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Isolation of two *N*-monosubstituted protoporphyrins, bearing either the whole drug or a methyl group on the pyrrole nitrogen atom, from liver of mice given griseofulvin*

Ann E. HOLLEY,† Yvonne FRATER,† Anthony H. GIBBS,† Francesco DE MATTEIS,†§ John H. LAMB,‡ Peter B. FARMER‡ and Steven NAYLOR‡

†Biochemical Mechanisms Section and ‡Biomonitoring Section, MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey, SM5 4EF, U.K.

1. A hepatic green pigment with inhibitory properties towards the enzyme ferrochelatase has been isolated from the liver of mice treated with griseofulvin and identified as *N*-methylprotoporphyrin. 2. All four structural isomers of *N*-methylprotoporphyrin have been demonstrated to be present, N_A, where ring A of protoporphyrin IX is *N*-methylated, being the predominant isomer. 3. In addition to *N*-methylprotoporphyrin, a second green pigment, present in far greater amounts, was also isolated from the liver of griseofulvin-treated mice. This second green pigment is also an *N*-monosubstituted protoporphyrin, but in this case the substituent on the pyrrole nitrogen atom appears to be intact griseofulvin rather than a methyl group. 4. The fragmentation of this adduct in tandem m.s. studies suggests that griseofulvin is bound to the pyrrole nitrogen through one of its carbon atoms and further suggests that *N*-methylprotoporphyrin may arise as a secondary product from the major griseofulvin pigment.

INTRODUCTION

The porphyrogenic compounds 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), griseofulvin and isogriseofulvin all promote the accumulation in rodent liver of an abnormal porphyrin (or green pigment) which has strong inhibitory activity towards mitochondrial ferrochelatase (protohaem ferrolyase, EC 4.99.1.1) both *in vivo* and *in vitro* (Tephly *et al.*, 1979, 1980; De Matteis & Gibbs, 1980). The increased production in the liver of this inhibitory porphyrin and the consequent inhibition of liver ferrochelatase represent the primary mechanism by which these drugs induce hepatic protoporphyria [see De Matteis *et al.* (1987) and Marks *et al.* (1988) for two recent reviews].

The DDC-derived pigment has been conclusively identified as *N*-methylprotoporphyrin by comparing its absorption spectrum, chromatographic behaviour and n.m.r. and m.s. data with those of authentic *N*-methylprotoporphyrin and by showing that the authentic porphyrin was a powerful inhibitor *in vitro* of mitochondrial ferrochelatase (De Matteis *et al.*, 1980*a*; Tephly *et al.*, 1981). Subsequent work has shown that *N*-methylprotoporphyrin is produced during oxidative metabolism of DDC by cytochrome *P*-450, resulting in the transfer of the intact 4-methyl group of the drug to a pyrrole nitrogen of the haem prosthetic group of cytochrome *P*-450 (De Matteis *et al.*, 1981; Ortiz de Montellano *et al.*, 1981*a*; Tephly *et al.*, 1981; Marks *et al.*, 1985). All four possible structural isomers of *N*-methylprotoporphyrin are formed biologically after DDC treatment, with isomer N_A predominating (Ortiz de Montellano *et al.*, 1981*b*).

The ferrochelatase-inhibitory pigments obtained after treatment with griseofulvin or isogriseofulvin have been studied less extensively. Although they have previously been reported (De Matteis & Gibbs, 1980) to exhibit electronic spectra very similar to those of authentic *N*-methylprotoporphyrin, they have not yet been conclusively identified, neither has the mechanism of their production been elucidated. In the present paper we report evidence that the inhibitory pigment produced by griseofulvin treatment is in fact *N*-methylprotoporphyrin. In addition, we report the isolation and characterization of a second green pigment obtained from the liver of griseofulvin-treated mice: this second pigment is also a *N*-monosubstituted protoporphyrin, but in this case the intact griseofulvin moiety appears to be present as a substituent on the pyrrole nitrogen atom.

