Induction of Fenarimol-efflux Activity in *Aspergillus nidulans* by Fungicides Inhibiting Sterol Biosynthesis

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Fungicides inhibiting sterol biosynthesis belong to chemically distinct classes such as imidazole, morpholine, pyridine, pyrimidine and triazole derivatives. Incubation of mycelium of *Aspergillus nidulans* for 90 min with representatives of these fungicides induced an efflux activity which prevented accumulation of fenarimol, a pyrimidine derivative, into the mycelium. Induction of this efflux activity reduced the fungitoxicity of fenarimol. Addition of oligomycin to mycelium in which fenarimol-efflux activity was induced immediately increased the uptake of fenarimol, indicating that the efflux activity is energy-dependent. Subsequent disruption of membrane permeability with sodium lauryl sulphate instantaneously caused leakage of fenarimol from the mycelium into the medium. The ability to induce fenarimol efflux is rather specific for inhibitors of sterol biosynthesis: except for pimarinic acid, fungicides with unrelated mechanisms of action did not have this ability.

INTRODUCTION

Fenarimol [α -(2-chlorophenyl)- α -(4-chlorophenyl)-5-pyrimidinemethanol] is a fungicide used for control of several leaf pathogens such as powdery mildews and scab (Brown *et al.*, 1975). Its antifungal action is attributed to inhibition of demethyl sterol biosynthesis (Buchenauer, 1977; Ragsdale & De Waard, 1977). Uptake of this fungicide by mycelium of *Aspergillus nidulans* is characterized by a rapid initial accumulation followed by a subsequent gradual excretion (De Waard & Van Nistelrooy, 1980). Uptake appears to be the result of rapid, passive influx and active efflux. Active efflux is energy-dependent since it is inhibited by low temperature, anaerobiosis, starvation of mycelium and incubation with respiratory inhibitors. Efflux also has an inducible character, since upon addition of fenarimol to mycelium, efflux activity increases with time until after a certain period a steady-state equilibrium between influx and efflux is established. Once operation of efflux activity has been fully induced, addition of more fenarimol does not result in additional uptake of the fungicide (De Waard & Van Nistelrooy, 1980).

In this study we report on the ability of fungicides, which, though chemically non-related, all inhibit ergosterol biosynthesis, to induce the energy-dependent efflux of fenarimol. In order to put this ability in its proper perspective we also studied the effect of fungicides with an unrelated mechanism of action.

METHODS

Organism and culture conditions. *Aspergillus nidulans* *biA1 acrA1* (strain 003), requiring biotin, resistant to acriflavin, and with wild-type sensitivity to fenarimol, was used in all experiments. The strain was maintained on malt-extract medium. Mycelium in liquid culture was grown in a synthetic glucose-nitrate medium on a Gallenkamp orbital shaker for 17 h (De Waard & Van Nistelrooy, 1979).

Preparation of mycelial suspension. Liquid cultures of *A. nidulans* were harvested by filtration on a Büchner filter and washed twice by resuspending 1 g of the wet mycelium in 50 ml 23.4 mM-potassium phosphate buffer pH 6.0 containing 0.1 mM- CaCl_2 and 1% (w/v) glucose. Standard mycelial suspensions with an average dry weight of 3.0 mg ml^{-1} were made by resuspending 1 g of the washed mycelium in 50 ml of the same medium.

Induction of [^{14}C]fenarimol efflux. Standard mycelial suspensions of *A. nidulans* (100 ml) were shaken in 300 ml flasks in a reciprocal waterbath shaker at 37°C for 30 min. Fenarimol efflux was induced by incubation of mycelium with the test fungicides at 0, 30, 100, and $300 \mu\text{M}$ for 90 min. Then [^{14}C]fenarimol (sp.act. $100 \mu\text{Ci mmol}^{-1}$; $3.7 \text{ MBq mmol}^{-1}$) was added to a final concentration of $30 \mu\text{M}$. In control treatments equal amounts of the test fungicide and of [^{14}C]fenarimol were added simultaneously. All fungicides used were added as concentrated solutions in methanol, the final methanol concentration being 1.1% (v/v). Uptake of [^{14}C]fenarimol was measured at intervals by filtering 5 ml mycelial samples through Whatman GF/A glass filter paper, using a Millipore sampling manifold apparatus. The mycelial residues were washed five times within 30 s with 5 ml of the incubation medium without fungicides. [^{14}C]Fenarimol in the mycelium was extracted with scintillation fluid for 1 d and counted in a liquid scintillation spectrometer (De Waard & Van Nistelrooy, 1979).

