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Differential inhibition of fungal oxidosqualene cyclase by 6*E* and 6*Z* isomers of 2,3-epoxy-10-aza-10,11-dihydrosqualene

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Inhibitory properties of 6*E* (compound 1) and 6*Z* (compound 2) isomers of 2,3-epoxy-10-aza-10,11-dihydrosqualene against oxidosqualene-lanosterol cyclase were assayed on microsomes and whole cells of *Saccharomyces cerevisiae* and *Candida albicans*. Only the 6*E* isomer (compound 1), bearing a correct substrate-like configuration, strongly inhibited the enzyme both in microsomes and cell cultures. The difference between compounds 1 and 2 (which had an unfavorable geometry) was especially evident when measuring [^{14}C]acetate incorporation into non-saponifiable lipids extracted from treated cells. While isomer *Z* was totally ineffective at up to 30 μM, in cells treated with 5 μM isomer *E*, labelled oxidosqualene, the level of which was negligible in the control, rose to over 60% of the non-saponifiable lipids.

Oxidosqualene cyclase; Sterol biosynthesis inhibitor; *Saccharomyces cerevisiae*; *Candida albicans*

1. INTRODUCTION

Enzymatic cyclization of 2,3-oxidosqualene into lanosterol is a multistep process involving a series of transient carbocationic high energy intermediates [1–3].

In the last few years, several successful attempts to inhibit oxidosqualene cyclizing enzymes, and several other enzymes involved in sterol or terpenoid biosynthesis, have been made by designing high energy intermediate analogues, bearing a stable positive charge at appropriate positions [4–8]. Among the intermediate carbocations formed during enzymatic cyclization of oxidosqualene, the pro C-8 ion seemed to play a central role in the interaction between the substrate and the active site of the enzyme [9]. We therefore supposed that an oxidosqualene-like molecule such as (6*E*)-2,3-epoxy-10-aza-10,11-dihydrosqualene (compound 1) (Fig. 1), bearing a nitrogen group at the pro C-8 position, should behave as a high energy intermediate analogue and act as a powerful inhibitor of oxidosqualene cyclase. This molecule, belonging to the azasqualene series [6], would have the advantage in its interaction with the enzyme, both by the presence of an epoxide group at the end of the isoprenic skeleton and by the strict configurational resemblance (i.e. *all trans* squalenoid structure) with the substrate. This latter feature was, in our opinion, the essential requirement for the specific action of an azasqualene bearing the nitrogen group in the central part of the molecule. For this reason we decided to compare the inhibitory properties of the two isomers *E* (compound 1) and *Z* (compound 2) of 2,3-epoxy-10-aza-10,11-dihydrosqualene (Fig. 1) against oxidosqualene cyclases in microsomes and in cell cultures of *Candida albicans* and *Saccharomyces cerevisiae*.

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2. EXPERIMENTAL

Compounds 1 and 2 were synthesized as reported [10]. Synthesis of [^{3}H]2,3-oxidosqualene has also been described elsewhere [5]. Microsomes from *Saccharomyces cerevisiae* (ATCC 12361) and *Candida albicans* were prepared by following a procedure previously described [11], except for the disruption of *Candida* cells which was performed using a Dyno-mill (WAB) homogenizer. Microsomal oxidosqualene cyclase from both yeast strains was assayed as reported [11]. Assay of sterol biosynthesis in whole cells of *S. cerevisiae* and *C. albicans* by incubation with [2-^{14}C]acetate, extraction of the non-saponifiable lipids and separation by thin layer chromatography has been reported [7].

^{14}C radioactivity associated with different chromatographic bands was measured directly by scanning plates in a System 2000 Imagine Scanner (Packard), equipped with an MPM Computer.

3. RESULTS

Both the isomers 6*E* (compound 1) and 6*Z* (compound 2) of 2,3-epoxy-10-aza-10,11-dihydrosqualene failed to inhibit the growth of *C. albicans*, whereas their effect on *S. cerevisiae* was comparable with that reported for other aza-squalene derivatives [6,7] (Table I).

