 H, dq, CH=C*H*$_2$), 4.34 (4 H, t, 2 × C*H*$_2$$CH_2$), 3.65 (6 H, s, 2 × OCH_3), 3.61, 3.60, 3.55, and 3.55 (12 H, 4 s, 4 × ring CH_3), 3.24 (4 H, t, 2 × CH_2C*H*$_2$) and −4.01 (2 H, br s, 2 × NH).

α-*Benzoyloxyoctaethylporphyrin*.—Benzoyl peroxide (100 mg, 0.4 mmol) in chlorobenzene (5 ml) was added in portions over a period of 5 min to a solution of octaethylporphyrin (100 mg, 0.18 mmol) in the same solvent (30 ml) at 95 °C. After 1 h the solvent was removed under reduced pressure and the residue chromatographed on alumina (Grade III) to give octaethylporphyrin (12.5 mg) (eluted with benzene–light petroleum, 1:1) and *meso*-benzoyloxyoctaethylporphyrin (eluted with benzene). The latter crystallised from chloroform–methanol as purple prisms (23 mg, 0.03 mmol, 19%), m.p. 282 °C (lit.,[16] m.p. 280—283 °C); δ (360 MHz; $CDCl_3$) 10.09 (2 H, s, 10- and 20-=CH), 9.94 (1 H, s, 15-=CH), 8.75 (2 H, d, *o*-ArH), 7.85 (1 H, t, *p*-ArH), 7.76 (2 H, t, *m*-ArH), 4.06 (12 H, m, 6 × C*H*$_2$$CH_3$), 3.83 and 3.67 (4 H, 2 m, 2 × C*H*$_2$$CH_3$), 1.90 (12 H, t, 4 × CH_2C*H*$_3$), 1.84 (6 H, t, 2 × CH_2C*H*$_3$), 1.73 (6 H, t, 2 × CH_2C*H*$_3$), and −3.44 (2 H, br s, 2 × NH).

Octaethyloxophlorin.—(*a*) 2M-Aqueous sodium hydroxide (3 ml) was added to a solution of 5-benzoyloxyoctaethylporphyrin (20 mg, 0.03 mmol) in pyridine (50 ml) and the solution was heated under reflux under nitrogen for 1 h. The pyridine was removed under reduced pressure and the dark blue-green residue was washed with water, dried, dissolved in benzene, and chromatographed without delay on alumina (Grade V, alkaline) in the dark. A deep blue fraction was eluted with benzene, this was concentrated and the oxophlorin crystallised as dark blue prisms (6.6 mg, 0.012 mmol, 42%), m.p. 250 °C [lit.,[16] 255 °C (decomp.)]; $\lambda_{max.}$ ($CHCl_3$) 404, 547, 587, and 632 nm.

(*b*) 5-Benzoyloxyoctaethylporphyrin (10 mg, 0.015 mmol) in chloroform (5 ml) was applied to a basic alumina column (Laporte type UGI, Grade I) and slowly eluted with benzene. The column and collecting flasks were protected from the light with aluminium foil. After slow elution for 2 h unhydrolysed starting material (3.2 mg, 0.0006 mmol, 40%) was collected, leaving a blue oxyporphyrin on the column. Elution with chloroform gave a dark blue solution which was concentrated and afforded the oxophlorin as dark blue prisms (4.4 mg, 0.008 mmol 53%), m.p. 253 °C identical with the preceding sample.

Preparation of a Mixture of the Four meso-*Benzoyloxyprotoporphyrin Dimethyl Esters, IXα, IXβ, IXγ, and IXδ* (**13a**—**d**).—Benzoyl peroxide (100 mg, 0.4 mmol) in chlorobenzene (5 ml) was added in portions over 5 min to a solution of protoporphyrin-IX dimethyl ester (**12**) (100 mg, 0.16 mmol) in chlorobenzene (30 ml) at 95 °C in the dark. The reaction was monitored by u.v. and after 10 min the reaction mixture was poured into chloroform (50 ml). The solvents were removed under reduced pressure and the residue was chromatographed on alumina (Grade III) eluting with dichloromethane–benzene (1:3, v/v) to give initially the unchanged protoporphyrin-IX (71 mg, 0.12 mmol, 73%) followed by the *meso*-benzoyloxyprotoporphyrin-IX dimethyl esters (23 mg, 0.032 mmol, 20%); $\lambda_{max.}$ (in $CHCl_3$) 407 (5.2), 505 (4.3), 536 (3.72), 578 (3.70), and 629 nm (3.3); δ (360 MHz; $CDCl_3$) 10.09—9.6 (4 H, m, 4 × =CH), 8.6 (2 H, m, *o*-ArH), 8.0 (1 H, m, C*H*=CH_2), 7.8—7.6 (4 H, m, 1 × C*H*=CH_2, *m*- and *p*-ArH), *ca.* 6.15 (2 H, m, CH=C*H*$_2$), *ca.* 5.4 (2 H, m, CH=C*H*$_2$), 4.4—3.6 (4 H, m, C*H*$_2$$CH_2$), 3.5—3.0 (18 H, m, ring CH_3's and OCH_3's), 3.3—3.0 (4 H, m, 2 × CH_2C*H*$_2$), −3.4 to −3.7 (2 H, m, 2 × NH).