Survival of mycelium. Survival of mycelium during experiments on [^{14}C]fenarimol uptake was determined by resuspending washed mycelial residues in 5 ml sterile water. Small droplets of the suspension were transferred on to malt agar. Radial growth was measured after 1 and 2 d incubation at 37°C .

Demonstration of antagonistic activity. Antagonistic activity between different fungicides was tested by the cross-paper technique. Filter paper strips (width 1 cm) were dipped in 3 or 10 mM methanolic solutions of the test fungicides or in 3 mM-fenarimol solution, dried and transferred on to glucose-nitrate agar medium pH 6 in Petri dishes (diam. 14 cm) seeded with 0.5 ml spore suspensions of *A. nidulans* (10^7 spores ml^{-1}). Each Petri dish contained a strip treated with the test fungicide and one treated with fenarimol (Fig. 1). The strips with the test fungicides were applied on the dried surface of the medium, either immediately before incubation of the plates at 37°C , or after 10 h incubation; the fenarimol strip was always applied after 10 h incubation. Growth was determined after 3 d incubation at 37°C by measuring the width of the inhibition zones at the edge of the Petri dish and near the crossing of the paper strips (Fig. 1).

Chemicals. The fungicides used were generous gifts: bitertanol [1-(biphenyl-4-yloxy)-3,4-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)butan-2-ol], triadimefon [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)butan-2-one], and triadimenol [1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)butan-2-ol] from Bayer, Leverkusen, Germany; carbendazim (methyl benzimidazol-2-ylcarbamate) from Du Pont de Nemours, Wilmington, Del., U.S.A.; carboxin (5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide) from Uniroyal, Naugatuck, Conn., U.S.A.; etaconazole {1-[2-(2,4-dichlorophenyl)-4-ethyl-1,3-dioxolan-2-ylmethyl]-1*H*-1,2,4-triazole} from Ciba Geigy, Basel, Switzerland; chloroneb (1,4-dichloro-2,5-dimethoxybenzene) from the Institute for Organic Chemistry T.N.O., Utrecht, The Netherlands; diclobutrazol [(2*R*,3*R*)- and (2*S*,3*S*)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pentan-3-ol] from I.C.I., Bracknell, Berks., U.K.; fenarimol [α -(2-chlorophenyl)- α -(4-chlorophenyl)-5-pyrimidinemethanol], [α - ^{14}C]fenarimol, and nuarimol [α -(2-chlorophenyl)- α -(4-fluorophenyl)-5-pyrimidinemethanol] from Lilly Research Centre, Erl Wood Manor, U.K.; fenpropimorph [*cis*-4-[3-(4-*tert*-butylphenyl)-2-methylpropyl]-2,6-dimethylmorpholine] from Dr Maag, Dielsdorf, Switzerland;

Fig. 1. Demonstration of antagonistic activity of triadimefon towards toxicity of fenarimol to *Aspergillus nidulans* in a cross-paper experiment. Treatment I, paper strips with triadimefon and fenarimol applied simultaneously after 10 h incubation; treatment II, paper strips with triadimefon and fenarimol applied after 0 and 10 h incubation, respectively. Fenarimol strip vertical; triadimefon strip horizontal.

imazalil {1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole} from Janssen Pharmaceutica, Beerse, Belgium; phenapronil (α -butyl- α -phenyl-1*H*-imidazole-1-propane nitrile) from Rohm & Haas, Philadelphia, Pa, U.S.A.; pimaricin from Gist-Brocades, Delft, The Netherlands; thiabendazole [2-(4'-thiazolyl)benzimidazole] from Merck, Rahway, N.Y., U.S.A.; and tridemorph (2,6-dimethyl-4-tridecylmorpholine) from BASF, Limburgerhof, Germany. Oligomycin and sodium lauryl sulphate (SLS) were obtained from Sigma. All chemicals used were of technical or crystalline pure quality.

