ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1996, p. 443–447 Vol. 40, No. 2
0066-4804/96/$04.00+0

Characterization of Squalene Epoxidase Activity from the Dermatophyte *Trichophyton rubrum* and Its Inhibition by Terbinafine and Other Antimycotic Agents

BERTRAND FAVRE AND NEIL S. RYDER*

Department of General Dermatology, SANDOZ Research Institute, A-1235 Vienna, Austria

Received 13 July 1995/Returned for modification 10 October 1995/Accepted 29 November 1995

Squalene epoxidase (SE) is the primary target of the allylamine antimycotic agents terbinafine and naftifine and also of the thiocarbamates. Although all of these drugs are employed primarily in dermatological therapy, SE from dermatophyte fungi has not been previously investigated. We report here the biochemical characterization of SE activity from *Trichophyton rubrum* and the effects of terbinafine and other inhibitors. Microsomal SE activity from *T. rubrum* was not dependent on soluble cytoplasmic factors but had an absolute requirement for NADPH or NADH and was stimulated by flavin adenine dinucleotide. Kinetic analyses revealed that under optimal conditions the K_m for squalene was 13 μM and its V_{max} was 0.71 nmol/h/mg of protein. Terbinafine was the most potent inhibitor tested, with a 50% inhibitory concentration (IC_{50}) of 15.8 nM. This inhibition was noncompetitive with regard to the substrate squalene. A structure-activity relationship study with some analogs of terbinafine indicated that the tertiary amino structure of terbinafine was crucial for its high potency, as well as the *tert*-alkyl side chain. Naftifine had a lower potency (IC_{50}, 114.6 nM) than terbinafine. Inhibition was also demonstrated by the thiocarbamates tolciclate (IC_{50}, 28.0 nM) and tolnaftate (IC_{50}, 51.5 nM). Interestingly, the morpholine amorolfine also displayed a weak but significant effect (IC_{50}, 30 μM). *T. rubrum* SE was only slightly more sensitive (approximately twofold) to terbinafine inhibition than was the *Candida albicans* enzyme. Therefore, this difference cannot fully explain the much higher susceptibility (≥100-fold) of dermatophytes than of yeasts to this drug. The sensitivity to terbinafine of ergosterol biosynthesis in whole cells of *T. rubrum* (IC_{50}, 1.5 nM) is 10-fold higher than that of SE activity, suggesting that the drug accumulates in the fungus.

The allylamine antimycotic agents terbinafine and naftifine act by the specific and selective inhibition of fungal squalene epoxidase (SE) (22, 28, 31) (for reviews, see references 1 and 30), a key enzyme in the ergosterol biosynthesis pathway. A similar mode of action has been described for the thiocarbamates, which include the topical antimycotic agents tolnaftate and tolciclate (3, 13, 29). Other major classes of antifungal agents act at later steps in the ergosterol pathway. Imidazoles and triazoles inhibit the cytochrome P-450-dependent 14α-sterol demethylase (37, 38), whereas morpholines, such as amorolfine, inhibit sterol-Δ^{14}-reductase and sterol-Δ^{7}-Δ^{8}-isomerase (37) (for a general review, see reference 7).

Among the SE inhibitors, terbinafine is the most potent agent against a wide variety of pathogenic fungi and has a primary fungicidal action (20). In vitro, dimorphic and filamentous fungi, especially dermatophytes, are more susceptible to terbinafine than are yeasts (30). Clinically, terbinafine is active when administered either topically or orally and appears to have a strong affinity for the skin and its appendages (39). Consequently, the dermatophytes are a major clinical target for this drug.

Despite the clinical significance of dermatophytes and their high degree of susceptibility to terbinafine, SE from these microorganisms has not been previously described. By contrast, SE from yeasts, including *Saccharomyces cerevisiae* (9, 11, 34), *Candida albicans* (27), and *Candida parapsilosis* (28), has been studied, and its properties have been compared with those of the rat liver enzyme. Mammalian SE has been purified to apparent homogeneity and consists of two components, a terminal oxidase (EC 1.14.99.7) and a flavoprotein NADPH–cytochrome P-450 reductase (EC 1.6.2.4) (2, 5, 18).

We report here the isolation and biochemical characterization of microsomal SE from the dermatophyte *Trichophyton rubrum* and the inhibitory properties of terbinafine and other clinical antimycotic agents. *T. rubrum* SE is more sensitive to terbinafine than is the *C. albicans* enzyme; however, this difference in affinity cannot completely explain the much higher level of susceptibility of the dermatophytes to this drug.

