Determination of the structure of an N-substituted protoporphyrin isolated from the livers of griseofulvin-fed mice

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Feeding mice with griseofulvin, a widely used anti-fungal agent which induces protoporphyria as a side-effect, leads to the formation in the liver of two green pigments which have been shown to be porphyrin adducts. In this work, the major porphyrin adduct isolated from the livers of griseofulvin-fed mice has been characterized structurally using one- and two-dimensional NMR spectroscopy. The adduct was shown to be an N-alkylated protoporphyrin IX in which the whole of griseofulvin (less a hydrogen atom) is attached to a pyrrole ring nitrogen of the porphyrin. It was shown that the drug-to-porphyrin linkage is an $-O-CH_2-N_{pyrrole}=$ linkage, to either the 4- or 6-position of ring *a* of griseofulvin. In an attempt to identify which pyrrole nitrogen is involved in this linkage, the ^1H spectra of the free base and zinc complex of the adduct were compared with the corresponding spectra of the four regioisomers of *N*-methylprotoporphyrin. These comparisons indicated that the adduct isolated from the livers of griseofulvin-fed mice is either the N_C or the N_D regioisomer, although a clear distinction between these two could not be made on the available evidence. The mechanism of formation of the adduct and its relation to griseofulvin-induced protoporphyria are discussed.

INTRODUCTION

Griseofulvin is a widely used anti-fungal agent that has long been known to induce protoporphyria [1], a disorder of liver haem biosynthesis caused by inhibition of ferrochelatase, the enzyme responsible for inserting iron into protoporphyrin to form haem. Recently, two green pigments have been isolated from the livers of mice fed griseofulvin [2] and they have been shown to be porphyrin adducts. These are believed to be formed by reaction of a species derived from griseofulvin with the porphyrin of cytochrome P_{450}, griseofulvin acting as a suicide substrate of the enzyme.

The pigment that is formed only in small amounts (termed the minor adduct) has been identified as *N*-methylprotoporphyrin (*N*-MePP), which is a potent ferrochelatase inhibitor both *in vivo* and *in vitro* [3–5]. The properties of the minor adduct have been compared with those of synthetic *N*-MePP, showing that all four regioisomers of *N*-MePP are formed *in vivo* following administration of griseofulvin, with the N_A regioisomer predominating [6]. *N*-MePP is also formed on administration of another porphyrogenic compound 3,5-diethoxy-1,4-dihydrocollidine (DDC) to rodents [3–5], again by suicide inhibition of cytochrome P_{450}. The predominant regioisomer formed on DDC administration was shown by NMR also to be the N_A regioisomer [5,7].

The second, major, pigment formed on griseofulvin administration has no equivalent in the DDC-induced porphyria. This adduct has been shown by MS to have a mass consistent with a structure in which the whole of the griseofulvin drug (less a hydrogen atom) is attached to the protoporphyrin ring [2]. It has not yet been possible, however, to identify the point of attachment of the drug to the porphyrin ring, or to show which regioisomer is present. We now report the use of NMR spectroscopy to establish the structure of the major griseofulvin–protoporphyrin adduct.

MATERIALS AND METHODS

Materials

Formation and isolation of the major griseofulvin–protoporphyrin adduct (*N*-GfPP)

Griseofulvin (7-chloro-2′,4,6-trimethoxy-6′-methylgris-2′-ene-3,4′-dione) was given to MF1 mice in a powdered diet as described previously [8]. The major griseofulvin–protoporphyrin adduct (*N*-GfPP) was isolated and purified from the livers of the mice as the dimethyl ester (in the discussion of the analysis of the NMR spectra of the isolated pigment, the abbreviation *N*-GfPP refers to the dimethyl ester of the griseofulvin–protoporphyrin adduct) by procedures involving column chromatography on alumina and TLC on silica [8]; the pigment was crystallized from methyl acetate by gradual addition of heptane and stored at −70 °C under nitrogen. A sample of the pigment in which the methyl ester groups were deuterated was prepared by hydrolysing off the ester groups (25% HCl in methanol overnight at room temperature, followed by drying over KOH) and then re-esterifying using $C^2H_3O^2H$ containing 5% $^2H_2SO_4$.

Synthesis of individual regioisomers of *N*-MePP

The free-base forms of these compounds were synthesized and separated as described previously [9] and the zinc complexes were formed as described by Kunze and Ortiz de Montellano [7].

All other chemicals were of analytical grade, purchased from Sigma Chemical Co.

NMR spectroscopy

NMR samples contained approx. 0.7 mM solutions of *N*-GfPP (free base) in deuterated chloroform, with 0.1% tetramethylsilane

Abbreviations used: DDC, 3,5-diethoxy-1,4-dihydrocollidine; *N*-GfPP, major griseofulvin–protoporphyrin adduct; *N*-MePP, *N*-methylprotoporphyrin; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; TMS, tetramethylsilane.

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(TMS) as internal reference. Samples of the *N*-MePP regioisomers (3–6 mM) were dissolved in C^2HCl_3 with 0.1 % TMS and 1.25 % [^{2}H]pyridine to deter monocation formation. ^{1}H-NMR spectra were obtained by using a Bruker AM500 spectrometer at a sample temperature of 298 K. Two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) and COSY used a 2048 × 1024 data matrix. The mixing time for the NOESY was 1.5 s, with a 3 s gated presaturation pulse. Spin-lattice relaxation times were measured using the inversion recovery method.

