On the Nature of ' Haematoporphyrin Derivative '

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The components of haematoporphyrin derivative (a preparation used as a photosensitiser in clinical applications, and made by treating haematoporphyrin with sulphuric acid–acetic acid) have been separated by preparative h.p.l.c. and identified by comparison with authentic porphyrindicarboxylic acids. The composition of the mixture is somewhat variable but the main components are *O,O'*-diacetylhaematoporphyrin (6) and *O*-acetylhaematoporphyrin (2)/(3) with smaller amounts of the 8(3)-(1-acetoxyethyl)-3(8)-vinyldeuteroporphyrin isomers (7) and (8) and the corresponding alcohols (4) and (5).

CERTAIN porphyrins are known to have a photodynamic effect in mammals, that is, they cause a sensitivity to light which can result in considerable damage to exposed tissue.[1] The reaction requires oxygen, and there is evidence for the view that singlet oxygen is involved, at least in certain circumstances.[2] The phenomenon was originally observed by Hausmann (1911) [3] in mice and by Meyer-Betz (1913) in Man (himself).[4] In biological studies the solubilisation of the porphyrin in aqueous media prior to injection often presents a problem: one way in which this has been overcome has involved the use of ' haematoporphyrin derivative ', a porphyrin mixture formed by treating haematoporphyrin dihydrochloride [1; as $(HCl)_2$] with 5% sulphuric acid in acetic acid.[5]

There is now a considerable body of evidence which supports the conclusion that some porphyrins, including haematoporphyrin derivative, are, with respect to surrounding skin and muscle, to some degree preferentially taken up by tumour tissue (high accumulations also appear to occur in liver, spleen and kidney [6]). Since the porphyrins are brilliantly fluorescent in u.v. light (365 nm) this offers a way of detecting tumours.[7,8] It was not until 1972 that the two ideas (localisation and photodynamic effect) came together and the possibility that a porphyrin could be used to photosensitise the preferential degradation of tumour tissue was demonstrated.[9] Subsequently haematoporphyrin derivative has been shown to be effective in causing photo-degradation of tumour tissue both in experimental animals and in Man.[10]

Although haematoporphyrin derivative has been referred to as ' recrystallised ',[8] it is prepared by precipitation, and proves to be a complex mixture of porphyrins.[11] The mixture is somewhat variable in composition: this arises not only because the components are sensitive (hydrolysis, elimination, see below) but also because haematoporphyrin dihydrochloride, as commercially supplied, is itself usually not a single substance. It was clearly important to establish the structures of the components of haematoporphyrin derivative, and to determine their individual biological activities. Because of the latter aim we have worked with the porphyrindicarboxylic acids throughout. Although these substances are more difficult to manipulate than are the corresponding dimethyl esters,[12] they have the distinct advantage that they can be used directly in biological assays.[13] An independent approach to this problem using the dimethyl esters has been described by Clezy and his colleagues.[12]

Mixtures of porphyrin free carboxylic acids may conveniently be analysed by high pressure liquid chromatography employing a reverse-phase system.[11] With haematoporphyrin derivative several components were detected, and the major ones were recognised as the *O*-acetyl and *O,O'*-diacetyl derivatives of haematoporphyrin.[11] Figure 1 shows a typical analytical separation.

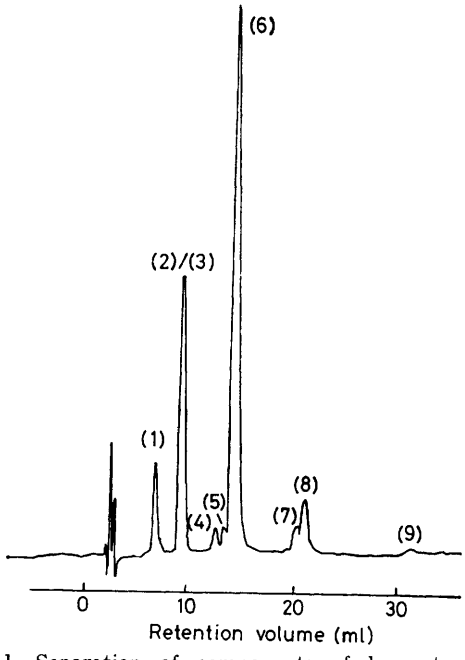


FIGURE 1 Separation of components of haematoporphyrin derivative by analytical h.p.l.c. using the DuPont Zorbax™ ODS column eluted with aqueous tetrahydrofuran–acetate buffer. The identified components are designated with the numerals used in Table 1. The two sharp peaks at *ca.* 2 ml are artefacts arising from the injection system

This approach has now been developed into a preparative method.

