Biochem. J. (1989) 260, 231–235 (Printed in Great Britain)

Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides

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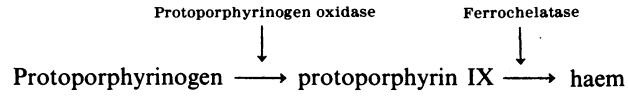
Diphenyl ether herbicides induce an accumulation of protoporphyrin IX in plant tissues. By analogy to human porphyria, the accumulation could be attributed to decreased (Mg or Fe)-chelatase or protoporphyrinogen oxidase activities. Possible effects of acifluorfen-methyl on these enzymes were investigated in isolated corn (maize, *Zea mays*) etioplasts, potato (*Solanum tuberosum*) and mouse mitochondria, and yeast mitochondrial membranes. Acifluorfen-methyl was strongly inhibitory to protoporphyrinogen oxidase activities whatever their origins [concn. causing 50% inhibition (IC_{50}) = 4 nM for the corn etioplast enzyme]. By contrast, it was roughly 100000 times less active on (Mg or Fe)-chelatase activities (IC_{50} = 80–100 μM). Our results lead us to propose protoporphyrinogen oxidase as a cellular target for diphenyl ether herbicides.

INTRODUCTION

Diphenyl ether herbicides have been in use since 1962 for the control of annual grasses and dicotyledonous weeds in soybean (*Glycine max*), peanut (*Arachis hypogaea*), cotton (*Gossypium*), rice (*Oryza sativa*) and other crops (Matsunaka, 1976). The phytotoxicity of these molecules is light-dependent and results in peroxidative degradation of cellular constituents, especially membrane lipids (Orr & Hess, 1982). As some diphenyl ethers do not absorb visible radiation, their activity appears to be mediated by cellular photodynamic pigment(s), and carotenoids have long been proposed to play this role (Matsunaka, 1969). Accordingly, it is generally conjectured that photoexcited carotenoids can induce the formation of nitrodiphenyl ether free radicals, which in turn readily react with membrane lipids to give unstable addition products (Orr & Hess, 1982).

However, in spite of numerous studies aimed at supporting this theory [for a review, see Kunert *et al.* (1987)], the postulated mechanism has never been convincingly demonstrated to occur *in vivo*, and diphenyl ethers have even been found to exert their characteristic light-dependent phytotoxicity on cells devoid of carotenoids (Matringe & Scalla, 1987*a*; Gaba *et al.*, 1988). Recently, we have found evidence that a pigment with the fluorescence spectrum of protoporphyrin IX accumulates in diphenyl ether-treated tissues (Matringe & Scalla, 1987*b*, 1988), and that finding has since been confirmed by others (Lydon & Duke, 1988; Witkowski & Halling, 1988). We were thus able to propose an entirely different explanation for the phytotoxicity of diphenyl ethers (Matringe & Scalla, 1987*b*, 1988). Since porphyrins are known as powerful singlet-oxygen generators in the light (Hopf & Whitten, 1978), their accumulation in treated plants can lead to the light-dependent peroxidation of membrane lipids and the ensuing membrane disruptions which are responsible for the herbicidal effects (Orr & Hess, 1982).

Accumulation of protoporphyrin is known to be associated with some human inherited diseases (protoporphyria and porphyria variegata) [for a review, see Kappas *et al.* (1983)], and with certain mutations in yeasts (Bassel *et al.*, 1975; Urban-Grimal & Labbe-Bois, 1981). The accumulation can originate from two abnormalities in the functioning of the terminal enzymes of the haem-biosynthesis pathway:



In the case of protoporphyria, the biochemical defect has been traced back to a deficiency of ferrochelatase, which catalyses the insertion of a ferrous-iron atom into protoporphyrin IX. In porphyria variegata and some yeast mutants (Brenner & Bloomer, 1980; Deybach *et al.*, 1981; Camadro *et al.*, 1982) protoporphyrin IX accumulation results from a defect in protoporphyrinogen oxidase, which normally carries out the enzymic oxidative aromatization of protoporphyrinogen to yield protoporphyrin IX. In the latter case, protoporphyrinogen molecules presumably diffuse out of their site of synthesis and further metabolization, and are then subjected to non-enzymic oxidation. This leads to the paradoxical situation where the right product (protoporphyrin IX) is accumulating in the cells, but is no longer accessible to ferrochelatase.