H.p.l.c. (t_R) on Hypersil-5μ column (4.5 × 250 mm) using acetone–1,2-dichloroethane (1:99 by volume as eluant), isomer I (5.5 min), isomer II (6.15 min), isomer III (6.55 min), and isomer IV (6.85 min).

Separation of the Four meso-*Benzoyloxyprotoporphyrin Dimethyl Esters, IXα, IXβ, IXγ, and IXδ* (**13a**—**d**).—A solution of the four *meso*-benzoyloxyprotoporphyrin-IX dimethyl esters (30 mg) in chloroform was chromatographed on alumina (150 g, Grade III) and slowly eluted with benzene–dichloromethane (2:1). The column and collecting flasks were protected from the light with aluminium foil. As the porphyrin was eluted from the column, it was collected in 50 ml fractions, each fraction being examined by h.p.l.c. (250 × 4 mm Hypersil 5 column), acetone–1,2-dichloroethane (1:99) solvent, 1 ml/min flow rate). The fractions containing the two least-polar isomers (isomer I and isomer II) were combined and the solvent removed to give fraction A (11.2 mg). The fractions containing the two more polar isomers (isomer III isomer IV) were combined and the solvent removed to give fraction B (10.3 mg). Any fractions that contained more than two isomers or were a mixture of isomer II and isomer III (as shown by h.p.l.c.) were set aside for re-chromatography.

Fraction A and fraction B were each then separated by h.p.l.c. to give the four individual *meso*-benzoyloxyprotoporphyrin-IX dimethyl esters in isomerically pure form. The conditions for both separations were identical: a 250 × 4 mm Hypersil 5 column, acetone–1,2-dichloroethane (1:99) solvent, 1 ml/min flow rate and the u.v. detector set at 400 nm. Fraction A (11.2 mg) was taken up in chloroform (2 ml) and applied onto the column as 50 μl injections. Each *meso*-benzoyloxyprotoporphyrin was collected individually in a flask protected from the light. Removal of the solvent gave isomer I (5.1 mg) and isomer II (4.3 mg). Both were crystallised from chloroform–light petroleum. Fraction B was dissolved in chloroform (2 ml) and separated in a similar manner, 20 μl injections being applied to the h.p.l.c. column and each *meso*-benzoyloxyprotoporphyrin being collected individually as above. Removal of the solvent gave isomer III (4.1 mg) and isomer IV (3.9 mg). Both were recrystallised from chloroform–light petroleum. The separated *meso*-benzoyloxyprotoporphyrin isomers were individually re-examined by h.p.l.c. using analytical conditions to confirm their isomeric purity, and then characterised.

Isomer I, γ-Benzoyloxyprotoporphyrin-IX Dimethyl Ester (**13c**), m.p. 243—246 °C, $\lambda_{max.}$($CHCl_3$) 407 (log ε 5.20), 505 (4.12), 537 (3.75), 576 (3.71), and 630 nm(3.32); *m/z*(f.d) 710 (M^+, 100%), and 711 ($M^+ + 1$, 66%) (Found: M^+, 710.3111. $C_{43}H_{42}N_4O_6$ requires *M*, 710.3094); δ (360 MHz; $CDCl_3$) 10.17, 10.07, 10.01 (3 H, 3 s, 3 × CH), 8.70 (2 H, d, *o*-ArH), 8.19 (2 H, m, 2 × C*H*=CH_2), 7.88 (1 H, t, *p*-ArH), 7.75 (2 H, t, *m*-ArH), 6.34 (2 H, qd, CH=CH_2), 6.17 (2 H, qd, CH=CH_2), 4.26 and 3.91 (4 H, 2 m, 2 × C$H_2$$CH_2$–), 3.65, 3.61, 3.57, and 3.56 (12 H, 4 s, 4 × ring CH_3), 3.51 (6 H, s, 2 × OCH_3), 3.29 and 2.99 (4 H, 2 m, 2 × CH_2CH_2), and −3.41 (2 H, br s, 2 × NH).

