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Original article

Antifungal activity of chalcones: A mechanistic study using various yeast strains

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Abstract

We reported the synthesis, antifungal evaluation and study on substituent effects of 21 chalcones. A lot of genetically defined strains belonging to different yeast genera and species, namely *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Kluyveromyces lactis*, were used as test organisms. Concerning the mode of the antifungal action of chalcones it was shown that DNA was probably not the main target for the chalcones. It was revealed that the yeast's intracellular glutathione and cysteine molecules play significant role as defence barrier against the chalcone action. It was also shown that chalcones may react with some proteins involved in cell separation.

Keywords: Chalcones; Yeasts; Antifungal activity; Substituent effects

1. Introduction

Chalcones (1,3-diaryl-2-propen-1-one) are natural or synthetic flavonoids displaying an impressive array of biological properties [1–6]. Their antimicrobial activity and particularly the antifungal action have been largely attributed to the reactive enone moiety [1,3]. As a Michael reaction acceptor the enone unit binds thiol groups of certain proteins. Probably in that manner, most chalcones inhibit biosynthesis of yeast cell wall and thus unfold their antifungal potential [7]. The Michael reactions of chalcones are facilitated by electron withdrawing (EW) groups at *p*-position in ring B. Such substituents increase the electron deficiency at C-β transforming it into an attractive electrophilic centre for the thiol attack. The alternative *p*-electron donating (*p*-ED) groups hamper this reaction. Therefore, introduction of *p*-EW but not *p*-ED substituents is effective for the synthesis of chalcones with antifungal properties [1,3]. Some exceptions of this rule are known. For example, *p*-nitrochalcone (strong *p*-EW group) showed the same or weaker antifungal activity if compared to *p*-chlorochalcone (poor EW group) and *p*-methylchalcone (ED group) [1]. Besides, the ED hydroxyl groups are considered to play a significant role, though it is not clear if and how exactly the number and precise locations of the phenolic groups correlate with the antifungal activity of chalcones [1,5,8–11].

Previously we reported the effects of various substitutions of a series of synthetic chalcones against the pathogenic yeast *Candida albicans* [12]. It was shown that the potency of the chalcones against *C. albicans* to a large extent depended on their ability to interact with sulfhydryl groups. However, additional studies are needed to determine the discrete cellular targets for the chalcone action.

For deeper insight of the antifungal mechanisms of chalcones, it is necessary to employ suitable organisms. Yeasts

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are appropriate and useful eukaryotic models for such studies. They allow for rapid and low-cost experiments and avoid the ethical questions inherent in the use of animals. To date, chalcones have been tested mainly against clinical isolates of various medically important yeasts such as *Cryptococcus neoformans* [1,3,11], *Candida* [1,8,9,12,13], *Microsporum* [1,3], *Trichophyton* [1,3] and *Penicillium* species [1,13]. However, these strains have been with unknown ploidy and carried many unknown natural determinants giving rise to resistance against chemicals. The employment of such strains allows determination of compounds with very strong activity only and hampers elucidation of mechanisms of antifungal action of chalcones. These problems can be avoided by employment of genetically defined strains belonging to well-investigated yeast species.

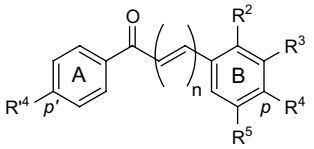
In this study a series of 21 chalcones with various substituents were synthesized and examined in vitro against 33 yeast strains of *Saccharomyces cerevisiae*, *Hansenula polymorpha* and *Kluyveromyces lactis*. All the strains employed were with well-defined ploidy and carrying specific mutations in either the biosynthetic pathways or defence systems.

2. Results and discussion

2.1. Chemistry

A series of 7 recently synthesized and 14 known chalcones (Table 1) were prepared by a cold or hot variant of the Claisen–Schmidt condensation between acetophenones (acetophenone or *p*-chloroacetophenone) and appropriate aryl aldehydes or cinnamaldehyde (Scheme 1). The desired products were obtained in average yield of 80% after purification. Their structures were established with UV, IR, NMR, mass spectrometry and elemental analysis. ^{1}H NMR spectra showed that only *E*-chalcones were obtained. The spectral data of the recently synthesized compounds are presented in Sections 4.1.1.1 and 4.1.1.2.

Table 1
Substitution patterns of the chalcones studied for the antifungal effects



Compound	*n*	R′4	R2	R3	R4	R5
1	1	H	H	H	H	H
2	1	H	H	OH	H	H
3	1	H	H	H	OH	H
4	1	H	H	OH	OH	H
5	1	H	H	OH	OCH_3	H
6	1	Cl	H	H	H	H
7	1	Cl	H	OH	H	H
8	1	Cl	H	H	OH	H
9	1	Cl	H	OH	OH	H
10	1	Cl	H	OH	OCH_3	H
11	1	Cl	H	OCH_3	OH	H
12	1	Cl	H	OCH_3	OH	OCH_3
13	1	Cl	H	OCH_3	H	H
14	1	Cl	OH	OCH_3	H	H
15	1	Cl	H	H	Cl	H
16	1	Cl	H	H	CN	H
17	1	Cl	H	H	NO_2	H
18	1	Cl	H	H	$NHCOCH_3$	H
19	1	Cl	H	H	CH_3	H
20	1	Cl	H	H	OCH_3	H
21	2	Cl	H	H	H	H

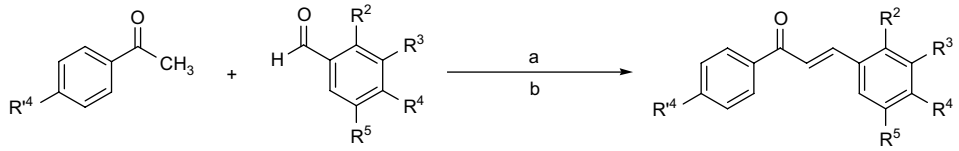
2.2. Microbiology

The chalcones were tested for their growth inhibitory activity against 33 strains belonging to *S. cerevisiae* (13 strains), *H. polymorpha* (17 strains) and *K. lactis* (3 strains). Their MICs were determined by the agar dilution method. The most active chalcones were **1**, **2**, **5**, **7** and **8** (Tables 2–4). The rest of the compounds exhibited antifungal activity at very high concentrations (>40 μg/ml) and were taken into consideration only for the analysis of the substituent effects.

K. lactis appeared to be the most sensitive species among the tested yeasts (Table 4). On the other side, *S. cerevisiae* was more resistant to the parent chalcone **1** than *H. polymorpha* and the opposite was true for compound **7** bearing a *m*-hydroxyl group in ring B (Tables 2 and 3).