MATERIALS AND METHODS

Source of special chemicals

Griseofulvin was either a gift from ICI Pharmaceuticals, Macclesfield, Cheshire, U.K., or was purchased from Sigma Chemical Co., Poole, Dorset, U.K. DDC and *N*-methylprotoporphyrin IX dimethyl ester were both prepared as described (De Matteis *et al.*, 1981). The copper chelate of protoporphyrin IX was prepared from protoporphyrin dimethyl ester and cupric acetate, as described by Falk (1964) for the synthesis of Co^{2+}-protoporphyrin. The product was purified by silica-column chromatography, using chloroform as the developing solvent. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals. The Nucleosil 5 analytical h.p.l.c. column (4.6 mm × 250 mm) and the Partisil 10-PAC semi-preparative h.p.l.c. column (4.6 mm × 250 mm) were obtained from Macherey–Nagel, Düren, Germany, and Whatman respectively. Methanol and dichloromethane (Rathburn Chemicals), hexane (Fisons Scientific Apparatus) and tetrahydrofuran (Chromatographic Services) were all h.p.l.c. grade. Tetrahydrofuran was made peroxide-free and dried immediately before use by filtration through aluminium oxide (type UG; Koch–Light Laboratories), previously activated by heating at 100 °C overnight.

Abbreviations used: DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; NBA, 3-nitrobenzyl alcohol; f.a.b., fast atom bombardment.

* This paper is dedicated to the memory of the late Professor A. H. Jackson (deceased 12 September 1990) in appreciation of his contributions in the field of *N*-alkylated porphyrins.

§ To whom correspondence and reprint requests should be addressed.

Treatment of animals

Male and female mice of the MF1 strain (from these laboratories; 6–10 weeks old) were allowed food (expanded R & M no. 3; Special Diet Services, Witham, Essex) and water *ad libitum*. Griseofulvin and DDC were fed for 3 days in a powdered diet, containing 2% (v/w) arachis oil, at a concentration of 1% and 0.1% (w/w) respectively. Food consumption was measured and found to be similar in control and treated groups. Mice were killed by cervical dislocation, and their livers were quickly removed and homogenized in ice-cold 0.25 M-sucrose.

Isolation and characterization of hepatic green pigments

Green pigments were extracted from liver homogenates, isolated by column chromatography on Sephadex LH-20 and purified by t.l.c. on silica gel as described by De Matteis *et al.* (1980*b*). The dimethyl esters were prepared by reaction with BF_3 in methanol (Smith & Francis, 1979) and further purified by t.l.c. with a developing system of chloroform/methanol (20:3, v/v). After elution from silica with methanol/11.6 M-HCl (40:1, v/v), the dimethyl ester derivatives were chromatographed by isocratic h.p.l.c. on a Nucleosil 5 column using a mobile phase consisting of dichloromethane/methanol/conc. aq. NH_3 (relative density 0.88) (500:500:1, by vol.), a flow rate of 2 ml/min with monitoring at 417 nm. Eluted fractions were collected, evaporated to dryness and dissolved in chloroform for spectral studies. Zinc complexes were prepared as described by De Matteis & Gibbs (1980), and absorption spectra were obtained by using a Varian Cary 2200 recording spectrophotometer calibrated with a holmium filter. The drug-induced *N*-methylprotoporphyrin isolated by Nucleosil 5 h.p.l.c. was further resolved into the individual isomers by h.p.l.c. on a Partisil 10-PAC column as described by Ortiz de Montellano *et al.* (1981*b*), using isocratic elution with tetrahydrofuran/hexane/methanol (97:97:6, by vol.) and a flow rate of 1 ml/min. Cu^{2+}-catalysed dealkylation of *N*-alkylporphyrin dimethyl esters was studied at 36 °C in stoppered absorption cells with the use of a Kontron Unikon 860 spectrophotometer as described by De Matteis *et al.* (1985). Green-pigment dimethyl esters were tested for inhibitory activity on ferrochelatase *in vitro* by the method of Tephly *et al.* (1979) after hydrolysis of the methyl ester to the free carboxylate derivative.

M.s. studies

Porphyrin methyl esters for m.s. studies were purified by Nucleosil 5 h.p.l.c. immediately before use. A developing system consisting of dichloromethane/methanol (9:1, v/v) and a flow rate of 2 ml/min was also used in some cases, as this was found to achieve better separation of the major griseofulvin pigment from traces of contaminating protoporphyrin.

