Effect of Allylamine Antimycotic Agents on Fungal Sterol Biosynthesis Measured by Sterol Side-chain Methylation

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Sterol side-chain (C-24) methylation was assayed by incorporation of radioactivity from [Me - ^{14}C]methionine into the ergosterol fraction in cells of the pathogenic fungi *Candida albicans*, *Candida parapsilosis* and *Trichophyton mentagrophytes*. Methylation at C-24 occurred after nuclear demethylation in all cases. The method was used to measure ergosterol biosynthesis inhibition by the allylamine antimycotics naftifine and SF 86-327, which are known to block squalene epoxidation. In *C. albicans* cells treated with SF 86-327 (1 mg l^{-1}) to fully inhibit squalene epoxidation, C-24 methylation continued for several hours at about 40% of the control rate. This residual biosynthesis was probably due to methylation of endogenous sterol precursors. The degree of residual biosynthesis in the three fungi correlated well with their susceptibility to SF 86-327. The highly susceptible dermatophyte *T. mentagrophytes* had negligible residual sterol biosynthesis. These differences were not due to inhibition of methionine uptake. For naftifine (100 mg l^{-1}) there was evidence of a second inhibitory action in *C. albicans*. A cell-free assay indicated that this was due to direct inhibition of the C-24 methyltransferase.

INTRODUCTION

The allylamines, a recently developed class of antifungal agents, include the topical antimycotic naftifine (Georgopoulos *et al.*, 1981) and the more potent, orally active derivative SF 86-327 (Petranyi *et al.*, 1984). Initial studies showed that naftifine inhibited ergosterol biosynthesis in several fungi, acting at the point of squalene epoxidation (Paltauf *et al.*, 1982; Ryder & Troke, 1982). Inhibition of the enzyme squalene epoxidase has been confirmed as the primary mode of action of both naftifine and SF 86-327 (Ryder, 1984; Petranyi *et al.*, 1984). The activity of the allylamines is exceptionally high against dermatophytes and rather variable against yeasts such as *Candida* species (Georgopoulos *et al.*, 1981; Petranyi *et al.*, 1984). This variability was not fully explained by differences in the sensitivity of squalene epoxidation in these fungi (Ryder, 1984, 1985). To help clarify this point, an assay was required to measure ergosterol biosynthesis at the end of the pathway in fungal cells. In fungi and plants, but not in mammals, alkylation occurs at C-24 of the sterol side-chain late in the pathway (Goad *et al.*, 1974). The reaction is catalysed by a methyltransferase with *S*-adenosylmethionine as the methyl donor (Parks, 1958). There are a number of reports on C-24 methylation by cells of *Saccharomyces cerevisiae* using [Me - ^{14}C]methionine as a methyl donor (Starr & Parks, 1972; Osumi *et al.*, 1978; McCammon & Parks, 1981), but no report of this reaction occurring in pathogenic fungi. The present communication describes the characterization of an assay for C-24 methylation in cells of *Candida albicans* and other pathogens, and its use in studying the mechanism of action of the allylamines.

METHODS

Organisms and growth conditions. The strains used were *Candida albicans* $\Delta 63$ (a clinical isolate), *C. parapsilosis* ATCC 46589 and *Trichophyton mentagrophytes* CBS 56066. Stock cultures were maintained in liquid nitrogen as described by Georgopoulos *et al.* (1981). Yeast cells of the *Candida* strains were grown in shake cultures on

Sabouraud dextrose broth, pH 6.5, as described previously (Ryder *et al.*, 1984). Late exponential phase cells (18 h) were harvested and washed by centrifugation. *T. mentagrophytes* shake cultures were grown for 90 h and harvested by gravity filtration on a glass sinter.

Antimycotic agents. The allylamines naftifine and SF 86-327 were synthesized at the Sandoz Forschungsinstitut, Vienna (Stutz & Petranyi, 1984).

Measurement of ergosterol biosynthesis in whole cells. Washed cells were incubated with radiolabelled substrates as described by Ryder *et al.* (1984). The incubation medium A was yeast nitrogen base (Oxoid), without amino acids or $(\text{NH}_4)_2\text{SO}_4$, containing 1% (w/v) glucose and adjusted to pH 6.5. Cells were incubated at different pH values in medium B, which contained 25 mM-potassium phosphate buffer and 1% (w/v) glucose. Test compounds were added dissolved in ethanol (final concentration 1%, v/v) and preincubated for 10 min with the cells.