RESULTS

Comparison of the ^{1}H spectrum of *N*-GfPP with those of *N*-MePP and griseofulvin

The 500 MHz ^{1}H-NMR spectrum of *N*-GfPP dimethyl ester in chloroform solution is shown in Figure 1. A convenient starting point for the assignment of the resonances in this spectrum, and hence for the determination of the structure of the adduct, is a comparison with the spectra of its components, *N*-MePP and griseofulvin. The structures of these two compounds are also given in Figure 1, along with the 500 MHz ^{1}H-NMR spectrum of griseofulvin.

Porphyrins have very distinctive NMR spectra because of the substantial magnetic anisotropy of the aromatic π-system of the tetrapyrrole, which can be induced by an externally applied magnetic field. The proton assignments of all the regioisomers of *N*-MePP are already known [7], and resonances appear in similar positions in the ^{1}H-NMR spectrum of the major adduct. We can thus identify, in Figure 1, resonances attributable to protons of the porphyrin moiety of *N*-GfPP, specifically the *meso* protons (10.0–10.2 p.p.m.), proximal vinyl (8.1–8.4 p.p.m.) and distal vinyl protons (6.0–6.5 p.p.m.), the methyl protons (3.4–3.8 p.p.m.) and the protons of the proximal (4.2–4.3 p.p.m.) and distal (approx. 3.2 and 2.9 p.p.m.) methylene groups of the propionate side-chains.

On the other hand, comparison of the spectrum of *N*-GfPP with that of griseofulvin (Figure 1) shows that only a limited number of assignments of the resonances of the griseofulvin moiety of *N*-GfPP can be made by inspection. For example, in the region 5.5–6.5 p.p.m., only one singlet is seen in the spectrum of *N*-GfPP, rather than the two singlets (at 6.1 and 5.5 p.p.m.) in the spectrum of griseofulvin. Clearly, a number of the griseofulvin protons in the adduct are affected by the magnetic anisotropy of the porphyrin ring, and their resonances have been shifted from their positions in griseofulvin itself.

Identification of the point of attachment of griseofulvin to the porphyrin ring

The metabolism of griseofulvin by cytochrome P_{450} involves demethylation of the methoxy groups (see below); in light of the postulated role of cytochrome P_{450} in the formation of *N*-GfPP this suggests that one of these three sites might form the point of attachment of griseofulvin to the porphyrin [2].

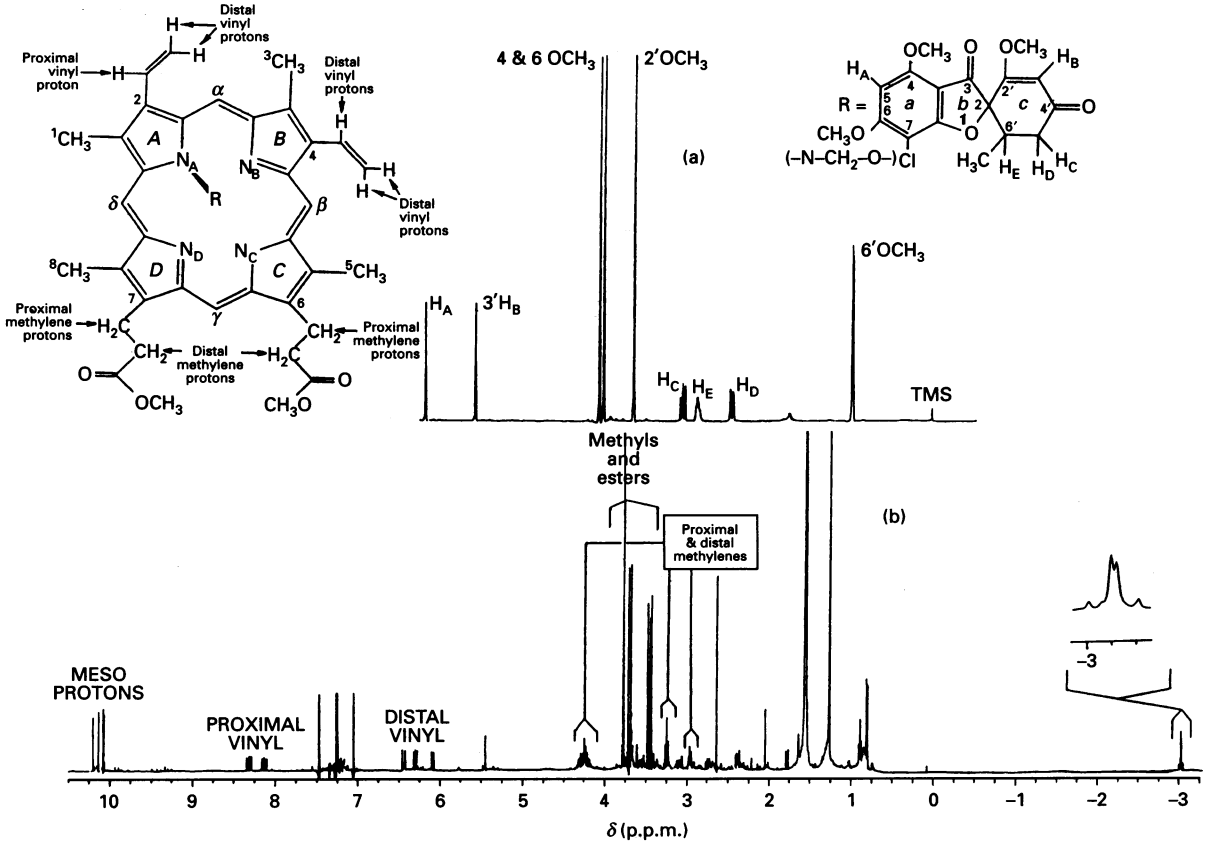

Figure 1 A comparison of the 500 MHz spectra of (a) griseofulvin and (b) the griseofulvin–protoporphyrin adduct

Samples were 1 mM and 0.74 mM in deuterochloroform (TMS as internal standard) respectively. The structures and nomenclature are shown for *N*-methylprotoporphyrin dimethyl ester and for griseofulvin. Some important resonance assignments are indicated.

