The Effect of Clotrimazole and Triadimefon on 3-Hydroxy-3-Methyl-Glutaryl-CoA-Reductase-[EC 1.1.1.34]-Activity in *Saccharomyces cerevisiae*

Dieter Berg and Wilfried Draber

Bayer AG, Agrochemical Division, Wuppertal, Bundesrepublik Deutschland

Hasso von Hugo

Bayer AG, Pharmaceutical Division, Wuppertal, Bundesrepublik Deutschland

Werner Hummel and Dieter Mayer

Institut für Klinische Biochemie und Physiologische Chemie, Medizinische Hochschule Hannover, Bundesrepublik Deutschland

Z. Naturforsch. **36 c,** 798–803 (1981); received July 17, 1981

Dedicated to Prof. Dr. Grünewald *on Behalf of his 60th Birthday*

HMG-CoA-Reductase, Sterol Biosynthesis, Antifungals, Antimycotics, Mode of Action

Clotrimazole and triadimefon are known as potent inhibitors of ergosterol synthesis in pathogenic yeasts and fungi, respectively. As their mode of action generally the inhibition of sterol desmethylation reactions is accepted. We report about a second effect, a "feed-back" inhibition of 3-hydroxy-3-methyl-glutaryl (HMG)-CoA-reductase by accumulation of ergosterol precursors. Addition of lanosterol to intact cells leads to an inhibition of HMG-CoA-reductase as well, but not to fungistatic effects. From the reported data the influences of clotrimazole and triadimefon have to be considered as an inhibition of desmethylation reactions involved in ergosterol synthesis of yeasts and fungi with a concomitant decreased production of mevalonate.

Introduction

During the last decade a number of N-substituted imidazole as well as triazole derivatives, effective against human pathogenic and phytotoxic fungi and yeasts, have appeared on the market. The first compound from this series is the broad-spectrum antimycotic clotrimazole (Canesten® (**I**)) [1]. Clotrimazole is an imidazole derivative as well as miconazole (**II**) [2] and econazole (**III**) [3], which is chemically closely related to miconazole. Active ingredients against phytopathogenic fungi are the imidazole imazalil (**IV**) [4–6] and the triazole derivatives triadimefon (Bayleton® (**V**)) and fluotrimazole (**VI**) [7, 8]. Besides this the fungicides fenarimol (**VII**) and denmert (**VIII**) have been reported to inhibit ergosterol biosynthesis in fungi as well [4–6].

All these fungicides retard fungal growth by causing morphologic abnormalities in a very similar manner [4, 8–13]. They hardly inhibit growth of conidiae but suppress extension of hyphae. The latter swell and branch extensively in presence of those fungicides. Another similarity is their effectiveness against powdery mildew.

Within the last years a number of investigations dealing with the mode of action have been carried out. All these compounds (**I-VIII**) have been reported to inhibit desmethylation at 24-methylene-dihydrolanosterol in ergosterol biosynthesis [14]. An exception from this mechanism is the fungicide tridemorph (**IX**) which attacks at a later stage of ergosterol synthesis, *i. e.* Δ^8 to Δ^7-isomerisation [15].

The now presented investigations deal with the problem of the physiological consequences which result from accumulation of trimethylsterols. From studies with the mammalian liver system as well as from plants it had become evident that exogenously applied sterols may regulate the HMG-CoA-reductase by a "feed-back-control" [16–23]. We demonstrate that the regulation of the enzyme in yeasts and fungi depends on similar conditions.