MATERIALS AND METHODS

Chemicals. Allylamines terbinafine, naftifine, SDZ 87-469, and compound I, as well as the analog compounds II and III, were synthesized by SANDOZ (14–16, 32, 35, 36). Tolciclate was a gift from Montedison, tolnaftate was a gift from Lundbeck and Co., and amorolfine was a gift from F. Hoffmann-La Roche Ltd. They were all dissolved in dimethyl sulfoxide and stored at −20°C. The detergents Triton X-100, Mega-8, Mega-9, and octyl β-D-glucopyranoside were purchased from Pierce, Fluka, Cambridge Research Biochemicals, and Sigma, respectively. Other biochemicals were from Sigma unless otherwise stated. Ergosteryl acetate was prepared by incubating 0.5 g of ergosterol dissolved in 5 ml of pyridine with 5 ml of acetic anhydride at 60°C for 1 h. After the addition of 200 ml of water, ergosteryl acetate was extracted with three 100-ml volumes of petroleum ether (bp, 40 to 60°C). The organic fraction was washed with two 100-ml volumes of 1 N hydrochloric acid, 100 ml of 5% sodium bicarbonate, and two 100-ml volumes of water, dried by the addition of anhydrous sodium sulfate, and concentrated with a rotavapor. [4,8,12,13,17,21-^{3}H]-squalene (1,100 GBq/mmol) (ARC or NEN) and [24,30-^{14}C]squalene (7.0 mCi/mmol) (obtained from Glenn D. Prestwich, Department of Chemistry, State University of New York, Stony Brook, N.Y.) were purified by chromatography through a column of hydrated silica gel prepared as follows. A 0.5-ml volume of water was thoroughly mixed with 5 g of silica gel 60 (Merck). Hydrated silica gel was resuspended in

* Corresponding author. Mailing address: Department of General Dermatology, SANDOZ Research Institute, Brunner Strasse 59, P.O. Box 80, A-1235 Vienna, Austria. Phone: 43 1 86 634 324. Fax: 43 1 86 634 354.

petroleum ether (bp, 40 to 60°C; Fluka), and the slurry was poured into an Econo column (Bio-Rad) (1 by 10 cm) and washed with the same solvent. The [^{3}H]- or [^{14}C]squalene was applied to the column, eluted with 50 ml of petroleum ether, concentrated with a rotavapor, and stored at −20°C. Squalene was purified by crystallization in methanol saturated with thiourea as described by Moreau et al. (12).

***T. rubrum* culture.** *T. rubrum* SFI 1895 was grown on Kimmig agar (Merck) at 30°C for 3 weeks. The mycelium-bearing conidia were collected with a spatula, homogenized in a small volume of 2% glucose Sabouraud's medium (pH 6.5) supplemented with 5% dimethyl sulfoxide, aliquoted, and frozen in liquid nitrogen. The number of CFU per milliliter, determined on Kimmig agar, was usually $\geq 1 \times 10^8$. These preparations were used to inoculate 500 ml of 2% glucose Sabouraud's medium (pH 6.5) in 2-liter Erlenmeyer flasks at 0.5×10^5 CFU/ml. The cultures were incubated on a rotary shaker at 100 rpm for 4 days at 30°C. Mycelium was harvested by filtration through filter paper (Schleicher & Schuell) washed exhaustively with 0.1 M potassium phosphate (pH 7.4) and frozen in liquid nitrogen.

Preparation of microsomes. All procedures were carried out in a cold room at 4°C. Frozen mycelium (80 g, fresh weight) was pulverized with a Moulinex blender (Moulinette SE). It was further homogenized in the same apparatus after addition of 200 ml of 0.1 M potassium phosphate (pH 7.4)–1 mM dithiothreitol–2 mM EDTA–1 mM phenylmethylsulfonyl fluoride–1 μM leupeptin–1 μM pepstatin (homogenization buffer) to the powder. The preparation was centrifuged at 10,000 × *g* for 20 min to remove the mycelial debris. The supernatant was filtered under gravity through two layers of filter paper (Schleicher & Schuell) to avoid any contamination with mycelium. An aliquot of the homogenate was saved and frozen in liquid nitrogen. The rest of the homogenate was further centrifuged at 170,000 × *g* for 60 min. Lipids floating on the surface of the supernatant were removed with a pipette, and the soluble fraction was saved. The pellet was resuspended in about 20 ml of homogenization buffer by using a glass-Teflon homogenizer. Both the soluble and the particulate fractions were aliquoted and frozen in liquid nitrogen. Protein concentrations were determined with the Bradford reagent from Bio-Rad, using bovine plasma gamma globulin (Bio-Rad) as a standard. The particulate fraction was incubated for 10 min with 0.5 M (final concentration) sodium hydroxide at room temperature prior to protein determination. Approximately two-thirds of the total protein in the homogenates was soluble, the remainder being recovered in the microsomal fraction.