2.3. DNA is not the main target for the antifungal action of chalcones

The antifungal activity of chalcones was examined against 9 laboratory employed haploid strains of *S. cerevisiae* and 4 artificially constructed or naturally occurring diploids. No clear relationship was observed between the ploidy level and the strain sensitivity (Table 2). Similar results were found for the 16 *H. polymorpha* haploids and a diploid strain designated as D22 (Table 3). These results show that the ploidy has no significant influence on the observed antifungal effects and that probably DNA is not the main target for the chalcone action. To verify the last statement chalcones were examined for their ability to induce nuclear and mitochondrial mutations. For this purpose, two diploid strains of *S. cerevisiae* were used: PV-2 [MATa/MATα *his7-1*/*his7-1 ade2-475*/*ade2-475 lys2-67*/*lys2-68 rad1-5*/*RAD1*] and its ultraviolet-light-sensitive (homozygous for the *rad1-5* mutation) derivate designated as PV-3 [14]. These strains give a possibility for detection of various genetic events such as reversion of ochre mutation *his7-1* [*his7-1* → His+], reversion of frame-shift mutation *ade2-475* [*ade2-475* → Ade+], mitotic gene conversion at LYS2 gene [*lys2-67*/*lys2-68* → Lys+], illegitimate mating, etc. Chalcones **1**, **6**, **7** and **13** were tested without metabolic activation by the method of Fink and Lowenstein [25]. No cases of enhanced appearance of Ade+, His+ and Lys+ colonies were detected suggesting that these substances cannot act as strong mutagens or recombinogens at the used conditions. In separate experiments, chalcones **1** and **7** were tested for their ability to induce loss of mitochondrial DNA. In *S. cerevisiae* such alternations cause appearance of respiratory deficient 'petite' mutants, which form invariable small sized



Scheme 1. General routes used for the chalcone synthesis. Reagents and conditions: (a) 6 M NaOH, MeOH, keeping in refrigerator overnight; (b) KOH, MeOH, reflux for 1.5 h.

colonies. The strains PV-2 and PV-3 were grown on liquid YPD supplemented with 20 μg/ml of the chalcones **1** and **7**. After 2 days of incubation yeast cells were washed, serially diluted and plated onto YPD plates. The lack of 'petite' colonies in the experimental plates compared to the controls indicated that the chalcones did not react with mitochondrial DNA.

2.4. Role of chromosomal mutations

For better evaluation of the antifungal activity of chalcones we used strains carrying many chromosomal mutations controlling specific metabolic pathways. Those with mutations impaired in purine, pyrimidine and some amino acid biosynthetic pathways did not show high sensitivity against the chalcones.

Some of the *H. polymorpha* strains revealed great differences in their sensitivities depending on the cellular genotype. The strain CTA1 carrying mutation *cat1* (lack of catalase activity as a result of disruption of the structural gene for catalase) showed increased resistance against the chalcones. This might be due to accumulation of hydrogen peroxide because of the lack of catalase activity. Hydrogen peroxide is highly reactive and may transform chalcones into less effective epoxides [5]. The mutants R3 and M4 are with altered morphology of colonies and cells. They were very sensitive to chalcones **2** and **7**. The phenotype of R3 (chains of undivided cells) is caused by mutation *rgh3*, whereas M4 carries mutation *rpm2* leading to pseudohyphal and invasive growth [21]. Both mutations are involved in the process of cell separation, suggesting that the compounds **2** and **7** acted on some proteins involved in the cell division cycle. The strains MC1, 2a, AS1 and AS2 were very sensitive to most of the chalcones regardless of their cell density and the chalcone concentration (Figs. 1 and 2). Their increased susceptibility was due to a diminished pool of thiol compounds as this can be judged by the specificity of the carried mutations. MC1 and 2a possess *cys1-1* allele, which strongly requires cysteine and encodes the enzyme *O*-acetyl homoserine sulfhydrylase. The strain AS1 is impaired in biosynthesis of *S*-adenosyl-L-methionine uroporphyrogen III methyltransferase. This enzyme is responsible for sirohem biosynthesis and it is required for the sulfite/sulfide reduction due to the mutation *gsh1* [20]. The most sensitive strain AS2 is impaired in the gene *GSH2* encoding γ-glutamylcysteine synthetase, the enzyme controlling the first step of glutathione biosynthetic pathway. An increased mortality of AS2 cells was observed upon their treatment with various concentrations of chalcone **1**. This was due to the *gsh2* mutation (Fig. 1). The obtained results show that at least one of the steps involved in chalcone detoxification is based on the attack from the available thiol molecules. The higher sensitivity of *gsh2* deletion mutant AS2 in comparison with the strains carrying *cys1* and *gsh1* mutations pointed at the cellular glutathione as the major yeast barrier towards chalcones.

Table 2
MIC values (μg/ml) of chalcones acting against *S. cerevisiae* strains

Strains	Ploidy level	Source	Chalcones				
			1	**2**	**5**	**7**	**8**
1S	Haploid	[14]	>40	25	>40	20	>40
PV-2	Diploid	[14]	>40	25	>40	18	35
PV-3	Diploid	[14]	>40	25	>40	15	35
1169	Haploid	[15]	>40	30	>40	20	>40
2180	Haploid	[15]	>40	25	>40	16	>40
1a	Diploid	[16]	>40	25	>40	20	35
29a	Diploid	[16]	>40	25	>40	25	>40
Σ1278b	Haploid	[15]	>40	25	>40	15	>40
D3C	Haploid	[15]	>40	25	>40	20	>40
FY4	Haploid	[15]	>40	25	>40	18	>40
10560-6B	Haploid	[17]	>40	25	>40	15	>40
YSH1170	Haploid	[17]	>40	25	35	15	>40
YSH1171	Haploid	[17]	>40	20	>40	18	>40

2.5. Effect of the chalcone concentration and strain cell density

The yeast survival depended on the chalcone concentration (Fig. 1) and the strain cell density. At high chalcone concentration (>40 μg/ml) and low cell density (10^3 cells/ml), a strong killing effect was observed. At concentration of 40 μg/ml, growth was observed only for strains with high cell density (10^9 cells/ml). Moderate concentrations of the chalcones (20–30 μg/ml) caused a clear growth inhibition after 1 day of incubation and restoration of the normal growth after 2 days of incubation. Such a stasis effect was observed regardless of the manner of the cell plating (spotted, Fig. 2 or streaked, Fig. 3) and the nature of the cultures (solid, Figs. 2 and 3 or liquid, Fig. 4). The observed effect may be due to structural alterations of the chalcones during the experiments and/or development of yeast cellular resistance. These possibilities were verified as follows. The stability of chalcones **1** and **5** was examined via UV spectrophotometrically determination of their concentrations in the culture media (liquid YPD) at 37 °C. No change in their quantities was registered after 24 h incubation. After 48 h the quantity of compound