Haematoporphyrin derivative, prepared from a batch

of haematoporphyrin dihydrochloride of superior quality (>95%), was fractionated on a column of reverse-phase silica gel (Merck LiChroprep RP-18, 25—40 μm) using aqueous methanol containing 4% acetic acid as the elutriant. Appropriate fractions were combined and the amorphous porphyrin free acids were isolated without delay. Conditions for the separation and isolation were such that solvolytic or addition reactions involving methanol, which might be expected to occur at the reactive side-chains at C-3 and C-8, were minimised and usually could not be detected. Thus, after 5 h in the elutriant solution (70% aqueous methanol + 4% HOAc) at room temperature neither haematoporphyrin nor *O,O'*-diacetylhaematoporphyrin was appreciably affected: it was estimated that <2% of the latter compound had reacted. However, when haematoporphyrin derivative was kept in the elutriant for 7 days, new components (methoxy-derivatives) appeared.

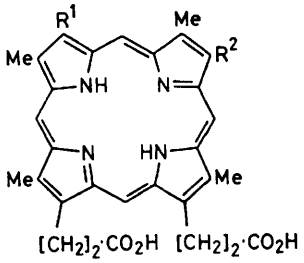
The components isolated, and the structures assigned to them, are shown in Table 1 in the order of decreasing

TABLE 1

Preparative separation of the components of haematoporphyrin derivative (294 mg)

Component	Weight isolated (mg)
Haematoporphyrin (1)	12
O-Acetylhaematoporphyrin isomers (2) and (3)	42
8(3)-(1-Hydroxyethyl)-3(8)-vinyldeuteroporphyrin isomers (4) and (5)	13
O,O'-Diacetylhaematoporphyrin (6)	66
8(3)-(1-Acetoxyethyl)-3(8)-vinyldeuteroporphyrin isomers (7) and (8)	20

polarity, *i.e.*, the order in which they were eluted from the column. The minor components eluting ahead of haematoporphyrin were very polar, and under our procedures were not obtained free from sodium chloride. This polar fraction (see Experimental section, Table 3) is



(1) $R^1 = R^2 = CH(OH)Me$
(2) $R^1 = CH(OH)Me$; $R^2 = CH(OAc)Me$
(3) $R^1 = CH(OAc)Me$; $R^2 = CH(OH)Me$
(4) $R^1 = CH{=}CH_2$; $R^2 = CH(OH)Me$
(5) $R^1 = CH(OH)Me$; $R^2 = CH{=}CH_2$
(6) $R^1 = R^2 = CH(OAc)Me$
(7) $R^1 = CH{=}CH_2$; $R^2 = CH(OAc)Me$
(8) $R^1 = CH(OAc)Me$; $R^2 = CH{=}CH_2$
(9) $R^1 = R^2 = CH{=}CH_2$
(10) $R^1 = R^2 = CO[CH_2]_2{\cdot}CO_2H$

viewed with some interest, since it might contain porphyrins with alkyl hydrogen sulphate side-chains. Such substances would be expected to show modification in parameters (solubility, partition coefficient, detergency) of importance for biological transport and localisation.

The major products were those of acetylation of the 1-hydroxyethyl side-chains at C-3 and C-8, the diacetate (6) being the main product. Elimination was a minor pathway, and, indeed, only a trace of protoporphyrin (9) was detected (Figure 1). On an earlier occasion [11] a larger proportion of protoporphyrin was found: this illustrates the variability that may be encountered in the composition of haematoporphyrin derivative. The individual isomers of *O*-acetylhaematoporphyrin (2) and (3) were not separated by h.p.l.c. under either analytical or preparative conditions. However, the other two pairs of positional isomers (4), (5) and (7), (8) while not resolved in the preparative run, were clearly separated on the analytical column. The individual isomeric structures of (4), (5), (7), and (8) were assigned by reference to the authentic dimethyl esters of porphyrins (4) and (5) which have been prepared by rational synthesis,[14] and which were kindly furnished by Professor P. S. Clezy. The recovery of material from the preparative separation was *ca.* 70%. This includes a number of minor components (see Experimental section, Table 3), but does not include material strongly bound to the top of the column. These components have not been identified, but are available for testing.

The compounds in Table 1 were identified by comparison [analytical h.p.l.c., n.m.r. in $(CD_3)_2SO$] with authentic samples of the dicarboxylic acids which were specially prepared for this work. Because of solubility problems, and the ease with which they pick up metal ions (forming salts and co-ordination compounds), the porphyrin-dicarboxylic acids are generally avoided, the diesters being preferred. We have developed procedures for obtaining these free acids in an amorphous but essentially pure condition (purity being judged by h.p.l.c. and elemental analysis) which allows the major components of haematoporphyrin derivative to be made more conveniently.