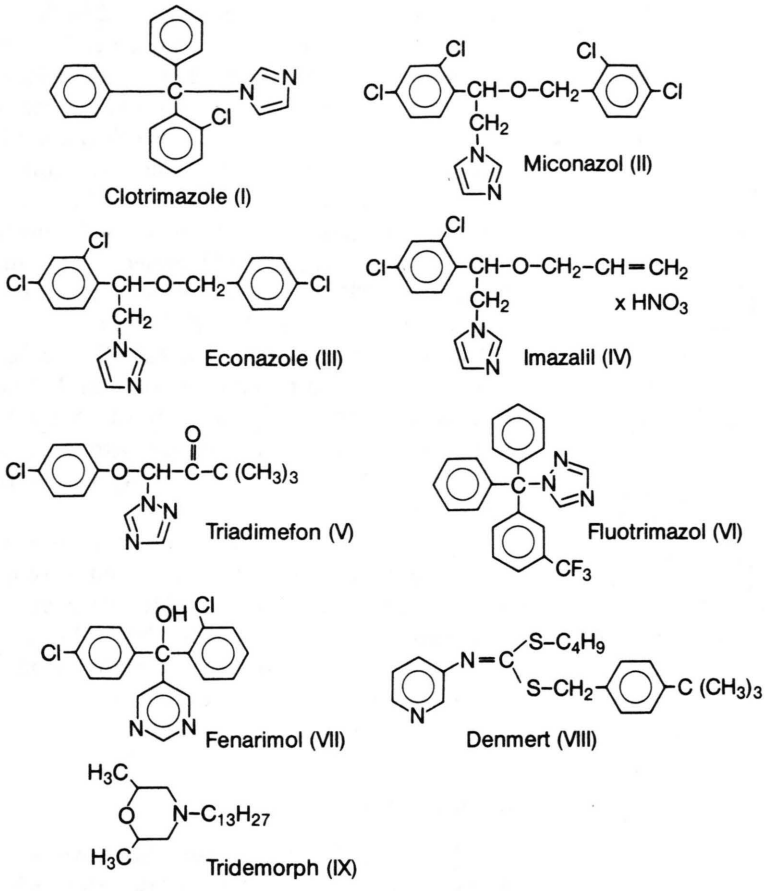
Materials and Methods

Chemicals: Chemicals used were reagent grade. Radioactively labelled HMG-CoA and mevalonate were purchased form NEN.

Abbreviations: HMG-CoA-reductase, 3-hydroxy-3-methyl-glutaryl-CoA-reductase-EC 1.1.1.34.

Reprint requests to Dr. D. Berg.

0341-0382/81/0900-0798 $ 01.00/0



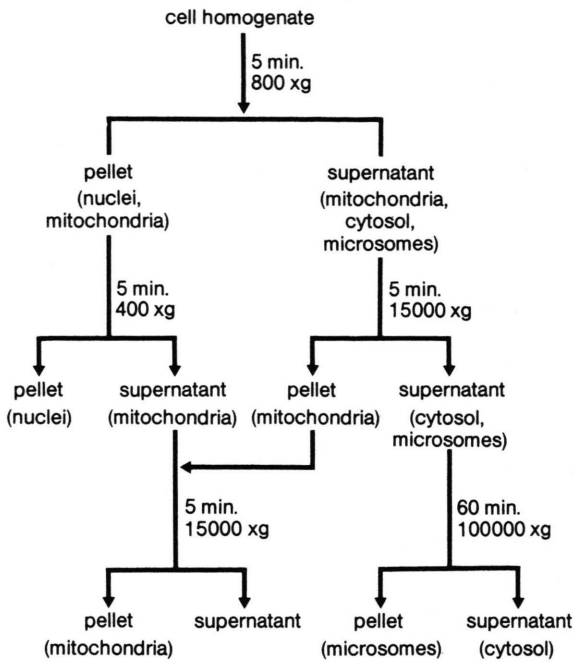
Organism and culture conditions: Fermentation of *Saccharomyces cerevisiae*: precultures of *Saccharomyces cerevisiae* are grown on the following media: glucose 1%, $(NH_4)_2SO_4$ 0.3%, KH_2PO_4 0.2%, citric acid 0.2% K_2CO_3, 0.2%, $MgSO_4$ 0.07%, NaCl 0.05%, yeast extract (Bacto) 0.25%, vitamin B_6 0.001% (aqua dest.).

After sterilisation (20 min, 110 °C) the pH-value is 5.7. 100 ml of medium in 1 l-Erlenmeyer-flasks are inoculated from agar slants with an inoculation loop and incubated on a shaker at 240 rpm for 48 h at 28 °C.

