Inhibition of Sterol Biosynthesis in *Saccharomyces cerevisiae* and *Candida albicans* by 22,23-Epoxy-2-Aza-2,3-Dihydrosqualene and the Corresponding N-Oxide

**GIANNI BALLIANO,** 1 **PAOLA MILLA,** 1 **MAURIZIO CERUTI,** 1 **LUCIA CARRANO,** 2 **FRANCA VIOLA,** 1 **PAOLA BRUSA,** 1 and **LUIGI CATTEL** 1*

Istituto di Chimica Farmaceutica Applicata, 10125 Torino, Italy and Lepett Research Center, Marion Merrell Dow Research Institute, 21040 Gerenzano (Varese), Italy

Received 10 February 1994/Returned for modification 30 March 1994/Accepted 15 June 1994

The abilities of 22,23-epoxy-2-aza-2,3-dihydrosqualene and the corresponding N-oxide, 22,23-epoxy-2-aza-2,3-dihydrosqualene-N-oxide, to inhibit sterol biosynthesis were studied in microsomes and cells of *Saccharomyces cerevisiae* and *Candida albicans*. 22,23-Epoxy-2-aza-2,3-dihydrosqualene, which differs from the other inhibitor only in lacking oxygen at position 2, exhibited higher inhibitory properties in all preparations tested. The different levels of effectiveness of the two azasqualene derivatives were evident mostly in microsomes from *S. cerevisiae* (the 50 inhibitory concentrations of the 2-aza derivative and the corresponding N-oxide on oxidosqualene cyclase were 30 and 120 μM respectively) and in cell cultures of the same strain (1 order of magnitude separated the inhibitory activities of the two compounds on sterol biosynthesis). A possible explanation for the differences between 22,23-epoxy-2-aza-2,3-dihydrosqualene and the corresponding N-oxide arose from the study of their metabolic fates in vivo and in vitro. While the 2-aza derivative did not undergo any transformation, the N-oxide compound was actively reduced to the corresponding amine in microsomes and in cells of both yeast strains. 22,23-Epoxy-2-aza-2,3-dihydrosqualene-N-oxide seems to behave as a proinhibitor of sterol biosynthesis, becoming active only after transformation into the active form 22,23-epoxy-2-aza-2,3-dihydrosqualene.

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2,3-Oxidosqualene-lanosterol cyclase (OS cyclase; EC 5.4.99.7) is a key enzyme in the biosynthesis of sterols in animals and fungi because it catalyzes the connection step between the acyclic and cyclic precursors of sterols (1, 9, 14). In the last 20 years this enzyme has attracted the attention of several research groups interested in unraveling the catalytic process of OS cyclization or developing new inhibitors potentially useful as hypocholesterolemic or antifungal drugs (1). Among the inhibitors of OS cyclase, the azaterpene series represents one of the most numerous and explored classes (5–7, 10, 13). Since the synthesis of 2-aza-2,3-dihydrosqualene (squalene dimethylamine), several terpenoid derivatives bearing nitrogen atoms in different positions of the isoprenoid skeleton (aza analogs of substrate or high-energy intermediates) have been synthesized, and some have been shown to have strong inhibitory properties on oxidosqualene cyclases (5, 10). Recently, azasqualenes bearing an oxirane ring at the end of the isoprenic molecule have been prepared (2, 8). These molecules have been designed with the aim of making the inhibitor more easily recognizable by the enzyme during formation of the enzyme-inhibitor complex.

Here we report the inhibitory properties of 22,23-epoxy-2-aza-2,3-dihydrosqualene (compound 1) and its N-oxide derivative (compound 2) (Fig. 1), which were recently proved to be efficient inhibitors of animal OS cyclase (16), on sterol biosynthesis in *Saccharomyces cerevisiae* and *Candida albicans*. The activities of these compounds were evaluated directly on microsomal OS cyclases from these yeasts and by estimating their effects on ergosterol biosynthesis in proliferating cells. The metabolic fates of the inhibitors were also studied in microsomes and whole cells in order to find a possible correlation between biological activity and metabolic involvement.

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**MATERIALS AND METHODS**

Compounds 1 and 2 were synthesized as reported previously (16). Syntheses of [2-H]-2,3-oxidosqualene (20 μCi/μmol) (10) and [1-3H]squalene dimethylamine (0.26 mCi/μmol) (17) have been described previously. [1-3H]squalene dimethylamine N-oxide was synthesized by treating 77 μCi of [1-3H]squalene dimethylamine with 1 ml of 35% H2O2 in methanol (8:2, vol/vol) for 12 h at 20°C under argon. After the addition of MnO2, the solution was extracted with CH2Cl2 (3 ml, three times); the combined organic phases were dried over anhydrous sodium sulfate and were evaporated under N2. The crude extract was then purified by thin-layer chromatography (MeOH) to give labelled [1-3H]squalene dimethylamine N-oxide (59 μCi; 0.26 mCi/μmol).