SE assay. The assay of SE from *T. rubrum* was based on methods described previously (27, 28), with some modifications. The substrate was prepared by mixing 10 μl of 1% Tween 80 in cyclohexane-acetone-ethanol (85:10:5) per assay, 2 μl of 1 mM squalene in cyclohexane-ethanol (95:5) per assay, and a volume of [^{3}H]squalene corresponding to about 80,000 cpm per assay. An aliquot of this substrate mixture corresponding to the volume for one assay was spotted onto a piece of thin-layer chromatography (TLC) aluminium sheet in duplicate, tested for radioactivity, and used in calculation of the true substrate concentration as described below. After the evaporation of the solvent under nitrogen, the lipid-detergent mixture was dispersed in a volume of 0.1 M potassium phosphate (pH 7.4), corresponding to 20 μl per assay, which was then vigorously agitated. From this solution, 20 μl was spotted onto a piece of TLC sheet in duplicate, and the rest was used for the assays. The standard assay contained 0.1 M potassium phosphate (pH 7.4), 0.02% dimethyl sulfoxide with or without inhibitor, 0.02% Tween 80, 4 μM squalene, 80,000 cpm of [^{3}H]squalene per assay, 1 mM NADPH, 0.1 mM flavin adenine dinucleotide (FAD), and the particulate fraction (final protein concentration, 2 mg/ml) in a final volume of 0.5 ml in screw-cap 10-ml glass tubes. For the blanks, the protein sample was heat denatured at 95°C for 10 min. The assay was started by the addition to the mixture of cofactors NADPH and FAD, which was then incubated for 45 min at 30°C, and stopped by the addition of 1 ml of 15% potassium hydroxide in water-ethanol (10:90) and 0.2 ml of 2% 1,2,3-benzenetriol (pyrogallol) in ethanol. The tubes were tightly capped, incubated at 80°C for 10 min, and left to cool at room temperature. To each tube, 1 ml of water and 2 ml of petroleum ether (bp, 40 to 60°C) were added, and the nonsaponifiable lipids were extracted into the organic phase by strong agitation. After centrifugation at 500 × *g* for 5 min, the organic phase was saved and the extraction was repeated with 2 ml of petroleum ether. The two organic phases were pooled and washed with 2 ml of water. The organic phase was transferred to another tube and evaporated with a rotavapor. Lipids were dissolved in 0.3 ml of cyclohexane-ethanol (95:5), reconcentrated, and finally dissolved in 50 μl of cyclohexane-ethanol (95:5) containing as standard lipids squalene, ergosterol, lanosterol, and ergosteryl acetate. They were separated by TLC on silica gel 60 F_{254} sheets (Merck) run in dichloromethane. The positions of the standard lipids were determined under UV light, and regions corresponding to lanosterol and squalene epoxide (R_f, ~0.50), migrating just below ergosteryl acetate (R_f, ~0.58), were cut out and put in scintillation vials. The region corresponding to squalene was also cut out and put in a separate vial. The TLC pieces were covered with the scintillant Opti-fluor (for aqueous samples) (Packard) and agitated for 60 min on a shaker, and then radioactivity was counted. Since squalene is poorly soluble in aqueous solutions and may easily adsorb to the glassware, the real concentration of squalene dissolved in phosphate buffer was determined by comparing the radioactivity of the substrate prepared in organic solvent (corresponding to 4 μM under standard conditions) with that of squalene after solubilization in phosphate buffer. The loss of radioactivity during the extraction and TLC of lipids was also determined by comparing the radioactivity of squalene solubilized in phosphate buffer with the sum of untransformed and transformed squalene after TLC. Activity values were then corrected to 100% recovery. Results were analyzed with the curve-fitting program Table Curve Windows 1.0 (Jandel). When [^{14}C]squalene was used as the substrate, TLC sheets were autoradiographed with Hyperfilm-MP (Amersham) for 2 weeks. All experiments were performed in duplicate, or triplicate for kinetic analyses, and repeated at least twice with different microsomal preparations.

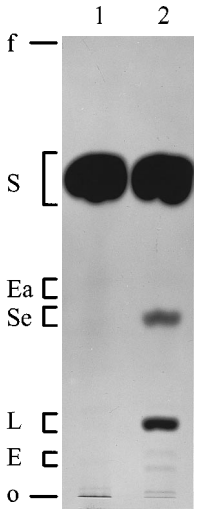

FIG. 1. Autoradiography of TLC separation of nonsaponifiable lipids from an SE assay performed with *T. rubrum* homogenates in the presence of 40 μM [^{14}C]squalene and incubated for 1 h. Lane 1, control (boiled homogenates); lane 2, untreated homogenates. The positions of the origin (o), front (f), and lipids ergosterol (E), lanosterol (L), squalene epoxide (Se), ergosteryl acetate (Ea), and squalene (S) are indicated.

RESULTS

Isolation of *T. rubrum* SE. Previous attempts to detect SE activity in cell extracts by disrupting the mycelium with glass beads were not successful (25). Therefore, we tried the technique of blending frozen mycelium as described in Materials and Methods and could detect SE activity after further homogenization of the disrupted mycelium in a phosphate buffer. Conversion of [^{14}C]squalene by homogenates was analyzed by autoradiography of the TLC sheets, revealing that, in addition to squalene epoxide, lanosterol was also formed, indicating the presence of active oxidosqualene cyclase (Fig. 1). However, no clear evidence for a further conversion (to 4-demethyl sterols) was observed. The positions of minor radioactive bands migrating more slowly than lanosterol (Fig. 1) were variable, and their intensity did not depend on SE activity.