Table 3
MIC values (μg/ml) of chalcones acting against *H. polymorpha* strains

Strains	Genotype	Source	Chalcones				
			1	**2**	**5**	**7**	**8**
WT1	*ade1 ura3*	[18]	30	35	>40	25	>40
WT2	*ade1 met6*	[18]	30	25	>40	<20	>40
CD1	*ade1 leu2 cds1*	[19]	30	25	>40	<20	>40
CD2	*ade1 leu2 cds2*	[19]	30	25	>40	<20	>40
CD3	*ade1 leu2 cds3*	[19]	30	25	>40	<20	>40
WA1[a]	*ade1 leu2 gsh1*	[20]	<20	<20	30	<20	30
2a[a]	*arg1 leu2 ura3 cys1*	[21]	25	25	30	<20	35
2b	*arg1 ade1 leu2*	[21]	30	25	>40	25	>40
D22	*arg1 ade1 leu2 URA3 CYS1* / *arg1 ADE1 leu2 ura3 cys1*	[19]	30	25	>40	<20	>40
M2	*met2*	[21]	30	25	>40	25	>40
MC1[a]	*met2 cys1*	[21]	20	20	30	<20	30
AS1[a]	*arg1 gsh2*Δ	[20]	20	20	30	<20	30
M4	*met4*	[21]	<20	30	>40	25	>40
AU05	*ade3 ura3*	[21]	<20	35	>40	25	>40
CTA1	*leu2 cat1*	[21]	30	35	>40	25	>40
R3[a]	*rgh3*	[21]	>40	20	>40	<20	~40
CRD2	*leu2 CDR2*	[19]	>40	20	>40	<20	~40

[a] Strain is very sensitive to the chalcone action when plated at low cell density.

1 decreased to 7.1 ± 0.3 μg/ml while that of the compound **5** retained. When the YPD media were inoculated with cells of *H. polymorpha* strain (WT1) the concentration of the chalcone **1** dropped to 1.4 ± 0.2 μg/ml and was not detected after 48 h. The quantity of chalcone **5** was 17.5 ± 0.1 μg/ml after 24 h and decreased to 0.41 ± 0.01 μg/ml after 48 h. These results indicate that both compounds interact with yeast cells but in different ways. The development of cellular resistance against chalcones' action was evident from the second group of experiments. Yeast cells were pre-incubated on YPD plates supplemented with 35 μg/ml of chalcone **1** for 3 days and then streaked onto plates containing a freshly prepared solution of **1**. The pre-incubated strains were found to be more resistant than the control cells. Taken collectively these experiments indicated that the observed stasis effect was due to depletion of chalcone molecules during the incubation, which tolerated the growth restoration of surviving yeast cells and development of cellular resistance.

2.6. Substituent effects

The antifungal effects of the substituted chalcones were compared with those of the parent chalcone **1**. The following correlations were observed.

1. Introduction of EW substituents (Cl, CN and NO_2 groups) in *p*-position in ring B yielded less active chalcones (compounds **15**–**17**) than the parent chalcone **1**.
2. Introduction of ED substituents (OH, CH_3 and OCH_3 groups) in *p*-position in ring B produced inactive chalcones (compounds **3**, **19** and **20**).
3. Presence of a single hydroxyl group was effective at *m*-position in ring B. Introduction of a single methoxy group at *m*-position in ring B led to inactive compound.
4. The combination of *m*-hydroxyl and *p*-methoxy groups in ring B was effective (compound **5**). Loss of activity was observed with the interchange of the positions of the hydroxyl and methoxy groups (compound **11**) and when the hydroxyl group was placed in *o*-position and the methoxy group was in *m*-position (compound **14**).
5. Introduction of *p*′-chloro atom in ring A was beneficial only for the chalcones with a single hydroxyl group at *m*- and *p*-positions (compounds **7** and **8**). The *m*-position was more favourable than the *p*-position. Presence of *m*- and *p*-hydroxyl groups together led to the inactive chalcone **9**.
6. Elongation of the conjugated system by introduction of one additional double bond between the ketovinyl moiety and the ring B did not produce an active compound (compound **21**).

Table 4
MIC values (μg/ml) of chalcones acting against *K. lactis* strains

Strains	Genotype	Source	Chalcones				
			1	**2**	**5**	**7**	**8**
2-18B	*MATa metA1 ura3 leu2*	[22]	<20	<20	25	<20	35
1-6A	*MATα uraA lys arg*	[23]	<20	<20	25	<20	>40
2-19B	*MATα ura lys arg*	[24]	25	<20	25	<20	35

Based on these observations it could be concluded that the electronic effects of the *p*-substituents in ring B of chalcones are not crucial for displaying antifungal activity towards the tested fungi. This is contradictory to the antifungal effects, which chalcones with EW and ED substituents in ring B have shown against several dermatophytes and the yeast *C. albicans* [1,3,12]. Besides, in this study the position of the hydroxyl group in ring B was

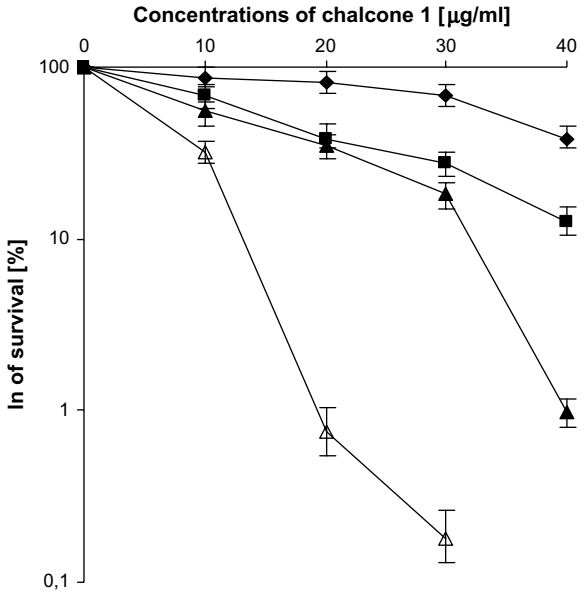


Fig. 1. Survival rates of *H. polymorpha* strains under the action of various concentrations of chalcone **1**: (◆) WT1; (■) *cys1*(2a); (▲) *gsh1*(AS1); (△) *gsh2*(AS2).

found important for the chalcone activity as opposed to some other antifungal studies [5,11]. Interestingly, the favoured location for the hydroxyl group was the *m*-position in ring B. In contrast, in our previous study we showed that *o*-position was the most favourable for the antifungal activity of hydroxyl-chalcones against *C. albicans* [12]. The reason for these effects is unknown.