Isomer II, δ-Benzoyloxyprotoporphyrin-IX Dimethyl Ester (**13d**), m.p. 203—205 °C, $\lambda_{max.}$($CHCl_3$) 406 (log ε 5.23), 504 (4.10), 5.36 (3.62), 575 (3.72), and 631 nm(3.20); *m/z*(f.d.) 710 (M^+, 100%) and 711 ($M^+ + 1$, 82%) (Found: M^+, 710.3103. $C_{43}H_{42}N_4O_6$ requires M^+, 710.3094); δ (360 MHz; $CDCl_3$) 10.11, 9.97, and 9.94 (3 H, 3 s, 3 × =CH), 8.67 (2 H, d, *o*-ArH), 8.18 and 8.03 (2 H, q, 2 × C*H*=CH_2), 7.84 (1 H, t, *p*-ArH), 7.74 (2 H, t, *m*-ArH), 6.34 and 6.17 (4 H, md, 2 × CH=CH_2), 4.32 (4 H, m, 2 × C$H_2$$CH_2$), 3.65 and 3.64 (6 H, 2 s, 2 × OCH_3), 3.61, 3.53, 3.31, and 3.25 (12 H, 4 s, 4 × ring CH_3), 3.24 (2 H, m, CH_2CH_2), 3.16 (2 H, t, CH_2CH_2), and −3.60 (2 H, br s, 2 × NH).

Isomer III, β-Benzoyloxyprotoporphyrin-IX Dimethyl Ester (**13b**), m.p. 220—223 °C, $\lambda_{max.}$($CHCl_3$) 407 (log ε 5.15), 502 (4.09), 538 (3.67), 575 (3.70), all 630 nm(3.33); *m/z*(f.d.) 710 (M^+, 100%) and 711 ($M^+ + 1$, 55%) (Found: M^+, 710.3111. $C_{43}H_{42}N_4O_6$ requires M^+, 710.3094); δ (360 MHz; $CDCl_3$) 10.11, 9.96, and 9.84 (3 H, 3 s, 3 × =CH), 8.62 (2 H, d, *o*-ArH), 8.19 and 7.80 (2 H, 2 m, 2 × C*H*=CH_2), 7.8 (3 H, m, *m*- and *p*-ArH), 6.25 and 5.57 (4 H, 2 dq, 2 × CH=CH_2), 4.32 (4 H, m, 2 × C$H_2$$CH_2$), 3.65 and 3.64 (6 H, 2 s, 2 × OCH_3), 3.61, 3.53, 3.27, and 3.53 (12 H, 4 s, 4 × ring CH_3), 3.23 and 3.16 (4 H, 2 t, 2 × CH_2CH_2), and −3.72 (2 H, br s, 2 × NH).

Isomer IV, α-Benzoyloxyprotoporphyrin-IX Dimethyl Ester (**13a**), m.p. 262—264 °C (lit.,[11] m.p. 262—265 °C), $\lambda_{max.}$($CHCl_3$) 408 (log ε 5.17), 505 (4.10), 539 (3.78), 576 (3.75), and 630 nm(3.19) [Found: *m/z*(f.d.) M^+, 710.3097. $C_{43}H_{42}N_4O_6$ requires *M*, 710.3094]; δ (360 MHz; $CDCl_3$) 10.07, 9.97, and 9.82 (3 H, 3 s, 3 × =CH), 8.59 (2 H, d, *o*-ArH), 8.04 and 7.78 (2 H, 2 m, 2 × C*H*=CH_2), 7.78 (3 H, m, *m*- and *p*-ArH), 6.18 (2 H, dd), 5.69 (1 H, d), and 5.43 (1 H, d) (2 × CH=CH_2), 4.31 (4 H, m, 2 × C$H_2$$CH_2$), 3.66 (6 H, s, 2 × OCH_3), 3.55, 3.54, 3.32, and 3.24 (12 H, 4 s, 4 × ring CH_3), 3.24 (4 H, t, 2 × CH_2CH_2), and −3.65 (2 H, br s, 2 × NH).