Fractionation of these homogenates by ultracentrifugation revealed that both SE and oxidosqualene cyclase activities were associated with the microsomes (Fig. 2). The pattern of conversion of [^{14}C]squalene by microsomes was identical to that shown in Fig. 1. SE activity, both in homogenates and in the particulate fraction, was often not linear at low protein concentrations (Fig. 2), a phenomenon previously observed with the yeast enzyme (9, 27, 28). Rat liver SE is strongly stimulated (up to 10-fold) by the soluble fraction, which contains a protein factor which catalyzes the intermembrane transfer of squalene (6). In contrast, the yeast SE is activated to a negligible extent (≤2-fold) (17, 27). Similar to the yeast enzyme, *T. rubrum* microsomal SE activity was only slightly increased by the soluble fraction, and usually only at low microsomal protein concentrations, at which the activity was not directly proportional to the protein concentration, suggesting

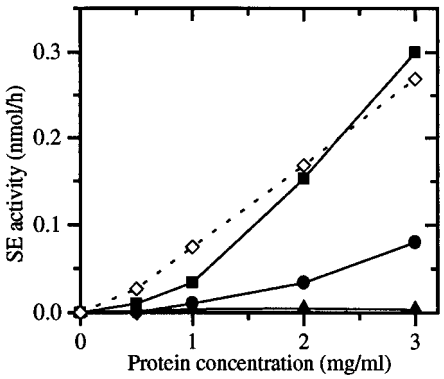

FIG. 2. Protein concentration-dependent SE activity in different fractions from *T. rubrum* assayed under standard conditions. ●, homogenates; ▲, soluble fraction; ■, microsomes; ◇, microsomes plus 2 mg of soluble fraction per ml.

that the total protein concentration in the assay might influence the linearity of the activity (Fig. 2). The concentration of 2 mg of microsomal protein per ml was adopted for all subsequent assays because it was situated in a linear portion of the curve. Furthermore, at this microsomal concentration, the soluble fraction had no significant effect on SE activity. Microsomal SE activity was perfectly linear with incubation for up to 75 min under the standard conditions (data not shown).

Biochemical characterization of microsomal SE. Rat liver and yeast SE require, in addition to squalene, molecular oxygen and NADPH or NADH to be active and are stimulated by FAD (9, 11, 18, 27, 28, 34). *T. rubrum* SE activity was also completely dependent on the addition of NADPH or NADH, NADPH being slightly superior. FAD also stimulated the activity but was not essential (Table 1). Higher concentrations of NADPH (5 mM) or FAD (0.5 mM) or both or a combination of all three cofactors did not increase the activity, demonstrating that the concentrations used were saturating. Four buffers were tested at pH 7.4. Phosphate buffer (100%) was slightly better than TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) (94% ± 2%), HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (88% ± 1%), and Tris (85% ± 6%) buffers (± standard errors of the means [SEM]; $n = 3$). Acidification or alkalinization of the assay solution negatively affected the activity in comparison with that obtained with the respective buffer at pH 7.4 (54% ± 1% [± SEM; $n = 4$] with phosphate buffer at pH 6.4 and 68% ± 37% [± SEM; $n = 4$] with Tris buffer at pH 8.3).

The effects of detergents were also tested, including Triton X-100, which strongly stimulates mammalian SE (2, 17) but inhibits the *C. albicans* enzyme (23, 25), and Mega-8, Mega-9, and octyl β-D-glucopyranoside, which are weak activators of the *C. albicans* enzyme (23, 25). At 0.3%, Triton X-100 and Mega-8 caused inhibition of 71% ± 14% and 19% ± 9%, respectively, while Mega-9 and octyl β-D-glucopyranoside were slightly stimulatory by 44% ± 24% and 42% ± 20%, respectively (± SEM; $n = 3$). These effects are all with reference to the standard assay conditions, which included 0.02% Tween 80 (see Materials and Methods). At 0.5%, all the detergents were inhibitory.

TABLE 1. Dependence of microsomal SE on cofactors

Cofactor(s)[a]	Mean activity (%) ± SEM[b]
None	0.2 ± 0.2
FAD	0.8 ± 0.2
NADH	34.0 ± 10.4
NADPH	41.1 ± 14.8
FAD + NADH	89.4 ± 8.1
FAD + NADPH	100.0[c]

[a] 0.1 mM FAD; 1 mM NADPH or NADH.
[b] Values are the means of three experiments performed in duplicate, each with a different microsomal preparation.
[c] Specific activity, 0.189 ± 0.067 nmol/h/mg of protein (mean of three experiments performed in duplicate ± SEM).