Thus, the mono-hydroxyethyl mono-vinyl isomers (4) and (5) were prepared by partial dehydration of haematoporphyrin (1) in *N,N*-dimethylformamide (65 °C, 1 h) followed by separation by preparative h.p.l.c. Acetylation of the isomers (4) and (5) thus obtained gave the individual mono-acetoxyethyl mono-vinyl compounds (7) and (8). The mixed isomers of the *O*-acetylhaematoporphyrins (2) and (3) were not separated in this work, and were prepared by partial acetylation (HOAc–HCl) of haematoporphyrin, followed again by preparative h.p.l.c. The *O,O'*-diacetyl derivative (6) was prepared by acetylation of haematoporphyrin: in an analogous reaction haematoporphyrin was converted into the *O,O'*-disuccinoyl derivative (10) by reaction with succinic anhydride in pyridine.

Clearly haematoporphyrin derivative is a complex mixture, and one that may be anticipated to give irreproducible results in clinical trials [10,15] unless special precautions are taken. Two further observations emphasise this conclusion. Firstly, the progress of the

reaction of haematoporphyrin with HOAc–H_2SO_4 has been followed (Figure 2) and shows the build up of the monoacetate followed by that of the diacetate, as would be expected. Clearly the composition of the mixture will vary with time: reaction times of 15 min,[5] 1 h,[6] and overnight [15] have been described for the preparation of haematoporphyrin derivative.

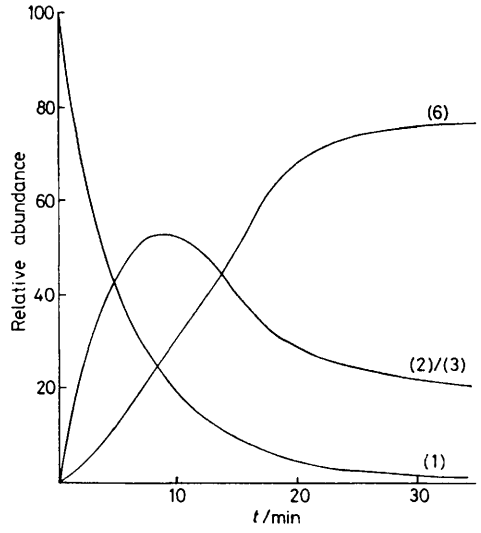


FIGURE 2 Course of the formation of the main products (2)/(3) and (6) when haematoporphyrin dihydrochloride is treated with 5% H_2SO_4–HOAc at *ca.* 20 °C

Secondly, haematoporphyrin derivative appears usually to be dissolved for injection with the aid of base. Often this treatment is for an unrecorded duration. Treatment with 0.1N-NaOH for 1 h (which has been used to prepare haematoporphyrin derivative for injection) [6,10,15] causes elimination and hydrolysis of the reactive (pseudobenzylic) acetoxy-groups. According to h.p.l.c. analysis the product which emerges from the column consists principally of haematoporphyrin, the mixed mono-hydroxyethyl mono-vinyl isomers (4) and (5) and protoporphyrin. Again, however, some porphyrinic material is retained rather strongly on the column.

The component fractions of haematoporphyrin derivative and its hydrolysis products are being subjected to an *in vivo* assay for tumour photonecrosis, the results of which study will be reported elsewhere.

EXPERIMENTAL

General.—Analytical h.p.l.c.: Waters M6000A pump with a UK6 injector, and a Cecil CE212 variable wavelength detector set at 400 nm. Samples were dissolved in the elutriant being used or in tetrahydrofuran–water, the injection volume usually being in the range 1—20 μl. A reverse-phase column (Waters μ-Bondapak C_{18}, i.d. 3.9 mm × 300 mm, or DuPont Zorbax ™ ODS, i.d. 4.6 mm × 250 mm) was used with a solvent flow of *ca.* 1 ml min^{-1}. Product ratios were estimated from peak areas, corrected for ε_{400} where known.

Preparative h.p.l.c.: Jobin-Yvon Chromatospac 10, with a column (i.d. 40 mm) of Merck LiChroprep RP-18 reverse-phase silica gel (25—40 μm, 200 g). The column was packed in methanol at 10 atm, and was conditioned by passing through the column 1.5—2 l of the elutriant to be used. The mixture to be chromatographed was dissolved in *ca.* 3 ml of elutriant (containing a little dimethyl sulphoxide if needed) and was injected on to the top of the column. Elution was carried out with flow rates in the range 15—38 ml min^{-1}. Fractions were collected, analysed by analytical h.p.l.c. (above, direct injection of *ca.* 50 μl) and then combined appropriately.

Elutriant solutions were prepared as follows. Acetate buffer: aqueous sodium acetate (0.2 M; 25 ml) was mixed with aqueous acetic acid (0.2 M; 975 ml). Solvent A: methanol : water = 17 : 3 (v/v), 0.001 M in $[Bu_4N][H_2PO_4]$.[11] Solvent D: tetrahydrofuran : water = 1 : 1 (v/v) + 10% acetate buffer.