For measurement of sterol side-chain methylation, the substrate was L-[$\text{Me-}^{14}\text{C}$]methionine [Amersham, 1 μCi (37 kBq) per 5 ml incubation]. Unlabelled L-methionine was added to give a final concentration of 1 mmol l^{-1} with *Candida* cells or 0.25 mmol l^{-1} with *T. mentagrophytes*. Parallel incubations were done using as substrate [U- ^{14}C]acetate (Amersham), final specific activity 0.4 Ci (14.8 GBq) mol^{-1} (Ryder *et al.*, 1984). The standard incubation period was 3 h. Subsequent procedures for harvesting cells, extracting non-saponifiable lipids, isolating sterols by thin layer chromatography and counting radioactivity were as previously described (Ryder *et al.*, 1984; Ryder, 1985).

Methionine uptake by cells. Incubations with [$\text{Me-}^{14}\text{C}$]methionine were as described above. At intervals, 1 ml samples were removed and collected on glass fibre filters (Whatman GFA, 25 mm) under vacuum. Filters were washed with medium containing 1 mM-methionine, dried and counted for radioactivity, with correction for zero time controls.

Sterol side-chain methylation in cell-free extracts. Two assays were used, similar to those described by Nishino *et al.* (1980, 1981). (a) Cell-free extracts of *C. albicans* and *C. parapsilosis* were prepared and incubated with cofactors as described by Ryder *et al.* (1984), except that the substrate was either L-[$\text{Me-}^{14}\text{C}$]methionine [56.7 Ci (2.1 TBq) mol^{-1} , 1 μCi (37 kBq) ml^{-1}] or S-adenosyl-L-[$\text{Me-}^{14}\text{C}$]methionine [Amersham, 0.5 Ci (18.5 GBq) mol^{-1} , 0.125 μCi (4.625 kBq) ml^{-1}]. (b) Washed microsomal membranes of *C. albicans* were prepared in 50 mM Tris/HCl buffer, pH 7.5, containing 0.5 mM-dithiothreitol, by the method of Ryder & Dupont (1984). Samples (0.5 ml) for incubation contained 2.2 mg microsomal protein, 0.2 μCi (7.4 kBq) S-adenosyl-L-[$\text{Me-}^{14}\text{C}$]methionine and 5 mM- MgSO_4 in Tris buffer as above. Subsequent procedures were as for cell-free extracts (Ryder *et al.*, 1984).

Experimental design. For measurement of the incorporation of label into sterols, a suitable quantity of [1,2- ^3H]cholesterol (Amersham) was added as an internal standard to correct for losses. The ^{14}C -labelled products were measured by dual-label scintillation counting (Ryder *et al.*, 1984). All experiments included at least three replicate incubations per treatment, with standard deviations routinely within 10% of the mean value. All experiments were repeated to obtain consistent results.

RESULTS

Sterol side-chain methylation in C. albicans cells

C. albicans cells rapidly incorporated radioactivity from [$\text{Me-}^{14}\text{C}$]methionine into sterols, the rate being linear with time for at least 3 h (Fig. 1). More than 95% of the radioactivity incorporated into the total non-saponifiable lipids was recovered in the ergosterol fraction, with only trace incorporation into squalene and 4-methyl sterols (Table 1). This pattern of labelling was confirmed by autoradiography of thin-layer separations of the non-saponifiable lipids as described by Ryder *et al.* (1984); only the band corresponding to ergosterol was labelled. The lack of significant incorporation into squalene (Table 1) indicates that randomization of the label did not occur and that incorporation was solely into the sterol side-chain. Treatment of *C. albicans* cells with SF 86-327 (1 mg l^{-1}) fully inhibited squalene epoxidation (see below) so that any randomized label would have accumulated in the squalene fraction in these cells. Treatment of cells with ketoconazole (1 mg l^{-1}), which blocks lanosterol 14 α -demethylation (Van den Bossche *et al.*, 1980), caused only a slight accumulation of label in the 4,4-dimethylsterol fraction (Table 1).