Experimental incubation (semianaerobic): 1 l-Erlenmeyer-flasks containing 400 ml of the following medium are inoculated with 1 ml of the well grown preculture and incubated on a shaker at 50 rpm for 24 h at 28 °C: glucose 20%, yeast extract (Bacto) 1.0%, NaCl 0.5%, peptone (Bacto) 2.0% (aqua dest.). Glucose was sterilizied separately (30 min, 121 °C) and added to the medium after cooling. The pH-value was adjusted to 5.7 before sterilisation.

Isolation of subcellular fractions: The isolation of subcellular fractions was carried out according to Middleton and Apps [24] with some modifications according to Shimizu *et al.* [25]. After incubation the cells were isolated by centrifugation ($6000 \times g$) and washed twice with 0.1 M Sørensen-buffer, pH 6.2. The resulting pellet amounts to 0.5 to 1 g wet weight. The cells are incubated for 30 min at 28 °C in 10 ml 0.2 M mercaptoethanol, 25 nM EDTA, 0.01 M Tris/HCl-buffer, pH 8.0. After centrifugation at $6000 \times g$ the cells are resuspended in 5 ml 0.2 M citric acid/phosphate-buffer, pH 5.6, 0.6 M mannitol, together with 200 µl arylsulfatase/β-glucuronidase (Boehringer) and incubated for 2.5 h at 28 °C. Cells are centrifugated and disrupted by pottering (Potter S, Fa. Braun) in 4 ml 50 mM Sørensen-buffer, pH 7.2, 0.6 M mannitol, 5 mM EDTA. The degree of cell disruption can be observed microscopically.

The following fractionated centrifugation is carried out according to Shimizu *et al.* [26] at 4 °C.



The pellets are dissolved in 50 mM Sørensen-buffer, pH 7.2, 0.6 M mannitol. The efficiency of the isolation procedure is proved with succinate-dehydrogenase as marker-enzyme for mitochondria, according the method of Sisakyan *et al.* [27].

Estimation of HMG-CoA-reductase [EC 1.1.1.34] activity

Incubation was carried out after the method of Hummel [28] with some modifications according to Huber [29]. Radioactively labelled HMG-CoA was incubated in presence of a NADPH generating system, *i.e.* glucose-6-phosphate/$NADP^+$/glucose-6-phosphate dehydrogenase. Enzymatic reaction was started by addition of 0.2 ml cell fraction to 1 ml incubation solution containing 167 mM Sørensen-buffer, pH 7.2, 25 mM sucrose, 75 mM nicotinamide, 20 mM EDTA, 33 µM unlabelled HMG-CoA, 11 µM DL-3-[methyl-^{3}H]-HMG-CoA (spec. act. 8.2 Ci/mM), 10 mM glutathion-SH, 1 µM $NADP^+$ and 5 µl glucose-6-phosphate-dehydrogenase suspension.

Method 1. After 20 min at 28 °C the reaction is stopped by addition of 1 ml 3 N H_2SO_4. R/S-mevalonic-2-[^{14}C]acid, DBED-salt (spec. act. 3.7 Ci/mM) is added (30000 cpm). In order to isolate the mevalonolactone, which is generated under these conditions, 1 ml of the test solution is applied to a Na_2SO_4/sea-sand (2/1)-column (2.5 cm × 15 cm) which is equilibrated with diethylether. The elution is carried out with 100 ml diethylether. The ether fraction is evaporated to dryness and the mevalonolacton is redissolved in 5 ml water, applied to a Dowex 1 × 8-column (1 cm × 4 cm, Cl^--form) and eluted twice with 10 ml water. The eluate is evaporated to dryness and redissolved with two times 7 ml scintillator-cocktail (1 l toluene, 0.5 dimethyl-POPOP, 5 g PPO). The double labelled fraction is counted in a β-LSC (Philips PW 4700).

Method 2. After incubation (20 min, 28 °C), the reaction is stopped by addition of 1 ml 3 N H_2SO_4. 10 µl of the test solution is applied to a HPTLC-plate (silicagel 60, Merck), eluted with isopropanol/water (3/1 = v/v) and counted with a Berthold-Friesecke-scanner.

In case of inhibition studies the microorganism is cultivated in presence of the inhibitor (ethanolic solution) in culture medium. Measurements of direct inhibition are carried out by addition of the reagents to the test solution. Protein contents of the subcellular fractions are estimated according to Lowry *et al.* [30].