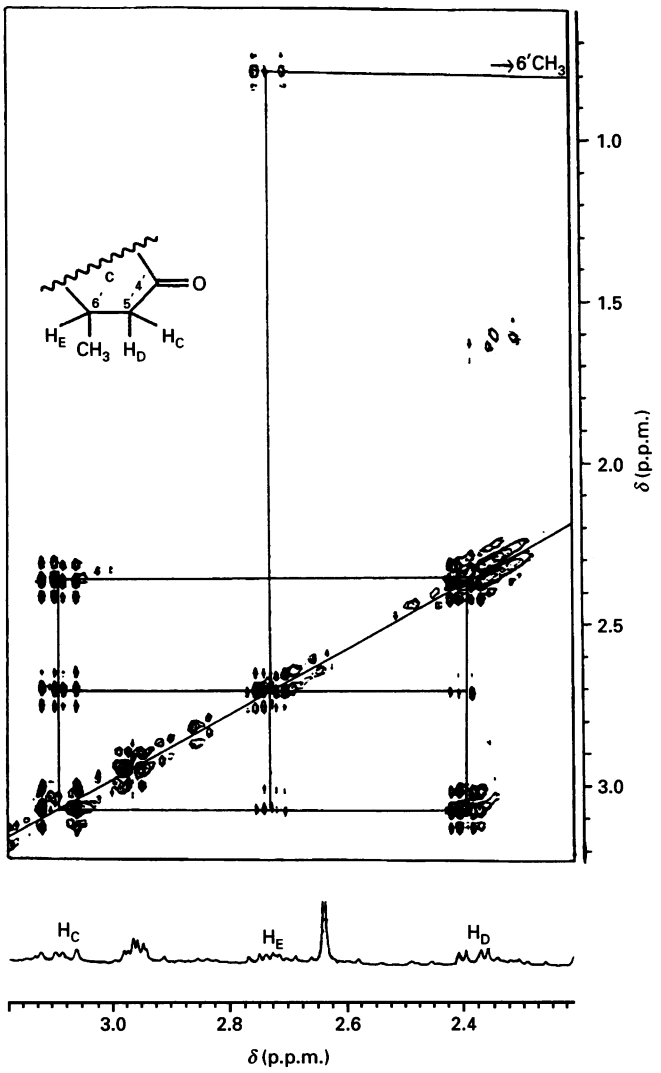

Figure 2 Portion of the ^{1}H–^{1}H double-quantum-filtered-COSY spectrum of *N*-GfPP showing the through-bond connectivities defining the spin-system of ring *c* of griseofulvin