The radiolabelled product of [$\text{Me-}^{14}\text{C}$]methionine incorporation (ergosterol fraction) was confirmed as a sterol by precipitation as the sterol digitonide by the method of Popjak (1969). Using [^3H]cholesterol as an internal standard, more than 98% of the product was recovered by this procedure.

Fig. 1. Time course of sterol side-chain methylation in *C. albicans* cells. Incorporation of radioactivity from [*Me*-¹⁴C]methionine into the ergosterol fraction was measured as described in Methods. ○, Control; ●, plus SF 86-327 (1 mg l⁻¹).

Table 1. Distribution of radioactivity incorporated from [*Me*-¹⁴C]methionine into non-saponifiable lipid fractions in *C. albicans* cells

Cells were incubated for 3 h with [*Me*-¹⁴C]methionine in the presence of inhibitors (1 mg l⁻¹) as described in Methods. The non-saponifiable lipids were extracted and the components separated by thin-layer chromatography. Values are means of triplicate incubations.

Treatment	10 ⁻³ × Radioactivity incorporated (d.p.m.)			
	Ergosterol	4 α -Methylsterol	4,4-Dimethylsterol	Squalene
Control	48.4	0.6	0.2	0.1
SF 86-327	14.5	0.7	0.1	0.1
Ketoconazole	7.2	0.6	2.7	0.1

Table 2. Effect of sinefungin and SF 86-327 on sterol synthesis in *C. albicans* cells

Sterol synthesis was measured by incorporation of either [*U*-¹⁴C]acetate or [*Me*-¹⁴C]methionine into the ergosterol fraction as described in Methods. Results are means of triplicate incubations. Control values (d.p.m.) were 49900 (methionine) and 253800 (acetate).

Treatment	Radioactivity incorporated (percentage of control)	
	Acetate	Methionine
Sinefungin (0.1 mmol l ⁻¹)	75.6	62.1
Sinefungin (1.0 mmol l ⁻¹)	88.5	18.4
Sinefungin (5.0 mmol l ⁻¹)	76.2	11.2
SF 86-327 (1 mg l ⁻¹)	2.0	30.6
SF 86-327 (1 mg l ⁻¹) + sinefungin 5.0 mmol l ⁻¹)	0.5	7.1

Sinefungin, a specific inhibitor of transmethylation reactions (McCammon & Parks, 1981) strongly inhibited the side-chain methylation reaction at concentrations > 0.1 mM (Table 2), but had little effect on the biosynthesis of 4-desmethylsterols as measured by acetate incorporation, indicating that side-chain methylation occurred after nuclear demethylation. The inhibitory effects of sinefungin and SF 86-327 were additive when using either substrate (Table 2).

Inhibition by SF 86-327 and naftifine in different fungi

Table 3 shows the inhibitory effect of SF 86-327 and naftifine on sterol biosynthesis in *C. albicans* cells, measured either from the beginning of the pathway (acetate incorporation) or

Table 3. *Effect of naftifine and SF 86-327 on sterol biosynthesis in C. albicans cells*

Sterol synthesis was measured by incorporation of either [U - ^{14}C]acetate or [Me - ^{14}C]methionine into the ergosterol fraction as described in Methods. Results are means of triplicate incubations in medium B. Control values (d.p.m.) were 15400 (methionine) and 45900 (acetate).

Compound	Concn (mg l ⁻¹)	Radioactivity incorporated (percentage of control)	
		Acetate	Methionine
Naftifine	1	12.7	54.8
	10	2.7	37.0
	100	1.0	13.5
SF 86-327	1	1.7	54.5
	10	1.0	41.9
	100	0.9	37.2

Table 4. *Comparison of growth inhibition and residual ergosterol biosynthesis in fungal cells treated with SF 86-327*

Ergosterol biosynthesis was measured by incorporation of radioactivity from [Me - ^{14}C]methionine as described in Methods. Values represent the degree of biosynthesis in cells treated with SF 86-327 (1 mg l⁻¹) as a percentage of that in control cells over a 3 h incubation. They are expressed as means $\pm$ SD (number of separate experiments in parentheses). Susceptibility to *in vitro* growth inhibition by SF 86-327 is expressed as the minimum inhibitory concentration (MIC); the values are from Ryder (1985).