Results and Discussion

Gibbons *et al.* [31] reported the HMG-CoA reductase activty in *Saccharomyces cerevisiae* to be found in the microsomal fraction after isolation of subcellular fractions. We confirm this observation. Table I shows the specific activities of the subcellular fractions. From these data the specific HMG-CoA reductase activity in *Saccharomyces* is within the same range as compared to the isolation from mouse liver [17]. In the following the estimation of HMG-CoA-reductase activity was carried out with the microsomal fraction.

Table I. HMG-CoA-reductase activity in subcellular fractions (determination of activity according to method 1, 24 h fermentation).

Fraction	Spec. activity [pmol min^{-1} mg_{Prot}^{-1}]
nuclei	n. d.
mitochondria	12.5 ± 5
supernatant	n. d.
microsomes	240 ± 25
microsomal supernatant	20 ± 8

First the question should be answered whether the addition of triadimefon or clotrimazole has an effect on the specific activity of the HMG-CoA reductase. For this purpose we demonstrate the enzyme activity of untreated *Saccharomyces cerevisiae* as well as the activity after fermentation in presence of clotrimazole and triadimefon, respectively. Fig. 1 shows the specific activity of HMG-CoA-reductase as a function of fermentation time. The estimation of cell number as a function of fermentation time showed an analogous course (Fig. 2). Additionally, one should consider that, on a quantitative level, there is a difference between sensitivity of the HMG-CoA-reductase and the inhibition of growth due to the primary effect of the compounds towards desmethylation reactions, *i. e.* that growth of the microorganism is more effectively inhibited than the specific HMG-CoA-reductase activity. Generally, these two curves clearly indicate that incubation of *Saccharomyces cerevisiae* with fungistatic azoles leads to fungistatic effects and to the inhibition of HMG-CoA-reductase as well.

In order to investigate some more details about the mode of inhibition of the enzyme we incubated the microsomal fraction directly with clotrimazole and triadimefon. Table II shows the results. From these experiments it is obvious that a direct interaction between the enzyme and the azoles cannot be observed. So the question is wether the reduction of

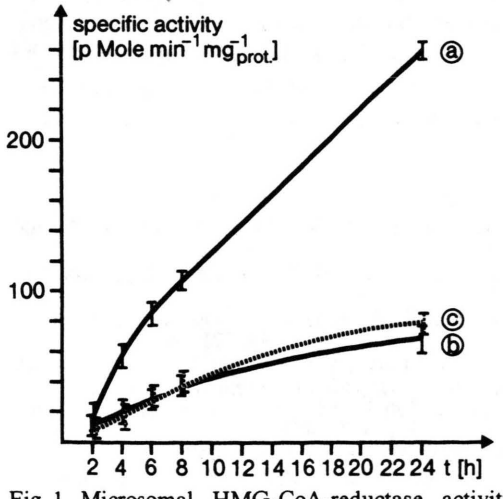


Fig. 1. Microsomal HMG-CoA-reductase activity as a function of fermentation time; a) untreated; b) in presence of 2.9×10^{-5} M clotrimazole; c) in presence of 3.4×10^{-5} M triadimefon; addition of inhibitors at $t = 0$.

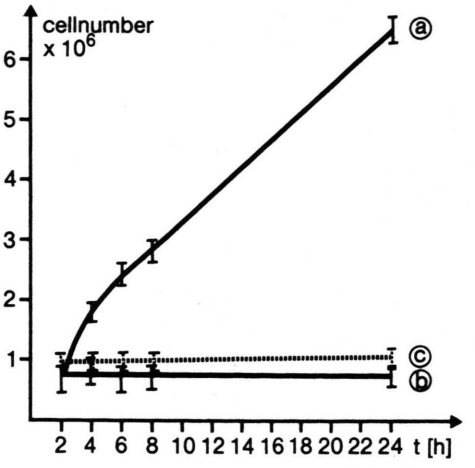


Fig. 2. Cellnumber as a function of fermentation time; a) untreated; b) 2.9×10^{-5} M clotrimazole; c) 3.4×10^{-5} M triadimefon; addition of inhibitors at $t = 0$.

enzyme activity, observed by incubation with intact cells, depends on the primary action of the azoles, *i. e.* inhibition of sterol desmethylation and the concomitant accumulation of 4,4,14-trimethylsteroles. So the more detailed question was, whether the accumulation of trimethylsteroles leads to an inhibition of HMG-CoA-reductase as well.