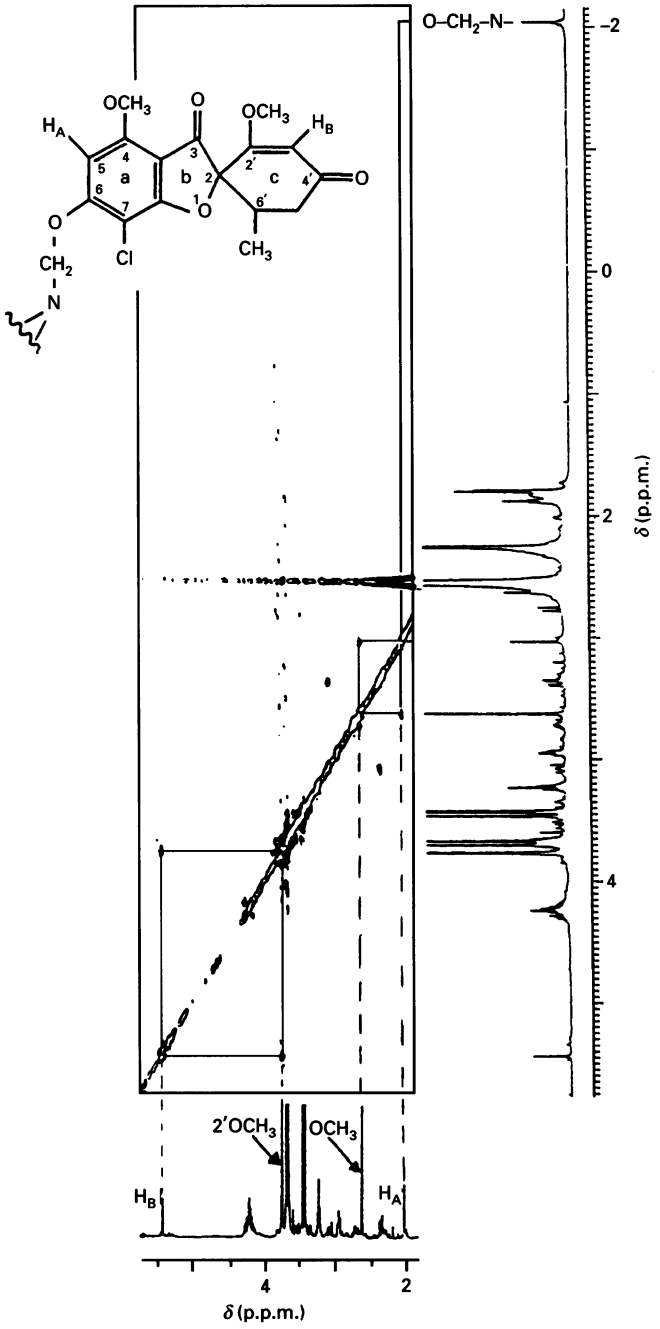

Figure 3 Portion of the ^{1}H–^{1}H NOESY spectrum of *N*-GfPP showing the identification of the -O-CH_2-N- linkage between the griseofulvin and porphryin moieties and of the methoxy group in ring *c* of griseofulvin

For details see the text.

Assignment of the resonances of ring *c* of griseofulvin

The characteristic spin system of ring *c* involving H_C, H_D, H_E and 6′-CH_3 can be clearly identified in a COSY spectrum of *N*-GfPP as shown in Figure 2. The remaining proton signals from this ring, H_B and the neighbouring 2′-OCH_3, can be identified from a NOESY spectrum (Figure 3). (The signals of the H_B and 2′-OCH_3 can be distinguished from those protons of ring *a* by the fact that the singlet H_B shows an NOE to only one other resonance; see below.) All these resonances from ring *c* have chemical shifts which are very similar to those in griseofulvin itself (see Table 1). This clearly implies that the position of the drug relative to the porphyrin macrocycle is such that these protons are not, on average, experiencing significant porphyrin ring current shielding effects, and hence that ring *c* is not located above the centre of the macrocycle. This in turn, together with the fact that resonances for all the protons on ring *c* (in particular that of 2′-OCH_3), have been identified, demonstrates that ring *c* cannot be the site of attachment of griseofulvin to the porphyrin.

Assignment of protons of the linking group

The spectrum of *N*-GfPP (Figure 1) displays a very high-field resonance at −3.03 p.p.m. In *N*-MePP, the N-methyl resonance appears at approximately −4.5 p.p.m., being very strongly shielded by the porphyrin ring-current. We conclude that the signal at −3.03 p.p.m. in *N*-GfPP must be similarly placed with respect to the porphyrin ring, and must therefore arise from the group linking griseofulvin to the porphyrin. The integral of this

Table 1 ^{1}H chemical shifts (δ) of the griseofulvin–protoporphyrin adduct

These chemical shifts are compared with those of griseofulvin and the N_C regioisomer of *N*-methylprotoporphyrin. Chemical shifts, for the free-base form, are given in p.p.m. from tetramethylsilane; for numbering see Figure 1.

Group	δ *N*-GfPP	Multiplicity[a]	δ Griseofulvin	δ *N*-MePP
1-CH_3	3.48[b]	s	–	3.75
3-CH_3	3.77[c]	s	–	3.58
5-CH_3	3.43[c]	s	–	3.47
8-CH_3	3.45[b]	s	–	3.49
6-CH_2 (proximal)	4.23[d]	m	–	4.05
6-CH_2 (distal)	2.94[e]	m	–	2.76
6-OMe	3.68[f]	s	–	3.86
7-CH_2 (proximal)	4.20, 4.30[d]	m, m	–	4.25
7-CH_2 (distal)	3.25[e]	m	–	3.24
7-OMe	3.71[f]	s	–	3.70
2-Vinyl (proximal)	8.32[g]	dd	–	8.34
2-Vinyl (distal)	6.30, 6.09[h]	dd	–	6.23, 6.39
4-Vinyl (proximal)	8.13[g]	dd	–	8.18
4-Vinyl (distal)	6.29, 6.44[h]	dd, dd	–	6.10, 6.25
α-meso	10.13[i]	s	–	10.10
β-meso	10.08[i]	s	–	10.02
γ-meso	10.20	s	–	9.94
δ-meso	10.07	s	–	9.94
N-CH_2-O	−3.03	q	(4.03 or 3.97)[j]	(−4.48)[j]
5-H_A	2.04	s	6.13	–
3′-H_B	5.45	s	5.53	–
6′-CH_3	0.81	dd	0.95	–
2′OCH_3	3.77	s	3.61	–
4- or 6-OCH_3[k]	2.64	s	4.03 or 3.97	–

[a] Abbreviations: s, singlet; d, doublet; m, multiplet; dd, doublet of doublets; q, AB quartet.
[b–i] Denote pairs of proton resonances in *N*-GfPP whose assignment may be interchanged.
[j] -O-CH_3 in griseofulvin, -N-CH_3 in *N*-MePP.
[k] Specific assignment not made in *N*-GfPP.

signal, together with its fine structure (that of an AB quartet; see Figure 1), indicates that it comes from a methylene group in which the two protons are magnetically slightly non-equivalent. This 'linking' methylene group, which is presumably derived from one of the methoxy groups of griseofulvin, can be identified from its NOEs to neighbouring protons in griseofulvin.