Esters were prepared on a microscale by dissolving the porphyrin acid in moist tetrahydrofuran (2—3 ml) and adding an excess of diazomethane in ether at 0 °C. After 10 min the solvent was removed in a stream of nitrogen, and the residue was dried *in vacuo*.

Electronic spectra were measured on a Perkin Elmer 552 spectrophotometer. The sample was dissolved in dimethyl sulphoxide (0.5 ml) before being diluted to 250 ml with methanol. N.m.r. spectra were recorded in $[^2H_6]$dimethyl sulphoxide using a Bruker WP80 instrument. Acetic acid refers to glacial acetic acid unless otherwise stated.

Haematoporphyrin.—Analytical h.p.l.c. of a commercial sample of haematoporphyrin dihydrochloride eluted from the Zorbax column with solvent D showed that haematoporphyrin was the main component (variable but *ca.* 90%) but that several minor impurities were present. These gradually increased when the sample was stored.

Haematoporphyrin Derivative.[5]—Haematoporphyrin dihydrochloride (345 mg, 95% pure by h.p.l.c.) was stirred rapidly with 5% (v/v) sulphuric acid in acetic acid (15 ml) in subdued light for 15 min at room temperature (*ca.* 20 °C). The liquid was gradually added to 3% aqueous sodium acetate (285 ml). The precipitate was collected and washed with water at the centrifuge and dried (P_2O_5, reduced pressure) to give ' haematoporphyrin derivative ' (HpD) as a dark purple solid (293 mg). The composition of this was determined by analytical h.p.l.c. (Figure 1, Table 2), the

TABLE 2

Composition of a typical sample of haematoporphyrin derivative. (Zorbax column with solvent D)

Component	Retention volume (ml)	Compound	Relative abundance (%)
(1)	7.0	Haematoporphyrin	5.1
(2)/(3)	9.3	*O*-Acetylhaematoporphyrin isomers	22.4
(4)	13.0	8-(1-Hydroxyethyl-3-vinyldeuteroporphyrin	1.4
(5)	13.5	3-(1-Hydroxyethyl)-8-vinyldeuteroporphyrin	1.8
(6)	14.5	*O*,*O*′-Diacetyl-haematoporphyrin	60.3
(7)	20.0	8-(1-Acetoxyethyl)-3-vinyldeuteroporphyrin	2.7
(8)	21.0	3-(1-Acetoxyethyl)-8-vinyldeuteroporphyrin	4.8
(9)	31.5	Protoporphyrin	1.4

identifications being made by h.p.l.c. comparison with the authentic materials prepared as indicated in the sequel.

Course of HpD Formation.—Haematoporphyrin dihydrochloride (20 mg) was dissolved in 5% H_2SO_4 in glacial acetic

acid (1 ml) at room temperature under subdued light with stirring. Aliquots (0.1 ml) were removed at intervals and poured into 3% aqueous sodium acetate, the porphyrin being isolated at the centrifuge as before. The porphyrin product was analysed by h.p.l.c. (μ-Bondapak C_{18} eluted with solvent A). The variation in the amount of the three major components (1), (2)/(3), and (6) is shown in Figure 2.

Hydrolysis of Haematoporphyrin Derivative.—Haematoporphyrin derivative (10 mg) was stirred in 0.1 M-NaOH (0.5 ml) for 1 h at room temperature. The solution was brought to pH 4 by dropwise addition of hydrochloric acid, and shaken with tetrahydrofuran (20 ml) and saturated sodium chloride (20 ml). Ethyl acetate (30 ml) was added, and the mixture was shaken. The organic layer was washed with water, separated, and taken to dryness. Analytical h.p.l.c. (Zorbax column, solvent D) showed that only trace amounts of the mono- and di-acetoxy-derivatives remained, and that the relative amounts of the major components which emerged from the column were as follows: haematoporphyrin, 45%; 8-(1-hydroxyethyl)-3-vinyldeuteroporphyrin, 16%; its isomer, 30%; and protoporphyrin 5%.

Fractionation of Haematoporphyrin Derivative by Preparative High Pressure Liquid Chromatography.—Haematoporphyrin derivative (294 mg) was applied in Me_2SO (0.2 ml) + elutriant (below, 2.8 ml) to the preparative column, which had been conditioned with methanol–water = 7 : 3 + 4% acetic acid. The composition of the elutriant was changed by increasing the methanol content in a stepwise manner. Fractions of *ca.* 300 ml were collected (F1-F37) and after analytical h.p.l.c. appropriate fractions were combined and worked up. All procedures were carried out without delay: where it was necessary to keep fractions overnight they were stored at −2 °C. Acetate fractions were not stored, but were worked up immediately.