The influence of compounds 1 and 2 on sterol biosynthesis was evaluated by incorporation of [^{14}C]acetate into non-saponifiable lipids extracted from yeast cells cultured in the presence and in the absence of the inhibitors (Tables II and III) (Fig. 2). Isomer *E* (compound 1) caused a dose-dependent accumulation of radioactiv-

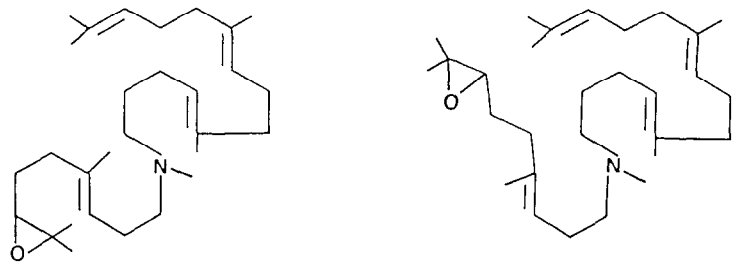

Fig. 1. 6*E* (compound 1) and 6*Z* (compound 2) isomers of 2,3-epoxy-10-aza-10,11-dihydrosqualene.

ity in oxidosqualene, accompanied by a sharp drop in the radioactivity incorporated into the 4,4-desmethyl sterol fraction (ergosterol fraction). In cultures of *S. cerevisiae* and *C. albicans*, the radioactivity associated with the oxidosqualene fraction, which was negligible in the controls, increased to represent over 60% of the non-saponifiable lipids in cells treated with 5 μM isomer *E*. The ergosterol fraction, which contained 70–80% of the radioactivity in the control cultures, fell to 10–20% in treated cells. Moreover, in *C. albicans*, accumulation of radioactivity in the oxidosqualene fraction was accompanied by the appearance of a fraction corresponding to dioxidosqualene. No increase in the radioactivity associated with the squalene fraction was noted, neither in *S. cerevisiae* nor in *C. albicans*, by treatment of cells with isomer *E* at concentrations up to 60 μM.

The two fungal strains responded differently to isomer *Z* (compound 2). In *C. albicans*, incorporation of radioactivity into components of the non-saponifiable fraction was not affected by exposure of cells to compound 2 at concentrations up to 30 μM (Table III). At higher concentrations the effect was similar to that previously observed at lower concentrations of isomer *E*, i.e. an increase in the ratio of labelled oxidosqualene to labelled ergosterol. In *S. cerevisiae* the main response to isomer *Z* seemed to be a significant accumulation of radioactivity in a fraction co-chromatographing with lanosterol, a response quite similar to that reported after the treatment of cells with *N,N*-diethyldodecylamine [7].

A clear difference between the effects of isomer *E* and isomer *Z* was confirmed by results obtained with the microsomal oxidosqualene cyclase(s) from *S. cerevisiae* and *C. albicans*. Only isomer *E*, which has a substrate-like conformation, was an effective inhibitor of oxidosqualene cyclase from both sources (Table IV). As in the case of the assay on whole cells, microsomal oxidosqualene cyclases prepared from the two fungal strains responded differently to treatment with isomer *Z*. While the enzyme from *S. cerevisiae* was totally in-

Table I

Effect of isomer *E* and *Z* on cell growth

Isomer	MIC (μM)	
	S. cerevisiae	*C. albicans*
E	30	> 200
Z	60	> 200

Table II

Effect of isomer *E* and *Z* on incorporation of [^{14}C]acetate into non-saponifiable lipid fractions in *S. cerevisiae* cells

Concentrations of inhibitors (μM)	% total radioactivity incorporated by				
	Ergosterol	Lanosterol	Dioxidosqualene	Oxidosqualene	Squalene
0 (control)	65 9	7.1	<0.1	0.1	26.7
Isomer *E* 5	16.2	8.5	<0 1	70.7	7 5
Isomer *E* 10	2.6	<0.1	<0.1	87.5	9.9
Isomer *E* 30	0.9	0.4	<0.1	86.6	10.1
Isomer *E* 60	<0.1	0.2	<0 1	82.0	17.8
Isomer *Z* 5	63 3	6 5	<0.1	<0.1	30.2
Isomer *Z* 10	27.5	28.1	<0 1	6.6	37.8
Isomer *Z* 30	49.1	18.8	<0.1	8.5	23.5
Isomer *Z* 60	27 7	25.2	<0.1	27.3	19.7

Results are means of 2 separate experiments each with duplicate incubations. Maximum deviations from the mean were less than 10%.