The resonance at −3.03 p.p.m. shows an NOE to a singlet at 2.04 p.p.m. (Figure 3). This singlet could be ascribed to either H_B (if the methylene were derived from the 2′-OCH_3) or H_A (if the methylene were derived from either the 4- or the 6-OCH_3). However, the resonance of H_B in ring *c* has already been assigned as the singlet at 5.45 p.p.m. In addition, the singlet at 2.04 p.p.m. shows a second NOE to a second singlet, at 2.64 p.p.m., which must also arise from a griseofulvin proton. This pattern of NOEs is readily explained if the linking methylene group is derived from one of the methoxy groups in ring *a*; there would be an NOE from this methylene (4- or 6-) to H_A, and one from H_A to the second methoxy group in the ring (6- or 4-).

Assignment of the porphyrin resonances of *N*-GfPP and identification of the predominant regioisomer

No NOEs have been observed between protons in the griseofulvin moiety of *N*-GfPP and protons in the porphyrin moiety. It is therefore necessary to use chemical-shift arguments in an effort to establish to which pyrrole nitrogen the griseofulvin moiety is attached.

We have synthesized and purified the four individual regioisomers of *N*-MePP using published methods [9]. Their ^{1}H-NMR spectra are compared with that of *N*-GfPP in Figure 4 (A: free-base forms and B: zinc-complex forms; for resonance assignments see [7,10]). By examining both the free-base and the zinc-complex forms, the spectra of the four regioisomers of *N*-MePP can be distinguished, and a comparison of these with the spectra of *N*-GfPP facilitates the identification of the regioisomer in the latter case.

The *meso* proton resonances of *N*-GfPP can be assigned by making use of NOEs between the protons around the periphery of the porphyrin ring; a relevant portion of the NOESY spectrum is shown in Figure 5. The *meso* proton at lowest field (10.20 p.p.m.), shows an NOE to the resonances ascribed to the proximal propionate methylene protons at approx. 4.22 p.p.m.; only H_γ would be expected to show such an NOE. (Some additional support for this assignment comes from relaxation measurements. Among the *meso* protons, H_γ would be expected to have a relatively short T_1, due to its proximity to the propionate methylene protons [11]. The proton giving rise to the resonance at 10.20 p.p.m. was found to have a T_1 of 0.66 s, compared with values in the range 0.95–1.02 s for the other *meso* protons.) The H_γ resonance thus shows a clear downfield shift from its position in protoporphyrin dimethyl ester (10.06 p.p.m.) on formation of the griseofulvin adduct. None of the regioisomers of *N*-MePP show this downfield shift; indeed in all the regioisomers of *N*-MePP the H_γ resonance, in common with the other *meso* proton resonances, is upfield of its position in protoporphyrin by 0.12–0.25 p.p.m., presumably due to the decrease in the ring-current of the macrocycle due to the out-of-plane distortion of the N-alkylated pyrrole [12–14]. The unusual shift of H_γ in *N*-GfPP can thus plausibly be attributed to the griseofulvin substituent, most probably to a ring-current effect. Since, as discussed