Fungus	Radioactivity incorporated (percentage of control)	MIC (mg l ⁻¹)
<i>C. albicans</i>	39.1 $\pm$ 10.8 (10)	3.1
<i>C. parapsilosis</i>	3.6 $\pm$ 1.0 (5)	0.4
<i>T. mentagrophytes</i>	0.5 $\pm$ 0.4 (6)	0.003

towards the end (side-chain methylation). SF 86-327 caused virtually total inhibition of squalene epoxidation, and hence acetate incorporation, at concentrations above 1 mg l⁻¹. However, a significant degree of ergosterol biosynthesis still occurred when measured by side-chain methylation, a feature also shown in Tables 1 and 2. This residual, reduced rate of biosynthesis was detectable for at least 5 h after addition of inhibitor (Fig. 1) and was not appreciably inhibited by SF 86-327 up to 100 mg l⁻¹ (Table 3). Naftifine had a similar effect (Table 3) but at 100 mg l⁻¹ appeared to cause a further inhibition of side-chain methylation, indicating the possible existence of a second inhibitory action at this higher concentration of naftifine. A lower level of residual biosynthesis was seen in cells treated with ketoconazole (Table 1), which inhibits a later stage in the pathway.

Sterol side-chain methylation from [Me - ^{14}C]methionine in cells of *C. parapsilosis* and *T. mentagrophytes* was similar to the system described in *C. albicans*. The incorporation of label was linear with time for at least 3 h and was exclusively into the ergosterol fraction. SF 86-327 (1 mg l⁻¹) caused full inhibition of squalene epoxidation (acetate incorporation) in cells of all three fungi (Ryder, 1985). However, residual side-chain methylation was reproducibly low in *C. parapsilosis* and virtually absent in *T. mentagrophytes* (Table 4). In these two fungi, inhibition of sterol biosynthesis by SF 86-327 was dose-dependent when measured either by acetate incorporation or by side-chain methylation.

Effect of incubation medium and pH

In the two *Candida* species, measured rates of sterol side-chain methylation were about 1 nmol (mg cell dry weight)⁻¹ h⁻¹ in medium B (pH 6.5) and twice this value in medium A. The respective rates in *T. mentagrophytes* were about 25% of those in *Candida*, reflecting the lower growth rate of the dermatophyte. Choice of medium did not influence the linearity of incorporation with time, or the degree of inhibition by SF 86-327. However, in complete yeast nitrogen base medium (initial pH 6.5), side-chain methylation in *C. albicans* cells was inhibited for only about 1 h in the presence of SF 86-327 (1 mg l⁻¹) and then reverted to the control rate.

Fig. 2. Effect of pH on SF 86-327-induced inhibition of ergosterol biosynthesis in *T. mentagrophytes* cells, measured by sterol side-chain methylation. Inhibition was measured in the presence of SF 86-327 at 0.001 mg l⁻¹ (○), 0.01 mg l⁻¹ (●) and 0.1 mg l⁻¹ (□).

This was found to be due to a rapid acidification of the medium to pH 2.5 as a result of NH₄⁺ uptake into the cells. The pH sensitivity of the inhibitory action of SF 86-327 has previously been shown in *Candida* (Ryder, 1985), by measuring acetate incorporation into sterols. The side-chain methylation technique was used to demonstrate the pH sensitivity of inhibition in *T. mentagrophytes* at low concentrations of SF 86-327 (Fig. 2), with maximal inhibition at pH 8. Inhibition was maximal at pH 6.5 in *C. albicans* cells, measured either by acetate incorporation (Ryder, 1985) or side-chain methylation as described here. Maximal rates of incorporation of radioactivity from [*Me*-¹⁴C]methionine into sterols in control cells occurred at pH 6.5 in *T. mentagrophytes*, at pH 5.0 in *C. parapsilosis* and at pH 3.5 in *C. albicans*.

Role of methionine uptake by cells

In *C. albicans* cells, the rate of methionine uptake under standard assay conditions was linear with time for at least 3 h and with methionine concentrations within the range 0.1 to 1.0 mmol l⁻¹. Sterol side-chain methylation rate was independent of methionine concentration in the range 0.25 to 5.0 mmol l⁻¹. Percentage inhibition by SF 86-327 (1 mg l⁻¹) was constant in the range 0.1 to 5.0 mM-methionine.