3. Conclusion

We report a study on the antifungal mechanism of 21 chalcones with diverse substituents in their aryl ring B. A large number of genetically defined strains of various yeast genera and species were used as test organisms. In general, the antifungal activity of chalcones depended on their substitution pattern as well as on the yeast genotype and strain cell density.

The data obtained widen the knowledge about the mode of antifungal action of chalcone. The role of the ploidy level was clarified by utilization of haploid and diploid strains and according to the results DNA is probably not the main target for the chalcones. At least one of the steps involved in chalcone detoxification is based on thiol alkylation. This was proved by the increased sensitivities of strains carrying mutations in genes impaired in sulphur (*GSH1*), cysteine (*CYS1*) and glutathione (*GSH2*) metabolism. For the first time a precise link of chalcone action to particular protein targets encoded by the genes *GSH1*, *GSH2* and *CYS1* was experimentally demonstrated. Our data also showed that chalcones may react with proteins involved in cell separation as estimated by the increased sensitivity of strains with mutation *rgh3* to the lethal action of chalcones **2** and **7**.

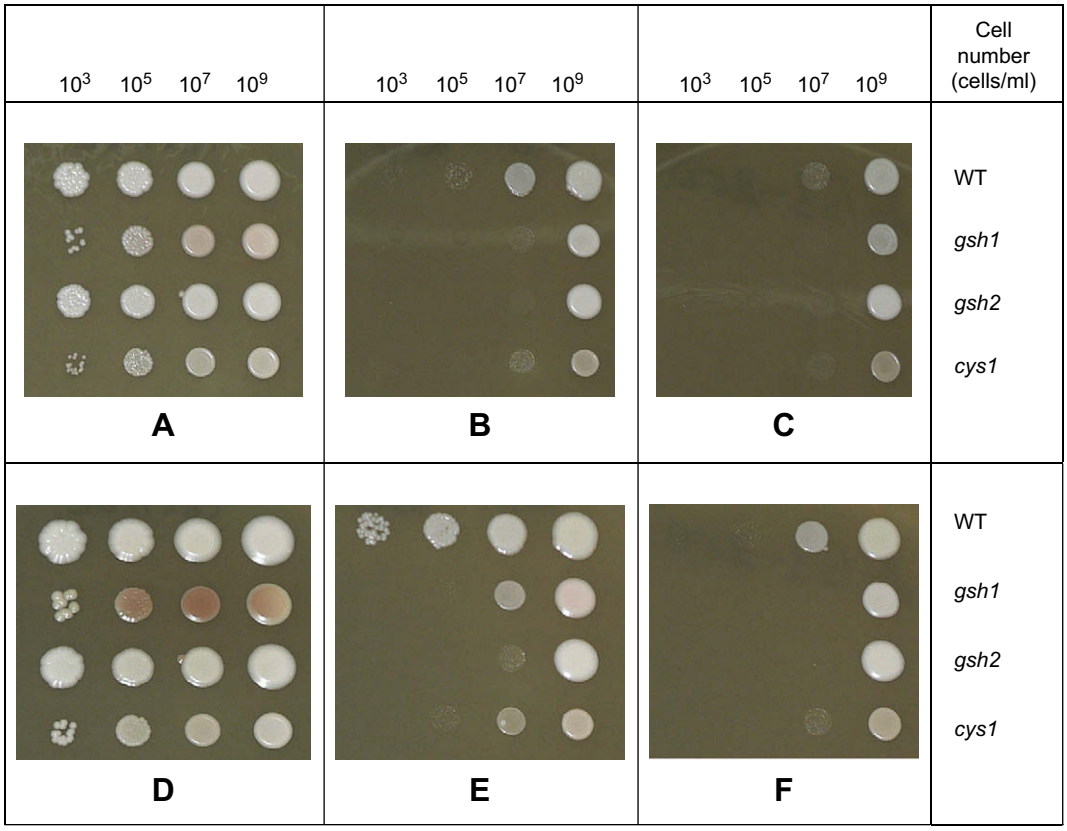


Fig. 2. Growth of wild type and mutant strains of *H. polymorpha* on rich media supplemented with 15 (B and E) and 20 µg/ml of chalcone **1** (C and F). The strains WT1 (wild type), AS1 (*gsh1*), AS2 (*gsh2*) and 2a (*cys1*) were used; yeast cells were plated at 100-fold dilutions indicated on the top. Results were scored after 48 h (A–C) and 72 h (D–F) incubations at 37 °C.