TABLE 2. Comparison of IC_{50}s of various allylamines and analogs, thiocarbamates, and amorolfine for microsomal SEs from *T. rubrum* and *C. albicans*

Inhibitor	*T. rubrum* enzyme		*C. albicans* enzyme	
	Mean IC_{50} (nM) ± SEM	n	IC_{50} (nM)	Reference
Terbinafine	15.8 ± 1.6	6	30	28
SDZ 87-469	19.9 ± 4.7	3	11	32
Naftifine	114.6 ± 29.7	4	1,100	32
Compound II	129.4 ± 41.9	3	2,160	32
Compound III	201.4 ± 51.6	3	260[a]	32
Compound I	636.0 ± 42.7	3	320[a]	32
Tolciclate	28.0 ± 2.9	3	120	29
Tolnaftate	51.5 ± 8.9	4	1,040	29
Amorolfine	30,400 ± 13,900	4	>100,000	

[a] Determined with cell extracts.

The affinity of the enzyme for squalene was determined, and classical Michaelis-Menten curves were obtained. The K_m for squalene was 13.3 ± 5.3 μM (± SEM; $n = 5$), and the V_{max} was 0.71 ± 0.12 nmol/h/mg of protein (± SEM; $n = 5$), as deduced from double-reciprocal plots (not shown). Under the standard conditions, with a real concentration of squalene of 3.74 ± 0.04 μM (± SEM; $n = 19$), the specific activity was 0.19 ± 0.01 nmol/h/mg of protein (± SEM; $n = 19$). It was previously demonstrated that the second substrate of the enzyme, O_2, is in large excess in air-saturated aqueous solutions and does not constitute a rate-limiting factor under normal conditions (6, 11).

Characterization of SE inhibition by terbinafine. Once the assay conditions had been optimized, the effect of terbinafine on SE activity was tested. Terbinafine was a potent inhibitor, with a 50% inhibitory concentration (IC_{50}) of 15.8 nM (Table 2). The mode of inhibition by terbinafine was analyzed by varying the concentrations of both squalene and the inhibitor. Double-reciprocal plot analyses of the data indicated that the affinity of SE for squalene was not significantly affected by the presence of terbinafine, demonstrating that the mode of inhibition was noncompetitive towards the substrate (Fig. 3).

The relationship between structure and inhibitory activity was investigated with some analogs of terbinafine. The naphthalene ring could be replaced by the 3-chloro-7-benzo[*b*]thienyl residue, SDZ 87-469, without having a strong effect on the affinity for SE (Fig. 4 and Table 2), in agreement with results found for whole fungal cells (15). On the other hand, the structure of the tertiary amino group of terbinafine could not be modified without drastically affecting the affinity for *T. rubrum* SE. For instance, removal of the methyl group on the tertiary amine (compound I) or replacement of the allylamine nitrogen by an oxygen (compound II) or a carbon (compound III) (Fig. 4) markedly increased the IC_{50}s in comparison with those of terbinafine (Table 2). Moreover, naftifine (Fig. 4) was also less potent than terbinafine (Table 2).

Inhibition of SE by other antimycotic agents. In addition to the allylamines, the thiocarbamates, tolciclate and tolnaftate,

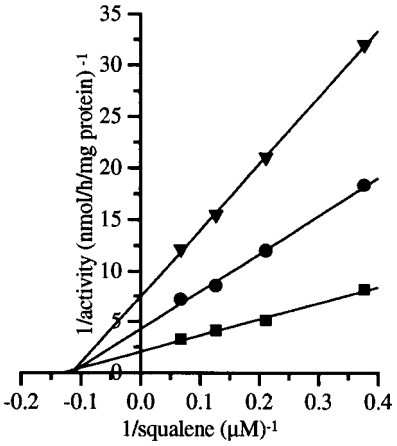

FIG. 3. Analysis of the mode of inhibition of microsomal SE by terbinafine (double-reciprocal plot). The activity was determined with four concentrations of squalene in the absence of inhibitor (■) or in the presence of terbinafine at 12.3 (●) or 30 (▼) nM.

were also found to be potent inhibitors (Table 2). Amorolfine has been reported to cause accumulation of squalene in the dermatophyte *Trichophyton mentagrophytes*, at high concentrations (21). Therefore, its action was also investigated. It was indeed inhibitory for *T. rubrum* SE but exhibited a much lower affinity than allylamines and thiocarbamates, with significant effects only in the micromolar range (Table 2).

DISCUSSION

Inhibition of fungal SE is the primary mechanism of action of terbinafine as well as other allylamine and thiocarbamate antimycotic agents (24, 30). Within the fungi, SE has previously been characterized only from yeasts, *S. cerevisiae* (9, 11, 34), *C. albicans* (27), and *C. parapsilosis* (28). However, the fungi most susceptible to terbinafine and other SE inhibitors both in vitro and in vivo are the dermatophytes (4, 30, 40). A straightforward explanation for this difference could be that the affinity of dermatophyte SE for inhibitors is much higher than that of the yeast enzyme. To test this hypothesis, we characterized SE enzymatic activity from *T. rubrum*. Its main features are the following: it is (i) a microsomal enzyme; (ii) completely dependent on the presence of a reducing cofactor, NADPH or NADH; (iii) stimulated by FAD; (iv) almost independent of soluble factors; and (v) inhibited in a noncompetitive manner by terbinafine.