The work-up procedure was as follows. Saturated sodium hydrogencarbonate (5 ml per 100 ml) was added and the bulk of the methanol was removed under reduced pressure. The residue was shaken with ethyl acetate (300 ml for 1 l of eluate) and saturated sodium chloride was added with shaking until the porphyrin was transferred to the organic layer. The aqueous layer was extracted with a second portion of ethyl acetate, and the extracts were combined and washed with water several times. The organic layer was separated, taken to dryness under reduced pressure, and dried *in vacuo*. Because of their solubility in water the minor polar porphyrin components in fractions 1, 2, 3, 9, and 10 were washed in ethyl acetate solution with saturated sodium chloride rather than with water. Inevitably these fractions became contaminated with sodium chloride.

The results are presented in Table 3.

Preparation of Authentic Porphyrindicarboxylic Acids, 8-(1-Hydroxyethyl)-3-vinyldeuteroporphyrin and 3-(1-Hydroxyethyl)-8-vinyldeuteroporphyrin.—Haematoporphyrin dihydrochloride (0.5 g) was kept in nitrogen-flushed *N,N*-dimethylformamide (200 ml) at 65 °C for 1 h in subdued light and then poured into saturated sodium chloride (600 ml). The solution was extracted with dichloromethane (3 × 200 ml): the combined organic extracts were washed in turn with water (2 × 150 ml), with ice-cold aqueous acetic acid (20%, 4 × 100 ml), and again with water (4 × 100 ml). The dichloromethane layer was taken to dryness under reduced pressure, and the residue was chromatographed in the preparative procedure, being eluted with methanol–ethanol–water (20 : 45 : 35) + 10% acetate buffer for 7.25 l and then with methanol–ethanol–water (20 : 46 : 35) + 10% acetate buffer. The eluate was examined by analytical h.p.l.c. and the appropriate fractions (isomer 1, 5050—7250 ml; isomer 2, 7920—9240 ml) were combined, and the porphyrin recovered as follows. The eluate (1 l) was diluted with ethyl acetate (300 ml) and solid sodium chloride was added, and the mixture was shaken until saturated. Addition of saturated sodium chloride gave two layers, which were separated, the aqueous phase being re-extracted with ethyl acetate (200 ml). The combined ethyl acetate extracts were washed with water (6 × 200 ml), and then taken to dryness and dried *in vacuo*.

TABLE 3

Preparative separation of the components of haematoporphyrin derivative

Fractions	% MeOH in elutriant [a]	Component present [b]	Wt. isolated (mg)
1—3, 9, 10	70	Minor polar components	23
4—8	70	Haematoporphyrin [c]	12 [d]
11—13	72	*O*-Acetylhaematoporphyrin isomers [c]	42
14—16	74	Mixture of flanking components	18
17	74	3(8)-(1-Hydroxyethyl)-8(3)-vinyldeuteroporphyrin isomers	13
18, 19	75		
20—22	75	*O,O'*-Diacetyl-haematoporphyrin [c]	66
23—25	77		
26—29	78	Minor components	17
30, 31	85		
32, 33	90		
34	90	3(8)-(1-Acetoxyethyl)-8(3)-vinyldeuteroporphyrin isomers [c]	4
35	90	3(8)-(1-Acetoxyethyl)-8(3)-vinyldeuteroporphyrin isomers [c] + minor components	16
36, 37	100		

[a] Solvent system: *x* MeOH, (100 − *x*) H_2O; +4% HOAc. [b] Identified by comparison (h.p.l.c.) with authentic compounds. [c] Identification confirmed by n.m.r. spectroscopic comparison with authentic compounds. [d] Estimated spectroscopically.

Isomer 1, 8-(1-*hydroxyethyl*)-3-*vinyldeuteroporphyrin*, was obtained as an amorphous dark red solid (54 mg, 12%-(Found: C, 70.55; H, 6.4; N, 9.45; O, 13.35%. $C_{34}H_{36}N_4O_5$ requires C, 70.3; H, 6.25; N, 9.65; O, 13.8%), $\lambda_{max.}$ MeOH–Me_2SO (ε) 398 (110 000), 500 (8 300), 535 (6 200), 570 (4 100), and 622 nm (1 900); ν(Nujol) 1 694 cm^{-1}; δ[$(CD_3)_2SO$] 12.15 (bs, 2 × CO_2H); 10.72, 10.18 (2 H, and 10.15 (all s, 4 *meso*-H); 8.39 (q, J_{AX} 11 Hz, J_{BX} 17 Hz, C-3^{1}H); 6.50 (m, partly obscured, C*H*OHMe); 6.34 (q, J_{AB} 2 Hz, J_{BX} 17 Hz, *trans*-C-3^{2}H); 6.11 (q, J_{AB} 2 Hz, J_{AX} 11 Hz, *cis*-C-3^{2}H); 4.28 (m, 2 × Ar$CH_2CH_2CO_2H$); 3.70, 3.67, 3.58, and 3.56 (all s, 4 × Ar*Me*); 3.15 (bt, 2 × $ArCH_2CH_2CO_2H$); 2.13 (d, *J* 6.5 Hz, $CHOHCH_3$); and −3.95 (sb, 2 × NH).