All mass spectra were obtained on a tandem mass spectrometer (VG70-SEQ) of EBQ_1Q_2 geometry, where E is an electrostatic analyser, B is the magnet and Q_1 is a radio frequency-only quadrupole which acts as a collision cell. All samples were ionized by positive-ion fast atom bombardment (f.a.b.). Xenon atoms from a model B11N (Ion Tech, Teddington, Middx., U.K.) saddle-field fast-atom gun were used as the primary ionizing beam and impacted the sample at 8.5 keV. The secondary ions produced by the xenon atoms were accelerated to 8 keV from the source region and the magnet scanned at 5 s/decade over the mass range m/z 1350–50. All samples were dissolved in dichloromethane and added to 2 μl of matrix, which was usually 3-nitrobenzyl alcohol (NBA) or thiodiglycol on the stainless-steel probe tip.

In the tandem-m.s. studies, parent ions $(MH)^+$ were selected with a resolution of approx. 1000 using EB (equivalent to the first mass-spectrometer MS_1) and subjected to collision-activated dissociation using argon as the collision gas. Collision energies (laboratory frame-of-reference) were varied from 5 to 460 eV, and target gas pressures in the collision cell were typically ~ 10 mPa ($\sim 10^{-6}$ mbar). Daughter-ion spectra were acquired by scanning Q_2 (equivalent to the second mass spectrometer MS_2) usually over mass range m/z 1000–40, and 15 scans were acquired under data system control in the multi-channel analysis mode.

RESULTS AND DISCUSSION

Chromatographic properties, electronic absorption spectra and ferrochelatase-inhibitory activity of the griseofulvin pigments

The hepatic green pigments accumulating in the liver of mice treated with griseofulvin were isolated by Sephadex LH-20

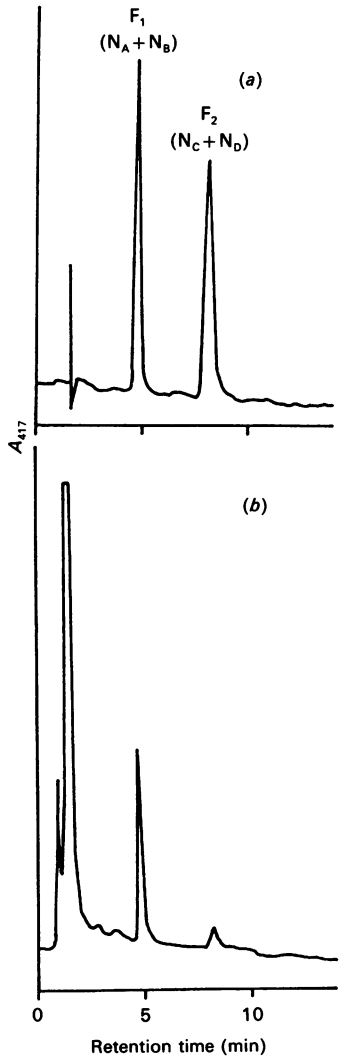

Fig. 1. H.p.l.c. analysis on Nucleosil 5 of authentic *N*-methylprotoporphyrin and green pigments isolated from the liver of griseofulvin-treated mice

A Nucleosil 5 column eluted with dichloromethane/methanol/conc. NH_3 (500:500:1, by vol.) was used for the analysis of the dimethyl esters of (*a*) authentic *N*-methylprotoporphyrin and (*b*) the griseofulvin-derived green pigments. These pigments were resolved into four components with retention times (in order of elution) of 1.03, 1.53, 4.76 and 8.00 min respectively. Note that the retention time of the third and fourth peaks are identical with those of isomeric fractions of authentic *N*-methylprotoporphyrin.

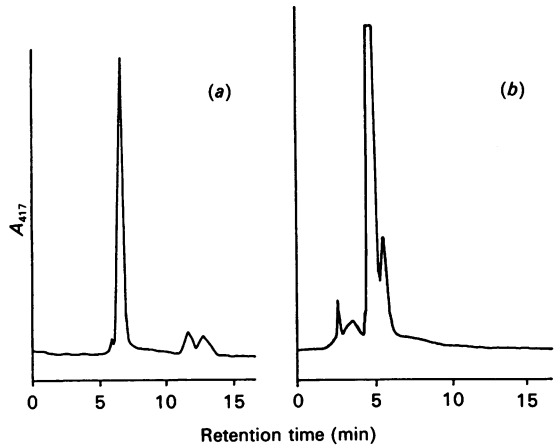

Fig. 2. H.p.l.c. analysis on Partisil 10-PAC of the minor and the major pigments isolated from the liver of griseofulvin-treated mice