For this purpose we incubated whole cells of *Saccharomyces cerevisiae* with lanosterol. In order to look for a possible direct interaction of trimethylsteroles with the enzyme we incubated the subcellular fraction with lanosterol as well. Table III shows that the specific activity of HMG-CoA reductase is reduced after addition of lanosterol to the fermentation medium. On the other side a fungistatic effect cannot be observed as is readily seen from the cell number plot. Concerning the microsomal fraction a direct inhibition of the enzyme by lanosterol as well as by azoles is not detectable (Table II).

From the reported results the conclusion is obvious that the observed inhibition of HMG-CoA

Table II. Influence on the microsomal HMG-CoA reductase by *direct incubation* with clotrimazole and triadimefon (determination of activity according to method 2 after 24 h fermentation).

Addition	Spec. activity [pmol min^{-1} mg$_{Prot}^{-1}$]
–	236 ± 18
clotrimazole (2.9×10^{-5} M)	231 ± 26
triadimefon (3.4×10^{-5} M)	224 ± 23
lanosterol (2.3×10^{-5} M)	234 ± 27

Table III. Specific activity of the microsomal HMG-CoA reductase after fermentation in presence of different inhibitors (determination of activity according to method 2 after 24 h fermentation).

Fermentation in presence of	Spec. activity [pmol min^{-1} mg_{Prot}^{-1}]	Cell number $\times 10^6$
[8]	248 ± 21	6.5 ± 0.2
clotrimazole (2.9×10^{-5} M)	67 ± 13	0.7 ± 0.2
clotrimazole (2.9×10^{-6} M)	62 ± 15	0.6 ± 0.1
clotrimazole (2.9×10^{-7} M)	97 ± 19	1.4 ± 0.2
clotrimazole (2.9×10^{-8} M)	228 ± 22	6.1 ± 0.4
triadimefon (3.4×10^{-5} M)	78 ± 14	1.1 ± 0.1
triadimefon (3.4×10^{-6} M)	112 ± 17	2.1 ± 0.3
triadimefon (3.4×10^{-7} M)	238 ± 27	6.4 ± 0.5
triadimefon (3.4×10^{-8} M)	254 ± 26	6.6 ± 0.5
lanosterol (2.3×10^{-5} M)	104 ± 15	6.4 ± 0.5
lanosterol (2.3×10^{-6} M)	102 ± 17	6.2 ± 0.4
lanosterol (2.3×10^{-7} M)	121 ± 15	6.8 ± 0.4
lanosterol (2.3×10^{-8} M)	142 ± 16	6.6 ± 0.4
lanosterol (2.3×10^{-9} M)	190 ± 21	6.4 ± 0.6
lanosterol (2.3×10^{-11} M)	256 ± 27	6.6 ± 0.4
ergosterol (2.3×10^{-5} M)	257 ± 24	6.6 ± 0.5

reductase in intact cells, caused by the azoles, depends on a "feed-back" control of the enzyme which is responsible for an early step in ergosterol synthesis. Similar effects have been described for the mammalian enzyme previously [16–23]. So Gibbons [19] reported an inhibition by cholesterol precursors, oxygenated in position 32, and Schröpfer [16, 20] found a similar effect with 9-α-fluoro- and 9-α-hydroxysteroles. Mitropoulos [17] even observed an inhibition after addition of cholesterol. In these cases a "feed-back"-control of the enzymes should be responsible for a decreased ergosterol biosynthesis as well.

For mammalian systems this kind of regulation of HMG-CoA reductase seems well established. But even for a plant system, Brooker and Russell [22] were able to show that the HMG-CoA reductase in pea seedlings could be regulated by addition of isoprenoids. Our own results indicate that in yeast the regulation of HMG-CoA reductase follows similar principles.

As we are interested in the physiological consequences of the addition of sterol biosynthesis inhibitors to microorganisms, we want to conclude, that in this special case of compounds, which generally are considered as single-site effectors, an additional physiological consequence can be observed. The primary attack of the compounds on sterol desmethylation reactions is still out of discussion. In addition, by a partial inhibition of the HMG-CoA reductase, the rate of ergosterol biosynthesis decreases even more.