The first three features are shared by all SEs studied so far, both fungal and mammalian (2, 9, 11, 18, 27, 28, 34). The specificity for the reducing cofactor is strong in the case of SE activity from mammals and *S. cerevisiae* (requiring NADPH) but is weaker with *T. rubrum* SE (which also prefers NADPH) and with the *C. albicans* enzyme (which prefers NADH) (11, 27, 28, 34). It is not yet known whether certain fungal NADPH-cytochrome P-450 reductases are less specific for the reducing cofactor or whether other reductases are able to supply the fungal enzyme with electrons. The partial dependence on FAD of all SEs is apparently not directly related to NADPH-cytochrome P-450 reductase (17), although this reductase is a flavoprotein containing one molecule each of FAD and flavin mononucleotide (8). It has therefore been suggested that SE itself might be a flavoprotein (17, 19). This suggestion has recently been confirmed by the analysis of the sequence of the cloned rat SE sequence (33). Both mammalian and yeast SE sequences contain a β1-αA-β2 motif characteristic for an FAD-binding domain (10, 33).

The features most specific to fungal SEs are their lack of dependence on a soluble protein factor(s) and their potent noncompetitive inhibition by terbinafine (11, 27, 28, 34). An additional feature which seems to distinguish fungal and mammalian SEs is their sensitivity to detergents, especially Triton X-100. The latter can replace the soluble cytoplasmic factor which is indispensable for the activity of mammalian SE (2, 17) but strongly inhibits both *C. albicans* (27) and *T. rubrum* SEs. The exact mechanism of action of Triton X-100 on mammalian SE has not yet been elucidated, since the detergent not only substitutes for a soluble factor protein(s) but also solubilizes the mammalian enzyme (2, 17). Comparison of the behavior of *C. albicans* and *T. rubrum* SEs in the presence of detergents revealed some subtle differences (23, 25), which are probably related to the different lipid and protein compositions of the microsomal membranes from the respective microorganisms.

Of all SE inhibitors tested against *T. rubrum* SE, terbinafine was the most potent in comparison with other clinical antimycotic agents. Interestingly, comparison of the compounds tested against *T. rubrum* and *C. albicans* SEs reveals two categories of inhibitors: (i) those which have similar affinities for the two enzymes, including terbinafine, SDZ 87-469, and compounds I and III, and (ii) those which have a much higher specificity for the dermatophyte enzyme, consisting of naftifine, compound II, tolciclate, tolnaftate, and amorolfine (Table 2). This correlates with the antimycotic efficacies of the compounds, with those of the second category effective only against dermatophytes, while terbinafine is active against a wide range of fungi. From the results in Table 2, it appears that the *Trichophyton* SE is less discriminating than the *Candida* enzyme with regard to structural requirements for inhibition. This is particularly obvious for naftifine, the ether derivative of terbinafine compound II, and the morpholine amorolfine (Table 2). Amorolfine has been reported to cause squalene accu-

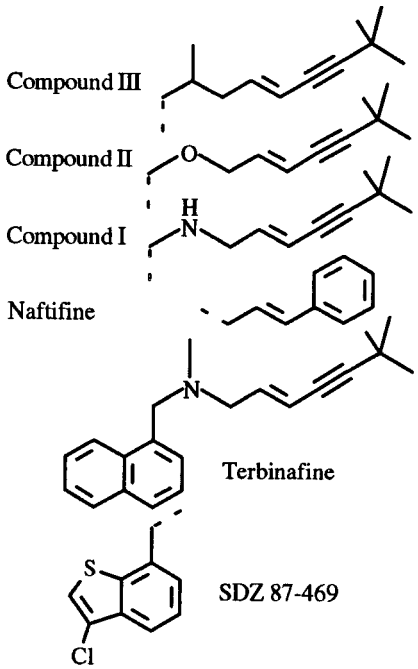

FIG. 4. Structure of allylamines and analogs tested on microsomal SE, the IC_{50}s of which are presented in Table 2. Dotted lines indicate that the rest of the structure is identical to that of terbinafine.

mulation in *Trichophyton* cells but not in *C. albicans* (21), in agreement with its effects on the respective SEs.

In terms of in vitro MICs, terbinafine is 2 to 4 orders of magnitude more potent against *Trichophyton* species than *Candida* species (20, 30). However, the affinity of the dermatophyte SE for terbinafine appeared to be only slightly higher (~2-fold) than that for the yeast enzyme (IC_{50}s, 16 and 30 nM [28], respectively). It can thus be concluded that the greater susceptibility of dermatophytes to terbinafine is not solely related to a higher affinity of their SE for the inhibitor. Previous measurements of the inhibition of ergosterol biosynthesis in the same strain of *T. rubrum* by terbinafine gave an IC_{50} of 0.5 ng/ml (1.5 nM) (26), 10-fold lower than the IC_{50} of the microsomal SE (15.8 nM). In contrast, the IC_{50}s of terbinafine for both SE activity and ergosterol biosynthesis in *C. albicans* agree closely at 30 nM (26). As the assay conditions for the two fungal enzymes are very similar, it might be suggested that terbinafine is accumulated by *T. rubrum* hyphae, leading to higher intracellular drug levels.