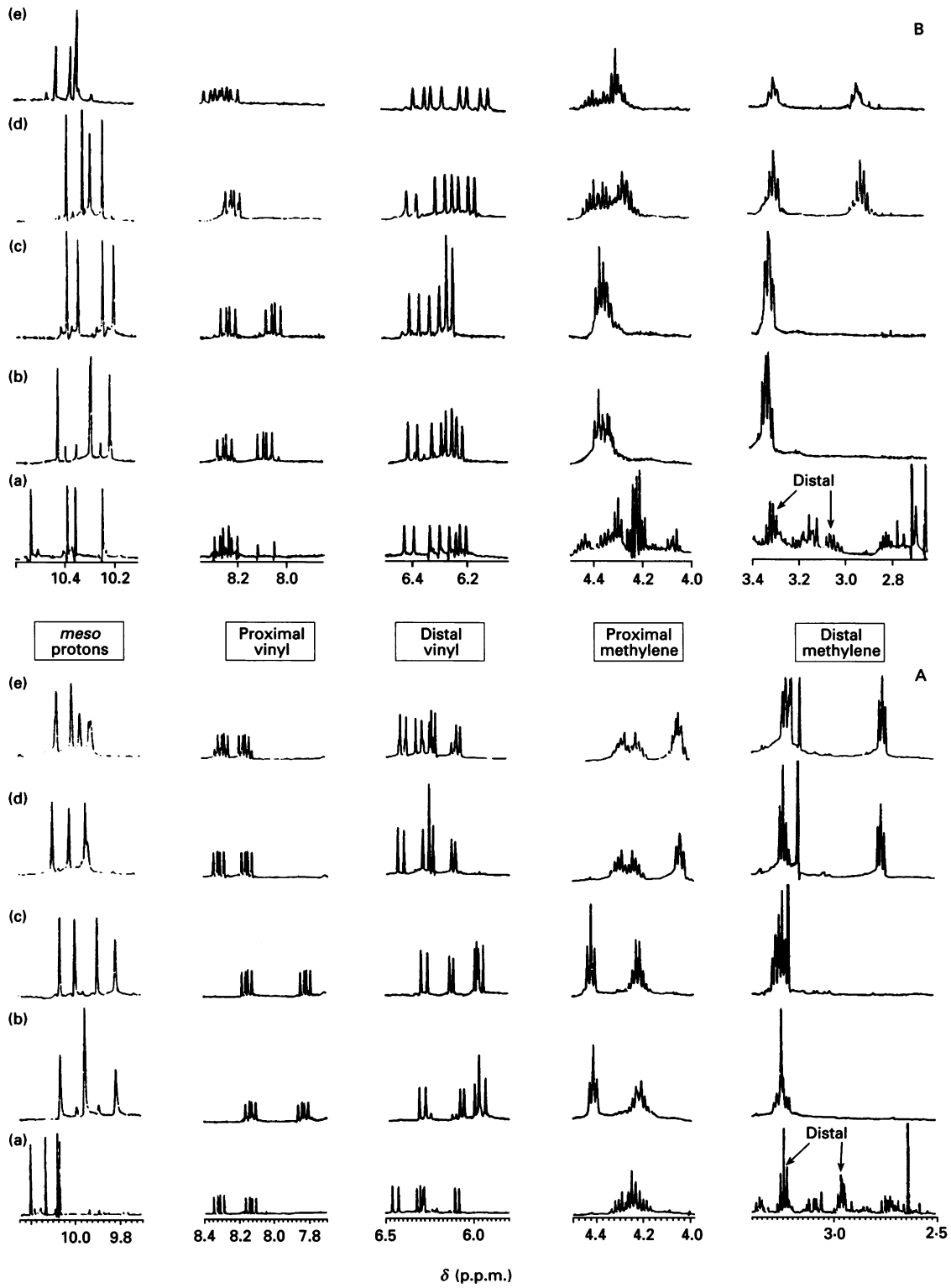

Figure 4 Comparison of the proton spectra of *N*-GfPP and the four regioisomers of *N*-MePP

Panel A in the free-base forms, and Panel B as the zinc complexes. In both A and B, spectra are shown for (a) *N*-GfPP, (b) N_A-methylprotoporphyrin, (c) N_B-methylprotoporphyrin, (d) N_C-methylprotoporphyrin and (e) N_D-methylprotoporphyrin. Sections of the spectra are shown to illustrate the resonances of, from low to high field, the *meso*, proximal and distal vinyl, and proximal and distal methylene groups; refer to the full spectrum of *N*-GfPP in Figure 1.

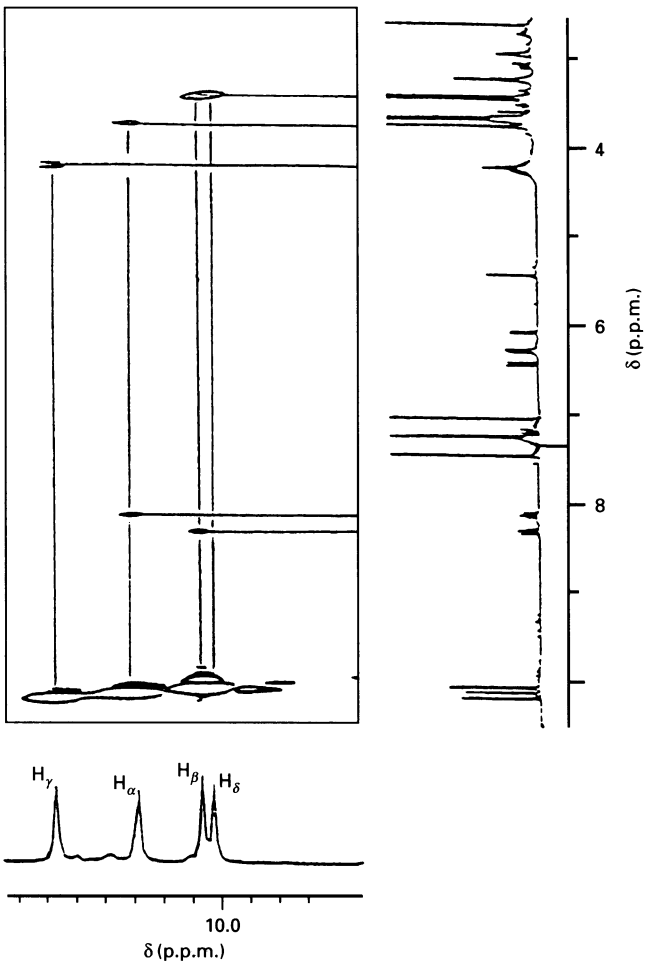

Figure 5 Portion of the NOESY spectrum of *N*-GfPP showing NOEs between the *meso* protons (H_α, H_β, H_γ, H_δ) and the proximal vinyl (8.13 and 8.32 p.p.m.), proximal methylene (centred at 4.22 p.p.m.) and methyl/methoxy (in the range 3.43–3.77 p.p.m.) protons

For details see the text.

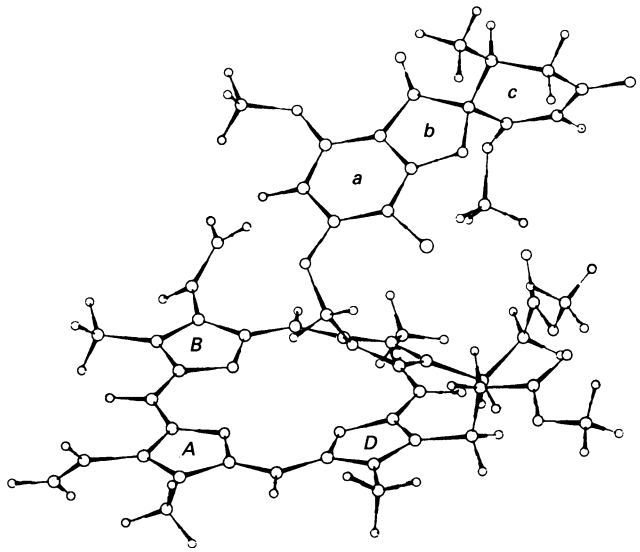

Figure 6 Proposed structure of the *N*-GfPP adduct

The linkage point shown is from the 6-OCH_3 group of griseofulvin to N_C of protoporphyrin; as discussed in the text, it is not yet possible to distinguish between linkages involving the 4- or 6-OCH_3 groups on the one hand, or the N_C or N_D pyrrole nitrogens on the other. The conformation shown, depicted using Insight II (Biosym), is essentially arbitrary, but is consistent with the observation that the resonances of protons in ring *c* of griseofulvin are not affected by the ring current of the porphyrin.