[1] M. Plempel, K. Bartmann, K. H. Büchel, and E. Regel, Dtsch. med. Wschr. **94,** 1356 (1969).
[2] J. M. van Cutsem and D. Thienpont, Chemotherapy **17,** 392 (1972).
[3] D. Thienpont, J. M. van Cutsem, J. M. van Nueten, C. J. E. Wiemegus, and R. Marsboom, Arzneim. Forsch. (Drug Res.) **25,** 224 (1975).
[4] H. Buchenauer, Z. Pfl. Krankh. Pfl. Schutz **84,** 440 (1977).
[5] P. Leroux and M. Gredt, Ann. Phytopathol. **10,** 45 (1978).
[6] M. R. Siegel and N. N. Ragsdale, Pestic. Biochem. Physiol. **9,** 48 (1978).
[7] H. Buchenauer, Z. Pfl. Krankh. Pfl. Schutz **83,** 363 (1976).
[8] H. Buchenauer, Pestic. Biochem. Physiol. **8,** 15 (1978).
[9] H. Buchenauer, Pestic. Biochem. Physiol. **7,** 309 (1977).
[10] H. Buchenauer, Z. Pfl. Krankh. Pfl. Schutz **84,** 286 (1977).
[11] M. R. Siegel, A. Kerkenaar, and A. Kaars Sijpesteijn, Neth. J. Plant Pathol. **83,** 121 (1977).
[12] T. Kato, S. Tanaka, S. Yamamoto, Y. Kawase, and M. Ueda, Ann. Phytopath. Soc. Japan **41,** 1 (1975).
[13] T. Kato, S. Tanaka, M. Ueda, and Y. Kawase, Agric. Biol. Chem. **38,** 2377 (1974).
[14] M. S. Mariott, J. Gen. Microbiol. **117,** 253 (1980).
[15] T. Kato, M. Schoami, and Y. Kawase, J. Pesticide Sci. **5,** 69 (1980).
[16] G. J. Schroepfer, E. J. Parish, M. Tsuda, D. L. Raubston, and A. A. Kandutsch, J. Lipid Res. **20,** 994 (1979).

[17] K. A. Mitropoulos, B. L. Knight, and B. E. A. Reeves, Biochem. J. **185,** 435 (1980).
[18] E. H. Goh, Lipids **15,** 624 (1980).
[19] G. F. Gibbons, C. R. Pullinger, H. W. Chen, W. K. Cavenee, and A. A. Kandutsch, J. Biol. Chem. **255,** 395 (1980).
[20] G. J. Schroepfer, E. J. Parish, M. Tsuda, and A. A. Kandutsch, Biochem. Biophys. Res. Commun. **91,** 606 (1979).
[21] Shi-Kanng Peng, P. Tham, C. B. Taylor, B. Mikkelson, J. Chin. Nutrit. **32,** 1033 (1979).
[22] J. D. Brooker and D. W. Russell, Arch. Biochem. Biophys. **198,** 323 (1979).
[23] D. Mayer and W. Hummel, 19th Int. Conference on Biochemistry of Lipids, Paris 1976.
[24] B. Middleton and D. K. Apps, Biochim. Biophys. Acta **177,** 276 (1969).
[25] J. Shimizu and J. Nagai, Biochim. Biophys. Acta **296,** 310 (1973).
[26] J. Shimizu and J. Nagai, J. Biochem. **70,** 175 (1971).
[27] Y. Sisakyan, *et al.*, Biokliniya **22,** 761 (1975).
[28] W. Hummel, Diplomarbeit 1976, Medizinische Hochschule Hannover.
[29] I. Huber, S. Latzin, and B. Hamprecht, Hoppe Seyler's Z. Physiol. Chem. **354,** 1645 (1973).
[30] O. H. Lowry, N. L. Rosebrough, A. L. Farr, and R. Randall, J. Biol. Chem. **193,** 265 (1951).
[31] G. F. Gibbons and K. A. Mitropoulos, Biochem. J. **127,** 315 (1972).