In addition to this possible accumulation of terbinafine in *T. rubrum*, the morphology of filamentous fungi is probably an important factor. The highly polarized growing tip of hyphae is likely to be highly sensitive to disturbances in membrane composition. This is demonstrated by the greater susceptibility to terbinafine of the filamentous form than of the yeast form of the same *C. albicans* strain (26). In addition, dermatophytes appear to be unusually sensitive to the effects of intracellular squalene accumulation resulting from SE inhibition (26).

In conclusion, the high potency of terbinafine against dermatophytes appears to be based on a combination of factors, including the high degree of sensitivity of their SEs, accumulation of the drug in the fungal cell, and the inherent susceptibility of filamentous growth to this mechanism of inhibition.

ACKNOWLEDGMENTS

We thank Henrietta D. Moore and Peter Nussbaumer for critical reading of the manuscript.

REFERENCES

1. **Abe, I., J. C. Tomesch, S. Wattanasin, and G. D. Prestwich.** 1994. Inhibitors of squalene biosynthesis and metabolism. Nat. Prod. Rep. **11:**279–302.
2. **Bai, M., and G. D. Prestwich.** 1992. Inhibition and activation of porcine squalene epoxidase. Arch. Biochem. Biophys. **293:**305–313.
3. **Barrett-Bee, K., A. C. Lane, and R. W. Turner.** 1986. The mode of antifungal action of tolnaftate. J. Med. Vet. Mycol. **24:**155–160.
4. **deCarneri, I., G. Monti, A. Bianchi, S. Castellino, G. Meinardi, and V. Mandelli.** 1976. Tolciclate against dermatophytes. Drug Res. **26:**769–772.
5. **Dennerancona, P., M. Bai, H. K. Lee, I. Abe, and G. D. Prestwich.** 1995. Purification of pig and rat liver squalene epoxidase by affinity chromatography. Bioorg. Med. Chem. Lett. **5:**481–486.
6. **Friedlander, E. J., I. J. Caras, L. F. H. Lin, and K. Bloch.** 1980. Supernatant protein factor facilitates intermembrane transfer of squalene. J. Biol. Chem. **255:**8042–8045.
7. **Georgopapadakou, N. H., and T. J. Walsh.** 1994. Human mycoses—drugs and targets for emerging pathogens. Science **264:**371–373.
8. **Iyanagi, T., R. Makino, and F. K. Anan.** 1981. Studies on the microsomal mixed-function oxidase system: mechanism of action of hepatic NADPH-cytochrome P-450 reductase. Biochemistry **20:**1722–1730.
9. **Jahnke, L., and H. P. Klein.** 1983. Oxygen requirements for formation and activity of the squalene epoxidase in *Saccharomyces cerevisiae*. J. Bacteriol. **155:**488–492.
10. **Jandrositz, A., F. Turnowsky, and G. Hoegenauer.** 1991. The gene encoding squalene epoxidase from *Saccharomyces cerevisiae*: cloning and characterization. Gene **107:**155–160.
11. **M'Baya, B., and F. Karst.** 1987. In vitro assay of squalene epoxidase of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **147:**556–564.
12. **Moreau, J. P., P. J. Ramm, and E. Caspi.** 1975. Biosynthesis of sterols by a yeast homogenate. Incorporation of mevalonic acid into cholesta-5,7,24-trien-3beta-ol and 5alpha-cholesta-7,24-dien-3beta-ol. Eur. J. Biochem. **56:** 393–402.
13. **Morita, T., and Y. Nozawa.** 1985. Effects of antifungal agents on ergosterol biosynthesis in *Candida albicans* and *Trichophyton mentagrophytes*: differential inhibitory sites of naphthiomates and miconazole. J. Invest. Dermatol. **85:**434–437.
14. **Nussbaumer, P., I. Leitner, K. Mraz, and A. Stuetz.** 1995. Synthesis and structure-activity relationships of side-chain substituted analogs of the allylamine antimycotic terbinafine lacking the central amino function. J. Med. Chem. **38:**1831–1836.
15. **Nussbaumer, P., G. Petranyi, and A. Stuetz.** 1991. Synthesis and structure-activity relationships of benzo[*b*]thienylallylamine antimycotics. J. Med. Chem. **34:**65–73.
16. **Nussbaumer, P., N. S. Ryder, and A. Stuetz.** 1991. Allylamine antimycotics: recent trends in structure-activity relationships and syntheses. Pestic. Sci. **31:** 437–455.
17. **Ono, T., and K. Bloch.** 1975. Solubilization and partial characterization of rat liver squalene epoxidase. J. Biol. Chem. **250:**1571–1579.
18. **Ono, T., K. Nakazono, and H. Kosaka.** 1982. Purification and partial characterization of squalene epoxidase from rat liver microsomes. Biochim. Biophys. Acta **709:**84–90.