Microsomal OS cyclases from *S. cerevisiae* ATCC 12361 and *C. albicans* ATCC 10231 were assayed as reported previously (2, 3). Briefly, 0.5 ml of the reaction mixture, which consisted of (R,S)-[3-3H]squalene 2,3-oxide (100,000 dpm, 50 μM), Tween 80 (0.04%; wt/vol), 0.5 mM dithiothreitol, 0.1 M phosphate buffer (pH 7.3), and 3 mg of microsomal protein per ml, was incubated for 30 min at 35°C in a shaking water bath. The nonsaponifiable lipids were chromatographed on silica gel plates (Merck) with authentic samples of squalene oxide and lanosterol and were developed in CH2Cl2. Areas corresponding to squalene oxide and lanosterol were scraped off and counted directly in plastic scintillation minivials containing 3 ml of Ready Gel (Beckman) (2, 3). Ergosterol biosynthesis in whole yeast cells was measured by incorporation of [2-14C]acetate into nonsaponifiable lipids as described previously (2, 3). Briefly, washed cells (10 × 10^6 to 20 × 10^6 cells) resuspended
in 5 ml of 25 mM phosphate buffer (pH 6.5) containing 1% glucose and inhibitors at different concentrations were fed 2 μCi of [2-14C]acetate (specific activity, 50 mCi/mmol) and were shaken for 2 h at 30°C. Nonsaponifiable lipids were separated on silica gel plates (Merck) developed in n-hexane–ethyl acetate (85:15; vol/vol) with authentic references of ergosterol, lanosterol, oxidosqualene, and squalene. The 14C radioactivities of the chromatographed bands were evaluated by a System 2000 Imaging Scanner (Packard). The metabolism of azasqualene derivatives 1 and 2 was studied by incubating cells and microsomes with inhibitors under the same conditions chosen to test their inhibitory properties. Lipid extracts (i.e., nonsaponifiable lipids) were then chromatographed on silica gel plates (Merck) developed in methanol with authentic samples of compounds 1 and 2. Chromatographic bands were visualized with I2, and their relative amounts were directly evaluated by scanning chromatographic plates with a TLC–high-pressure thin-layer chromatographic scanner (Camag, Muttenz, Switzerland), an instrument for measuring the optical densities of chromatographic or electrophoretic layers. The identity of the metabolite that cochromatographed with compound 1, which was recovered from TLC plates, was established by 1H nuclear magnetic resonance and mass spectroscopy; data were coincident with those reported previously (16). Interconversion of [1-3H]squalene dimethylamine and [1-3H]squalene dimethylamine N-oxide in S. cerevisiae cells was evaluated by a similar procedure except for the scanning of the chromatographic plates, which was done with a System 2000 Imaging Scanner (Packard); the radioactive metabolite obtained by incubating cells with [1-3H]squalene dimethylamine N-oxide cochromatographed with an authentic sample of squalene dimethylamine in two different TLC systems: (i) silica gel and MeOH and (ii) silica gel and CH2Cl2-CH3OH-anhydrous NH3 (97:1:2; vol/vol).

RESULTS

Effects of compounds on yeast growth. The influences of compounds 1 and 2 on S. cerevisiae growth were comparable to those reported for other azasqualene derivatives (MICs, 45 ± 4 and 120 ± 10 μM, respectively) (2, 3). As has been observed for other pairs of azasqualenes and N-oxides, compound 1 was more effective than compound 2 as a cell growth inhibitor. Assay of the antifungal activities of compounds 1 and 2 on C. albicans gave virtually the same results obtained with S. cerevisiae (MICs, 30 ± 2 and 120 ± 10 μM, respectively).

Effect on yeast sterol biosynthesis. The effects of compounds 1 and 2 on sterol biosynthesis were evaluated by incubating yeast cells with [14C]acetate in the presence of different concentrations of inhibitors. The amount of radioactivity detected in different nonsaponifiable lipids gives indirect information on the enzymatic activities affected by the inhibitors. In S. cerevisiae the dose-dependent effect of compound 1 on sterol biosynthesis seemed to be directed to different enzymatic targets, depending on the inhibitor concentration; at 10 μM, the accumulation of the bulk of radioactivity in the oxidosqualene fraction, which was negligible in the control culture, clearly indicated that OS cyclase was the primary target of the inhibitory action. At the same concentration of compound 1, the increase in radioactivity associated with the 4,4-dimethylsterol fraction suggested that steps in sterol biosynthesis after OS cyclization might also be affected. At higher concentrations, compound 1 also inhibited squalene epoxidase, as suggested by the distribution of radioactivity among different components of the nonsaponifiable extract (Fig. 2). The N-oxide derivative (compound 2) was less efficient than compound 1 in inhibiting sterol biosynthesis, since its effect at 100 μM was comparable to that of the corresponding amine (compound 1) at 10 μM (Fig. 3). Nevertheless, it seemed completely ineffective against squalene epoxidase, even at the highest concentrations tested.

