Biochem. J. (1988) 256, 829–834 (Printed in Great Britain)



# Inhibition by the fungicide fenpropimorph of cholesterol biosynthesis in 3T3 fibroblasts

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Fenpropimorph {*N*-[3-(*p*-t-butylphenyl)-2-methylpropyl]-*cis*-2,6-dimethylmorpholine}, a morpholine fungicide known to be an inhibitor of sterol biosynthesis in fungi and in higher plants, was demonstrated to be an efficient inhibitor of cholesterol biosynthesis in cultured Swiss 3T3 fibroblasts. Treatment of the mammalian cells with fenpropimorph resulted in a dose-dependent inhibition of [$^{14}$C]acetate incorporation into the $C_{27}$ sterols [$IC_{50}$ (concentration causing half-maximal inhibition) = 0.5 $\mu$M], which was accompanied by an accumulation of polar sterols and a decrease in cellular hydroxymethylglutaryl-CoA reductase activity. Exposure of the cells to the drug affected cell growth. Analysis of the sterols in the growth-arrested and in the pulse-labelled cells indicate that fenpropimorph has, in the sterol-biosynthetic pathway, target enzymes in mammalian cells different from those in the other phyla. Whereas in plants and fungi fenpropimorph mainly affects sterol isomerases and reductases, in the fibroblasts its main target seems to be the demethylation of lanosterol.

## INTRODUCTION

Morpholine derivatives such as tridemorph and fenpropimorph have been widely used during recent decades as systemic agricultural fungicides, particularly against powderly mildew and rust diseases of cereals (Pommer, 1984). They act primarily on the sterol (i.e. ergosterol) biosynthesis in fungi by inhibiting the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and the $\Delta^{8,14}$-sterol $\Delta^{14}$ reductase steps (Leroux & Gredt, 1983; Baloch & Mercer, 1987). These fungitoxic molecules, however, were also found to interfere with the normal synthesis of the sterols of the host plants by blocking the cycloeucalenol:obtusifoliol isomerase and the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase (Benveniste *et al.*, 1984). Such results can be rationalized by assuming that the positive charge, carried by the morpholine ring at physiological pH, could mimic carbocationic intermediates occurring during these enzyme-catalysed reactions (Rahier *et al.*, 1986). Comparatively little has been reported on the effect of these compounds on mammalian systems (Mercer *et al.*, 1985), and it was of interest to determine their possible target(s) in the biosynthesis of cholesterol.

The present study describes the effect of fenpropimorph on the synthesis of cholesterol in cultured 3T3 fibroblasts. The fungicide was found to be a potent inhibitor of acetate incorporation into cholesterol; this effect was correlated with an accumulation of polar sterols and the repression of HMG-CoA reductase activity.

## MATERIALS AND METHODS

### Chemicals

Sodium [2-$^{14}$C]acetate (48 mCi/mmol) was purchased from C.E.A., Saclay, France; DL-[2-$^3$H]mevalonic acid lactone (1 Ci/mmol) and 3-hydroxy-3-methyl[3-$^{14}$C]-glutaryl-CoA (60 mCi/mmol) were from Amersham (Les Ulis, France). (*R,S*)-2,3-oxido[3-$^3$H]squalene was a gift from Dr. L. Cattel (University of Torino, Torino, Italy). (*R,S*)-Fenpropimorph and its *N*-oxide derivative were from Dr. E. H. Pommer (BASF, Ludwigshafen, Germany) and ketoconazole was a gift from Dr. H. Vanden Bossche (Janssen, Beerse, Belgium).