above, the griseofulvin must be oriented, on average, away from the macrocycle (cf. Figure 6), such an effect on H_γ would imply that the griseofulvin must be attached to N_C or N_D.

The two *meso* proton signals at 10.13 and 10.08 p.p.m. show NOEs, both to the proximal vinyl protons (8.13 p.p.m. and 8.32 p.p.m. respectively) and also to signals in the methyl region (3.77 p.p.m. and 3.43–3.48 p.p.m. respectively). The ester methyl resonances of *N*-GfPP dimethyl ester were assigned to the singlets at 3.68 and 3.71 p.p.m. by preparing a sample of *N*-GfPP in which these groups were deuterated and comparing its ^1H spectrum to that of normal *N*-GfPP dimethyl ester. The methyl resonances at 3.77 p.p.m. and 3.43–3.48 p.p.m. can thus be assigned to the pyrrole 3- and 5-methyl groups, and the two *meso* proton resonances at 10.13 and 10.08 p.p.m. to H_α and H_β. A distinction between the 2- and 4-vinyl proton resonances and between the 3- and 5-methyl resonances, and hence between these two *meso* proton resonances, cannot be made at present, since the small amounts of *N*-GfPP available have not allowed us to observe NOEs between the methyl and the vinyl or propionate methylene protons. The highest-field *meso* proton singlet (at 10.07 p.p.m.) shows an NOE to the group of three pyrrole methyl singlets (3.43, 3.45 and 3.48 p.p.m.); this *meso* proton resonance must thus arise from H_β, and the 1-CH_3 and 8-CH_3 protons must resonate at 3.43–3.48 p.p.m.

Among the resonances of the propionate methylene protons on rings *C* and *D*, the clearest difference in behaviour between the regioisomers of *N*-MePP (Figure 4) can be seen for the distal methylene protons. The resonances of these protons of the two propionate groups are essentially equivalent in protoporphyrin dimethyl ester itself (data not shown) and in both the free base and the zinc complexes of the N_A and N_B regioisomers of *N*-MePP. In the N_C and N_D regioisomers, on the other hand, the distal methylene proton resonance of the ring which is alkylated is shifted upfield by approx. 0.5 p.p.m. This clear distinction between (N_A, N_B) and (N_C, N_D) regioisomers is also seen in the spectra of the zinc-complex and free-base forms of *N*-ethylprotoporphyrin, *N*-1-propylprotoporphyrin and *N*-1-butylprotoporphyrin [10]. The spectrum of *N*-GfPP, like those of N_C-MePP and N_D-MePP, shows two distinct multiplets for the propionate distal methylene protons (at 2.94 and 3.25 p.p.m. in the free base), one of which is shifted significantly upfield from its postion in protoporphyrin dimethyl ester. This clearly suggests that the isolated *N*-GfPP is the N_C or N_D regioisomer. The smaller upfield shift seen in *N*-GfPP compared with *N*-MePP (0.35 versus 0.53 p.p.m.) can be rationalized by assuming a downfield ring-current shift arising from the griseofulvin moiety, as discussed above for H_γ.

In protoporphyrin dimethyl ester, the resonances of the distal protons of the two vinyl groups are equivalent, appearing at 8.30 p.p.m. In the N-alkyl derivatives, on the other hand, these protons become magnetically non-equivalent, due in part to the distortion of the macrocycle from planarity which accompanies N-alkylation [12–14]. In free-base forms of the N_A and N_B regioisomers of *N*-MePP the resonance of the proximal proton of the vinyl group on the alkylated ring (the 2-vinyl for the N_A and 4-vinyl for the N_B regioisomer) is shifted upfield by 0.38–

0.47 p.p.m., while there is a smaller (0.07–0.14 p.p.m.) upfield shift of the corresponding resonance on the other ring. In the free-base forms of the N_C and N_D regioisomers, alkylation has no effect on the resonance of the proximal vinyl proton on the ring opposite to the alkylated ring (2-vinyl in the case of N_C, 4-vinyl for N_D), while there is an upfield shift of 0.1 p.p.m. of the corresponding resonance on the neighbouring ring (4-vinyl in the case of N_C, 2-vinyl for N_D). Similar behaviour of the proximal vinyl resonances is also seen in the spectra of *N*-ethylprotoporphyrin, *N*-1-propylprotoporphyrin and *N*-1-butylprotoporphyrin [10]. The chemical shifts of the two proximal vinyl protons of *N*-GfPP (which cannot yet be assigned individually to the 2- and 4-vinyl groups) have chemical shifts of 8.13 and 8.32 p.p.m.; these are closely similar to the chemical shifts seen for the N_C and N_D regioisomers of *N*-MePP (8.18, 8.34 and 8.19, 8.31 p.p.m. respectively), but quite different from those of the N_A and N_B regioisomers (see Figure 4). This provides further evidence that *N*-GfPP is either an N_C or an N_D regioisomer. A similar distinction between N_C/N_D and N_A/N_B can be made from the chemical shifts of the proximal vinyl protons in the zinc complexes; here the 2- and 4-vinyl protons have similar chemical shifts in the N_C and N_D regioisomers, as observed for *N*-GfPP, but different shifts in the N_A and N_B regioisomers. These resonances in the zinc complexes also permit a distinction between N_C-MePP and N_D-MePP; there is a shift difference of 0.1 p.p.m. between the two regioisomers. A comparison with the zinc complex of *N*-GfPP (Figure 4) suggests that the latter is the N_C regioisomer, but this shift difference is too small for a reliable distinction to be made.