In C. albicans the inhibitory activity of compound 1 seemed to be shared between squalene epoxidase and oxidosqualene cyclase. Substrates of these two enzymes, which failed to incorporate significant amounts of acetate in the control cultures, in cells treated with increasing amounts of inhibitor accumulated radioactivity in a dose-dependent manner. With compound 1 at 50 μM, more than 60% of the radioactive nonsaponifiable extract was represented by squalene and oxidosqualene (Fig. 4). The action of compound 2 seemed to be less effective and less specific, as shown by the complex
distribution of radioactivity among the components of the nonsaponifiable extract (Fig. 5).

Inhibition of microsomal oxidosqualene cyclase. The direct effects of compounds 1 and 2 on yeast oxidosqualene cyclase were evaluated by incubating microsomes (microsomal protein concentration, 3 mg/ml) from S. cerevisiae and C. albicans with radioactive oxidosqualene in the presence of different amounts of inhibitors. The results confirmed the differences in the inhibitory activities of compounds 1 and 2, with compound 1 being more active than compound 2 in C. albicans (50% inhibitory concentrations [IC₅₀], 8 ± 0.5 and 19 ± 1 μM, respectively) and, mostly, in S. cerevisiae (IC₅₀, 28 ± 2 and 120 ± 10 μM, respectively).

Metabolism of compounds 1 and 2 in microsomes and cell cultures. Two possible features of the metabolic fates of compounds 1 and 2 were examined: (i) the conversion of the terminal tertiary amine of compound 1 into the corresponding N-oxide group of compound 2 or vice versa, and (ii) the possible cyclization of compound 1 or 2 into the corresponding aza derivatives of lanosterol, 25-azalano-sterol, or 25-azalano-sterol-N-oxide. Interconversion of the two inhibitors was initially tested by evaluating the abilities of the yeast cells (S. cerevisiae) either to oxidize a tertiary amine located at the end of a squalenoid skeleton lacking the terminal oxirane ring or to reduce a similarly located N-oxide group. Experiments were carried out with [³H]-2-aza-2,3-dihydroxyolane and [³H]-2-aza-2,3-dihydroxyolane-N-oxide, two squalene derivatives which had been proved in several studies to be good inhibitors of oxidosqualene cyclase (3, 6, 10). As shown by the results reported in Fig. 6, no N-oxidase activity was detected under the described conditions, whereas the labelled N-oxide derivative underwent an extensive reduction during the incubation period. The results of the preliminary experiments described above were confirmed by directly incubating microsomes or cells of S. cerevisiae and C. albicans with the cold inhibitors compounds 1 and 2. Assays were carried out under the same conditions used to test the inhibitory properties in microsomes and cells. TLC of the extracts revealed that compound 1 was never N-oxidized to compound 2, whereas compound 2 was transformed into a compound which cochromatographed with compound 1 to an extent dependent on the yeast strain and the biological system (Table 1).

Cyclization of the aza-oxidosqualene derivatives, compounds 1 and 2, can be ruled out, because neither in microsomes nor in cells treated with inhibitors were metabolites other than the starting compounds recovered.

**DISCUSSION**

The activity of 22,23-epoxy-2-aza-2,3-dihydroxyolane (compound 1) as an inhibitor of sterol biosynthesis in S. cerevisiae and C. albicans is comparable to those of other members of the azasqualene series (3, 5). The use of azasqualene derivatives as antifungal drugs has generally been ruled out since they either are less powerful than other known inhibitors of sterol biosynthesis in fungi (11, 15) or are less active against yeast enzymes than against mammalian enzymes (5–7, 16). However, the inhibitory activity of compound 1 on the OS cyclase from C.
albicans microsomes (IC_{so} 8 \mu M) was not far from the anti-OS cyclase activity observed in a cell extract of C. albicans treated with the inhibitor Ro-44-9985 (IC_{so} 2 \mu M), a known inhibitor of OS cyclase in fungi (11); furthermore, compound 1 has antifungal activity against C. albicans (MIC, 30 \mu M) that is 10-fold greater than that of Ro-44-9985 and activity similar to that exhibited by Ro-44-9985 against Histoplasma capsulatum and Trichophyton mentagrophytes (11).