### Cell cultures and sterol synthesis

Swiss 3T3 fibroblasts were routinely maintained as monolayers in Dulbecco's modified Eagle's medium and cultured in a delipidated growth medium for [2-$^{14}$C]-acetate-incorporation studies essentially as described previously (Gerst *et al.*, 1986). In brief, cells derived from stock cultures were seeded at about $3 \times 10^5$ cells per 60 mm-diameter plastic Petri dish in 5 ml of medium containing 10% (v/v) delipidated serum. On the second day of culture the cells were pretreated, in duplicate, for indicated times (generally 2 h) with given amounts of inhibitor. Labelled acetate (10 $\mu$Ci/dish; i.e., 0.21 $\mu$mol) was then added. After 2 h of incubation the medium was removed and the dishes washed three times with phosphate-buffered saline (6.48 mM-$Na_2HPO_4$/1.47 mM-$KH_2PO_4$/2.68 mM-KCl/136.75 mM-NaCl, pH 7.4). Isolation of the non-saponifiable lipids, separation of the sterols and squalene oxides by t.l.c., determination of radioactivity associated with the t.l.c. zones, acetylation of the sterols and analysis by g.l.c.–m.s. were performed according to published procedures (Gerst *et al.*, 1986; Costet *et al.*, 1987). Analysis of the $C_{27}$-sterol fractions by thin-layer argentation chromatography has been described elsewhere (Gerst *et al.*, 1988). Separation of the polar sterols was performed by t.l.c. on silica-gel plates using benzene/ethyl acetate/acetic acid (60:40:1, by vol.) as solvent (Maerker & Bunick, 1986). The results given in the Figures and Tables are means of duplicates which do not differ by more than 10%. The incorporation of [2-$^{14}$C]acetate into the fatty acid fraction was determined according to Kandutsch & Saucier (1969).

Abbreviations used: fenpropimorph, *N*-[3-(*p*-t-butylphenyl)-2-methylpropyl]-*cis*-2-6-dimethylmorpholine; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA.

### Treatment of the polar sterols with sodium borohydride

Fibroblasts which had been pretreated for 2 h with 1 μM-fenpropimorph or oxoconazole ('ketoconazole') were labelled for 2 h with [2-$^{14}$C]acetate. Polar sterols, i.e. sterols migrating below cholesterol, were first isolated from the non-saponifiable fractions by t.l.c. on silica-gel 60 $F_{254}$ plates (Merck) using a double migration in hexane/ethyl acetate (17:3, v/v). They were then reduced with $NaBH_4$ (10 mg/1 ml of methanol). After 3 h at room temperature, the reaction mixture was treated with 1 ml of $NaHCO_3$ (10%, w/v); the polar sterols were then extracted with n-hexane and re-chromatographed on silica plates using benzene/ethyl acetate/acetic acid (60:40:1, by vol.) as solvent.

### Enzyme activities

The HMG-CoA reductase activity of cell extracts was measured as described previously (Gerst *et al.*, 1988); the effect of fenpropimorph on the 2,3-oxidosqualene:lanosterol cyclase activity, associated to pig liver microsomes (microsomal fractions), was determined as described previously (Duriatti *et al.*, 1985).

## RESULTS

### Effect of fenpropimorph on sterol biosynthesis

Swiss 3T3 fibroblasts when grown in the presence of delipidated fetal-calf serum actively incorporated [2-$^{14}$C]acetate into cholesterol during their exponential phase of growth (Gerst *et al.*, 1986). When the cells were treated with 10 μM-fenpropimorph, the incorporation of the radiolabel into the $C_{27}$-sterol fraction was decreased by about 85%. This dramatic effect was observed whether the cells were pretreated with the drug between 0 and 8 h before receiving [$^{14}$C]acetate, indicating that fenpropimorph must be rapidly taken up by the fibroblasts. Half-maximal inhibition of cholesterol biosynthesis was determined by using a 2 h preincubation time (Fig. 1); a value for $IC_{50}$ of 0.5 μM was obtained.

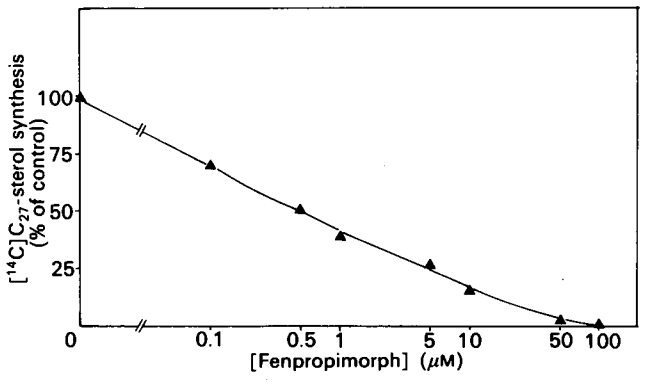


**Fig. 1. Influence of fenpropimorph on $C_{27}$-sterol biosynthesis in 3T3 fibroblasts**

The cells, seeded at $3 \times 10^5$ cells/60 mm dish, and cultured in 5 ml of a delipidated growth medium for 48 h were incubated for 2 h in the presence of the indicated concentrations of the drug. [2-$^{14}$C]Acetate (10 μCi) was then added to the medium and, after 2 h, the incorporation into $C_{27}$ sterols was determined. The results, which are means of duplicates, are expressed as percentages of [$^{14}$C]$C_{27}$ sterols measured in the absence of drug [100% corresponds to $3.0(\pm 0.2) \times 10^4$ d.p.m./mg of cell protein].