RESULTS

Fenarimol-efflux inducing potency of fungicides inhibiting sterol biosynthesis. Like fenarimol (De Waard & Van Nistelrooy, 1980), the structurally related fungicide nuarimol induced fenarimol-efflux activity in *Aspergillus nidulans* when added 90 min before addition of [^{14}C]fenarimol (Fig. 2*a*). When nuarimol was added simultaneously with [^{14}C]fenarimol to the mycelial suspension, uptake was about as high as in the control treatment (Fig. 2*b*). Structurally non-related inhibitors of sterol biosynthesis, such as the imidazole fungicides imazalil and phenapronil, the morpholine fungicides fenpropimorph and tridemorph, and the triazole fungicides etaconazole, diclobutrazol and triadimefon, gave similar results but varied in effective concentrations. However, incubation of mycelium with 300 μM -tridemorph gave abnormal uptake patterns. Selected data are presented in Fig. 2.

In order to show that low uptake or accumulation of [^{14}C]fenarimol after incubation with sterol biosynthesis inhibitors is not due to non-specific damage of cell membranes, mycelium was treated with oligomycin (0.1 mM) and SLS (10 mM) 65 and 105 min after addition of [^{14}C]fenarimol. Oligomycin treatment always resulted in an immediate enhanced uptake of [^{14}C]fenarimol. In contrast, SLS treatment instantaneously caused leakage of the fungicide from the mycelium into the medium. Addition of oligomycin to mycelium treated with fenpropimorph or tridemorph always resulted in relatively high levels of [^{14}C]fenarimol uptake (Fig. 2).

Fenarimol-efflux inducing potency of other site-specific fungicides. Similar experiments were carried out with fungicides with an unrelated mechanism of action, such as the benzimidazoles carbendazim and thiabendazole, and carboxin, chloroneb and pimaricin. All such fungicides tested, except pimaricin, failed to induce fenarimol-efflux activity. Examples are given in Fig. 3. Only at the highest carboxin concentration tested (300 μM) was [^{14}C]fenarimol uptake reduced to some extent. Incubation of mycelium with 100 or 300 μM -pimaricin resulted in relatively low [^{14}C]fenarimol uptake levels, even when the antibiotic was added simultaneously with the radiochemical (Fig. 3).

Survival of mycelium. During the [^{14}C]fenarimol-efflux induction experiments, the toxicity of the different fungicides was assessed by measuring growth of washed mycelium taken from the mycelial suspensions. Neither [^{14}C]fenarimol nor any of the test fungicides inhibited growth in comparison with the control treatment, indicating no lethal or highly toxic effect. However, treatment with oligomycin reduced radial growth by about 35%, and with additional SLS, by about 60%. Combined treatment with pimaricin and oligomycin was lethal.

Antagonism of toxicity of fenarimol. Induction of fenarimol-efflux activity by fungicides inhibiting sterol biosynthesis (including fenarimol) prevented subsequent uptake of [^{14}C]fenarimol. This may mean that under these conditions these fungicides have an antagonistic effect towards toxicity of fenarimol. To test this hypothesis, efflux activity was induced by placing paper strips containing the test fungicides on agar plates seeded with *A. nidulans* conidia, 10 h before the strips containing fenarimol were applied (treatment II). In control experiments the strips were applied simultaneously (treatment I). In treatment I almost all the sterol biosynthesis inhibitors tested did not interfere with toxicity of fenarimol while the same chemicals in treatment II always antagonized fenarimol toxicity. Fenpropimorph and

Fig. 2. Effect of the test fungicides nuarimol (a, b), phenapronil (c, d), triadimefon (e, f) and tridemorph (g, h) on uptake of ^{14}C fenarimol by *Aspergillus nidulans*. ^{14}C fenarimol was added at zero time. Test fungicides were added simultaneously (b, d, f, h), or 90 min before addition of ^{14}C fenarimol (a, c, e, g). Concentrations of test fungicides: 0 ($\times$), 30 μM (o), 100 μM ($\square$) and 300 μM (Δ). Oligomycin (0.1 mM) and SLS (10 mM) were added 65 and 105 min after addition of ^{14}C fenarimol, respectively (arrows).