A Partisil 10-PAC column eluted with hexane/tetrahydrofuran/methanol (97:97:6, by vol.) was used for the analysis of the dimethyl esters of (*a*) the minor and (*b*) the major pigment, both isolated by Nucleosil 5 h.p.l.c. (see Fig. 1) from the liver of griseofulvin-treated mice. The isomers of authentic *N*-methylprotoporphyrin were eluted in the following order [retention times (min) in parentheses]: N_B (5.75), N_A (6.30), N_C (11.56) and N_D (12.93). All four isomers were present in griseofulvin-derived *N*-methylprotoporphyrin (*a*), with isomer N_A predominating. The major griseofulvin pigment was also resolved into four components (*b*) with retention times of 2.61, 3.30, 4.61 and 5.54 min respectively. Note that retention times of all these peaks are shorter than those of authentic *N*-methylprotoporphyrin.

chromatography and purified by t.l.c. After methylation, the pigments were resolved by h.p.l.c. on Nucleosil 5 and their elution profiles compared with that of authentic *N*-methylprotoporphyrin. In this system *N*-methylprotoporphyrin is resolved into two isomeric fractions (F_1 and F_2), with retention times of 4.69 and 8 min, each containing two structural isomers, (N_A+N_B) and (N_C+N_D) respectively (Fig. 1*a*). In this notation, the suffixes A–D indicate the pyrrole ring that is *N*-methylated in each structural isomer. When subjected to h.p.l.c. on Nucleosil 5, the griseofulvin-derived green pigment afforded a major component with a very short retention time and, in addition, two peaks corresponding in retention times to the F_1 and F_2 components of *N*-methylprotoporphyrin (Fig. 1*b*). The major pigment and the combined F_1 and F_2 fractions (referred to below as the major and the minor griseofulvin pigment respectively) were estimated to be present in an approx. 10:1 ratio. They were subjected to further h.p.l.c. analysis on Partisil 10-PAC, a system capable of resolving the individual isomers of *N*-methylprotoporphyrin. The electronic absorption spectra (before and after incorporation of metals) and the ferrochelatase-inhibitory activities of both major and minor pigments were also determined.

On Partisil 10-PAC the combined F_1 and F_2 fractions collected from Nucleosil 5 (minor griseofulvin pigment) gave rise to four distinct peaks (Fig. 2*a*) with retention times identical with those of the individual isomers of *N*-methylprotoporphyrin. Isomer N_A clearly predominated, the N_C and N_D isomers were present in small and similar amounts, and only a trace of the N_B isomer could be demonstrated. The major griseofulvin pigment also gave rise to several peaks on Partisil 10-PAC, all peaks showing retention times shorter than exhibited by the isomers of *N*-methylprotoporphyrin (Fig. 2*b*): the first, minor, peak probably represented a small amount of contaminating protoporphyrin, as suggested by the similarity of its chromatographic behaviour and absorption spectrum with those of authentic protoporphyrin. The third peak accounted for most (approx. 90%) of the total injected material, and its neutral and zinc complex spectra were very similar to those discussed below for the unresolved major pigment (that is prior to Partisil 10-PAC chromatography).

Both griseofulvin pigments showed a neutral spectrum of the aetio-type with all absorption maxima shifted to longer wavelengths, as compared with the parent porphyrin, protoporphyrin (Table 1). Their zinc-complex derivatives also showed a bathochromic shift of all absorption maxima (as compared with the zinc complex of protoporphyrin) and exhibited, in addition to the α and β bands typical of a metalloporphyrin, a minor band (band α^1 in Table 1), approx. 200 nm towards the red from the Soret maximum. The absorption maxima of the two griseofulvin pigments were in fact quite close to the maxima of authentic *N*-methylprotoporphyrin, with the minor pigment showing a closer similarity. The presence of an α^1 band in the zinc-complex spectrum, as well as the relative intensity of the bathochromic shifts of the various absorption bands, are diagnostic for *N*-monosubstitution, and the absolute values of these shifts allow the identification of the particular porphyrin which bears the *N*-substituent, in this case

Table 1. Absorption spectra of the green pigments obtained after treatment with griseofulvin: comparison with protoporphyrin and with chemically synthesized or biologically produced *N*-methylprotoporphyrin

The neutral spectrum of all porphyrins listed here is of an aetio-type and was obtained with the dimethyl ester derivatives dissolved in chloroform. The spectra of zinc complexes of the various porphyrins were also determined in chloroform and those of the DDC pigment, and of the minor griseofulvin pigment are from previous work (De Matteis & Gibbs, 1980). Note the bathochromic shifts of all absorption maxima of both griseofulvin pigments (as compared with protoporphyrin) and similarity to those exhibited by authentic *N*-methylprotoporphyrin.

