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ERGOSTEROL DEPLETION AND 4-METHYL STEROLS ACCUMULATION IN THE YEAST *SACCHAROMYCES CEREVISIAE* TREATED WITH AN ANTIFUNGAL, 6-AMINO-2-*n*-PENTYLTHIOBENZOTHIAZOLE

Tomáš Kuchta[1], Katarína Bartková[2] and Róbert Kubinec[3]

[1] *Food Research Institute, Štefánikova 45, CS-90001 Modra, Czecho-Slovakia*

[2] *Drug Research Institute, Horná 36, CS-90001 Modra, Czecho-Slovakia*

[3] *Chemical Institute, Faculty of Science, Comenius University, Mlynská dolina Ch-2, CS-84215 Bratislava, Czecho-Slovakia*

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Summary: In *Saccharomyces cerevisiae* treated with an antifungal agent, 6-amino-2-*n*-pentylthiobenzothiazole, levels of ergosterol and other 4-desmethylsterols were found to be significantly reduced. Major sterols in treated yeast were lanosterol, 4,4-dimethylzymosterol, 4-methylzymosterol and 4-methylfecosterol. A hypothesis is stated that the antifungal agent inhibits sterol demethylation at C-4 and forces the biosynthesis to a blind pathway ending by 4-methylfecosterol.

Several currently used antifungal agents, antimycotics and fungicides, act as inhibitors of ergosterol biosynthesis. Following reactions in ergosterol biosynthesis pathway were found to be inhibited by antifungals: squalene epoxidation (e.g. by allylamines, [1]), sterol 14α-demethylation (e.g. by azoles, [2]), sterol Δ^{14}-reduction and Δ^{8}- Δ^{7}-isomerization (both by dimethylmorpholines, [3]). Block in any of the mentioned pathway steps causes ergosterol depletion in fungal cells, accompanied with accumulation of the intermediate(s) preceding the blocked reaction, or of their unusual metabolites.

Abbreviations used in the text: APB, 6-amino-2-*n*-pentylthiobenzothiazole; NSL, nonsaponifiable lipids; GC-MS, gas chromatography - mass spectrometry.

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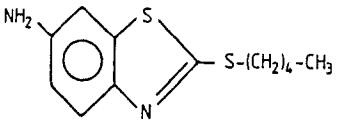

Fig. 1. 6-Amino-2-*n*-pentylthiobenzothiazole.

6-Amino-2-*n*-pentylthiobenzothiazole (Fig. 1) is a synthetically prepared compound exhibiting high antifungal activity with yeasts and yeast-like microorganisms *in vitro* [4,5] and *in vivo* (Bujdáková, H., personal communication). Its mode of action has already been studied and reduced proportion of ergosterol in treated yeast was observed [6]. Here we report the effects of APB on nonsaponifiable lipids pattern in *S. cerevisiae* and attempt to define the step in ergosterol biosynthetic pathway blocked by the compound.

MATERIALS AND METHODS

Antifungal agent. 6-Amino-2-*n*-pentylthiobenzothiazole was provided by Dr E. Sidóová, Chemical Institute, Faculty of Science, Comenius University, Bratislava.

Microorganism and growth conditions. Saccharomyces cerevisiae FL200 (ATCC 32119) was grown in 100 ml batches at 30 °C in a rotary shaker at 2 Hz in YPD medium containing yeast autolysate (Imuna) 0.5 % (w/v), peptone (Imuna) 1 % (w/v), glucose 2 % (w/v), and various quantities of APB dissolved in dimethyl sulfoxide. Solvent concentration in the media did not exceed 1 % (v/v). Growth of the cultures was stopped after 16 h by cooling them to 4 °C without interrupting shaking.

NSL extraction. Yeast biomass was collected by centrifugation and washed with cold distilled water. After adding cholesterol (Fluka) as an internal standard, samples were saponified in 10 % (w/v) KOH in 50 % (v/v) ethanol in the presence of 0.1 % (w/v) pyrogallol at 73 °C for 1 h. NSL were extracted to hexane, dried in the vacuum evaporator and taken up in 0.5 ml of cyclohexane.