At concentrations up to 10 mg l⁻¹, SF 86-327 and naftifine had no detectable effect on methionine uptake by cells of the three fungi used in this study. This rules out any possibility of the apparent variation in inhibition of side-chain methylation (Table 4) being due to effects on substrate uptake. Naftifine (100 mg l⁻¹) was previously reported to cause about 50% inhibition of methionine uptake in *C. albicans* (Ryder, 1984). SF 86-327 did not have this effect in any of the three fungi tested.

Sterol side-chain methylation in cell-free extracts

Cell-free assays using *Candida* were established to test for direct inhibition of the methyltransferase as a possible explanation for the effects in whole cells. In all cases incorporation of radioactivity was linear with time within the 3 h assay and was exclusively into the ergosterol fraction (confirmed by autoradiography). Table 5 shows the results of *C. albicans* cell-free assays. Cell-free extracts supplemented with cofactors are capable of synthesizing 4-desmethylsterols from precursors such as mevalonate (Ryder *et al.*, 1984) or squalene (Ryder & Dupont, 1984). Under these conditions therefore, part of the measured incorporation from [*Me*-¹⁴C]methionine was into sterols synthesized from endogenous precursors. This portion was sensitive to inhibitors such as ketoconazole and the allylamines (Table 5). At 100 mg l⁻¹, naftifine was more effective than either SF 86-327 or ketoconazole, confirming the second inhibitory effect seen in whole *C. albicans* cells (Table 3). The second cell-free assay used, with *S*-adenosyl-L-[*Me*-¹⁴C]methionine as substrate, measured only the methyltransferase reaction to an endogenous acceptor, since washed microsomes alone are incapable of effecting the later

Table 5. *Effect of antifungal agents on sterol side-chain methylation in cell-free extracts of C. albicans*

Side-chain methylation was measured as the incorporation of radioactivity from [$Me-^{14}C$]methionine by a cell-free extract, and from *S*-adenosyl[$Me-^{14}C$]methionine by a washed microsomal preparation as described in Methods. Control incorporations were 45 300 d.p.m. [$0.015 \text{ nmol h}^{-1} (\text{mg protein})^{-1}$] and 5270 d.p.m. [$0.73 \text{ nmol h}^{-1} (\text{mg protein})^{-1}$] respectively. Values are means $\pm$ SD for six incubations.

Compound	Concn (mg l^{-1})	Radioactivity incorporated (percentage of control)	
		[$Me-^{14}C$]methionine	<i>S</i> -adenosyl[$Me-^{14}C$]methionine
Naftifine	1	92.2 $\pm$ 5.5	100.0 $\pm$ 0.3
	10	90.4 $\pm$ 2.4	97.2 $\pm$ 1.5
	100	59.1 $\pm$ 1.6	78.9 $\pm$ 4.1
SF 86-327	1	93.1 $\pm$ 4.6	97.5 $\pm$ 2.2
	10	88.1 $\pm$ 2.1	98.6 $\pm$ 0.9
	100	75.1 $\pm$ 2.1	88.6 $\pm$ 5.4
Ketoconazole	1	75.8 $\pm$ 0.5	92.9 $\pm$ 4.2

stages of ergosterol biosynthesis (Ryder & Dupont, 1984). In this case, only naftifine (100 mg l^{-1}) caused significant inhibition (Table 5).

In *C. parapsilosis* cell-free extracts, SF 86-327 and naftifine at concentrations up to 10 mg l^{-1} had no significant effect on side-chain methyl transfer from *S*-adenosylmethionine. Effects in *T. mentagrophytes* could not be tested, due to the absence of detectable methyltransferase activity in cell-free extracts of this fungus.

DISCUSSION

Incorporation of [$Me-^{14}C$]methionine into the ergosterol side-chain proved to be a valuable technique for the measurement of ergosterol biosynthesis and its inhibition in fungal cells. The validity of the method described here was confirmed by the specific incorporation of radioactivity into the ergosterol fraction, the lack of incorporation into squalene and the recovery of the labelled product as sterol digitonide. In addition, the system was inhibited by several classes of compound (allylamines, imidazoles and simefungin) known to inhibit ergosterol biosynthesis at progressively later stages in the pathway (squalene epoxidase, lanosterol 14α -demethylation and C-24 methylation respectively).