	Absorption maxima (nm)									
	Neutral						Zinc complex			
Porphyrin	Soret	IV	III	II	Ia	I	Soret	β	α	α^1
Protoporphyrin IX	407	505	541	575	603	529.5	410	539	578	–
N-Methylprotoporphyrin IX										
(*a*) Obtained chemically	419	513	545	594	626	652	431	545	594	633
(*b*) Produced by treatment with DDC	419	513	545	594	625	653	431	547	596	633
Griseofulvin pigments										
Minor	419	512	545	594	628	652	431	547	596	634
Major	417	510	544	597	626	656	434	545	596	635

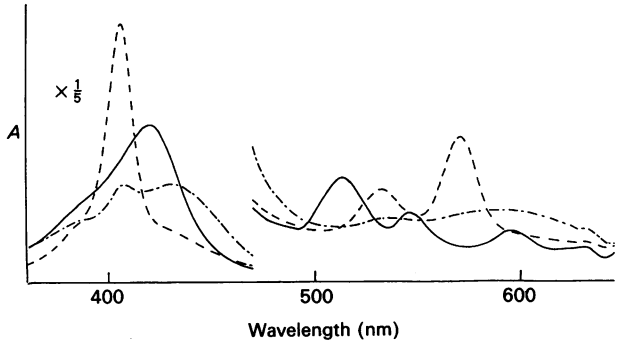

Fig. 3. Copper-dependent changes in the absorption spectrum of *N*-methylprotoporphyrin in chloroform

N-Methylprotoporphyrin dimethyl ester (approx. 3 nmol) was dissolved in chloroform (2.5 ml) and its absorption spectrum (——) determined. A 2 μl portion of a methanolic solution of 0.5% (w/v) cupric acetate monohydrate was then added to the sample cuvette (methanol alone to the reference cell). The absorption spectrum of the mixture was again determined after 1 min (– – – –) and 145 min (– – – –) incubation at 22 °C. Note the rapid disappearance of band IV of the neutral spectrum of the porphyrin, accompanied by decreased intensity and bathochromic shift of the Soret band (all indicative of copper incorporation into *N*-methylprotoporphyrin), followed by late appearance of the characteristic spectrum of the copper chelate of protoporphyrin IX, indicating loss of the *N*-alkyl group.

protoporphyrin IX (De Matteis & Cantoni, 1979; De Matteis & Gibbs, 1980). It can therefore be concluded that both the major and the minor griseofulvin pigments are *N*-monosubstituted derivatives of protoporphyrin IX.

This conclusion is strengthened by the finding that both griseofulvin pigments, like authentic *N*-methylprotoporphyrin, underwent rapid spectral changes in chloroform on addition of copper acetate, compatible with a copper-catalysed elimination of the *N*-alkyl group and formation of the copper chelate of protoporphyrin IX (Fig. 3). These spectral changes indicate the formation of two distinct types of copper-chelate derivatives of the porphyrin macrocycle: a transient, highly distorted copper chelate of the *N*-alkylprotoporphyrin is first formed, followed by ejection of the *N*-alkyl group and generation of the planar copper chelate of protoporphyrin IX. The final products of the reaction showed identical spectra in all cases, with absorption maxima identical with those of authentic Cu^{2+}-protoporphyrin IX (Table 2): they were also identified as Cu^{2+}-protoporphyrin dimethyl ester by f.a.b. m.s. giving an ion ($M^{+\cdot}$) at m/z 651 in all cases.