Biliverdin-IXα Dimethyl Ester (**7a**) *from the Dehydrogenation of Natural Bilirubin-IXα* (**10a**).—Commercial bilirubin was purified by recrystallisation from chloroform. The bilirubin (15 mg, 0.0025 mol) in methanol (20 ml) was heated under reflux with ferric chloride (20% solution, in 10M hydrochloric acid) (1 ml) on a water-bath for 30 min. The solution was cooled, filtered free from residual bilirubin, and neutralised with aqueous sodium acetate. The mixture was extracted with ether (3 × 10 ml) and the combined extracts were washed with 2M-sodium acetate and water and extracted with 1M-hydrochloric acid (3 × 10 ml). The pigment present in the acid was extracted into chloroform (3 × 10 ml) which was then washed with water (5 × 10 ml) to liberate the free pigment. The chloroform solution was then filtered and evaporated to dryness under reduced pressure, and the residual biliverdin taken up in boron trifluoride–methanol (14%; 25 ml). The solution was heated under reflux for 15 min under nitrogen and then kept overnight before being poured into water (100 ml). The pigment was extracted with chloroform (2 × 50 ml) and the combined extracts were washed with water (2 × 50 ml), dried (Na_2SO_4), and evaporated. The residue was chromatographed on a short column of alumina (grade V) eluting with chloroform–benzene (1:9). The blue eluate was evaporated to give biliverdin-IXα dimethyl ester (**7a**) (8 mg, 0.0013 mmol, 52%), m.p. 200 °C (lit.,[23] 205—207 °C).

When the biliverdin-IXα dimethyl ester sample was examined using h.p.l.c. a small additional peak was noted which was eluted slightly after the main peak. This indicates the probable presence of traces of biliverdin-IIIα or XIIIα dimethyl ester which has been shown to arise from a reversible acid-catalysed cleavage and subsequent recombination under these reaction conditions.[23] H.p.l.c. (t_R) on Hypersil-5 (4.5 × 250 mm), in acetone (1.2%), methanol (1.2%), and 1,2-dichloroethane (97.6%) biliverdin-IXα dimethyl ester (6.85 min); isomeric biliverdin dimethyl ester (6.95 min).

Preparation of a Mixture of the Four Biliverdin Dimethyl Esters IXα, IXβ, IXγ, and IXδ (**7a**—**d**) (*from Haemin*).—Oxygen was bubbled briskly through a solution of haemin (25 mg, 0.04 mmol) in aqueous pyridine (3:1, 400 ml) at 37 °C for 20 min. The oxygen inlet tube was then withdrawn to just above the surface of the solution, rapid stirring was started, and a solution of ascorbic acid (300 mg) in water (5 ml) was added. Three min (stopclock) after completion of the addition the solution was

poured into ice-cold chloroform (100 ml) under nitrogen. The mixture was shaken and the layers separated without delay. The aqueous layer was washed with the chloroform (2 × 30 ml), and the extracts were washed at once with water (100 ml, N_2, flush), and dried briefly (Na_2SO_4) before evaporation under reduced pressure (N_2). The green residue was dissolved in chloroform (5 ml) and the solution diluted with light petroleum (10 ml). The precipitate was collected by centrifugation, washed twice with light petroleum, and dried *in vacuo* to give crude amorphous verdohaemochrome ($\lambda_{max.}$ 1% pyridine in $CHCl_3$ 396, 502, 535, and 663 nm).

The crude verdohaemochrome in methanol (10 ml, N_2 flush) was treated with methanolic potassium hydroxide (2M; 2 ml) in the dark. Boron trifluoride in methanol (14%, 25 ml) was added after 1 min, and the solution was heated under reflux in nitrogen for 15 min and then kept at 20 °C overnight in the dark. The solution was diluted with water (100 ml) and extracted with chloroform (3 × 25 ml) and this extract was then washed with water (2 × 50 ml). The resulting chloroform solution was filtered and evaporated under reduced pressure to give the crude esters. This material was chromatographed on a short column of alumina (Grade V made up in benzene). The material was applied in chloroform–benzene (1:9) and the blue-green band was eluted without delay to give the purified mixture of biliverdin-IX dimethyl ester isomers (**7a—d**) (9.1 mg, 0.015 mmol, 37%). H.p.l.c. (t_R) on Hypersil-5 (4.5 × 250 mm) in acetone (1.2%), methanol (1.2%), and 1,2-dichloroethane (97.6%) gave biliverdin-IXβ dimethyl ester (5.6 min), biliverdin-IXα dimethyl ester (6.0 min), biliverdin-IXγ dimethyl ester (8.6 min), and biliverdin-IXδ dimethyl ester (10.4 min).