C. albicans cells of both wild type and polyene-resistant strains have previously been reported to contain 4α -methyl- and 4,4-dimethylsterols methylated at C-24 (Fryberg *et al.*, 1975*a*; Subden *et al.*, 1977; Pierce *et al.*, 1978). This indicates that *C. albicans* is able to effect side-chain methylation before nuclear demethylation at C-4 and C-14, a sequence known to occur in some filamentous fungi (Goulston *et al.*, 1967). Furthermore, *C. albicans* cells blocked at C-14 demethylation by treatment with azole antimycotic agents accumulate 24-methylenedihydrolanosterol and other 14α -methyl sterols methylated at C-24 (Van den Bossche *et al.*, 1978, 1980; Berg *et al.*, 1984). However, the results of the present study indicate that 24-methylation occurred predominantly after nuclear demethylation in normal *Candida* cells. Even under treatment with ketoconazole to block 14α -demethylation, C-24 methylation of the 4,4-dimethylsterol (lanosterol) fraction amounted to only about 5% of total sterol C-24 methylation in controls. The lack of effect of simefungin, a transmethylation inhibitor, on acetate incorporation into ergosterol supports this view. Radiolabelling studies with *S. cerevisiae* (Moore & Gaylor, 1970; Parks *et al.*, 1974; Osumi *et al.*, 1978) also showed that C-24 methylation occurred primarily at the level of the 4-desmethylsterols, the normal methyl acceptor being zymosterol. The occurrence under certain conditions of C-24 methylation earlier in the pathway may be explained by the results of analytical and labelling studies both with *S. cerevisiae* (Barton *et al.*, 1973; Fryberg *et al.*, 1973, 1975*b*) and with *C. albicans* (Fryberg *et al.*, 1975*a*). These provided evidence for a network of pathways in the late stages of ergosterol biosynthesis, the enzymes apparently having relatively low substrate specificity.

In the side-chain methylation assay, *C. albicans* cells continued ergosterol biosynthesis at about 40% of the control rate for several hours, in the presence of SF 86-327 concentrations which completely blocked incorporation of acetate into ergosterol. A similar effect has been described in *Acer pseudoplatanus* tissue culture cells treated with the sterol biosynthesis inhibitor compactin (Ryder & Goad, 1980). The residual biosynthesis in *C. albicans* was most probably due to methylation of sterol originating from precursors distal to squalene epoxidase, the primary point of inhibition by the allylamines (Ryder, 1984; Petranyi *et al.*, 1984). This is supported by the earlier finding (Ryder *et al.*, 1984) that ergosterol precursors disappear from *C. albicans* cells treated with a growth-inhibiting concentration of naftifine. This precursor pool may exist in the form of sterol esters, which in *S. cerevisiae* are utilized for ergosterol biosynthesis under conditions of sterol deficiency (Nagai *et al.*, 1977; Taketani *et al.*, 1978).

The phenomenon of residual ergosterol biosynthesis in SF 86-327-treated cells may provide a clue to the great variation in susceptibility of different fungi to the allylamine antifungal agents. The degree of residual biosynthesis occurring correlates very well with the *in vitro* susceptibility of the three fungi tested (Table 4). The continuation of sterol synthesis in *C. albicans* cells treated with SF 86-327 may enable them to withstand low concentrations of the drug by maintaining a very low level of growth. In contrast, the extremely effective and fungicidal action of the allylamines against dermatophytes such as *T. mentagrophytes* (Georgopoulos *et al.*, 1981; Petranyi *et al.*, 1984) is probably due to the immediate and total cessation of ergosterol biosynthesis induced by these drugs.

The low level of residual sterol synthesis in SF 86-327-treated cells of *C. parapsilosis*, and its absence in *T. mentagrophytes*, could be caused by a lack of precursor pools, an inability to utilize them, or the occurrence of a second point of inhibition later in the pathway. In *C. parapsilosis*, no evidence could be found for inhibition of steps distal to squalene epoxidase (Ryder, 1985). In addition, SF 86-327 and naftifine up to 10 mg l⁻¹ had no effect on the cell-free sterol side-chain methylation assay in this fungus. In *C. albicans* cells treated with SF 86-327 (1 mg l⁻¹), further inhibition of the residual side-chain methylation rate can be taken as an indication of inhibition distal to squalene epoxidase, as shown here by naftifine (100 mg l⁻¹). This effect was not due to the previously reported partial inhibition of methionine uptake (Ryder, 1984) since it could be reproduced in the cell-free methylation assays. In addition to squalene epoxidase inhibition, naftifine thus appears to possess at least two further actions – on cellular uptake and on C-24 methylation – when present at high concentrations. This may enhance the efficacy of naftifine as a topical antimycotic agent.