**Table 1. Analysis by t.l.c. of the incorporation of [$^{14}$C]acetate into sterols and polar sterols: effect of fenpropimorph**

3T3 Fibroblasts were grown in a delipidated medium. After 2 days they were preincubated for 2 h with the drug and labelled with [$^{14}$C]acetate (see the Materials and methods section and the legend to Fig. 1). After 2 h the cells were then harvested and the non-saponifiable fraction analysed by t.l.c. on silica plates using hexane/ethyl acetate (17:3, v/v) as solvent. The results, which are means of duplicates, are given as percentages of the radioactivity associated with the respective non-saponifiable fraction.

| Supplement | Associated radioactivity (%) | | [$^{14}$C]Acetate incorporation‡ |
|---|---|---|---|
| | Polar sterols* | Total sterols† | |
| None | 12 | 88 | 100 |
| Fenpropimorph | | | |
| 0.5 μM | 66 | 34 | 79 |
| 1.0 μM | 76 | 24 | 72 |
| 10 μM | 71 | 29 | 66.5 |

* Label associated with the chromatographic zone between the $C_{27}$ sterols and the baseline.

† Label associated with the sterols and squalene oxides; in the control experiment the following distribution was observed: $C_{27}$ sterols, 73%; 4α-sterols, 4%; 4,4-dimethylsterols, 5.5%; squalene epoxides, 5.5%.

‡ Label recovered in the non-saponifiable fraction compared with the control [%; 100% corresponds to $2.58(\pm 0.08) \times 10^4$ d.p.m./mg of cell protein].

In order to identify the step(s) where fenpropimorph interferes with the synthesis of cholesterol, we have analysed by t.l.c. the non-saponifiable lipid extracts of the treated cells. Exposure of the fibroblasts to increasing concentrations of the drug decreased the extent of incorporation of [$^{14}$C]acetate into the non-saponifiable fraction (Table 1) and provoked a redistribution of the label among the different sterol fractions. The major effect observed was a progressive decrease in [$^{14}$C]acetate incorporation into the sterols, which was correlated with an increased accumulation of sterols more polar than cholesterol (Table 1). Besides this accumulation of polar sterols, the drug affected the distribution of the sterols and their precursors after [$^{14}$C]acetate labelling; this is indicated in Fig. 2. Compared with the control, the relative percentages of the [$^{14}$C]$C_{27}$-sterols decrease to a limited extent in favour of 4α-methylsterols, 4,4-dimethylsterols and 2,3-oxidosqualene (very minor) and overwhelmingly in favour of 2,3:22,23-dioxidosqualene. However, because of the low incorporation of the label into these fractions, especially at the higher drug concentrations (see Table 1), this redistribution does not reveal a particular target of fenpropimorph. Moreover, because of the paucity of radiolabelled material, we could not fully identify the different sterols present in the chromatographic zones of Fig. 2 (see, however, below).

The polar sterols accumulated in cells treated with fenpropimorph could in principle originate from the inhibition of two main targets in the cholesterol-biosynthetic pathway: (i) the 2,3-oxidosqualene:lanosterol cyclase, which through the accumulation of 2,3-oxido-