The free carboxylic acid derivatives of both the major and minor griseofulvin pigments were tested for inhibitory activity on ferrochelatase *in vitro* and the results compared with the inhibitory activity of authentic *N*-methylprotoporphyrin. In agreement with previous findings (De Matteis & Gibbs, 1980; De Matteis *et al.*, 1980*a*), both authentic *N*-methylprotoporphyrin and griseofulvin-derived inhibitory pigment were found to be inhibitory towards ferrochelatase at concentrations as low as 10–20 nM. By contrast, the major griseofulvin pigment was consistently found to be inactive as an inhibitor of ferrochelatase at concentrations up to 170 nM. Since it is known from previous work that when the *N*-alkyl group is increased in size the inhibitory activity towards ferrochelatase decreases (De Matteis *et al.*, 1987), these results suggest that the *N*-alkyl group may differ in the two griseofulvin pigments and be of a much larger size in the major, non-inhibitory, pigment. In the m.s. experiments described below, direct confirmation of this was obtained and the identity of each of the two pigments was conclusively established.

M.s. studies

An f.a.b. mass spectrum obtained in the positive-ion mode for the minor griseofulvin pigment showed a monoprotonated ion $(M\text{H})^+$ at m/z 605 (Fig. 4*a*). This is compatible with an ion corresponding to *N*-methylprotoporphyrin [protoporphyrin dimethyl ester − 1 pyrrole hydrogen (589) + the CH_3 grouping (15) + one additional proton (1)]. Under similar conditions, synthetic *N*-methylprotoporphyrin dimethyl ester gave also an $(M\text{H})^+$ ion at m/z 605 and, in tandem-m.s. studies, the molecular ions of both the synthetic and the biological minor pigment produced identical fragmentation patterns, with daughter ions at m/z 590 and 518 in both cases (the first of these two ions corresponds to loss of the *N*-methyl group to afford, presumably, the radical cation ($M^{+\cdot}$) of protoporphyrin; the second corresponds to loss of the side chain ester $-CH_2-CH_2-CO_2CH_3$). Additional evidence that the minor griseofulvin pigment is *N*-methylprotoporphyrin was obtained by direct identification of the *N*-methyl group. In a recently developed technique (Gibbs *et al.*, 1990) the *N*-alkyl group released during copper-dependent dealkylation of *N*-alkylporphyrins is trapped by dodecylamine and the amine adduct is identified by f.a.b. m.s. By using this technique, transfer of a methyl group to the amine could be demonstrated, starting either from synthetic *N*-methylprotoporphyrin or from the minor griseofulvin pigment (Gibbs *et al.*, 1990), confirming the identification of the latter pigment as *N*-methylprotoporphyrin.

A positive-ion f.a.b. mass spectrum obtained for the major griseofulvin pigment gave a molecular ion ($M\text{H}^+$) at m/z 941 (Fig. 4*b*). The molecular ion is compatible with an *N*-alkylated porphyrin structure, where the whole of griseofulvin is bound

Table 2. Absorption maxima of the Soret, α and β bands of authentic Cu^{2+}-protoporphyrin and of the products of copper-catalysed dealkylation obtained from authentic *N*-methylprotoporphyrin and from minor and major griseofulvin pigments

For copper-catalysed dealkylation, 10 μl of a 0.1% (w/v) methanolic solution of cupric acetate monohydrate were added to 0.5 ml of a chloroform solution of each pigment (containing 0.3–0.9 nmol of *N*-methylprotoporphyrin equivalents). The reaction was allowed to proceed at 36 °C for at least 3 h, when the absorption maxima of the products were determined. The electronic absorption spectra of the various pigments listed were all obtained with the dimethyl ester derivatives dissolved in chloroform.

	Absorption maxima (nm)		
Pigment	Soret	α	β
Authentic Cu^{2+} protoporphyrin	406	533.5	570.5
Products of dealkylation obtained from:			
(*a*) Authentic *N*-methylprotoporphyrin	405.5	533	570.5
(*b*) Minor griseofulvin pigment	405.8	534	570
(*c*) Major griseofulvin pigment	406	533	570