Reasoning by analogy, we have investigated a possible involvement of these enzymes in the mechanism of action of diphenyl ethers. Studies about tetrapyrrole synthesis in plants are complicated, however, by the simultaneous functioning in the cell of two systems, one leading to the biosynthesis of haems in mitochondria, and the other to the synthesis of haems and chlorophylls in chloroplasts. In the present study we have taken advantage of the ability of isolated etioplasts to synthesize metalloporphyrins *in vitro* from exogenous protoporphyin IX or

Abbreviations used: ALA, δ-aminolaevulinic acid; AFM, acifluorfen-methyl {methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate}; PAR, photosynthetically active radiation; IC_{50}, concentration causing 50% inhibition; BSA, bovine serum albumin; DTT, dithiothreitol.

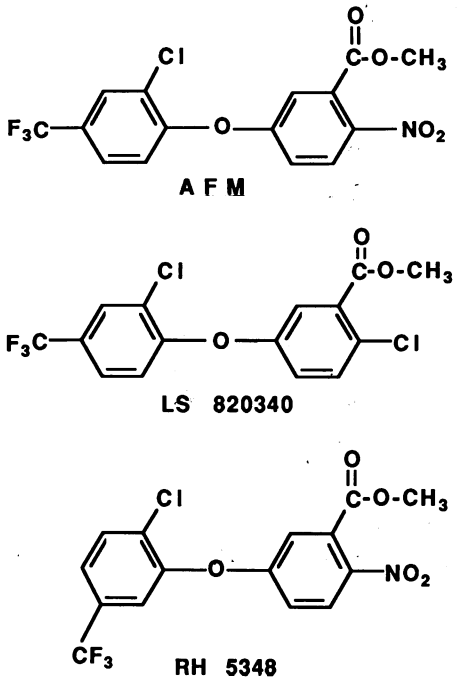


Fig. 1. Structures of AFM, LS 820340 and RH 5348

δ-aminolaevulinic acid (ALA) (Castelfranco *et al.*, 1979; Richter & Riehits, 1981) to discriminate between possible effects of diphenyl ethers on chelatases or on enzymic steps involved earlier in the biosynthetic pathway of tetrapyrroles. Moreover, in order to check the validity of our conclusions, we have examined the biochemical activity of three diphenyl ether molecules (Fig. 1): acifluorfen-methyl (AFM), which is a typical nitrodiphenyl ether herbicide; LS 820340, which differs from AFM in having a chlorine atom instead of a nitro substituent, but nevertheless retains the same type of light-dependent activity (Ensminger *et al.*, 1985); and RH 5348, in which the displacement of a CF_3 group results in a considerably lowered phytoxicity (Duke *et al.*, 1984).

MATERIALS AND METHODS

Chemicals

AFM and LS 820340 were provided by Rhône Poulenc Agrochimie, Lyon, France. RH 5348 was a gift from Rohm and Hass, Philadelphia, PA, U.S.A. Protoporphyrin IX (disodium salt) and ALA were purchased from Sigma, Zn-protoporphyrin IX (free acid) from Aldrich, and Mg-protoporphyrin IX dimethyl ester from Porphyrin Products, Logan, UT, U.S.A.

Etiolated corn (maize; *Zea mays* cv. Monclair) seedlings

These were grown as described by Clément *et al.* (1986). Seeds were surface-sterilized with calcium hypochlorite, soaked in water for 1 day and germinated on a stainless-steel screeen above distilled water for 6–7 days in darkness. Just before etioplast isolation, seedlings were allowed to green for 2–4 h in dim light [30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR)].

Preparation of plant organelles

Corn etioplasts were isolated as described by Prado *et al.* (1980). Mitochondria were isolated from potato (*Solanum tuberosum*) tubers (cv. *Bintje*) as described by Jackson & Moore (1979).

Yeast mitochondrial membranes

Laboratory strain FL 200 or commercially available baker's yeast (Fould Springer) were used. Cell growth and preparations of a fraction enriched in mitochondrial membranes were described by Urban-Grimal & Labbe-Bois (1981).

Mouse liver mitochondria

These were isolated from DBA/2 strain livers by standard procedures (Johnson & Lardy, 1967).