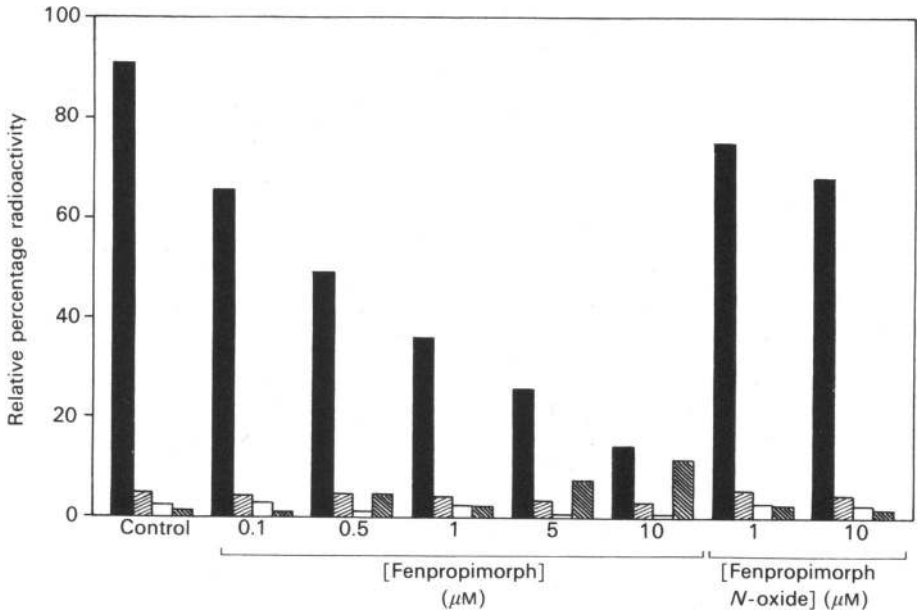


**Fig. 2. Influence of fenpropimorph and its *N*-oxide derivative on the incorporation of [$^{14}$C]acetate into sterols and precursors**

The non-saponifiable lipids obtained from 3T3 fibroblasts that had been treated with the drugs under conditions identical with those of Fig. 1 were analysed by t.l.c. and the different zones counted for radioactivity. The results are means for two experiments. In the control cells, the radioactivity associated with each fraction is given as the percentage of the sum of the radioactivities recovered in the different fractions: ■, $C_{27}$ sterols; ▨, 4α-methylsterols; □, 4,4-dimethylsterols; and ▧, 2;3:22,23-dioxidosqualene. This sum, i.e. 100%, corresponds to $3.05 (\pm 0.35) \times 10^4$ d.p.m./mg of cell protein. In the treated cells the results are given as relative percentages based on this 100% value.

squalene and 2,3:22,23-dioxidosqualene, its oxidized metabolite, can ultimately generate 24(*S*),25-epoxycholesterol (Saucier *et al.*, 1985) and (ii) the cytochrome *P*-450-dependent lanosterol 14α-methyl demethylase, whose inhibition, e.g. by oxoconazole, can yield oxidized intermediates such as the C-32-hydroxy and C-32-aldehyde derivatives (Trzaskos *et al.*, 1986). We have analysed, by t.l.c. (see the Materials and methods section), the polar sterols accumulated in the fibroblasts which had been treated for 2 h with fenpropimorph (1 μM) or oxoconazole (1 μM) before $^{14}$C labelling. Both compounds inhibited the synthesis of $C_{27}$-sterols to a similar extent (about 75%), and in both cases three major polar sterol bands were observed at $R_F$ 0.15 (broad band), 0.33 and 0.57 ($R_F$ of lanosterol 0.62). For both fenpropimorph and oxoconazole the band at $R_F$ 0.33 was the major one, i.e. it accounted for about 40% of the radioactivity recovered in the polar sterols. When the polar-sterol fractions were reduced by treatment with $NaBH_3$, the compound(s) migrating at $R_F$ 0.57 declined in favour of compound(s) at $R_F$ 0.33 (Table 2). The polar sterols mentioned above are known to be repressors of HMG-CoA reductase (Panini *et al.*, 1986), the regulatory enzyme of cholesterol biosynthesis. We therefore tested to see whether fenpropimorph could also inhibit cellular HMG-CoA reductase. This was found to be the case, i.e. in cells treated under the usual conditions for 4 h with 0.1 and 1.0 μM-fenpropimorph, the reductase activity was decreased to respectively 84 and 46% of control (the activity in the control cells was 34 pmol·min$^{-1}$·mg of protein$^{-1}$). In control experiments we showed that the drug did not in itself inhibit the enzyme *in vitro* (i.e. the microsomal enzyme). This rapid and important inhibition of the cellular HMG-CoA reductase could in part explain the decreased incorporation of labelled acetate observed in cells treated with the fenpropimorph concentrations mentioned above. In favour of this hypothesis, we have found that the drug did not, by contrast, affect the extent of [$^{14}$C]mevalonate incorporation into the non-saponifiable fractions (results not shown).