19. **Ono, T., S. Ozasa, F. Hasegawa, and Y. Imai.** 1977. Involvement of NADPH-cytochrome c reductase in the rat liver squalene epoxidase system. Biochim. Biophys. Acta **486:**401–407.
20. **Petranyi, G., J. G. Meingassner, and H. Mieth.** 1987. Antifungal activity of the allylamine derivative terbinafine in vitro. Antimicrob. Agents Chemother. **31:**1365–1368.
21. **Polak, A.** 1988. Mode of action of dimethylmorpholine derivatives. Ann. N. Y. Acad. Sci. **544:**221–228.
22. **Ryder, N. S.** 1985. Specific inhibition of fungal sterol biosynthesis by SF 86-327, a new allylamine antimycotic agent. Antimicrob. Agents Chemother. **27:**252–256.
23. **Ryder, N. S.** 1987. Squalene epoxidase as the target of antifungal allylamines. Pestic. Sci. **21:**281–288.
24. **Ryder, N. S.** 1988. Mechanism of action and biochemical selectivity of allylamine antimycotic agents. Ann. N. Y. Acad. Sci. **544:**208–220.
25. **Ryder, N. S.** 1990. Squalene epoxidase: enzymology and inhibition, p. 189–203. *In* P. J. Kuhn, A. P. J. Trinci, M. J. Jung, M. W. Goosey, and L. G. Copping (ed.), Biochemistry of cell walls and membranes in fungi. Springer-Verlag, Berlin.
26. **Ryder, N. S.** 1992. Terbinafine: mode of action and properties of the squalene epoxidase inhibition. Br. J. Dermatol. **126**(Suppl. 39)**:**2–7.
27. **Ryder, N. S., and M.-C. Dupont.** 1984. Properties of a particulate squalene epoxidase from *Candida albicans*. Biochim. Biophys. Acta **794:**466–471.
28. **Ryder, N. S., and M.-C. Dupont.** 1985. Inhibition of squalene epoxidase by allylamine antimycotic compounds. A comparative study of the fungal and mammalian enzymes. Biochem. J. **230:**765–770.
29. **Ryder, N. S., I. Frank, and M.-C. Dupont.** 1986. Ergosterol biosynthesis inhibition by the thiocarbamate antifungal agents tolnaftate and tolciclate. Antimicrob. Agents Chemother. **29:**858–860.
30. **Ryder, N. S., and H. Mieth.** 1991. Allylamine antifungal drugs. Curr. Top. Med. Mycol. **4:**158–188.
31. **Ryder, N. S., G. Seidl, and P. F. Troke.** 1984. Effect of the antimycotic drug naftifine on growth of and sterol biosynthesis in *Candida albicans*. Antimicrob. Agents Chemother. **25:**483–487.
32. **Ryder, N. S., A. Stuetz, and P. Nussbaumer.** 1992. Squalene epoxidase inhibitors: structural determinants for activity and selectivity of allylamines and related compounds, p. 192–204. *In* W. D. Nes, E. J. Parish, and J. M. Trzaskos (ed.), Regulation of isopentenoid metabolism. American Chemical Society, Washington, D.C.
33. **Sakakibara, J., R. Watanabe, Y. Kanai, and T. Ono.** 1995. Molecular cloning and expression of rat squalene epoxidase. J. Biol. Chem. **270:**17–20.
34. **Satoh, T., M. Horie, H. Watanabe, Y. Tsuchiya, and T. Kamei.** 1993. Enzymatic properties of squalene epoxidase from *Saccharomyces cerevisiae*. Biol. Pharm. Bull. **16:**349–352.
35. **Stuetz, A., and P. Nussbaumer.** 1989. SDZ 87-469. Drugs Fut. **14:**639–642.
36. **Stuetz, A., and G. Petranyi.** 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. J. Med. Chem. **27:**1539–1543.
37. **Troke, P. F.** 1993. In vitro and experimental in vivo activities of fluconazole against some fungi causing cutaneous mycoses, p. 199–214. *In* J. W. Rippon and R. A. Fromtling (ed.), Cutaneous antifungal agents. Selected compounds in clinical practice and development. Marcel Dekker, Inc., New York.
38. **Vanden Bossche, H., J. Heeres, L. J. J. Backx, P. Marichal, and G. Willemsens.** 1993. Discovery, chemistry, mode of action, and selectivity of itraconazole, p. 263–283. *In* J. W. Rippon and R. A. Fromtling (ed.), Cutaneous antifungal agents. Selected compounds in clinical practice and development. Marcel Dekker, Inc., New York.
39. **Villars, V. V., and T. C. Jones.** 1993. Clinical use of oral and topical terbinafine in the treatment of fungal diseases, p. 151–168. *In* J. W. Rippon and R. A. Fromtling (ed.), Cutaneous antifungal agents. Selected compounds in clinical practice and development. Marcel Dekker, Inc., New York.
40. **Wildfeuer, A., and T. Rozman.** 1984. Antimycotic efficacy of tolciclate. Mykosen **27:**142–152.