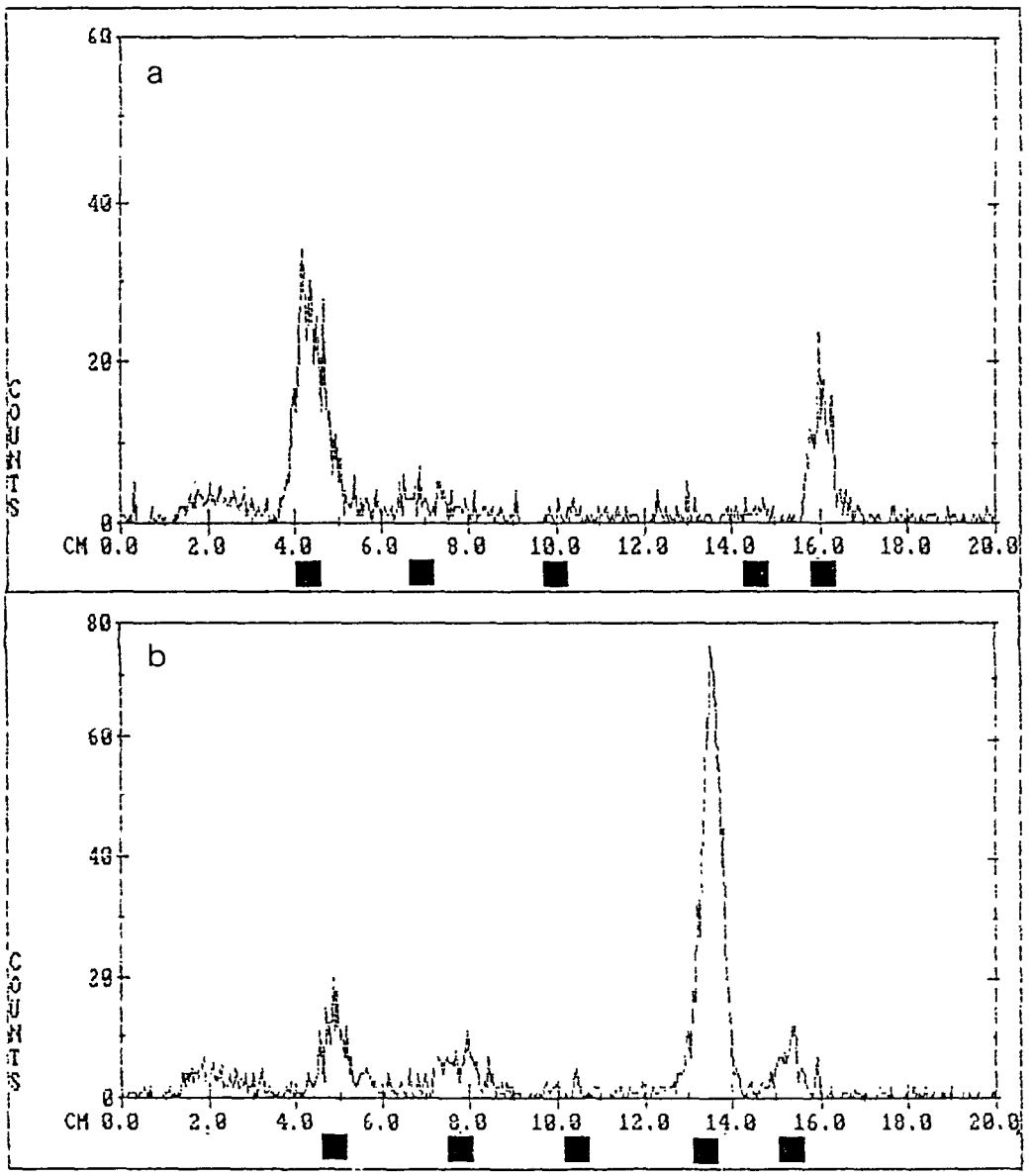

Fig. 2. Radiochromatograms of non-saponifiable lipids extracted from cells of *S cerevisiae* incubated with [^{14}C]acetate for 2 h. (a) Cells incubated without inhibitors. (b) Cells exposed to 5 μM isomer *E* (compound 1) during the incubation time. The markers from left to right were: ergosterol, lanosterol, dioxidosqualene, oxidosqualene and squalene.