To verify that the cellular effects of fenpropimorph were specific for the sterol-biosynthetic pathway or were

**Table 2. Analysis by t.l.c. of the polar sterols: effect of reduction by $NaBH_4$**

Cells grown in a delipidated medium were preincubated for 2 h with 1 μM-fenpropimorph or -oxoconazole and labelled with 20 μCi of [$^{14}$C]acetate (see the legend to Fig. 1). The polar-sterol fractions were isolated and analysed, before and after reduction by $NaBH_4$, by t.l.c. on silica plates using benzene/ethyl acetate/acetic acid (60:40:1, by vol.) as solvent (see the Materials and methods section). The results, which are means of duplicates, are given as percentages of total radioactivity recovered in the polar-sterol fractions [$2.97 (\pm 0.08) \times 10^4$ d.p.m. and $1.90 (\pm 0.02) \times 10^4$ d.p.m./mg of cell protein respectively in the control and reduced fractions].

| Polar-sterol fraction $R_F$ | Radioactivity recovered (%) | | | |
|---|---|---|---|---|
| | Fenpropimorph | | Oxoconazole | |
| | Control | Reduced | Control | Reduced |
| 0.15 | 37.5 | 30 | 21 | 26 |
| 0.33 | 42.5 | 67 | 42 | 64 |
| 0.57 | 20 | 3 | 37 | 10 |

due to a general toxicity, we examined the action of the drug on the incorporation of [$^{14}$C]acetate into fatty acids. No alterations were observed with fenpropimorph concentrations between 0.1 and 10 $\mu$M; in the 50–100 $\mu$M range a decreased incorporation was noted, e.g. 50% at 100 $\mu$M. This could be related to a toxic effect, since, when the cells were exposed to such concentrations for several hours, they started to detach from the substrate. Since fenpropimorph is an amphiphilic molecule, which might also act by disturbing membrane integrity, we have checked its effect on 5(6)-carboxyfluorescein-loaded phosphatidylcholine liposomes (Gerst *et al.*, 1986); no leakiness was induced by the drug up to 100 $\mu$M (results not shown).

We also investigated the effect on the sterol biosynthesis of longer exposures of the fibroblasts to fenpropimorph. When the cells were treated with the drug up to 24 h, no significant changes in [$^{14}$C]acetate incorporation were observed compared with the effects already measured after 2 h treatment. In principle, fenpropimorph could be metabolized by the cells into, e.g., its *N*-oxide, and alter its inhibitory properties. Interestingly, the effect of fenpropimorph *N*-oxide on the biosynthesis of cholesterol in the fibroblasts was less striking (Fig. 2). By contrast, in plants this molecule remains very inhibitory, both *in vivo* and *in vitro*, to the enzymic targets of fenpropimorph (Costet-Corio & Benveniste, 1988). At present we do not know why fenpropimorph *N*-oxide is only modestly active in mammalian cells; several possibilities could be envisaged, including: (i) this molecule does not readily enter the cells; (ii) the oxidized form has lost the inhibitory properties of the parent molecule (in this case we must assume that the fibroblasts are incapable of transforming fenpropimorph into its *N*-oxide).

### Effect of fenpropimorph on cellular sterols

Because, in the studies of the incorporation of [$^{14}$C]-acetate into cholesterol, we could not readily identify the enzymic targets of fenpropimorph, we investigated the effect of the drug treatment on the cellular sterol profile. When the fibroblasts were exposed for 48 h to 10 $\mu$M-fenpropimorph, a 35% decrease in cellular $C_{27}$ sterols was observed, i.e. 6.2 $\mu$g instead of 9.5 $\mu$g/mg of cell protein in control cells. Under similar conditions the *N*-oxide derivative of fenpropimorph was much less effective, i.e. a 7% decrease was found. The $C_{27}$-sterol fraction of the cells treated with fenpropimorph, when analysed by g.c.–m.s., revealed only the presence of cholesterol (99%) and desmosterol (1%), i.e. similar to the control cells, and no accumulation of $\Delta^8$ sterols could be detected. In the treated cells, in addition to the $C_{27}$ sterols, a 4,4-dimethylsterol band was detectable. It accounted for 32% of the total sterols, i.e. 2.8 $\mu$g/mg of protein, compared with 1.2 $\mu$g/mg of protein in the control cells. Analysis by g.c.–m.s. revealed that this fraction was composed of lanosterol and 24,25-dihydrolanosterol in equal amounts, and no $\Delta^{8,14}$-dienes were found.