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REFERENCES

- BARTON, D. H. R., CORRIE, J. E. T., MARSHALL, P. J. & WIDDOWSON, D. A. (1973). Biosynthesis of terpenes and steroids. VII. Unified scheme for the biosynthesis of ergosterol in *Saccharomyces cerevisiae*. *Bio-organic Chemistry* **2**, 363–373.
- BERG, D., REGEL, E., HARENBERG, H.-E. & PLEMPER, M. (1984). Bifonazole and clotrimazole. Their mode of action and the possible reason for the fungicidal behaviour of bifonazole. *Arzneimittel Forschung/Drug Research* **34**(I), 139–146.
- FRYBERG, M., OEHLISCHLAGER, A. C. & UNRAU, A. M. (1973). Biosynthesis of ergosterol in yeast. Evidence for multiple pathways. *Journal of the American Chemical Society* **95**, 5747–5757.
- FRYBERG, M., OEHLISCHLAGER, A. C. & UNRAE, A. M. (1975a). Sterol biosynthesis in antibiotic sensitive and resistant *Candida*. *Archives of Biochemistry and Biophysics* **173**, 171–177.
- FRYBERG, M., AVRUCH, L., OEHLISCHLAGER, A. C. & UNRAU, A. M. (1975b). Nuclear demethylation and C-24 alkylation during ergosterol biosynthesis in *Saccharomyces cerevisiae*. *Canadian Journal of Biochemistry* **53**, 881–889.
- GEORGOPOULOS, A., PETRANYI, G., MIETH, H. & DREWS, J. (1981). *In vitro* activity of naftifine, a new antifungal agent. *Antimicrobial Agents and Chemotherapy* **19**, 386–389.
- GOAD, L. J., LENTON, J. R., KNAPP, F. F. & GOODWIN, T. W. (1974). Phytosterol side chain biosynthesis. *Lipids* **9**, 582–595.
- GOULSTON, G., GOAD, L. J. & GOODWIN, T. W. (1967). Sterol biosynthesis in fungi. *Biochemical Journal* **102**, 15c–17c.
- MCCAMMON, M. T. & PARKS, L. W. (1981). Inhibition of sterol transmethylation by *S*-adenosylhomocysteine analogs. *Journal of Bacteriology* **145**, 106–112.
- MOORE, J. T. & GAYLOR, J. L. (1970). Investigation of an *S*-adenosylmethionine:Δ²⁴-sterol methyltransferase in ergosterol biosynthesis in yeast. *Journal of Biological Chemistry* **245**, 4684–4688.