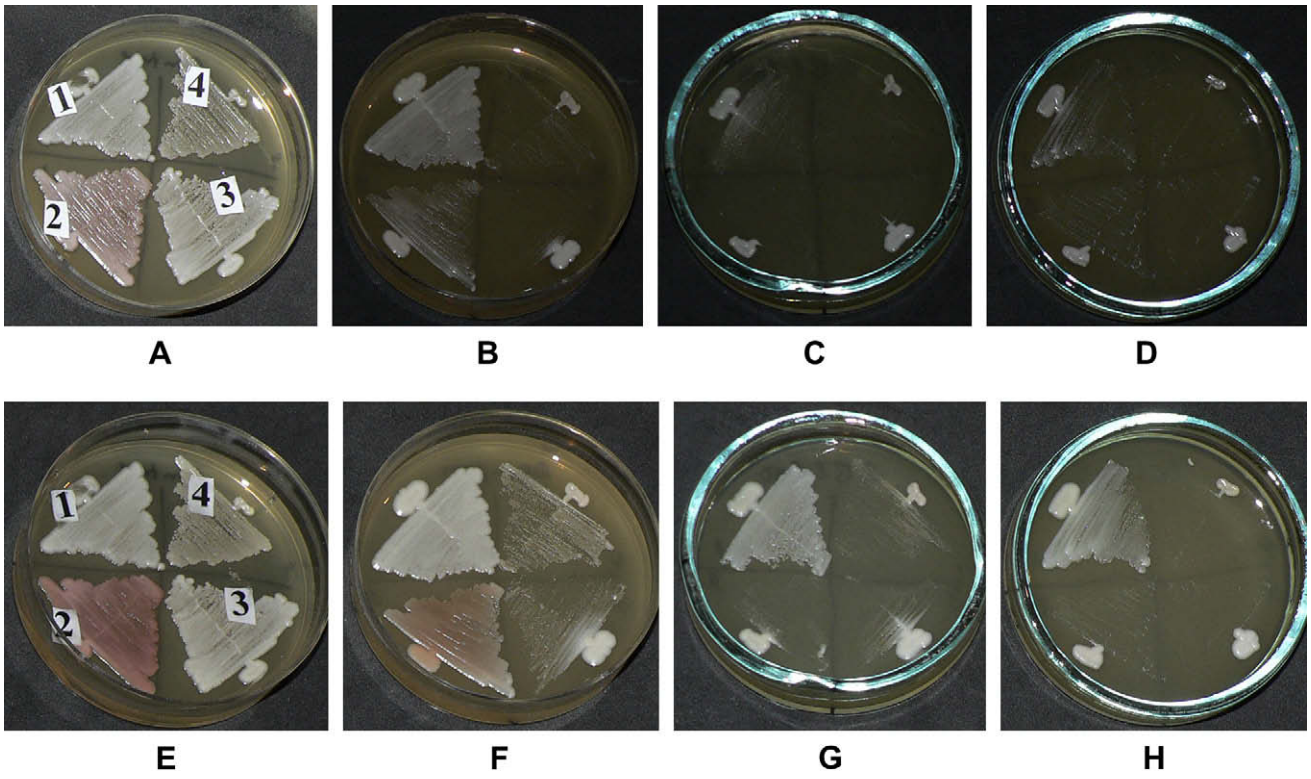


Fig. 3. Growth of wild type and mutant strains of *H. polymorpha* on rich media supplemented with 15 (B and F), 20 (C and G) and 30 μg/ml of chalcone **1** (D and H). 1 = WT1 (wild type), 2 = AS1 (*gsh1*), 3 = AS2 (*gsh2*) and 4 = 2a (*cys1*).

The approach used is suitable for studying the interaction of chalcones with living cells and finding new chalcone-like compounds with pharmacological activities.

4. Experimental protocols

Infrared and UV spectra were measured on Bruker IFS 113V and Helios gamma UV–visible spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AM 250 spectrometers with tetramethylsilane as internal reference. Chemical shifts are given in parts per million (δ-scale); coupling constants (J) are in hertz. Splitting patterns are described as singlet (s), doublet (d), triplet (t) and multiplet (m). Mass spectral analyses were accomplished on a Hewlett–Packard 5972 using Mass Selective Detector with EI (70 eV). The melting points were obtained using Mel-Temp 1102D-230 VAC and were uncorrected. The reactions were monitored on silica gel 60 F_{254} using PE:acetone 7:3 or toluene:Et_2O 4:1. Flash chromatography was performed for purification of the chalcones on silica gel 60 (230–400 mesh ASTM) using eluent PE:acetone 7:3.

4.1. Chemistry

4.1.1. Synthesis of chalcones

Preparation of the following chalcones was reported previously: chalcone (**1**) [3,26], 3-hydroxychalcone (**2**) [26], 4-hydroxychalcone (**3**) [26], 3,4-dihydroxychalcone (**4**) [27], 4′-chlorochalcone (**6**) [28], 4′-chloro-3-hydroxychalcone (**7**) [26], 4′-chloro-4-hydroxychalcone (**8**) [26], 4′-chloro-3,4-dihydroxychalcone (**9**) [29], 4′-chloro-3-methoxy-4-hydroxychalcone (**11**) [30], 4′,4-dichlorochalcone (**15**) [26], 4′-chloro-4-nitrochalcone (**17**) [31], 4′-chloro-4-methylchalcone (**19**) [12], 4′-chloro-4-methoxychalcone (**20**) [12] and 1-(4-chloro-phenyl)-5-phenyl-penta-2,4-dien-1-one (**21**) [32].

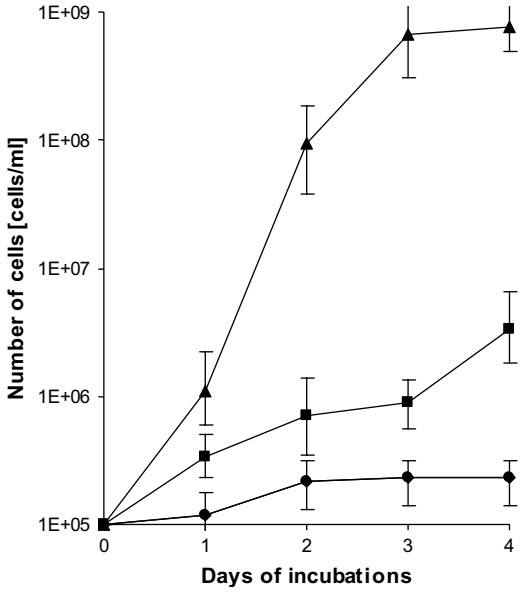


Fig. 4. Growth of wild type and mutant *H. polymorpha* strains in liquid YPD media supplemented with 20 μg/ml of chalcone **1**. (▲) WT1; (■) *cys1*(2a); (◆) *gsh1*(AS1) and *gsh2*(AS2).

Chalcones **4**, **9**, **12** and **14** were prepared applying a hot variant of the Claisen–Schmidt condensation, whereas the rest of the compounds were obtained by a cold variant of the same method.

4.1.1.1. A cold variant of the Claisen–Schmidt condensation. Acetophenone (97 μl, 0.8 mmol) or *p*-chloroacetophenone (84 μl, 0.7 mmol) was added to equimolar quantities of appropriate aryl aldehydes or cinnamaldehyde, dissolved in MeOH (0.8 ml). To this solution 6 M NaOH (0.4 ml) was added dropwise and the reaction mixture was stirred for 40 min and then kept in refrigerator overnight. The product crystals were filtrated and washed carefully with ice water and cold MeOH to neutral reaction. The resulting chalcones were purified by recrystallisation from MeOH. The reaction mixtures containing chalcones soluble in MeOH were evaporated and fractioned between EtOAc and water. The organic layer was evaporated and submitted to flash chromatography.

*4.1.1.1.1. 3-Hydroxy-4-methoxychalcone (**5**).* Yield 96%, yellow crystals, m.p. 92–94 °C (MeOH). UV (MeOH) λ/nm: 265, 360. IR (KBr) ν/cm^{-1}: 3260 (OH), 1646 (C=O), 1571 (C=C), 1260 (C–O). ^{1}H NMR ($CDCl_3$) δ: 7.99–8.03 (m, 2H, H-2′, H-6′), 7.74 (d, 1H, H-β, $J = 15.5$ Hz), 7.44–7.58 (m, 3H, H-3′, H-4′, H-5′), 7.40 (d, 1H, H-α, $J = 15.5$ Hz), 7.28 (d, 1H, H-2, $J = 2.3$ Hz), 7.13 (dd, 1H, H-6, $J = 8.2$, 2.2 Hz), 6.87 (d, 1H, H-5, $J = 8.5$ Hz), 5.77 (s, 1H, OH), 3.93 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ: 190.5 (C=O), 148.8 (C-4), 145.9 (C-3), 144.8 (C-β), 138.4 (C-1′), 132.6 (C-4′), 128.6, 128.4 (C-1, C-2′, C-3′, C-5′, C-6′), 122.7 (C-α), 120.3 (C-6), 113.0 (C-5), 110.6 (C-2), 56.0 (OCH_3). Mass (*m*/*z*, %): 254 (M^+, 100), 239 (55), 223 (14), 177 (36), 105 (33), 77 (47). Anal. Calc. for $C_{16}H_{14}O_3$ (%): C, 75.57; H, 5.55. Found C, 75.68; H, 5.82.