### Effect of fenpropimorph on cell growth

In Fig. 3(*a*) we show the effect of fenpropimorph (1 $\mu$M) on the growth curve of the fibroblasts. In the presence of the drug the cells have a limited growth capacity, and after day 3, some cell death occurs. In Fig. 3(*b*) we show the cholesterol levels in these cells; cell-growth arrest is correlated with a 50% decrease in cellular cholesterol content. Such cytostatic and cytotoxic effects of cholesterol-biosynthesis inhibitors are well documented (Chang *et al.*, 1979; Gerst *et al.*, 1986). It is noteworthy that the 4,4-dimethylsterols formed in the treated fibroblasts do not compensate for the cholesterol deprivation and do not support cell growth.

## DISCUSSION

This investigation represents the first demonstration that the morpholine fungicide fenpropimorph is a potent inhibitor of cholesterol biosynthesis in mammalian cells. In contrast with fungi and higher-plant systems, where

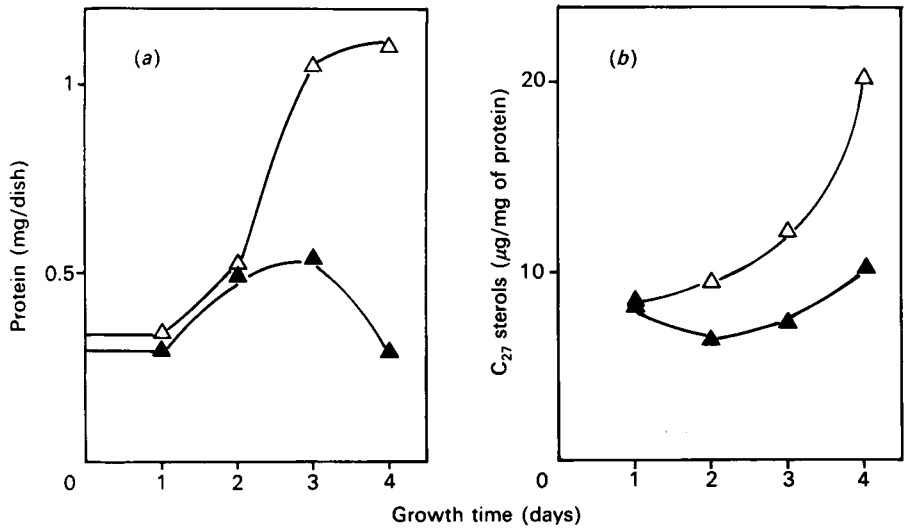


**Fig. 3. Influence of fenpropimorph on growth and on cholesterol content**

3T3 fibroblasts were seeded at $3 \times 10^5$ cells/60 mm dish and cultured in 5 ml of a delipidated growth medium in the absence (△) or presence (▲) of 1 $\mu$M-fenpropimorph. Dishes were harvested in duplicate every day, and cell growth (*a*) was determined. Cellular cholesterol content (*b*) was obtained by g.c. analysis of the $C_{27}$-sterol fractions.

the main enzymic targets of this molecule have been identified as sterol isomerases and reductases (see the Introduction), in the 3T3 fibroblasts fenpropimorph provokes primarily an accumulation of polar sterols and a repression of HMG-CoA reductase activity. Moreover, in 3T3 cells treated for several days with the drug, no accumulation of $\Delta^{8,14}$ sterols of $\Delta^{8}$ sterols could be detected, indicating that the $\Delta^{14}$-sterol reductase and the $\Delta^{8} \rightarrow \Delta^{7}$-sterol isomerase were not major targets of fenpropimorph in these cells. Such modifications in specificity, depending on the phyla, of several compounds interfering with the biosynthesis of sterols have been documented; e.g., with *N*-(1-n-dodecyl) heterocycles (Mercer *et al.*, 1985), decalins such as 4,4,10$\beta$-trimethyl-*trans*-decal-3$\beta$-ol (Duriatti *et al*, 1985) and with antimycotic agents such as oxoconazole (Vanden Bossche, 1986) or naftifine (Ryder & Dupont, 1985).