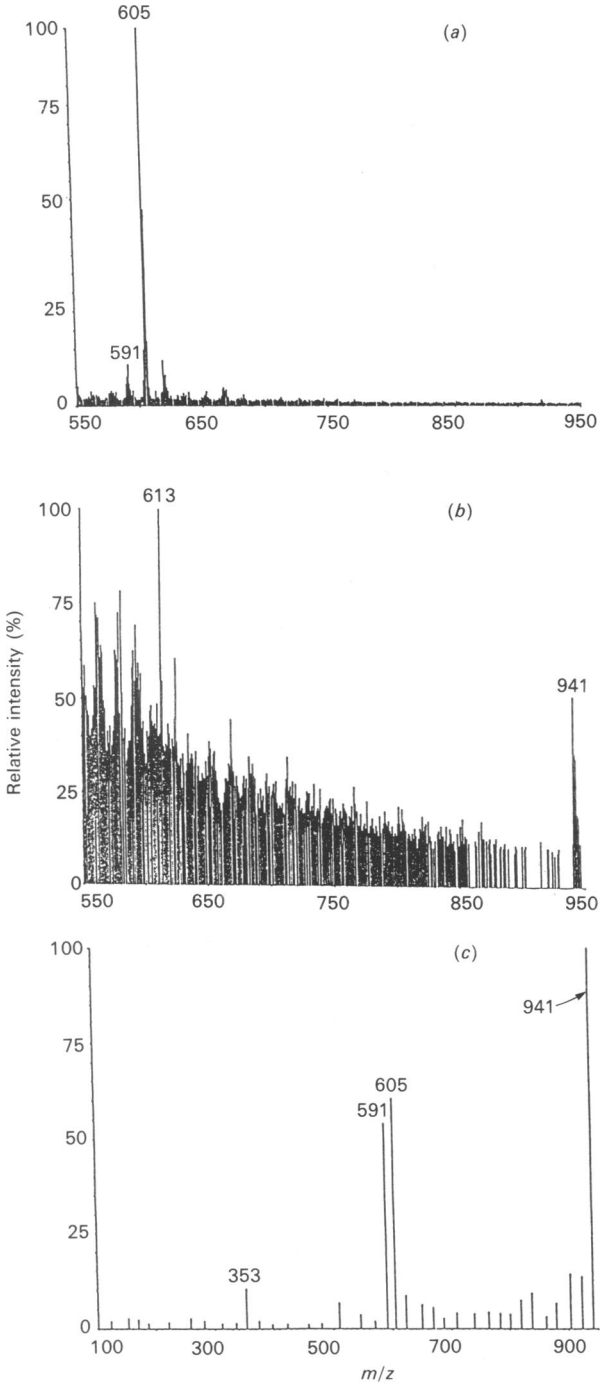

Fig. 4. Positive-ion f.a.b. mass spectra obtained, using NBA as matrix, with, (*a*) the minor griseofulvin pigment, (*b*) the major griseofulvin pigment (note that the ion at *m/z* 613 is matrix-related), and (*c*) daughter-ion tandem mass spectrum of the major griseofulvin pigment [collision carried out on the molecular ion $(MH)^+$ *m/z* 941]

to one pyrrole nitrogen of protoporphyrin, as the value of 941 can be accounted for by the sum: [protoporphyrin dimethyl ester − 1 pyrrole hydrogen (589)] + [griseofulvin − 1 hydrogen (351) + one additional proton].

When the molecular ion $(MH)^+$ at *m/z* 941 was subjected to tandem m.s. (see Fig. 4*c*), the two daughter ions predicted from *N*-dealkylation were seen, namely a peak at *m/z* 591, representing the protoporphyrin dimethyl ester moiety (see also Jackson & Dearden, 1973; Smith & Farmer, 1982), and an ion at *m/z* 353, representing griseofulvin, the putative *N*-alkyl group. An unexpected daughter ion was also detected, however, at *m/z* 605. One possible interpretation for this finding is that, in the gas-collision cell of the tandem mass spectrometer, facile loss of a portion of griseofulvin occurs, with concomitant proton transfer to the porphyrin moiety. This process affords an ion which is isobaric with protonated *N*-methylprotoporphyrin.

A similar fragmentation pattern was obtained with the zinc complex of the major griseofulvin pigment. In positive-ion f.a.b. m.s. this gave a molecular ion at *m/z* 1003, as expected [major griseofulvin pigment − 2 pyrrole hydrogen atoms (938) + Zn (65) = 1003]. When the molecular ion at *m/z* 1003 was subjected to tandem m.s., an ion at *m/z* 653 was seen, corresponding to the zinc complex of protoporphyrin dimethyl ester, predicted from *N*-dealkylation [protoporphyrin dimethyl ester − 2 pyrrole hydrogen atoms (588) + Zn (65) = 653]; in addition, a prominent ion was observed at *m/z* 667, a value corresponding to the zinc complex of *N*-methylprotoporphyrin [*N*-methylprotoporphyrin dimethyl ester − 2 pyrrole hydrogen atoms (602) + Zn (65) = 667].