The main difference between 22,23-epoxy-2-aza-2,3-dihydro-squalene (compound 1) and its N-oxide (compound 2) is the higher degree of effectiveness of compound 1 as an inhibitor of sterol biosynthesis in cell cultures and oxidosqualene cyclase in microsomes. Such a difference, which was observed both in S. cerevisiae and C. albicans, seems to agree with the results obtained with other pairs of aza and N-oxide derivatives of squalene (3). In the previous work (3), the lower level of activity of the N-oxide derivative (N,N-diethylazasqualene-N-oxide), which was mainly expressed in cell cultures, was interpreted by suggesting that the protonated amine derivative enters yeast cells more easily than the N-oxide. In the present work we attempted to explain the different inhibitory properties of compounds 1 and 2 by following their metabolic fates in whole cells and microsomes. It is well known that tertiary amines and the corresponding N-oxides can be transformed into each other by the reductase-oxygenase systems that occur in procaryotic and eucaryotic cells (4, 12). Indeed, 2-aza-2,3-
TABLE 1. Transformation of compound 2 into compound 1 in microsomes and cells of S. cerevisiae and C. albicans

<table>
<thead>
<tr>
<th>Biological system</th>
<th>Compound 2 in incubation mixture (µM)</th>
<th>% Transformation into compound 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
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<tr>
<td>C. albicans</td>
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<tr>
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* Microsomes and cells were incubated as described in the text. Nonextractable extracts were analyzed by TLC (silica gel, MeOH). Band corresponding to compounds 1 and 2 (Rf 0.25 and 0.4, respectively) were visualized by using I2, and their relative amounts were determined by a densitometric method.

The transformation of compound 2 into compound 1 was expressed as the ratio of the metabolite that cochromatographed with compound 1 to the starting compound 2 in the extract after the incubation. Results are the averages of two separate experiments (deviation was less than 10%).

dihydroxyxqualene proved to be efficiently N-oxidized by an NADPH-dependent oxidase in mammalian liver microsomes (17). In the experiments of the present work, neither in S. cerevisiae nor in C. albicans was N-oxidation of the two azasqualene tested observed, whereas the N-oxide derivatives were actively reduced in both yeast strains. This reductase activity was especially present in cell cultures, but it also occurred in C. albicans microsomes. Comparison of the inhibitory effect and the metabolic involvement of compound 2 in microsomes from C. albicans and S. cerevisiae suggests that compound 2 behaves as a proinhibitor of oxidosqualene cyclase and is active only after its transformation into compound 1, the true active form. In microsomes from S. cerevisiae, compound 2 would be a weaker inhibitor of oxidosqualene cyclase (IC50 120 µM) simply because it is poorly transformed into compound 1. In C. albicans, the apparently greater sensitivity of microsomal oxidosqualene cyclase to compound 2 would then be due to a more efficient reduction of compound 2 to compound 1. Metabolic transformation of compound 2 could also account for its inhibitory action in cell cultures. It cannot be ruled out, however, that compound 2 is an inhibitor per se of oxidosqualene cyclase, even though it is weaker than compound 1. In this case its inhibitory effect should depend on its intrinsic inhibitory properties and its metabolic transformation. To elucidate this problem, the evaluation of the effect of compound 2 on sterol biosynthesis in yeast cells and microsomes should be investigated in the presence of specific inhibitors of N-oxide reductases. Studies of the effects of inhibitors of N-oxide reductases on microsomes and cells treated with compound 2 are in progress to design new experimental procedures able to disclose the intrinsic inhibitory activity of compound 2 and other azasqualene N-oxides toward the enzymes of sterol biosynthesis.

Our results give strong evidence for the existence of highly effective N-oxide reductase activities in S. cerevisiae and C. albicans. An investigation of such enzymatic activities could contribute to the design of new specific inhibitors of sterol biosynthesis in yeasts and fungi. We are attempting to modify the isoprenoid structure of the squalenoid inhibitors in order to synthesize molecules which, although inactive as N-oxides in mammals and fungi, could be transformed into the active forms by the reductase activities of yeast cells. Such molecules should not be modified in mammalian cells because under normal aerobic conditions the N-oxide reductase activity of the mammalian system is generally inoperative (12). A good model for the described strategy could be the ammonium-containing compound Ro-44-9985 (11).

ACKNOWLEDGMENTS

This work was supported by grants from MURST and the Consiglio Nazionale delle Ricerche, Progetto Finalizzato Chimica Fine, Italy.

REFERENCES