Metalloporphyrin synthesis in corn etioplast preparations

The formation of Mg-protoporphyrin IX from protoporphyrin IX or ALA was monitored in preparations of corn etioplasts by the method of Castelfranco *et al.* (1979). Routine incubation media contained, in a final volume of 1 ml, 500 μmol of sucrose, 20 μmol of Tes, 10 μmol of Hepes, 1 μmol of $MgCl_2$, 1 μmol of EDTA, 10 μmol of ATP, 0.6 μmol of NAD^+, 4.0 μmol of GSH, 2.0 mg of BSA, 10 nmol of protoporphyrin IX (free acid) or 5 μmol of ALA, and 0.5–1 mg of plastid proteins (final pH: 7.6). After incubation for 2 h at 28 °C in dim light (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR), the reaction was stopped by rapid freezing.

Extraction of metalloporphyrins was carried out as described by Castelfranco *et al.*, (1979). A 3 ml portion of cold acetone was added to the incubation mixture before centrifuging at 12000 *g* for 10 min. The pellet was washed with 0.5 ml of 0.12 M-NH_3 plus 1.5 ml of acetone and the two supernatants were combined. Lipids and chlorophylls were removed by washing the extract successively with 7.5 and 2.5 ml of hexane. A 1.7 ml portion of a saturated NaCl solution and 0.15 ml of 0.25 M-monosodium maleate were then added to the aqueous acetone extract. After adjusting the pH to 6.8, porphyrins and metalloporphyrins were recovered from the aqueous phase by two extractions with 3.0 ml of diethyl ether. The fluorescence of the ether extract was recorded with a Jobin and Yvon 3D fluorimeter, and the amounts of Mg-protoporphyrin IX were estimated using excitation and emission wavelengths of 420 and 597 nm respectively. A reference curve was constructed with an authentic standard. Low-temperature (77 K) spectrofluorimetric analyses were done as described by Astier *et al.* (1986), with a cell holder equipped with filter paper to apply samples.

Protoporphyrinogen IX oxidase activity

This was assayed spectrofluorimetrically at 30 °C as described by Labbe *et al.* (1985) by measuring, under initial-velocity conditions, the rate of formation of protoporphyrin IX from chemically reduced protoporphyrinogen (Jacobs & Jacobs, 1982). Excitation and emission wavelengths were 410 and 633 nm respectively. The standard reaction medium contained in 1 ml consisted of 0.1 mmol of potassium phosphate buffer, 1 μmol of EDTA, 5 μmol of DTT, 0.3 mg of Tween 80, 0.1 μmol of protoporphyrinogen and 0.5–

1 mg of protein. The final pH was 7.2, and the reaction medium was saturated with air.

Mitochondrial chelatase activity

This was assayed spectrofluorimetrically at 30 °C by measuring, under initial-velocity conditions, the rate of Zn-protoporphyrin IX formation (Camadro *et al.*, 1984). Excitation and emission wavelengths were 420 and 587 nm respectively. The standard reaction medium contained, in 1 ml, 0.1 mmol of Tris/HCl buffer, 5 nmol of $ZnSO_4$, 0.3 mg of Tween 80, 1 nmol of protoporphyrin IX and 0.5–1 mg of protein. The final pH was 7.6.

Protein concentrations

These were determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Statistics

All experiments were done at least twice, with not less than two replicates.

RESULTS

Effects of diphenyl ethers on the synthesis of metalloporphyrins by isolated corn etioplasts

Upon incubation in complete medium with 5 mM-ALA or 10 μM-protoporphyrin IX, corn etioplasts accumulated a porphyrin pigment which was identified as Mg-protoporphyrin IX by its low-temperature fluorescence spectrum (Fig. 2). This product could be clearly differentiated from Zn-protoporphyrin IX, which was synthesized by yeast mitochondrial membranes in control experiments (Fig. 2). Formation of Mg-protoporphyrin IX by etioplasts occurred at a rate of 0.48–1.16 nmol·h^{-1}·mg of protein^{-1}. That synthesis was dependent upon the presence of ATP and exogenous precursors, i.e. ALA or protoporphyrin IX (Table 1).