Therefore daughter ions isobaric with *N*-methylprotoporphyrin or with its zinc complex were observed on fragmentation of the major griseofulvin pigment, depending on whether the pigment itself or its zinc complex were taken for tandem m.s. These results may therefore suggest that the attachment of griseofulvin to the pyrrole nitrogen is through a carbon atom and raise the possibility that the minor griseofulvin pigment, now conclusively identified as *N*-methylprotoporphyrin, may likewise arise *in vivo* by secondary degradation of the major griseofulvin pigment. It should be stressed, however, that such a sequential mechanism for the formation of the *N*-methylprotoporphyrin is still hypothetical, as it is only based on the m.s. fragmentation data, which are indirect and not conclusive. At this stage, alternative pathways for the formation of the *N*-methy' and *N*-griseofulvin derivatives should also be considered.

Previous work has shown that drugs can give rise to hepatic *N*-alkylated porphyrins by one of two different mechanisms, both involving reactive metabolites produced by cytochrome *P*-450 in a self-catalysed suicidal inactivation reaction [see Marks *et al.* (1988) for a recent review]. Either an alkylating derivative of the whole drug (plus an oxygen atom) is produced by mono-oxygenation, or a small alkylating moiety is generated from the drug by oxidative fragmentation. The present results appear to indicate the operation of yet another alkylating mechanism, involving as an alkylating species the whole of the drug (griseofulvin) without prior requirement for oxygen insertion. There is as yet no direct evidence that the *N*-alkylated porphyrins we have now isolated after griseofulvin treatment originate from cytochrome *P*-450; this is, however, suggested by the finding that after feeding griseofulvin to mice a loss of cytochrome *P*-450 is observed in the liver, which is accompanied by a loss of microsomal haem, but not of microsomal cytochrome b_5 (Wada *et al.*, 1968; Denk *et al.*, 1977; Williams & Simonet, 1986). Also, in recent experiments in which liver haem was prelabelled with radioactive 5-aminolaevulinate before administrating griseofulvin, both pigments were found to be radioactive, suggesting that they originate from pre-existing haem (F. De Matteis, A. H.Gibbs & R. Milek, unpublished work).

Griseofulvin is extensively metabolized by liver cytochrome *P*-450 through *O*-demethylation at one of its methoxy groups, all three methoxy groups participating, though to different extents (Chang *et al.*, 1973; Lin *et al.*, 1973; Zia *et al.*, 1979). This and other types of hydroxylation reactions catalysed by cytochrome *P*-450 are thought to operate through two discrete steps (Groves, 1986; Ortiz de Montellano, 1989): first, a carbon-centred radical intermediate is formed by hydrogen abstraction from the drug substrate to the activated ferryl oxygen of cytochrome *P*-450; in

a subsequent step the carbon radical undergoes recombination with the ferryl oxygen, leading to insertion of a hydroxy function in the substrate. With some drugs, particularly those where conjugated radicals are formed and the unpaired electron is therefore delocalized over more than one carbon atom, the half-life of the radical may be long enough to be measurable, for example by secondary rearrangement and loss of stereochemistry (Ortiz de Montellano, 1989). If the radical intermediate of griseofulvin were sufficiently long-lived to diffuse away, in part, before being trapped by the iron-bound oxygen, it could react, to some extent, with one of the pyrrole nitrogen atoms of the prosthetic group and give rise to the *N*-alkylporphyrin. This mechanism would be compatible with the whole drug (without an additional oxygen atom) being bound to the pyrrole nitrogen of the major griseofulvin pigment. In the very large number of *O*-demethylation reactions and other types of hydroxylations catalysed by cytochrome *P*-450 there is no known precedent for *N*-alkylation of the haem prosthetic group by a radical intermediate of the drug undergoing metabolism. However, this does not necessarily exclude a radical-mediated mechanism in the case of griseofulvin, as this drug may possess unusual structural features which make its interaction with mouse liver cytochrome *P*-450 potentially suicidal. Alternatively, it is possible that some degree of *N*-alkylation of the haem of cytochrome *P*-450 during mono-oxygenation of drugs may be of more common occurrence than suspected, and that the *N*-alkylated porphyrins produced have not yet been detected.

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