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Isocoproporphyrin: Nuclear-Magnetic-Resonance- and Mass-Spectral Methods for the Determination of Porphyrin Structure

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The use of 'shift reagents' in the determination of n.m.r. spectra, and of reductive alkylation in combination with g.l.c.–mass spectrometry, facilitates assignment of the order of substituents in porphyrins, and the application of these new techniques to isocoproporphyrin is described.

Most of the structural features of porphyrins can now be readily identified by the use of modern spectroscopic and analytical methods, especially n.m.r. and mass spectrometry. However, the determination of the precise order of substituents around the periphery of the macrocycle still presents a formidable challenge, especially if only small amounts of material are available, as is often the case with transient intermediates in normal, or abnormal, metabolism. In many cases this problem can only be solved by a combination of degradative methods and comparison with known porphyrins, although biogenetic considerations are usually very helpful; ultimately total synthesis may often be required to provide a definitive proof of structure (Kennedy *et al.*, 1970).

We now report partial solutions to these problems involving the use of two new modifications of existing spectral methods. The n.m.r. spectra of porphyrin methyl esters have proved very helpful in the structure determination of porphyrins, and, although position isomers ('type' isomers) often have different spectra (owing largely to aggregation phenomena), it has been possible in only a few instances to predict, or explain, the observed differences (Abraham *et al.*, 1966). However, a new dimension has recently been added to n.m.r. spectroscopy by the introduction of the lanthanide 'shift reagents', e.g. $Eu(dpm)_3$* or $Eu(fod\text{-}d_9)_3$, which complex strongly with basic centres in organic molecules, especially hydroxyl or amino functions etc. (Saunders & Williams, 1971; Bégné, 1972). These give rise to large upfield or downfield shifts of the neighbouring proton resonances, the magnitude of which is related to the distance and orientation of the protons from the metal. Ester groups also form complexes, and with certain porphyrin esters we have now observed some very striking effects that are markedly dependent on the substitution pattern, e.g. the spectra of simple alkyl porphyrins are virtually unaffected by $Eu(fod\text{-}d_9)_3$, but the spectra of the four isomeric coproporphyrin tetramethyl esters show marked downfield shifts in certain of the methoxyl, methyl, methylene and *meso*-proton resonances.

These effects are most simply explained by the assumption that the europium is strongly complexed by two neighbouring ester substituents but only weakly complexed by the other ester groups. The most striking shifts are undergone by the *meso*-protons situated between pairs of neighbouring propionate ester substituents (Figs. 1*a*–1*d*), and the four coproporphyrin isomers are thus readily differentiated from each other.

Comparable effects were observed with other porphyrins including uroporphyrins I and III and proto-, meso- and deutero-porphyrins; haematoporphyrin gave rather more complex results, owing to the strong complexing that also took place with the two hydroxyethyl side chains.

The results suggest that paramagnetic shift reagents might be of general value for the determination of the arrangement of the side chains in a porphyrin even when position isomers of known structure are not available for comparison. This is illustrated by the elucidation of the complete structure of a porphyrin recently isolated from human faeces, isocoproporphyrin, of which the nature, but not the relative positions, of the eight peripheral substituents has been established, although an arrangement was tentatively suggested (Elder, 1972). This porphyrin contains three propionate, one ethyl, one acetate and three methyl side chains. Addition of $Eu(fod\text{-}d_9)_3$ produced marked changes in the n.m.r. spectrum of the tetramethyl ester in deuterochloroform, and the original *meso*-proton doublet (cf. Elder, 1972) separated into four distinct singlets of equal area, indicating that

* Abbreviations: dpm, $(CH_3)_3C\text{–}CO\text{–}CH_2\text{–}CO\text{–}C(CH_3)_3$; fod-$d_9$, $(C^2H_3)_3C\text{–}CO\text{–}CH_2\text{–}CO\text{–}CF_2\text{–}CF_2\text{–}CF_3$.