As Fig. 3 shows, the inhibitory effect of diphenyl ethers on Mg-protoporphyrin synthesis depended on the nature of the precursor in the incubation medium. AFM, which was our model herbicide, strongly inhibited the formation of Mg-protoporphyrin from ALA

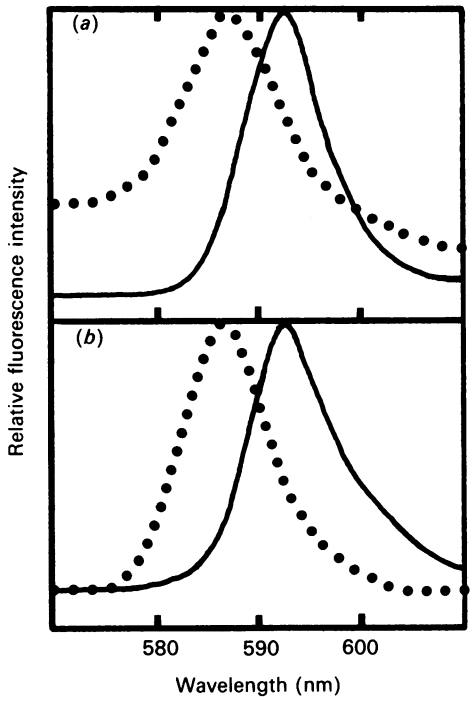


Fig. 2. Low-temperature (77 K) fluorescence emission spectra elicited at 420 nm

(*a*) ——, reaction product from corn etioplasts incubated for 2 h at 28 °C with 5 mM-ALA. Incubation with 10 μM-protoporphyrin IX gives the same result. ····, Yeast mitochondrial membrane preparation incubated at 30 °C for 1 h with 10 μM-protoporphyrin IX and 50 μM-zinc. Fluorescence spectra were recorded on unextracted materials. (*b*) Low-temperature emission fluorescence spectra of Zn-protoporphyrin IX (····) and Mg-protoporphyrin IX dimethyl ester (——), elicited at 420 nm in diethyl ether.

Table 1. Metalloporphyrin synthesis in corn etioplast preparations: requirement for etioplasts, ALA, protoporphyrin IX (Proto. IX) and ATP

The incubation medium was as described in the Materials and methods section, except for the above components, which were added as indicated in the Table. The values represent the ranges of results from three independent experiments.

Incubation conditions	Metalloporphyrin synthesis (nmol of Mg-Proto. IX·h^{-1}·mg of protein^{-1})
ALA or Proto. IX + ATP	–
Etioplasts + ATP	–
Etioplasts + ALA	0.02–0.03
Etioplasts + Proto. IX	0.01–0.03
Etioplasts + ALA + ATP	0.48–1.06
Etioplasts + Proto. IX + ATP	0.73–1.16

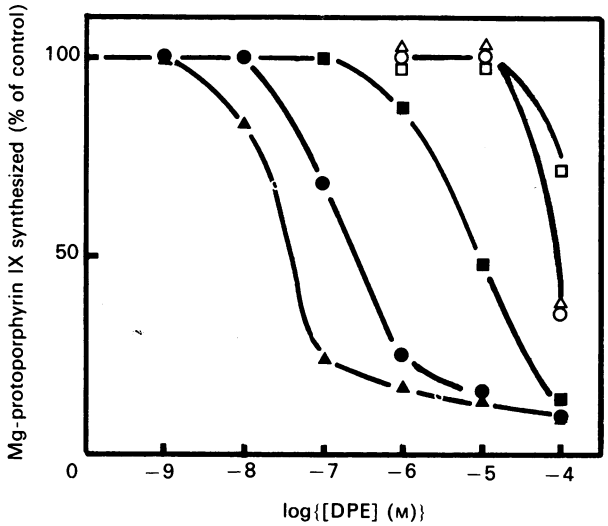


Fig. 3. Effect of diphenyl ethers (DPE) on the synthesis of Mg-protoporphyrin IX by corn etioplasts either from ALA (closed symbols) or protoporphyrin IX (open symbols)

Amounts of Mg-protoporphyrin IX are expressed as percentages of control values, which ranged from 0.48 to 1.16 nmol·h^{-1}·mg of protein^{-1}. △▲, AFM; ○●, LS 820340; ■□, RH 5348.