*4.1.1.1.2. 4′-Chloro-3-hydroxy-4-methoxychalcone (**10**).* Yield 97%, yellow crystals, m.p. 195–196 °C (96% EtOH). UV (MeOH) λ/nm: 265, 360. IR (KBr) ν/cm^{-1}: 3370 (OH), 1620 (C=O), 1563 (C=C), 1483, 1250 (C–O), 800 (C–Cl). ^{1}H NMR (CD_3OD) δ: 8.04 (d, 2H, H-2′, H-6′, $J = 8.5$ Hz), 7.70 (d, 1H, H-β, $J = 15.5$ Hz), 7.69 (s, 1H, OH), 7.52 (d, 2H, H-3′, H-5′, $J = 8.8$ Hz), 7.45 (d, 1H, H-α, $J = 15.5$ Hz), 7.09 (s, 1H, H-2), 6.77 (s, 2H, H-5, H-6), 3.82 (s, 3H, OCH_3). ^{13}C NMR (CD_3OD) δ: 191.4 (C=O), 158.1 (C-4), 156.7 (C-3), 149.8 (C-β), 139.9 (C-4′), 138.5 (C-1′), 131.1 (C-2′, C-6′), 129.9 (C-3′, C-5′), 129.3 (C-1), 119.8 (C-α), 117.9 (C-6), 116.8 (C-5), 111.2 (C-2), 55.7 (OCH_3). Mass (*m*/*z*, %): 288 (M^+, 100), 273 (50), 271 (36), 257 (13), 253 (49), 177 (26), 139 (27), 111 (32). Anal. Calc. for $C_{16}H_{13}ClO_3$ (%): C, 66.56; H, 4.54; Cl, 12.28. Found: C, 66.80; H, 4.64; Cl, 12.16.

*4.1.1.1.3. 4′-Chloro-3-methoxychalcone (**13**).* Yield 94%, white crystals, m.p. 65–66 °C (MeOH). UV (MeOH) λ/nm: 310. IR (KBr) ν/cm^{-1}: 1650 (C=O), 1576 (C=C), 1247 (C–O), 777 (C–Cl). ^{1}H NMR ($CDCl_3$) δ: 7.96 (d, 2H, H-2′, H-6′, $J = 8.5$ Hz), 7.77 (d, 1H, H-β, $J = 15.5$ Hz), 7.45 (d, 1H, H-α, $J = 15.5$ Hz), 7.44 (d, 2H, H-3′, H-5′, $J = 8.5$ Hz), 7.34 (t, 1H, H-5, $J = 7.8$ Hz), 7.22 (d, 1H, H-6, $J = 7.8$ Hz), 7.14 (s, 1H, H-2), 6.97 (dd, 1H, H-4, $J = 8.0$, 1.8 Hz), 3.85 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ: 189.1 (C=O), 159.9 (C-3), 145.2 (C-β), 139.2 (C-4′), 136.4 (C-1), 136.0 (C-1′), 130.0 (C-2′, C-6′), 129.9 (C-5), 128.9 (C-3′, C-5′), 121.8 (C-α), 121.1 (C-6), 116.4 (C-4), 113.5 (C-2), 55.3 (OCH_3). Mass (*m*/*z*, %) 272 (M^+, 100), 257 (14), 241 (98), 237 (56), 161 (28), 139 (55), 111 (39). Anal. Calc. for $C_{16}H_{13}ClO_2$ (%): C, 70.46; H, 4.80; Cl, 13.00. Found: C, 70.79; H, 4.90; Cl, 13.22.

*4.1.1.1.4. 4′-Chloro-4-cyanochalcone (**16**).* Yield 71%, yellow crystals, m.p. 139–141 °C (MeOH). UV (MeOH) λ/nm: 228, 310. IR (KBr) ν/cm^{-1}: 2210 (C≡N), 1656 (C=O), 1583 (C=C). ^{1}H NMR (DMSO-d_6) δ: 7.96 (d, 2H, H-2′, H-6′, $J = 8.8$ Hz), 7.78 (d, 1H, H-β, $J = 15.8$ Hz), 7.71 (s, 4H, H-2, H-3, H-5, H-6), 7.55 (d, 1H, H-α, $J = 15.8$ Hz), 7.49 (d, 2H, H-3′, H-5′, $J = 8.8$ Hz). ^{13}C NMR (DMSO-d_6) δ: 188.4 (C=O), 142.5 (C-β), 139.8 (C-4′), 138.9 (C-1), 135.9 (C-1′), 132.7 (C-3, C-5), 129.9, 129.1 (C-2′, C-3′, C-5′, C-6′), 128.7 (C-2, C-6), 124.4 (C-α), 118.3 (C≡N), 113.6 (C-4). Mass (*m*/*z*, %) 267 (M^+, 68), 232 (100), 156 (37), 139 (53), 128 (32), 111 (42). Anal. Calc. for $C_{16}H_{10}ClNO$ (%): C, 71.78; H, 3.77; Cl, 13.24; N, 5.23. Found: C, 72.15; H, 3.92; Cl, 13.02; N, 5.38.

*4.1.1.1.5. 4′-Chloro-4-acetamidochalcone (**18**).* Yield 84%, yellow crystals, m.p. 191–194 °C (96% EtOH). UV (MeOH) λ/nm: 260, 345. IR (KBr) ν/cm^{-1}: 3321 (NH), 1671 (NH–C=O, amide-I), 1639 (C=O), 1582 (C=C), 1504 (C–N–H, amide-II), 1307 (CH_3), 814 (C–Cl). ^{1}H NMR (DMSO-d_6) δ: 10.20 (s, 1H, NH), 8.14 (d, 2H, H-2′, H-6′, $J = 8.8$ Hz), 7.82 (d, 2H, H-3, H-5, $J = 8.8$ Hz), 7.78 (s, 1H, H-β), 7.71 (s, 1H, H-α), 7.65 (d, 2H, H-3′, H-5′, $J = 8.5$ Hz), 7.61 (d, 2H, H-2, H-6, $J = 8.8$ Hz), 2.06 (s, 3H, CH_3). ^{13}C NMR (DMSO-d_6) δ: 187.9 (C=O), 168.8 (CONH), 144.4 (C-β), 141.7 (C-4), 138.0 (C-4′), 136.4 (C-1′), 130.4 (C-2′, C-6′), 130.0 (C-3′, C-5′), 129.2 (C-1), 128.9 (C-2, C-6), 119.8 (C-α), 118.8 (C-3, C-5), 24.2 (CH_3). Mass (*m*/*z*, %) 299 (M^+, 100), 264 (20), 256 (53), 241 (26), 139 (22), 111 (27). Anal. Calc. for $C_{17}H_{14}ClNO_2$ (%): C, 68.12; H, 4.71; Cl, 11.83; N, 4.67. Found: C, 68.22; H, 5.00; Cl, 11.23; N, 4.41.