Isomer 2, 3-(1-*hydroxyethyl*)-8-*vinyldeuteroporphyrin*, was obtained as an amorphous dark brownish-red solid (57 mg, 13%), $\lambda_{max.}$ MeOH–$(CH_3)_2SO$ (ε) 398 (118 000), 500 (9 300), 535 (6 400), 570 (4 300), and 623 nm (2 200); ν(Nujol) 1 695 cm^{-1}; δ$(CD_3)_2SO$] 12.21 (bs); 10.62, 10.24, 10.21, and 10.18 (all s); 8.39 (q); 6.50 (m, partly obscured); 6.36 (q, J_{AB} 2 Hz, J_{BX} 18 Hz); 6.12 (q, J_{AB} 2 Hz, J_{AX} 11 Hz); 4.30 (m); 3.69, 3.67, 3.62, and 3.56 (all s); 3.16 (bt); 2.12 (d, *J* 6.5 Hz) and −3.94 (bs).

Comparison by h.p.l.c. (Zorbax column, system D) showed that these two compounds were isomerically pure and confirmed that they corresponded to two minor components of

haematoporphyrin derivative (Table 2, components 4 and 5). Methylation of each isomer, followed by t.l.c. comparison (Merck Kieselgel 60, $CHCl_3$–EtOAc = 19 : 1) with authentic ester samples,[14] established the structural assignments.

8-(1-*Acetoxyethyl*)-3-*vinyldeuteroporphyrin and* 3-(1-*Acetoxyethyl*)-8-*vinyldeuteroporphyrin*.—The title compounds were prepared from the individual corresponding alcohols by acetylation with pyridine–acetic anhydride using the method described below for the diacetate.

8-(1-Acetoxyethyl)-3-vinyldeuteroporphyrin was obtained as an amorphous dark red solid in 85% yield (Found: C, 69.55; H, 6.3; N, 9.0%. $C_{36}H_{38}N_4O_6$ requires C, 69.45; H, 6.15; N, 9.0%), $\lambda_{max.}$ MeOH–Me_2SO (ε) 399 (104 000), 502 (7 800), 536 (6 200), 572 (4 500), and 624 nm (1 800); ν(Nujol) 1 731 and 1 697 cm^{-1}; δ[$(CD_3)_2SO$] 10.43, 10.15, and 10.11 (2 H) (all s, 4 *meso*-H); 8.40 (q, J_{AX} 11 Hz, J_{BX} 18 Hz, 3^1-H); 7.50 (m, C*H*(OAc)); 6.38 (q, J_{AB} 2 Hz, J_{BX} 18 Hz, *trans*-C-3^2H); 6.16 (q, J_{AB} 2 Hz, J_{AX} 11 Hz, *cis*-C-3^2H); 4.26 (m, 2 × ArC$H_2$$CH_2CO_2H$) 3.72 (6 H), 3.56, and 3.52 (all s, Ar*Me*); 3.14 (m, 2 × $CH_2CH_2CO_2H$); *ca.* 2.29 [d, obscured, CH(OAc)CH_3]; 2.27 (s, CH_3CO_2); and −3.80 (s, 2 × NH).

3-(1-Acetoxyethyl)-8-vinyldeuteroporphyrin was obtained as an amorphous red solid (88% yield); $\lambda_{max.}$ MeOH–Me_2SO (ε) 396.5 (112 000), 501.5 (7 800), 535 (5 600), 572 (3 200), and 625.5 nm (1 600); ν(Nujol) 1 734 and 1 706 cm^{-1}; δ([$(CD_3)_2SO$] 12.22 (bs); 10.39, 10.27, 10.22, and 10.15 (all s); 8.42 (q); 7.47 (m); 6.39 (q, J_{AB} 2 Hz, J_{BX} 18 Hz); 6.17 (q, J_{AB} 2 Hz, J_{AX} 11 Hz); 4.31 (m); 3.72, 3.66, 3.63, and 3.58 (all s); 3.18 (m); 2.30 (d, obscured); 2.26 (s); and −3.98 (s).

Analytical h.p.l.c. (Zorbax column, system D) showed that these two components were indistinguishable from two minor components in haematoporphyrin derivative [Table 2, components (7) and (8)].