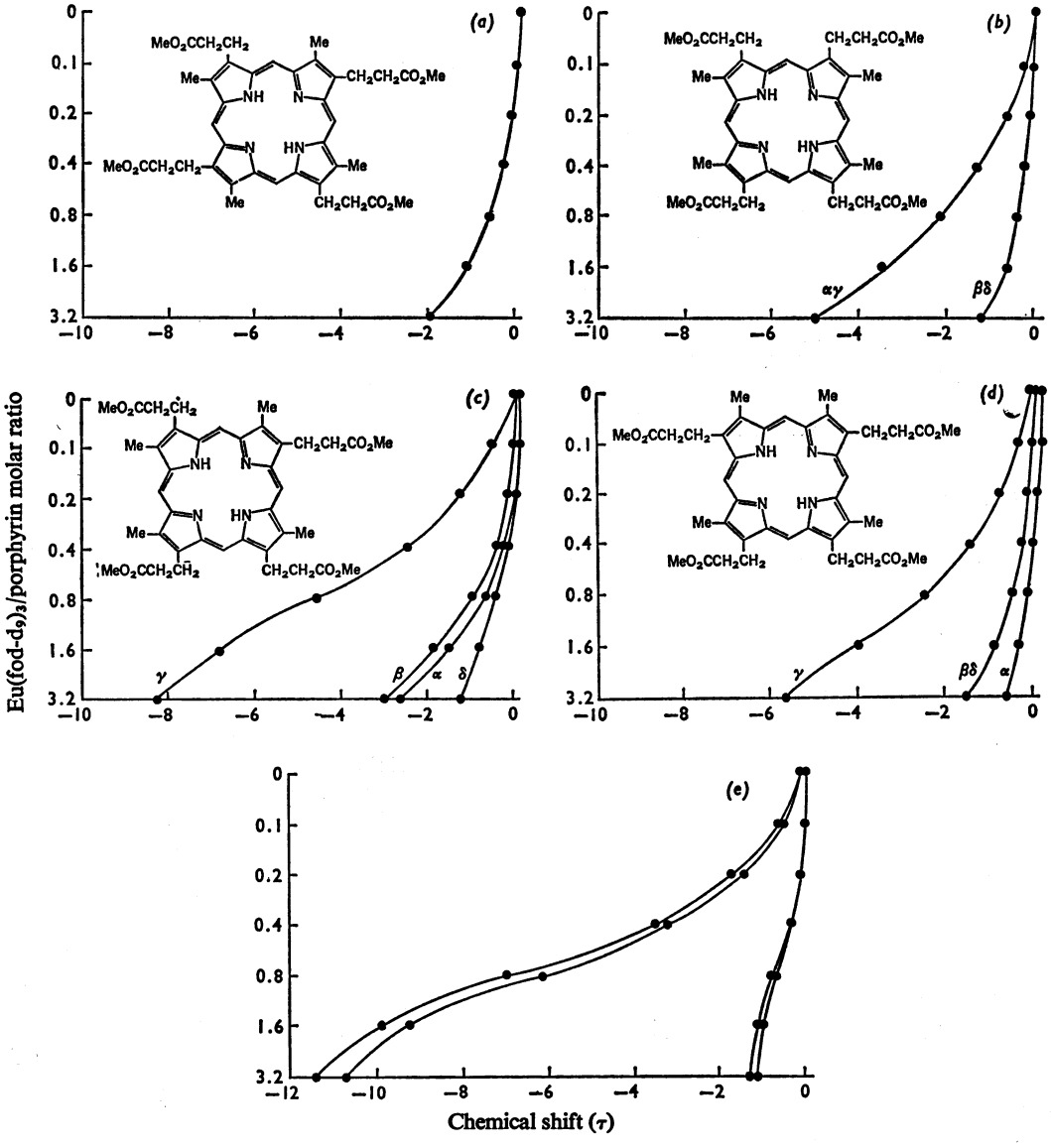

Fig. 1. *Variation of meso-proton chemical shifts in the n.m.r. spectra of the tetramethyl esters of coproporphyrins I–IV and isocoproporphyrin on the addition of increasing amounts of the shift reagent Eu(fod-d9)3*

Initial concentrations were: coproporphyrin tetramethyl esters, 67mM; isocoproporphyrin tetramethyl ester, 50mM.

in all probability only one positional isomer was present (Fig. 1*e*). Further, the signals from two of the *meso*-protons were shifted much farther downfield than the other two, and comparison of these findings with those described above suggests that two of the *meso*-protons lie between ester substituents and that two lie between alkyl substituents. Thus, on the reasonable assumption that isocoproporphyrin is derived from uroporphyrinogen I or III, the only possible structures are (1) and (2) (see Fig. 2). The somewhat lower field shifts exhibited by the β- and γ-*meso*-protons of the isocoproporphyrin tetramethyl ester (compared with those shown by coproporphyrin III tetramethyl ester) are paralleled by those observed