Conversion of the Mixture of the Four meso-*Benzoyloxyprotoporphyrin-IX Dimethyl Esters* (**13a—d**) *into a Mixture of the Four Biliverdin-IX Dimethyl Esters.*—A solution of the *meso*-benzoyloxyprotoporphyrin-IX dimethyl esters (5 mg, 0.007 mmol) in chloroform (5 ml) was applied to a basic alumina column (Laporte type UGI; Grade I). The column and collecting flasks were protected from the light with aluminium foil. Slow elution (2—3 h) with benzene–dichloromethane (1:1) removed unhydrolysed benzoate (1.2 mg, 0.0017 mmol) as a red band leaving the blue-green oxyporphyrin distributed over the column. Elution with chloroform gave a mixture of the oxyporphyrins [$\lambda_{max.}$ ($CHCl_3$) 412, 585, and 635—642 nm]. The solution was evaporated to dryness in the dark at 40 °C and the blue-green residue was quickly taken up in acetic acid (10 ml). Potassium carbonate (0.5 g) was then added and the solution was treated with saturated aqueous iron(II) sulphate and sodium chloride (1 ml). The mixture was heated under nitrogen (on a water-bath at 90–100 °C) for 30 min. The cooled solution of the oxyhaemins was poured into ether (50 ml) and the acetic acid was washed out with water (6 × 50 ml). The ether solution was dried (Na_2SO_4) and evaporated under reduced pressure.

The oxyhaems [$\lambda_{max.}$(ether) 410, 470, and 560 nm] were taken up in pyridine (10 ml) giving a green solution ($\lambda_{max.}$ 440 and 660br nm). The pyridine solution was stirred in a darkened flask which was flushed with oxygen for 2 h. After this time the visible spectrum showed no more formation of the verdohaemochromes [$\lambda_{max.}$ 400, 495, 530, 605, 655 nm]. The pyridine was removed under reduced pressure and the residual green gum taken up in nitrogen-flushed methanol (20 ml) before a solution of potassium hydroxide in methanol (2M; 2 ml) was added under nitrogen. After 1 min boron trifluoride in methanol (14%; 25 ml) was added to the solution which turned blue-green. The solution was heated under reflux for 15 min under nitrogen and then kept at 20 °C overnight before being poured into water (100 ml). The pigments were extracted with chloroform (2 × 50 ml) and the combined extracts washed with water (2 × 50 ml), dried (Na_2SO_4) and evaporated. The residue was chromatographed on a short column of alumina (Grade V) eluting with chloroform–benzene (1:9). The blue eluates were evaporated to give a mixture of the four biliverdin dimethyl esters (**7a—d**) (0.95 mg, 29%). H.p.l.c. (t_R) gave biliverdin-IXβ dimethyl ester (5.9 min.), biliverdin-IXα dimethyl ester (7.0 min), biliverdin-IXγ dimethyl ester (8.4 min), and biliverdin-IXδ dimethyl ester (10.4 min).

Conversion of the Chromatographically separated meso-*Benzoyloxyprotoporphyrin-IX Dimethyl Esters into the corresponding Biliverdin Dimethyl Esters.*—Each *meso*-benzoyloxyprotoporphyrin-IX dimethyl ester (1 mg) was converted into the corresponding biliverdin dimethyl ester following the same procedures outlined above for the mixed isomers. The products (*ca.* 20—25% yield) were crystallised from chloroform–methanol.

The benzoyloxyprotoporphyrin dimethyl esters of shortest retention time ('Isomer I') gave the deep blue biliverdin-IXγ dimethyl ester (**7c**), m.p. 204 °C (lit.,[20] m.p. 205—207 °C) $\lambda_{max.}$($CHCl_3$) 375 and 639nm. Isomer II gave biliverdin-IXδ dimethyl ester (**7b**), m.p. 170 °C (lit.,[20] 172—174 °C), $\lambda_{max.}$($CHCl_3$) 380 and 650—657 nm. Isomer III gave biliverdin-IXβ dimethyl ester (**7b**), m.p. 212 °C (lit.,[20] m.p. 212—214 °C) $\lambda_{max.}$($CHCl_3$) 380 and 647—654 nm. Isomer IV gave biliverdin-IXα dimethyl ester (**7a**), m.p. 207 °C (lit.,[20] 206—208 °C), $\lambda_{max.}$($CHCl_3$) 379 and 658nm.

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