The pattern of the distal vinyl proton resonances also differs characteristically between the four regioisomers of *N*-MePP (Figure 4), and again the patterns seen for the free base and the zinc complex of *N*-GfPP are most consistent with those of the N_C and N_D regioisomers. However, for these resonances, as for those of the proximal methylene protons and the methyl protons, the shift differences do not allow a clear-cut distinction between these two regioisomers to be made.

The chemical shifts of protons of the griseofulvin–protoporphyrin adduct are summarized in Table 1, together with those of griseofulvin and the N_C regioisomer of *N*-MePP.

DISCUSSION

The assignment of the ^{1}H-NMR spectrum of *N*-GfPP discussed above provides clear evidence that the griseofulvin–protoporphyrin adduct is formed by an N–CH_2–N= linkage between either the 4- or 6- substituent on ring *a* of the drug to a pyrrole ring nitrogen of the porphyrin ring. In order to identify the pyrrole ring nitrogen involved, the spectra of *N*-GfPP were compared with those of the regioisomers of *N*-alkylprotoporphyrins, in their free-base and zinc-complex forms. From these comparisons, we conclude that *N*-GfPP is clearly either the N_C or the N_D regioisomer; the N_C regioisomer is illustrated in Figure 6. In the absence of individual assignments of, particularly, the vinyl resonances of *N*-GfPP, a clear decision as to whether this adduct is an N_C or an N_D regioisomer cannot yet be made.

The porphyrogenic drug DDC is oxidatively metabolized by cytochrome P_{450} with loss of its 4-substituent as a reactive carbocation [15], or a methyl radical [16], which then reacts with the haem prosthetic group of the cytochrome to form an *N*-methylporphyrin. In this case, all four regioisomers of *N*-MePP are formed *in vivo*, but the N_A regioisomer predominates [5,6]. Cytochrome P_{450} has similarly been implicated in the porphyria produced by griseofulvin [8], although it has not yet been unequivocally established that the porphyrin component of the adduct arises from the haem of cytochrome P_{450}. Griseofulvin is metabolized by cytochrome P_{450} by demethylation of one of the two methoxy groups of ring *a* to form 6-desmethyl- and 4-desmethyl-griseofulvin [17,18]. It is thus easy to see that an adduct of the structure established in this present work could be formed on cytochrome P_{450} if an appropriate reactive intermediate, perhaps a carbon-centred radical, is formed during catalysis.

However, administration of griseofulvin leads to the formation not only of *N*-GfPP, which itself does not inhibit ferrochelatase [1], but also, to a lesser extent, of *N*-MePP [2] which does inihibit the enzyme and which seems likely to be the primary cause of the porphyria. In MS studies [2], evidence was obtained for a secondary fragmentation of *N*-GfPP to form *N*-MePP, raising the possibility that the major adduct *N*-GfPP might be a precursor of the inhibitory *N*-MePP. However, the regioisomer of *N*-MePP formed *in vivo* has been clearly identified as N_A [6], while the evidence presented here shows clearly that *N*-GfPP is either the N_C or the N_D regioisomer, making a precursor–product relationship very unlikely. We cannot, however, exclude the possibility that a small amount of the N_A regioisomer of *N*-GfPP is also formed *in vivo* and that this, rather than the predominant N_C or N_D regioisomer, is preferentially broken down to yield the N_A regioisomer of *N*-MePP.

An alternative explanation is that griseofulvin can interact with cytochrome P_{450} to produce two different reactive species, a methyl radical which leads to the formation of *N*-MePP and a 'griseofulvin radical', with a hydrogen abstracted from the 4- or 6-methoxy group of griseofulvin, which leads to the formation of *N*-GfPP. The predominant product of the action of cytochrome P_{450} on griseofulvin is the 6-desmethyl compound, but 4-desmethylgriseofulvin is also formed to a lesser extent. The two methoxy groups on ring *a* of griseofulvin are chemically very similar, making it likely that the group which is oxidatively demethylated is determined by the orientation of the drug relative to the haem in the active site of cytochrome P_{450}. These two orientations could arise from different modes of binding to a single cytochrome P_{450} species or, perhaps more probably, from the interaction of griseofulvin with two different isoenzymes of cytochrome P_{450}. In either case, the difference which leads to the formation of two different products may well be the difference which determines which product of 'suicide inactivation' is formed. If two different isoenzymes are indeed involved, the relative amounts of the porphyrogenic *N*-MePP and non-porphyrogenic *N*-GfPP which are formed, and hence the likelihood of porphyria, will depend upon the expression of the two isoenzymes in the individual.

This work was supported by an MRC Studentship to R.M.A.B.

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Received 20 July 1994/17 November 1994; accepted 23 November 1994