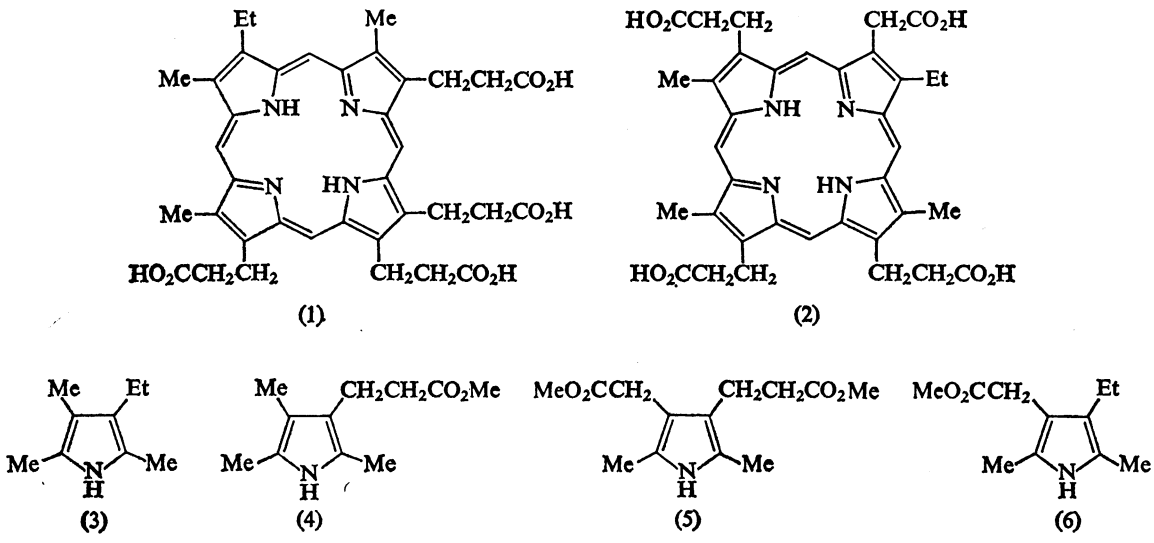

Fig. 2. *Possible structures of isocoproporphyrin* (1 *and* 2) *and their reductive alkylation products* (3–6)

for the *meso*-protons of uroporphyrin III octamethyl ester (concn. 45mM), which range from −11.4 to −13.2τ at an Eu(fod-d_9)$_3$/porphyrin molar ratio of 3.2:1.

To distinguish between these two possibilities, we subjected the isocoproporphyrin to reductive alkylation with HI and formaldehyde in acetic acid (Chapman *et al.*, 1971). Esterification of the resulting mixture of tetrasubstituted pyrroles followed by g.l.c.–mass-spectral analysis showed that only pyrroles (3), (4) and (5) were produced, in the approximate proportions 1:2:1. This result showed that isocoproporphyrin has structure (1), because a porphyrin of structure (2) would have given rise to pyrroles (4) and (6). The pyrroles produced were identified both by their g.l.c. retention times and by their mass spectra; these were identical with those of authentic materials obtained by reductive alkylation either of a mixture of meso- and uro-porphyrins or of appropriately substituted synthetic pyrrole α-carboxylic esters. The arrangement of side chains in structure (1) differs from that previously suggested (Elder, 1972).

The structure of isocoproporphyrin (1) is of considerable interest in relation to its biosynthesis and formation in symptomatic cutaneous hepatic porphyria and in experimental porphyria due to hexachlorobenzene poisoning. It has been suggested that isocoproporphyrin is formed by hydrogenation (by intestinal micro-organisms) of a vinyl-substituted precursor, which is produced in the liver by the action of coproporphyrinogenase on one of the propionate side chains of a pentacarboxylic porphyrinogen (Elder, 1972). If this is so, the structure (1) is in keeping with studies that suggest that coproporphyrinogenase degrades the 2-propionate side chain of coproporphyrinogen III more rapidly than that at the 4-position (Kennedy *et al.*, 1970). The absence of the 1-, 3- and 8-acetate isomers from the sample examined is noteworthy, and may indicate that sequential decarboxylation of the four acetate side chains of uroporphyrinogen III by uroporphyrinogen decarboxylase (EC 4.1.1.–) is an ordered process and/or that only the pentacarboxylate porphyrinogen with a 5-acetate side-chain can be a substrate for coproporphyrinogenase.

Porphyrin n.m.r. spectra were determined in deuterochloroform solution on Varian HA-100, Bruker HFX90 and Perkin–Elmer R14 spectrometers. Reductive alkylations of porphyrins and pyrrole esters were carried out by MacDonald's method (Chapman *et al.*, 1971) and the resulting pyrroles were subjected to g.l.c. on a column (2m×2mm internal diam.) of Gas-Chrom Q (100–120 mesh) coated with 3% OV-1 temperature-programmed at 8°C/min over the range 80–250°C, with a helium flow rate of 30ml/min. The gas chromatograph was connected through a two-stage Watson–Biemann separator to a Varian CH5D mass spectrometer, and mass spectra of the various components from the reductive alkylations were determined directly, corrected for background by using the Varian Spectro Systems 100 Data System and printed out on a Statos-1 recorder.

The coproporphyrins were isolated from natural sources or synthesized by modifications of published methods (Jackson *et al.*, 1972); the isocoproporphyrin used in this work was a mixture of material obtained from human faeces and from the faeces of rats poisoned with hexachlorobenzene (Elder, 1972).

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