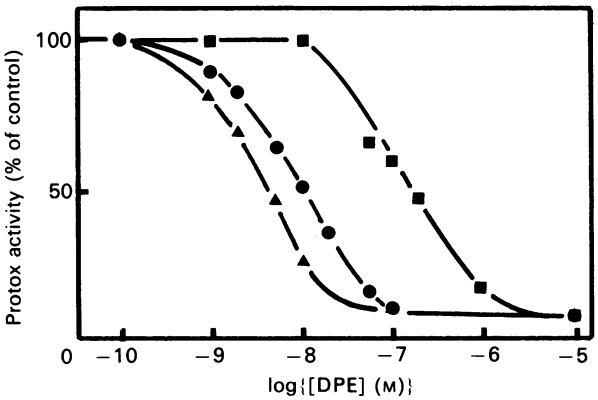


Fig. 4. Effect of diphenyl ethers (DPE) on the protoporphyrinogen oxidase activity of corn etioplasts measured under initial-velocity conditions

Protoporphyrinogen oxidase (Protox) activities are expressed as percentages of control values, which ranged from 8.1 to 9.5 nmol·h^{-1}·mg of protein^{-1}. ▲, AFM; ●, LS 820340; ■ RH 5348.

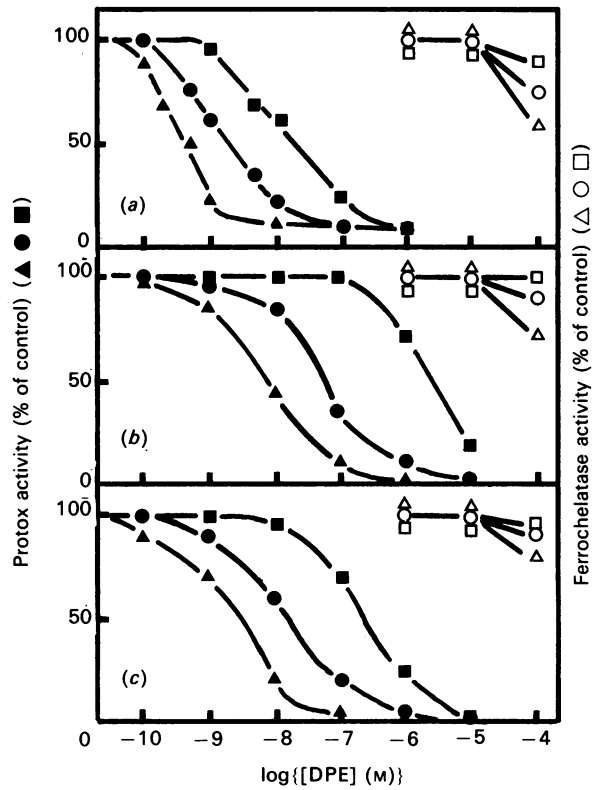


Fig. 5. Effect of diphenyl ethers (DPE) on protoporphyrinogen oxidase (closed symbols) and ferrochelatase (open symbols) activities of potato mitochondria (*a*), yeast mitochondria (*b*), and mouse liver mitochondria (*c*), measured under initial-velocity conditions

Protoporphyrinogen oxidase (Protox) and ferrochelatase activities are expressed as percentages of the control values, which were respectively 2.1 and 2.85 nmol·h^{-1}·mg of protein^{-1} for potato mitochondria; 7.4 and 13 nmol·h^{-1}·mg of protein^{-1} for yeast mitochondrial membranes, and 7.2 and 12 nmol·h^{-1}·mg of protein^{-1} for mouse liver mitochondria. ▲△, AFM; ●○, LS 820340; ■□, RH 5348.

(IC_{50} = 35 nM). By contrast, inhibition was much weaker (IC_{50} = 80 μM) when protoporphyrin IX was given as the direct substrate of Mg-chelatase; similar conclusions were reached with the AFM analogues. Fig. 3 also shows that AFM and LS 820340 were more active than RH 5348, in agreement with the known difference in phytotoxic potencies of these molecules (Duke *et al.*, 1984; Ensminger *et al.*, 1985). Since these results seemed to rule out Mg-chelatase as the primary target for diphenyl ethers, we investigated the effect of these herbicides on protoporphyrinogen oxidase.

Effect of diphenyl ethers on protoporphyrinogen oxidase activities

As shown in Fig. 4, AFM was a very potent inhibitor of etioplast protoporphyrinogen oxidase activity (IC_{50} = 4 nM). The inhibitory abilities of AFM analogues paralleled their herbicidal activities, the chlorinated analogue (LS 820340) being slightly less active than AFM (IC_{50} = 10 nM), whereas the less phytotoxic molecule (RH 5348) showed a much lower efficiency (IC_{50} = 180 nM).