O-*Acetylhaematoporphyrin* (*Mixed Isomers*).—To a mixture of glacial acetic acid (600 ml) and concentrated hydrochloric acid (12 ml) purged with nitrogen at room temperature was added haematoporphyrin dihydrochloride (300 mg). Purging was continued for 5 min and then the vessel was sealed and stirred for 3 h. The solution was poured into water (900 ml) and extracted with dichloromethane (4 × 100 ml); these extracts were rejected. To the aqueous layer was added saturated sodium chloride (1 800 ml), and the solution was again extracted with dichloromethane (6 × 100 ml). The dichloromethane extract was washed with water (3 × 200 ml) after which the aqueous extract was washed with ethyl acetate (400 ml). The ethyl acetate layer was then washed with water (4 × 200 ml). The dichloromethane and ethyl acetate extracts were taken to dryness, combined, and submitted to preparative h.p.l.c., the eluant was methanol–water (3 : 1) containing 4% acetic acid and then (at 1 650 ml) methanol–water (79 : 21) containing 4% acetic acid. The required fractions (from 1 650 ml→2 420 ml) were combined. Saturated sodium hydrogencarbonate was added to give a pH of 4—5, and most of the methanol was removed under reduced pressure. The residue (*ca.* 0.5 l) was treated with ethyl acetate (200 ml) and saturated sodium chloride was added until two layers formed. The aqueous layer was extracted with ethyl acetate (200 ml), and the combined organic extracts were washed with water (5 × 200 ml). The ethyl acetate was removed under reduced pressure, and the product was dried *in vacuo* to give O-*acetylhaematoporphyrin* as an amorphous purple-red solid (90.8 mg, 31%, purity by h.p.l.c. 98%) (Found: C, 67.45; H, 6.3; N, 8.6; O, 17.65%. $C_{36}H_{40}N_4O_7$ requires C, 67.5; H, 6.3; N, 8.75; O, 17.5%), $\lambda_{max.}$ MeOH (ε) 395 (176 000); 496 (13 300); 529 (8 100), 566 (6 000), and 618 nm (3 600). ν(Nujol) 1 735 and 1 710 cm^{-1}; δ[$(CD_3)_2SO$] 12.2 (bs, 2 × CO_2H); 10.69, 10.65, 10.41, 10.39, 10.24, 10.22, 10.19 (all s, *meso*-H); 7.42 [bq, *J* 7 Hz, C*H*(OAc)]; 6.51 (m, C*H*(OH)]; 6.14 (bs OH?); 4.32 (bm, 2 × $CH_2CH_2CO_2H$); 3.71, 3.69, 3.65, 3.63, 3.59, and 3.58 (all s, Ar*Me*); 3.21 (m, 2 × $CH_2CH_2CO_2H$); 2.26 (s, CH_3CO_2); 2.26 [d, obscured, CH(OAc)CH_3]; 2.14 [d, *J* 7 Hz, −CH(OH)CH_3]; and −3.97 (s, 2 × NH).

The isomeric mixture of *O*-acetyl derivatives was indistinguishable from component (2)/(3) (Table 2) of haematoporphyrin derivative on h.p.l.c. (μ-Bondapak C_{18}, eluted with MeOH–EtOH–H_2O = 2 : 2 : 1 + 10% acetate buffer).