The main target of fenpropimorph in the fibroblasts remains to be established. Its determination rests on the chemical identification of the polar sterols that are accumulated in the treated cells. Since a correlation exists between their occurrence and a repression of cellular HMG-CoA reductase, it is reasonable to assume, from data in the literature (Panini *et al.*, 1986; Rudney & Sexton, 1986), that they could originate *a priori* from the inhibition of 2,3-oxidosqualene:lanosterol cyclase and/or lanosterol 14$\alpha$-methyl demethylase. Partial inhibition of the cyclase leads to the formation of the regulatory metabolite 24(*S*),25-epoxycholesterol (Panini *et al.*, 1984). From our cellular data it does not seem that fenpropimorph is a good inhibitor of the mammalian cyclase. In contrast with results obtained recently with specific cyclase inhibitors in the same cellular system (Gerst *et al.*, 1986, 1988), the levels of 2,3-oxidosqualene and 2,3:22,23-dioxidosqualene in the fenpropimorph-treated cells represent only minor amounts of the total non-saponifiable fraction. Moreover, when tested *in vitro* on a pig liver microsomal 2,3-oxidosqualene:lanosterol cyclase, this fungicide was found to be a poor inhibitor, i.e. $K_i \gg 10\ \mu M$ (data not shown). Antimycotic azole compounds such as oxoconazole, which were recently also found to interfere with lanosterol 14$\alpha$-methyl demethylation in animal cells, promote the accumulation of oxygenated reaction intermediates (Favata *et al.*, 1987). 3$\beta$-Hydroxy-5$\alpha$-lanosta-8,24-dien-32-al, a putative regulator of the cellular HMG-CoA reductase, was identified in hepatocytes (Trzaskos *et al.*, 1987). It is tempting to speculate that fenpropimorph, which contains a morpholine residue, could also interfere with these oxidative processes. In a preliminary analysis by t.l.c. we have noted a similarity between the polar sterols accumulated in fenpropimorph- and oxoconazole-treated cells. Since the least polar one could be converted by reduction into a more polar compound (Table 2), it seems plausible that fenpropimorph could also provoke the accumulation of 3$\beta$-hydroxy-5$\alpha$-lanosta-8,24-dien-32-al. It remains, however, intriguing that fenpropimorph does not induce, in contrast with oxoconazole (Favata *et al.*, 1987), an important accumulation of lanosterol during the pulse-labelling experiments. Under our experimental conditions, 3T3 fibroblasts treated with 1 $\mu$M-oxoconazole also accumulate 4,4-dimethylsterols, which account for approx. 35% of the non-saponifiable fraction (results not shown). Lanosterol and 24,25-dihydrolanosterol are only accumulated after longer exposure of the cells to fenpropimorph. Only a full identification of the constituents of the polar sterols fraction and a determination of the inhibitory activity of fenpropimorph towards, e.g., the lanosterol 14$\alpha$-methyl demethylase will permit a better understanding of the mode of action of this drug. Finally, the fact that fenpropimorph affects targets in mammalian cells different from those in fungi and higher plants could also explain why its *N*-oxide derivative is, in contrast with the situation in these latter phyla, much less inhibitory towards cholesterol biosynthesis in the fibroblasts. In our hypothesis this would mean that the *N*-oxidation of fenpropimorph would decrease its inhibitory properties in the cytochrome *P*-450-dependent demethylation reaction and therefore constitute a detoxification process.

At concentrations lower than 10 $\mu$M, which already drastically affect cholesterol biosynthesis, fenpropimorph does not inhibit the incorporation of [$^{14}$C]acetate into fatty acids. It seems, therefore, that the only previous documented effects of this drug on mammalian systems, i.e. inhibition of mitochondrial succinate:cytochrome *c* oxidoreductase and of NADH oxidase (Müller & Schewe, 1976), which occur at elevated concentrations of the drug, are not relevant to our observations on cholesterol-biosynthesis inhibition. The cytotoxic effects we have seen at high concentrations of fenpropimorph could be due, however, to such an inhibition of electron-transfer reactions.

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Received 8 March 1988/24 June 1988; accepted 30 June 1988