- NAGAI, J., KAWAMURA, S. & KATSUKI, H. (1977). Occurrence of fatty acid esters of sterol intermediates in ergosterol synthesis by yeast during respiratory adaptation. *Journal of Biochemistry* **81**, 1665–1673.
- NISHINO, T., HATA, S., OSUMI, T. & KATSUKI, H. (1980). Biosynthesis of ergosterol in cell-free system of yeast. *Journal of Biochemistry* **88**, 247–254.
- NISHINO, T., HATA, S., TAKETANI, S., YABUSAKI, Y. & KATSUKI, H. (1981). Subcellular localization of the enzymes involved in the late stage of ergosterol biosynthesis in yeast. *Journal of Biochemistry* **89**, 1391–1396.
- OSUMI, T., TAKETANI, S., KATSUKI, H., KUHARA, T. & MATSUMOTO, I. (1978). Ergosterol biosynthesis in yeast. Pathways in the late stages and their variation under various conditions. *Journal of Biochemistry* **83**, 681–691.
- PALTAUF, G., DAUM, G., ZUDER, G., HÖGENAUER, G., SCHULZ, G. & SEIDL, G. (1982). Squalene and ergosterol biosynthesis in fungi treated with naftifine, a new antimycotic agent. *Biochimica et biophysica acta* **712**, 268–273.
- PARKS, L. W. (1958). *S*-Adenosylmethionine and ergosterol synthesis. *Journal of the American Chemical Society* **80**, 2023–2024.
- PARKS, L. W., ANDING, C. & OURISSON, G. (1974). Sterol transmethylation during aerobic adaptation of yeast. *European Journal of Biochemistry* **43**, 451–458.
- PETRANYI, G., RYDER, N. S. & STÜTZ, A. (1984). Allylamine derivatives: new class of synthetic antifungal agents inhibiting fungal squalene epoxidase. *Science* **224**, 1239–1241.
- PIERCE, A. M., PIERCE, H. D., UNRAU, A. M. & OEHLISCHLAGER, A. C. (1978). Lipid composition and polyene antibiotic resistance of *Candida albicans* mutants. *Canadian Journal of Biochemistry* **56**, 135–142.
- POPIAK, G. (1969). Enzymes of sterol biosynthesis in liver and intermediates of sterol biosynthesis. *Methods in Enzymology* **15**, 393–454.
- RYDER, N. S. (1984). Selective inhibition of squalene epoxidation by allylamine antimycotic agents. In *Microbial Cell Wall Synthesis and Autolysis*, pp. 313–321. Edited by C. Nombela. Amsterdam: Elsevier.
- RYDER, N. S. (1985). Specific inhibition of fungal sterol biosynthesis by SF 86-327, a new allylamine antimycotic agent. *Antimicrobial Agents and Chemotherapy* **27**, 252–256.
- RYDER, N. S. & DUPONT, M.-C. (1984). Properties of a particulate squalene epoxidase from *Candida albicans*. *Biochimica et Biophysica Acta* **794**, 466–471.
- RYDER, N. S. & GOAD, L. J. (1980). The effect of the 3-hydroxy-3-methylglutaryl CoA reductase inhibitor ML-236B on phytosterol synthesis in *Acer pseudoplatanus* tissue culture. *Biochimica et biophysica acta* **619**, 424–427.
- RYDER, N. S. & TROKE, P. F. (1982). The activity of naftifine as a sterol synthesis inhibitor in *Candida albicans*. In *Current Chemotherapy and Immunotherapy*, pp. 1016–1017. Edited by P. Periti & G. G. Grassi. Washington DC: American Society for Microbiology.
- RYDER, N. S., SEIDL, G. & TROKE, P. F. (1984). Effect of the antimycotic drug naftifine on growth of and sterol biosynthesis in *Candida albicans*. *Antimicrobial Agents and Chemotherapy* **25**, 483–487.
- STARR, P. R. & PARKS, L. W. (1972). Transmethylation of sterols in aerobically adapting *Saccharomyces cerevisiae*. *Journal of Bacteriology* **109**, 236–242.
- STÜTZ, A. & PETRANYI, G. (1984). Synthesis and antifungal activity of (*E*)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphthalenemethanamine (SF 86-327) and related allylamine derivatives with enhanced oral activity. *Journal of Medicinal Chemistry* **27**, 1539–1543.
- SUBDEN, R. E., SAFE, L., MORRIS, D. C., BROWN, R. G. & SAFE, S. (1977). Eburicol, lichesterol, ergosterol and obtusifolol from polyene antibiotic-resistant mutants of *Candida albicans*. *Canadian Journal of Microbiology* **23**, 751–754.
- TAKETANI, S., NAGAI, J. & KATSUKI, H. (1978). Quantitative aspects of free and esterified sterols in *Saccharomyces cerevisiae* under various conditions. *Biochimica et biophysica acta* **528**, 416–423.
- VAN DEN BOSSCHE, H., WILLEMSSENS, G., COOLS, W., LAUWERS, W. F. J. & LEJEUNE, L. (1978). Biochemical effects of miconazole on fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*. *Chemico-Biological Interactions* **21**, 59–78.
- VAN DEN BOSSCHE, H., WILLEMSSENS, G., COOLS, W., CORNELISSEN, F., LAUWERS, W. F. & VAN CUTSEM, J. M. (1980). *In vitro* and *in vivo* effects of the antimycotic drug ketoconazole on sterol synthesis. *Antimicrobial Agents and Chemotherapy* **17**, 922–928.