Fig. 3. Effect of the test fungicides carbendazim (a, b), carboxin (c, d), chloroneb (e, f) and pimarin (g, h) on uptake of $[^{14}\text{C}]$ fenarimol by *Aspergillus nidulans*. $[^{14}\text{C}]$ fenarimol was added at zero time. Test fungicides were added simultaneously (b, d, f, h), or 90 min before addition of $[^{14}\text{C}]$ fenarimol (a, c, e, g). Concentrations of test fungicides: 0 (x), 30 μM (O), 100 μM ($\square$) and 300 μM (Δ). Oligomycin (0.1 mM) and SLS (10 mM) were added 65 and 105 min after addition of $[^{14}\text{C}]$ fenarimol, respectively (arrows).

tridemorph were only antagonistic in treatment I and did not interfere with fenarimol toxicity in treatment II. The cross-paper experiment with triadimefon and fenarimol is shown in Fig. 1.

DISCUSSION

Incubation of mycelium of *A. nidulans* for 90 min with structurally unrelated fungicides inhibiting sterol biosynthesis caused a relatively low uptake of [^{14}C]fenarimol (Fig. 2). The low uptake cannot be ascribed to competition of these fungicides with influx of fenarimol from medium into mycelium since upon simultaneous addition of these fungicides with [^{14}C]fenarimol no effect on [^{14}C]fenarimol influx was observed. Neither is the low uptake due to non-specific damage of cell membranes, since fenarimol and the test fungicides did not affect the viability of the mycelium, and the addition of oligomycin was followed by a significant increase in uptake (Fig. 2), indicating that energy metabolism and membrane processes do operate under these conditions. Most probably, low uptake is caused by an induced energy-dependent efflux activity for fenarimol. Oligomycin is inhibitory towards this activity, as a result of which uptake is only determined by resulting passive influx (De Waard & Van Nistelrooy, 1980). Addition of SLS, which does damage cell membrane permeability, caused non-specific leakage of [^{14}C]fenarimol (Fig. 2) and is responsible for the slow growth of SLS-treated mycelium on agar.

The results of the cross-paper experiments substantiate the hypothesis, based on the results mentioned above, that induction of [^{14}C]fenarimol efflux activity by fungicides inhibiting sterol biosynthesis antagonizes fenarimol toxicity (Fig. 1).

The uptake patterns of [^{14}C]fenarimol upon incubation with tridemorph and fenpropimorph were slightly different from those obtained with other sterol biosynthesis inhibitors (Fig. 2). This may be related to the fact that tridemorph inhibits $\Delta^8\text{--}\Delta^7$ isomerization in sterol biosynthesis rather than C-14 demethylation (Kato *et al.*, 1980). Fenpropimorph may have a similar effect. Their antagonistic activity towards toxicity of fenarimol in cross-paper experiments was also distinct. However, this may simply be due to extraction of fenarimol into undissolved residues of fenpropimorph and tridemorph. Both chemicals are liquids and have a low water solubility.

The capability of fungicides inhibiting sterol biosynthesis to induce [^{14}C]fenarimol efflux is rather specific, since compounds with a non-related mechanism of action, except for pimarin, lack this ability (Fig. 3). The highest concentrations of pimarin tested reduced uptake of [^{14}C]fenarimol, irrespective of whether pimarin was added 90 min before or simultaneously with [^{14}C]fenarimol. Probably pimarin, like sterol biosynthesis inhibitors, reduces the effective ergosterol content of cell membranes by formation of pimarin-ergosterol complexes (De Kruyff *et al.*, 1974). In any case, reduced uptake after incubation with pimarin was not due to non-specific damage of cell membranes, since addition of oligomycin induced [^{14}C]fenarimol accumulation.

The nature of the energy-dependent efflux mechanism is not known. Pimarin and inhibitors of sterol biosynthesis, by interfering with membrane sterols or phospholipids, respectively (De Kruyff *et al.*, 1974; Buchenauer, 1980), might affect the membrane lipid physical state and hence membrane ATPase activity (De Kruyff *et al.*, 1973; Silvius & McElhaney, 1980). Membrane ATPase activity drives many transport processes (Slayman *et al.*, 1973; Scarborough, 1976). However, no evidence can, as yet, be provided for the view that enhanced ATP hydrolysis is responsible for increased [^{14}C]fenarimol-efflux activity. Circumstantial evidence in favour of the involvement of ATPase in [^{14}C]fenarimol efflux is suggested by observations that apparently reduced ATP levels inhibit [^{14}C]fenarimol-efflux activity (De Waard & Van Nistelrooy, 1980).

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