In order to determine the degree of specificity of these inhibitory properties, the effects of diphenyl ethers on protoporphyrinogen oxidase from a plant mitochondrial preparation was examined (Fig. 5*a*). AFM was found to be a strong inhibitor of potato mitochondrial protoporphyrinogen oxidase (IC_{50} = 0.43 nM), whereas the activities of its analogues again paralleled their phytotoxicities (IC_{50} of LS 820340 = 3 nM; IC_{50} of RH 5348 = 19 nM). By contrast, AFM and its analogues showed little activity towards mitochondrial ferrochelatase, with an IC_{50} well above 100 μM (Fig. 5*a*). Owing to the strong inhibition observed with etioplasts and mitochondria, it was of major interest to examine the inhibitory effect of diphenyl ethers on protoporphyrinogen oxidase activity from organisms other than plants. As shown in Figs. 5(*b*) and 5(*c*), similar inhibitions were observed with yeast and mouse liver mitochondrial protoporphyrinogen oxidase (IC_{50} of AFM = 7 and 2 nM respectively). Furthermore, the zinc-chelatase activities of ferrochelatases from yeast and mouse liver mitochondria were only slightly inhibited by diphenyl ethers (IC_{50} > 100 μM).

DISCUSSION

As mentioned in the Introduction, protoporphyrin IX accumulation in diphenyl ether-treated plants could be attributed to an effect on chelatase or protoporphyrinogen oxidase activities. Our results provide strong evidence that protoporphyrinogen oxidase is a primary target of diphenyl ether herbicides. This conclusion is supported by the marked inhibitory properties of these molecules on protoporphyrinogen oxidase of various origins. Our hypothesis is also strengthened by the fact that, at nanomolar concentrations, diphenyl ethers are 2000 to 100000 times more inhibitory to protoporphyrinogen oxidase than to chelatases.

By analogy with animal systems (Deybach *et al.*, 1981) or yeast mutants (Urban-Grimal & Labbe-Bois, 1981), the accumulation of protoporphyrin IX that occurs in diphenyl ether-treated plants may result from the non-enzymic oxidation of protoporphyrinogen [which does not fluoresce nor absorb visible light (Falk, 1964)], into the highly photodynamic protoporphyrin IX. Moreover,

as already described for a protoporphyrinogen oxidase-deficient yeast mutant (Camadro *et al.*, 1982), or human patients with porphyria variegata (Brenner & Bloomer, 1980; Deybach *et al.*, 1981), the membrane-bound ferrochelatase does not seem able to use the non-enzymically produced protoporphyrin IX, possibly because that accumulating molecule is sequestered in some other membrane structure and is no longer accessible to chelatases. That could explain why, in whole plants, inhibition of protoporphyrinogen oxidase by diphenyl ethers results in accumulation of protoporphyrin IX.

Concerning the structural requirements for protoporphyrinogen oxidase inhibition, our results with LS 820340 show that the NO_2 substituent of AFM can be replaced by a Cl atom without strongly lowering the inhibitory power. By contrast, the position of the CF_3 group appears of great importance, because its displacement in RH 5348 lowers the activity by roughly two orders of magnitude. Since LS 820340 possesses marked light-dependent herbicidal properties, whereas RH 5348 has a much lower toxicity *in vivo*, inhibition of protoporphyrinogen oxidase provides a likely explanation for the phytotoxic effects of diphenyl ethers.

On the other hand, the sensitivities of mammalian and yeast protoporphyrinogen oxidase to diphenyl ethers emphasize the need for precise assessment of the toxicity of these molecules.

A final remark is that AFM is about 5000 times more potent than the only inhibitor of protoporphyrinogen oxidase described so far, namely bilirubin, which has a K_i of 25 μM (Ferreira & Dailey, 1988). Diphenyl ethers thus represents a new family of protoporphyrinogen oxidase inhibitors. The great variety of chemical structures available should permit one to delineate the precise molecular interactions involved and perhaps to throw some light on the reaction mechanisms responsible for protoporphyrinogen oxidative aromatization.

We thank Rhône-Poulenc Agrochimie for supplying AFM and LS 820340, and Rohm and Haas for RH 5348. Thanks are also due to Dr. C. Vernotte (C.N.R.S., Gif-sur-Yvette, France) for her help with low-temperature spectrofluorimetric analyses. This work was supported by grants from C.N.R.S. Université Paris 7 and I.N.R.A.

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Received 10 October 1988/8 December 1988; accepted 16 December 1988