Table III

Effect of isomer *E* and *Z* on incorporation of [^{14}C]acetate into non-saponifiable lipid fractions in *C. albicans* cells

Concentrations of inhibitors (μM)	% total radioactivity incorporated by				
	Ergosterol	Lanosterol	Dioxidosqualene	Oxidosqualene	Squalene
0 (control)	86.3	1.4	<0.1	6.2	6.1
Isomer *E* 5	23.7	3.8	4.1	65.7	2.7
Isomer *E* 30	7.1	2.6	5.6	80.3	4.3
Isomer *E* 60	4.9	3.2	4.3	79 3	8.3
Isomer *Z* 30	83.2	5.8	<0.1	7.1	3.7
Isomer *Z* 60	30.3	6.9	3.0	53.6	6.2

Results are means of 2 separate experiments each with duplicate incubations. Maximum deviations from the mean were less than 10%

Table IV

I_{50} values (μM) of inhibition of microsomal oxidosqualene cyclase by isomer *E* and *Z*

Isomer	*S. cerevisiae*	*C. albicans*
E	5	3
Z	> 200	> 50

Results are means of two separate experiments. Microsomal protein concentration was 2 mg/ml for *S. cerevisiae* and 3 mg/ml for *C. albicans*. I_{50} values were determined at a substrate concentration of 25 μM. K_m of oxidosqualene cyclase from *S. cerevisiae* was 34 ± 8 μM (mean ± S.D for five separate experiments) [7].

sensitive to the inhibitor, the enzyme from *C. albicans* was weakly inhibited.

4. DISCUSSION

Results obtained with (6*E*)-10-azasqualene (compound 1) on microsomes and whole cells of *S. cerevisiae* and *C. albicans* indicate that this approach represents a new step in the strategy for designing azasqualene derivatives as inhibitors of fungal oxidosqualene cyclase. This inhibitor, which was designed as an analogue of the pro C-8 high energy intermediate formed during transformation of oxidosqualene into lanosterol, is the most powerful and specific of the azasqualenes tested so far [6,7,12,13]. This conclusion is based, in particular, on the experiments with whole cells, in which a dramatic accumulation of oxidosqualene occurred in the presence of (6*E*)-10-azasqualene at concentrations at which other azasqualene derivatives are inactive.

The inhibitory properties of (6*E*)-10-azasqualene might be due to the presence on the inhibitor molecule of a stable positive charge at a position (pro C-8) which is thought to strongly interact with the active site of the enzyme [5,9]. Moreover, the compound also has a 2,3-epoxide group, which increases its similarity to the substrate and may favor enzyme–inhibitor recognition.

Squalene epoxidase does not seem to be affected by the inhibitor. Neither in *S. cerevisiae* nor in *C. albicans* did treatment of cells with (6*E*)-10-azasqualene (compound 1) induce any accumulation of squalene in non-saponifiable lipids.

The difference in inhibitory activity between the 6*E* and 6*Z* isomers of 10-azasqualene provides direct evidence of the dependence of activity on structural similarity with the substrate. The *Z* isomer, which has an unfavorable geometry, did not significantly affect the sterol biosynthesis at up to 30 μM in whole cells, nor did it significantly inhibit oxidosqualene cyclase in microsomes. In *S. cerevisiae*, the inhibitory activity of isomer *Z* appeared to affect other enzymes of sterol biosynthesis, as indicated by the increase of the lanosterol fraction in treated cells.

In conclusion, our work supports the view that azasqualenes bearing the nitrogen group in the central part of the isoprenic skeleton, need a correct *all trans* configurational geometry to specifically inhibit fungal oxidosqualene cyclase. Azasqualenes having different geometrical structures, such as (6*Z*)-2,3-epoxy-10-aza-10,1-dihydrosqualene, do not have the specific ability to inhibit oxidosqualene cyclase; other enzymes of sterol biosynthesis seem, in this case, to become targets of their action. It can be supposed that the presence of a stable positive charge within the isoprenic skeleton enables the 6*Z* isomer to affect enzymes like Δ^8–Δ^7-sterol isomerase and Δ^{14}-sterol reductase, which catalyze reactions that also involve high energy intermediate carbocations. Similar inhibitory properties have been reported for azadecaline derivatives, which inhibit Δ^8–Δ^7-isomerase, in addition to oxidosqualene cyclase [12].

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