4.1.1.2. A hot variant of the Claisen–Schmidt condensation [33]. Solid KOH (4.2 g, 75.0 mmol) was added to a mixture of *p*-chloroacetophenone (274 μl, 2.1 mmol) and aryl aldehyde (2.1 mmol) in MeOH (4.2 ml) and H_2O (2.1 ml). The resulting solution was refluxed for 1.5 h, then cooled in an ice-water bath and acidified with 8.4 ml conc. HCl. The solution was diluted with water (20 ml) and stored in a refrigerator overnight. The formed crystals were filtrated, washed with water, dried out and recrystallised.

*4.1.1.2.1. 4′-Chloro-4-hydroxy-3,5-dimethoxychalcone (**12**).* Yield 83%, yellow crystals, m.p. 126–128 °C (MeOH). UV (MeOH) λ/nm: 213.5, 261.0, 371.5. IR (KBr) ν/cm^{-1}: 3432 (OH), 1643 (C=O), 1590 (C=C), 1287 (C–O–C), 821 (C–Cl). ^{1}H NMR (DMSO-d_6) δ: 8.15 (d, 2H, H-2′, H-6′, $J = 8.8$ Hz), 7.77 (d, 1H, H-β, $J = 15.5$ Hz), 7.67 (d, 1H, H-α, $J = 15.5$ Hz), 7.61 (d, 2H, H-3′, H-5′, $J = 8.8$ Hz), 7.19 (s, 2H, H-2, H-6), 3.82 (s, 6H, $2 \times OCH_3$). ^{13}C NMR

(DMSO-d_6) δ: 187.9 (C=O), 148.1 (C-3, C-5), 146.0 (C-β), 139.1 (C-4′), 137.9 (C-1′), 136.6 (C-4), 130.4 (C-2′, C-6′), 128.9 (C-3′, C-5′), 125.0 (C-1), 118.7 (C-α), 107.1 (C-2, C-6), 56.2 ($2 \times OCH_3$). Mass (*m*/*z*, %): 318 (M^+, 100), 303 (9), 287 (74), 207 (11), 139 (27), 111 (25). Anal. Calc. for $C_{17}H_{15}ClO_4$ (%): C, 64.06; H, 4.74; Cl, 11.12. Found: C, 64.20; H, 4.86; Cl, 10.85.

*4.1.1.2.2. 4′-Chloro-2-hydroxy-3-methoxychalcone (**14**).* Yield 80%, yellow crystals, m.p. 129–131 °C (MeOH). UV (MeOH) λ/nm: 215, 321. IR (KBr) ν/cm^{-1}: 3279 (OH), 1655 (C=O), 1595 (C=C), 1210 (C–O–C), 834 (C–Cl). ^{1}H NMR ($CDCl_3$) δ: 8.03 (d, 2H, H-β, $J = 15.8$ Hz), 7.97 (d, 2H, H-2′, H-6′, $J = 8.8$ Hz), 7.71 (d, 1H, H-α, $J = 15.8$ Hz), 7.46 (d, 2H, H-3′, H-5′, $J = 8.8$ Hz), 7.14–7.19 (m, 1H, H-5), 6.83–6.91 (m, 2H, H-4, H-6), 6.36 (s, 1H, OH), 3.92 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$) δ: 189.85 (C=O), 146.8 (C-3), 145.9 (C-2), 140.7 (C-β), 138.9 (C-4′), 136.7 (C-1′), 129.9 (C-2′, C-6′), 128.8 (C-3′, C-5′), 122.9 (C-α), 121.8 (C-5), 121.0 (C-1), 119.7 (C-6), 112.0 (C-4), 56.2 (OCH_3). Mass (*m*/*z*, %): 288 (M^+, 100), 271 (75), 257 (28), 177 (32), 149 (56), 139 (66). Anal. Calc. for $C_{16}H_{13}ClO_3$ (%): C, 66.56; H, 4.54; Cl, 12.28. Found: C, 66.66; H, 4.75; Cl, 12.56.

4.2. Microbiology

4.2.1. Strains

The number, genotypes and sources of yeast strains used are listed in Tables 2–4. They included 16 haploids and 1 diploid of *H. polymorpha*, three haploids of *K. lactis* as well as 9 haploid and 4 diploid strains belonging to *S. cerevisiae*. Diploid *S. cerevisiae* strain PV-3 was used in experiments for testing of some chalcones for their mutagenic and recombinogenic activities. All the *S. cerevisiae* and *K. lactis* strains were grown at 30 °C and those belonging to *H. polymorpha* at 37 °C. The isolation and genetic properties of strains belonging to *H. polymorpha* genetic breading stock were described previously [21]. Auxotrophic mutations *arg1*, *ade1*, *ade3*, *leu2*, *ura3*, *met2*, *met4* and *cys1* require arginine, adenine, leucine, uracil, methionine and cysteine for growth, respectively. Mutations *cds1*, *cds2* and *cds3* are recessive alleles conferring sensitivity to cadmium ions, whereas *CDR1* is a dominant mutation conferring increased resistance to cadmium ions. Mutation *cat1* abolishes the function of catalase enzyme and mutation *rgh3* affects cell separation. Mutation *gsh1* is involved in glutathione biosynthesis [20]. With *MATa* and *MATα* the alleles of mating type locus for *K. lactis* and *S. cerevisiae* are designated.

4.2.2. Media

All the strains were maintained and propagated onto solid YPD media (0.5% yeast extract, 0.5% bacto peptone, 0.5% glucose). The chalcones were dissolved into 96% ethanol and incorporated into YPD medium by the addition of appropriate amounts into hot and sterile media. In experiments with detection for mutagenic and recombinogenic action three kinds of omission media containing 0.7% yeast nitrogen base and 1% glucose were used. These media were supplemented with 50 μg/ml adenine, histidine or lysine but lacked one of the listed compounds.

4.2.3. Antifungal assays

The antifungal activity of chalcones was evaluated by the agar dilution method using rich YPD medium for all yeast strains. Stock solutions of compounds prepared in 96% EtOH were added to each medium resulting in concentrations ranging from 20 to 50 μg/ml. Yeast cells were suspended into water and serially diluted to cell densities of 10^9, 10^7, 10^5 and 10^3 cells/ml. These cell suspensions were filled into wells of metal replicator and plated onto agar surfaces. The MIC for each compound was defined as the lowest concentration, which completely inhibited the yeast growth after incubation time. Drug-free solution was used as a blank control and ketokonazol was used as a positive control.