NSL analysis. NSL were analysed in a Hewlett-Packard HP 5890 Series II gas chromatograph employing OV-1 (0.4 μm film) capillary column 20 m x 0.32 mm i. d., He carrier gas, splitless injection (3 min), 280 °C injection port, temperature programmed from 150 °C (3 min) to 300 °C (7 min) at 8 °C/min, connected to a Hewlett-Packard HP 5971A mass selective detector, operating at ionizing potential 70 eV.

RESULTS

Batches of yeast were grown for 16 h in the presence of various APB concentrations in the order of 10^{-5} M, or in the absence of the antifungal

(control). 20 μM, 40 μM and 60 μM APB allowed 17 %, 8 % and 3 % culture growth, respectively, based upon dry weight measurements, relative to the control over the culture period. At the given concentrations, culture growth rates (μ) were 0.49 h^{-1}, 0.37 h^{-1} and 0.29 h^{-1}, respectively, while the control exhibited $\mu = 0.55\ h^{-1}$, based upon absorbance readings.

NSL were then extracted from the yeast and were analysed by gas chromatography - mass spectrometry. The sterol pattern in treated yeast wa: markedly different from the control (Fig. 2). It also clearly differed from that observed with *S. cerevisiae* treated with sterol 14α -demethylase inhibitors, econazole and clotrimazole, where mainly component ***7*** (lanosterol**) accumulated (not shown). Components ***1***, ***2***, ***5*** which made up a large proportion in the control, gradually disappeared when yeast was treated with increasing APB concentrations, and components ***3***, ***6***, ***7***, ***8*** became dominant. Relative proportions of NSL components in yeast cultures treated with various APB concentrations are given in Table 1.

Peaks were identified by GC-MS on the basis of mass spectra and retention times relative to cholesterol, using available standards and literature data [7-10]. Component ***6*** was identified on the basis of comparison of its mass spectrum to that of 4-methylzymosterol ***(3)***. Mass spectra of both sterols ***(3, 6)*** contain common $[M - \text{side chain} - 2H]^+$ and $[M - \text{side chain} - 42 - H_2O]^+$ ions, which indicates the same molecule nucleus. $[M]^+$, $[M - CH_3]^+$ and $[M - CH_3 - H_2O]^+$ ions are shifted up by 14 in ***6***, which indicates additional methylene group in the side chain of ***6***. These properties suggest that ***6*** is 4-methylfecosterol***. Characteristic ion species in mass spectra of the sterols accumulated in APB treated yeast cells are given in Table 2.

DISCUSSION

APB markedly blocks formation of ergosterol in *S. cerevisiae* FL200 and causes accumulation of squalene, lanosterol, 4,4-dimethylzymosterol, 4-methylzy-

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** *Trivial names used in the text:*
lanosterol, 4,4,14α-trimethylcholesta-8,24-dien-3β-ol;
zymosterol, cholesta-8,24-dien-3β-ol;
fecosterol, ergosta-8,24(24^1)-dien-3β –ol;
ergosterol, ergosta-5,7,22-trien-3β -ol.

*** Orientation of the 4-methyl group in 4-methylfecosterol as well as in 4-methylzymosterol could not be determined. Thus α or β designations for these are not given in the text.