O,O′-*Diacetylhaematoporphyrin*.—(*a*) A mixture of glacial acetic acid (20 ml), acetic anhydride (4 ml), and concentrated hydrochloric acid (0.8 ml) was kept for 1 h at room temperature. This acetylating mixture was cooled in ice (but not allowed to solidify) and powdered haematoporphyrin dihydrochloride (110 mg) was added. The cooled mixture was stirred under nitrogen until all the porphyrin had dissolved (*ca.* 1 h). Dichloromethane (3 ml, to prevent partial crystallisation) was added, and the solution was kept at 4 °C for 72 h. The solvent was removed under reduced pressure at or below room temperature. The residue was dissolved in dichloromethane (100 ml) and was washed without delay with the following sequence of ice-cold solvents: water (100 ml), aqueous acetic acid (20%, 100 ml, to remove unchanged haematoporphyrin), and water (3 × 100 ml). The organic layer was filtered and at once evaporated to dryness under reduced pressure to give *O,O′*-diacetylhaematoporphyrin as an amorphous purple solid (98 mg, 87%) (Found: C, 65.45; H, 6.0; N, 8.25; O, 20.0%. $C_{38}H_{42}N_4O_8 \cdot H_2O$ requires C, 65.15; H, 6.35; N, 8.0; O, 20.55%), $\lambda_{max.}$ MeOH (ε) 396 (188 000), 498 (14 100), 531 (8 600), 567 (5 700), and 618 nm (4 300); ν(Nujol) 1 730 and 1 705 cm^{-1}; δ[$(CD_3)_2SO$] 12.00 (bs, 2 × CO_2H); 10.49, 10.42, 10.21, and 10.13 (all s, 4 × *meso*-H); 7.54 (m, 2 × C*H*OAc); 4.42, 4.38 (overlapping t, 2 × $CH_2CH_2CO_2H$); 3.81, 3.78, 3.70, and 3.65 (all s, 4 × Ar*Me*); 3.25 (m, 2 × $CH_2CH_2CO_2H$); 2.34 [two d, overlapping, CH(OAc)*Me*]; 2.31 (two s, overlapping 2 × CH_3CO_2); and −3.86 (s, 2 × NH). (*b*) Haematoporphyrin dihydrochloride (65 mg) in pyridine–acetic anhydride (9 : 1, 3 ml) was kept under nitrogen for 4 h. The mixture was frozen in an acetone–CO_2 bath, and treated with glacial acetic acid (4 ml). The solid was allowed to warm up in an ice-bath while being constantly agitated with a spatula. Ice-cold water (30 ml) was added in portions with continued stirring, and the precipitated porphyrin was collected, and washed twice with ice-cold water, at the centrifuge. The product was dried (P_2O_5, reduced pressure) to give the diacetylhaematoporphyrin as an amorphous purple solid (61 mg, 86%; purity 94% by h.p.l.c. analysis).

Analytical h.p.l.c. (μ-Bondapak C_{18} column, solvent A) showed that the *O,O′*-diacetylhaematoporphyrin was indistinguishable from component (6) of haematoporphyrin derivative (Table 2).

A small sample of each preparation was esterified with diazomethane. The comparison (Merck Kieselgel 60; $CHCl_3$: EtOAc = 9 : 1) showed the esters to be the same, and indistinguishable from an authentic sample of *O,O′*-diacetylhaematoporphyrin dimethyl ester prepared by acetylation of haematoporphyrin dimethyl ester.

O,O′-*Disuccinoylhaematoporphyrin*.—Succinic anhydride (1 g) and haematoporphyrin dihydrochloride (200 mg) were dissolved in nitrogen-purged dry pyridine (10 ml) and the sealed mixture was kept at room temperature for 114 h. The mixture was cooled in an acetone–CO_2 bath, glacial acetic acid (12 ml) was added, and the agitated mixture was allowed to warm up to 0 °C (ice-bath). When melted, the mixture was treated with ethyl acetate (100 ml) and saturated sodium chloride (100 ml). The ethyl acetate layer was washed with a second portion of saturated sodium chloride. Glacial acetic acid (20 ml) was added to the ethyl acetate solution, which was quickly washed with saturated sodium chloride (100 ml), and then with half-saturated sodium chloride (2 × 70 ml). The ethyl acetate layer was separated, diluted with ethyl acetate to its original volume, and the above washing procedure repeated after the addition of glacial acetic acid (10 ml). The ethyl acetate solution was separated, evaporated under reduced pressure, and subjected to preparative h.p.l.c., eluting with MeOH–EtOH–H_2O = 7 : 5 : 8 containing 1% citric acid. The appropriate fractions (2 583 ml→4 293 ml) were combined, and were saturated with sodium chloride. Ethyl acetate (300 ml) was added, followed by saturated sodium chloride to give two layers. The ethyl acetate layer was removed, and the aqueous layer was re-extracted with ethyl acetate (200 ml). The organic extracts were combined and washed with several small portions of water (this inevitably caused some loss of porphyrin). The solvent was removed, and the product dried *in vacuo* to give O,O′-*disuccinoyl haematoporphyrin* as an amorphous dark red-purple solid (86 mg, 36%) (Found: C, 63.2; H, 5.75; N, 6.85%. $C_{42}H_{46}N_4O_{12}$ requires C, 63.1; H, 5.8; N, 7.0%), $\lambda_{max.}$ MeOH (ε) 395 (141 000), 497 (10 400), 530 (6 600), 568 (4 400), and 620 nm (2 800). δ[$(CD_3)_2SO$] 11.98 (very broad s); 10.42, 10.37, 10.17, and 10.13 (all s, 4 × *meso*-H); 7.45 (bq, 2 × C*H*OR); 4.29 (bm, 2 × Ar$CH_2CH_2CO_2H$); 3.76, 3.73, 3.64, and 3.57 (all s, ArMe); 3.16 (m, 2 × Ar$CH_2CH_2CO_2H$); 2.79, 2.60 (overlapping m, 4 × CH_2CO of succinic residues); 2.29 (d, *J* 6 Hz, 2 × CHORCH_3); and −3.70 (s, 2 × NH).

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