4.2.4. Mutagenicity assays

The PV-2 and PV-3 strain cells were grown on liquid YPD medium and spread onto YPD plates. In the centre of each plate was placed filter paper and solutions containing 30 and 40 μg/ml of compounds tested were poured. These plates were grown at 30 °C for 2 days and replica-plated onto omission media lacking adenine, histidine and lysine. After another 2 days of incubation, plates were inspected for the appearance of ring of prototrophic colonies around the filter paper.

4.2.5. Determination of the concentrations of the chalcones *1* and *5* in the liquid YPD media

Stock solutions of chalcones **1** and **5** prepared in 96% EtOH were added to liquid YPD medium resulting in concentrations of 30 μg/ml. The media were inoculated with 10^5 cells/ml of *H. polymorpha* strain (WT1). The obtained solutions were incubated at 37 °C. Samples were taken after 24 and 48 h incubation and centrifuged. The concentrations of the chalcones in the culture supernatants were measured at 308 nm (for compound **1**) and at 361.5 nm (for compound **5**). Solutions of chalcones **1** and **5** in YPD media (30, 25, 15, 10 and 5 μg/ml) were used as references. Blank samples were YPD media. The control experiments were conducted in YPD media without yeast cells. Every assay was carried out in triplicates.

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References

[1] P. Boeck, P.C. Leal, R.A. Yunes, V.C. Fiiho, S. Lopez, M. Sortino, A. Escalante, R.L.E. Furlan, S. Zacchino, Arch. Pharm. Chem. Life Sci. 338 (2005) 87–95.

[2] L. Ni, C.Q. Meng, J.A. Sikorski, Expert Opin. Ther. Pat. 14 (2004) 1669–1691.

[3] S.N. Lopez, M.V. Castelli, S.A. Zacchino, J.N. Dominguez, C. Lobo, J. Charris-Charris, J.C.G. Cortes, J.C. Ribas, C. Devia, A.M. Rodriguez, R.D. Entriz, Bioorg. Med. Chem. 9 (2001) 1999–2013.

[4] V. Opletalova, P. Ricicarova, D. Sedivy, D. Meltrova, J. Krivakova, Folia Pharm. Univ. Carol. XXV (2000) 21–33.

[5] J.R. Dimmock, D.W. Elias, M.A. Beazely, N.M. Kandepu, Curr. Med. Chem. 6 (1999) 1125–1149.

[6] V. Opletalova, D. Sedivy, Ceska Slov. Farm. 48 (1999) 252–255.

[7] K. Bowden, A. Dal Pozzo, C.K. Duah, J. Chem. Res., Synop. 12 (1990) 2801–2830.

[8] M. Sato, H. Tsuchiya, M. Akagiri, S. Fujiwara, T. Fujii, N. Takagi, N. Matsuura, M. Iinuma, Lett. Appl. Microbiol. 18 (1994) 53–55.

[9] H. Tsuchiya, M. Sato, M. Akagiri, N. Takagi, T. Tanaka, M. Iinuma, Pharmazie 49 (1994) 756–758.

[10] M. Sato, H. Tsuchiya, M. Akagiri, N. Takagi, M. Iinuma, Aust. Dent. J. 42 (1997) 343–346.

[11] A.A. Bilgin, N. Yulug, S. Sarac, A. Tayhan, E.D. Berkman, Hacettepe Ecz. Fak. Der. 7 (1987) 39–50.Chem. Abstr. 109, 89593k.

[12] D. Batovska, St. Parushev, A. Slavova, V. Bankova, I. Tsvetkova, M. Ninova, H. Najdenski, Eur. J. Med. Chem. 42 (2007) 87–92.

[13] S. Alam, J. Chem. Sci. 116 (2004) 325–331.

[14] S.G. Inge-Vechtomov, Y.I. Pavlov, V.N. Noskov, M.V. Repnevskaya, T.S. Karpova, N.N. Khromov-Borisov, L. Chekuolene, D. Chitavichus, in: D.L. Ashby, D.L. de Serres (Eds.), Progress in Mutation Research, Elsevier, Amsterdam, 1985, pp. 243–255.

[15] R.K. Mortimer, J.R. Johnson, Genetics 113 (1986) 35–43.

[16] K.L. Lahtchev, M. Pesheva, O. Tzvetanov, J. Wine Res. 2 (1991) 191–201.

[17] N. Pettersson, C. Filipsson, E. Becit, L. Brive, S. Hohmann, Biol. Cell 97 (2005) 487–500.

[18] H.R. Waterham, V.I. Titorenko, I.-J. van der Klei, W. Harder, M. Veenhuis, Yeast 8 (1992) 961–972.

[19] This study.

[20] V.M. Ubiivovk, T.Y. Nazarko, E.G. Stasyk, A.A. Sibirnyi, Microbiologiya 71 (2002) 829–835.

[21] K.L. Lahtchev, V.D. Semenova, I.I. Tolstorukov, I.-J. van der Klei, M. Veenhuis, Arch. Microbiol. 177 (2002) 150–158.

[22] D. Staneva, D. Uccelletti, F. Farina, P. Venkov, C. Palleschi, Yeast 21 (2004) 41–51.

[23] D. Staneva, D. Uccelletti, N. Petrova, P. Venkov, C. Palleschi, C. R. Acad. Bulg. Sci. 58 (2005) 233–238.

[24] D. Uccelletti, D. Staneva, S. Rufini, P. Venkov, C. Palleschi, FEMS Yeast Res. 5 (2005) 735–746.

[25] G.R. Fink, R. Lowenstein, J. Bacteriol. 100 (1969) 1126–1127.

[26] J.F. Miquel, Memoires Presentes a la Societe Chimique 7–12 (1961) 1369–1376.

[27] C.-N. Lin, J. Pharm. Pharmacol. 49 (1997) 530–536.

[28] P. Wang, S.J. Wu, Photochem. Photobiol. A: Chem. 77 (1994) 127–131.

[29] G.J. Martin, J.M. Beiler, S. Avakian, J.N. Moss, U.S. Patent 2,647,148, 1953; Chem. Abstr.

[30] I.S. Arty, H. Timmerman, M. Samhoedi, S. Sastrohamidjojo, H. Van der Goot, Eur. J. Med. Chem. 35 (2000) 449–457.

[31] W. Davey, J.R. Gwilt, J. Chem. Soc. (1957) 1008–1014.

[32] J.-T. Li, W.-Z. Yang, S.-X. Wang, S.-H. Li, T.-S. Li, Ultrason. Sonochem. 9 (2002) 237–239.

[33] F.A. Davis, B.-C. Chen, J. Org. Chem. 58 (1993) 1751–1753.