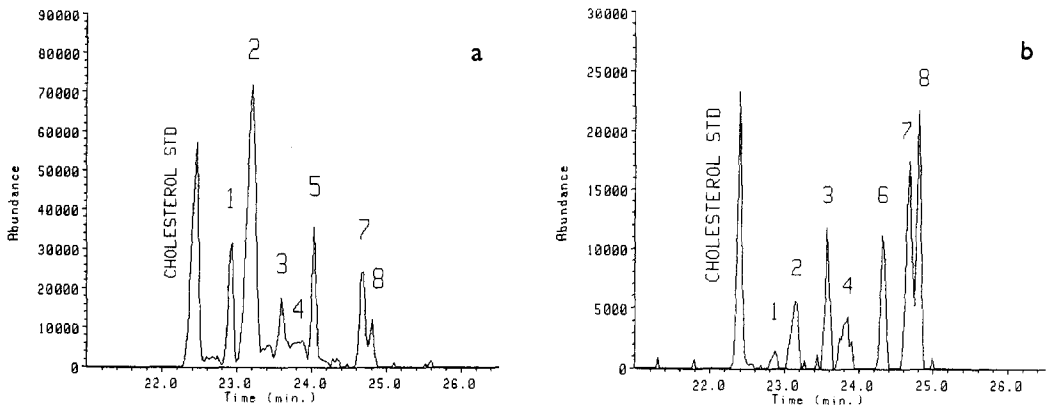

Fig. 2. Chromatograms of selected ions (384, 386, 396, 398, 400, 411, 412) from untreated control (a) and from the yeast treated with 60 μM APB (b).

mosterol and 4-methylfecosterol. These effects are concentration-dependent and closely accompany the overall culture growth inhibition. Contents of zymosterol and of its metabolites downstream the ordinary pathway are severely reduced in yeast cells treated with 60 μM APB. On the other hand, an unusual metabolite, 4-methylfecosterol, occurs then in significant quantity.

Table 1. Relative proportions of NSL in control and in APB-treated yeast

NSL	% total NSL[a]			
	control	20 μM APB	40 μM APB	60 μM APB
squalene	5	11	16	26
zymosterol *(1)*	20	4	0	0
ergosterol *(2)*	40	15	8	2
4-methylzymosterol *(3)*	5	12	13	10
ergosta-5,7-dienol *(4)*	5[b]	8	10	0
ergost-7-enol *(5)*	12	0	0	0
4-methylfecosterol *(6)*	0	10	12	15
lanosterol *(7)*	7	18	17	20
4,4-dimethylzymosterol *(8)*	6	22	24	27

[a] Calculated by the area method from records of total ion counts (m/z from 50 to 500).
[b] Mixed with other diunsaturated sterols with m/z = 398.

Table 2. Characteristic ion species in mass spectra of sterols extracted from *S. cerevisiae* treated with 60 μM APB [4-methylzymosterol (**3**), 4-methylfecosterol (**6**), lanosterol (**7**), 4,4-dimethylzymosterol (**8**)]

Fragmentation	**3**	**6**	**7**	**8**
$[M]^+$	398 (100)[c]	412 (100)	426 (47)	412 (100)
$[M - CH_3]^+$	383 (74)	397 (65)	411 (100)	397 (59)
$[M - CH_3 - H_2O]^+$	365 (38)	379 (30)	393 (56)	379 (31)
$[M - sc^a - 2H]^+$	285 (27)	285 (66)	313 (3)	299 (23)
$[M - sc - r^b - H_2O]^+$	227 (47)	227 (66)	255 (25)	241 (52)

[a] Loss of the side chain.
[b] Loss of C-15 to C-17 in **3**, **6**, **8**, and C-15 to C-17 plus C-14[1] in **7**.
[c] Figures in parentheses are the relative intensities of ions (m/z from 200 to 500).

The impact of these results can be explained in the context of ergosterol biosynthetic pathway which has been previously described in *S. cerevisiae* [11] (Fig. 3). Similar to the control, the first step of lanosterol (**7**) conversion in the presence of APB appears to be the demethylation at C-14, yielding 4,4-dimethylzymosterol (**8**). The next reaction taking place is the demethylation of 4,4-dimethylzymosterol (**8**) at C-4, yielding 4-methylzymosterol (**3**). However, increased level of 4,4-dimethylzymosterol (**8**) in APB-treated yeast may indicate considerable inhibition of this reaction by APB. 4-Methylzymosterol (**3**) generated can serve as a substrate for another demethylation, but also for a concurrent reaction, 24-methylation. *S*-Adenosylmethionine:Δ^{24}-sterol methyltransferase from *S. cerevisiae* is known to accept a variety of sterol substrates showing with zymosterol (**1**), 4-methylzymosterol (**3**), 4,4-dimethylzymosterol (**8**) and lanosterol (**7**) 100 %, 5 %, 2 % and 0 % activity, respectively [12]. Since no 4,4-dimethylfecosterol was found in APB-treated yeast, it seems that the first C-4 demethylation is only partially inhibited by APB and the substrate is still preferentially 4-demethylated rather than 24-methylated. On the other hand, the generated 4-methylzymosterol (**3**) is no further 4-demethylated to zymosterol (**1**) in the presence of APB but it undergoes 24-methylation instead. 4-Methylfecosterol (**6**) formed by this reaction seems the final product of the pathway in

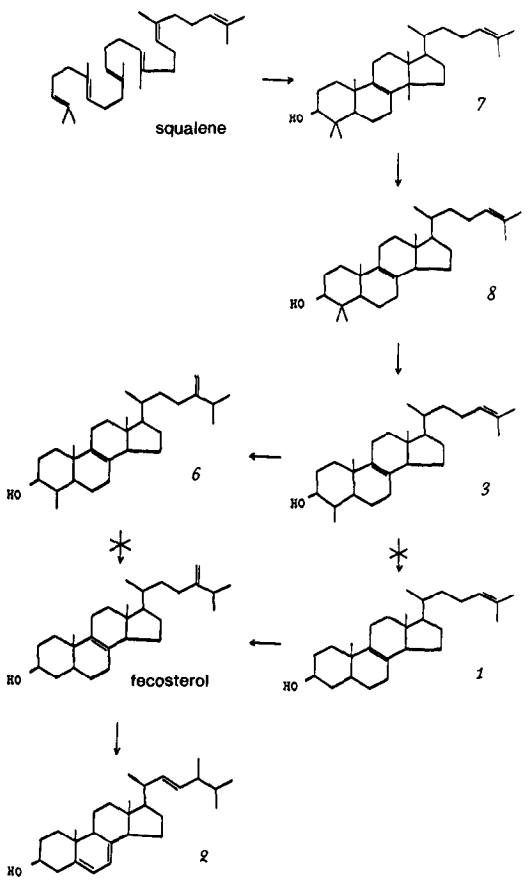

Fig. 3. A scheme of the biosynthetic pathway from squalene to ergosterol in *S. cerevisiae*. Lanosterol *(**7**)*, 4,4-dimethylzymosterol *(**8**)*, 4-methylzymosterol *(**3**)*, 4-methylfecosterol *(**6**)*, zymosterol *(**1**)*, ergosterol *(**2**)*. Arrows represent one or several reactions. Crossed arrows represent reactions which seem inhibited by APB.

APB-treated yeast, probably because a methyl group at C-4 makes the sterol molecule unacceptable for the following reaction, Δ^8-Δ^7-isomerization. This situation may be similar to that with yeast 14α-demethylation mutants which accumulate 14α -methylfecosterol as a final product of the altered pathway [13].

Besides the mentioned 4-methyl sterols, APB-treated yeast cells accumulate considerable amounts of squalene and lanosterol. It is, of course, possible that also squalene and lanosterol conversions are affected by APB treatment. However, more probable explanation could be that higher levels of these intermediates are just a consequence of a block further in the pathway. This would be similar to the situation in yeast sterol 14α-demethylation mutants in which also squalene accumulated although only a further step in the biosynthetic pathway was blocked [13].

Results very similar to these here reported were obtained with another *S. cerevisiae* strain, CCY 28-3 (Kuchta, T. and Kubinec, R., unpublished results), which suggests that the observed effects of APB are not restricted to strain FL200 used in this study.

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