Antifungal unsaturated cyclic Mannich ketones and amino alcohols: Study of mechanism of action

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · DECEMBER 2008

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2008.10.038 · Source: PubMed

European Journal of Medicinal Chemistry 44 (2009) 1823–1829

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Antifungal unsaturated cyclic Mannich ketones and amino alcohols: Study of mechanism of action

Béla Kocsis [a], Ildikó Kustos [a], Ferenc Kilár [b], Adrien Nyul [a], Péter B. Jakus [c], Szilárd Kerekes [c], Victor Villarreal [d], László Prókai [d], Tamás Lóránd [c,*]

[a] University of Pécs, Faculty of Medicine, Department of Medical Microbiology and Immunology, H-7624 Pécs, Szigeti út 12, Hungary
[b] University of Pécs, Faculty of Medicine, Institute of Bioanalysis, 7624 Pécs, Szigeti út 12, Hungary
[c] University of Pécs, Faculty of Medicine, Department of Biochemistry and Medical Chemistry, H-7624 Pécs, Szigeti út 12, Hungary
[d] Department of Molecular Biology and Immunology, University of North Texas Health Science Center, Fort Worth, TX 76107, USA

ARTICLE INFO

Article history:
Received 26 April 2008
Received in revised form
16 October 2008
Accepted 30 October 2008
Available online 7 November 2008

Keywords:
Mannich ketone
Antifungal activity
Protein synthesis
Pseudohypha
Chitin synthase
QSAR

ABSTRACT

The antifungal activity of some known unsaturated Mannich ketones and their amino alcohols has been reported and the mechanism of antifungal action has been studied. The inhibition of the fungal ergosterol, chitin, protein synthesis and pseudohypha formation was investigated. According to our results, Mannich ketones can influence the development of pseudohyphae of *Candida albicans* strains. In addition, they are able to induce significant changes in the protein composition of this fungal strain. Some of our Mannich ketones have shown inhibitory effect on the fungal chitin synthase enzyme. QSAR studies were also carried out.

1. Introduction

The number of both local and systemic fungal infections has increased. The superficial and deep local mucocutaneous cases are mostly nonaesthetic and unpleasant, but the systemic invasive processes (candidiasis, aspergillosis, etc.) are especially important in medicine because they are life threatening in immunocompromised, hospitalized patients. New effective antifungal drugs (voriconazole, caspofungin, etc.) have been introduced recently. Nevertheless, there is a demand for less toxic topical and systemic antifungal drugs with broader spectrum of activities.

Previously, we have reported our studies on cyclic Mannich ketones (**1**–**23**) regarding their antibacterial effect [1]. Some of them have been reduced to the corresponding amino alcohol derivatives [2]. The parent Mannich ketones and the amino alcohols have been screened on pathogenic yeasts (including *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 3916, *Candida krusei* ATCC 30068, *Candida parapsilosis* ATCC 22019 international standard strains; *Saccharomyces cerevisiae* and *Aspergillus* sp. were isolated from clinical samples in our laboratory). The present work addresses the mechanism of antifungal action for **1**–**23**.

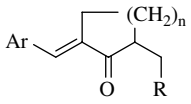
There are several known mechanism of actions for commercial antifungal agents such as inhibition of squalene epoxide, ergosterol, folic acid and protein biosyntheses, inhibition of DNA replication, chitin and β-(1,3)-glucan synthase inhibition, etc. Our aim has been to find a class of compounds having a new target specific only for fungi, and started the investigations with our Mannich ketones. First, we wished to study their possible inhibition of the ergosterol biosynthesis, pseudohypha formation, and effect on the protein composition. We also explored the influence of our Mannich ketones on the chitin synthase enzyme. In addition, we compared the antifungal activity of the parent Mannich ketones with that of the amino alcohols in order to get more insight into structure–activity relationships. To survey the relationship between the chemical structure of antifungal agents and their biological activity, a QSAR study has been carried out.

Dimmock et al. have demonstrated that some Mannich bases of conjugated ketones react with low-molecular weight and protein-associated thiols [3] to show, thereby, activity against pathogenic fungi [4]. Compound NC1175 examined by this group, inhibited

* Corresponding author. Tel.: +36 72 536 000; fax: +36 72 536225.
E-mail addresses: bela.kocsis@aok.pte.hu (B. Kocsis), lidiko.kustos@aok.pte.hu (I. Kustos), ferenc.kilar@aok.pte.hu (F. Kilár), nyu202@freemail.hu (A. Nyul), peter.jakus@aok.pte.hu (P.B. Jakus), vvillarr@hsc.unt.edu (L. Villarreal), lprokai@hsc.unt.edu (L. Prókai), tamas.lorand@aok.pte.hu (T. Lóránd).

0223-5234/$ – see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2008.10.038

H^+-ATP-ase mediated proton pumping in *Candida* spp. [5]. Our unsaturated Mannich ketones examined contain two potential thiol alkylating groups. On one hand, the C=C bond is a site for alkylation. On the other hand, Mannich ketones are also considered latent thiol alkylators. Under biological conditions for 1,2-elimination, they can yield reactive vinylketones, if there is a possibility for such elimination. They can also undergo addition reaction with thiols [6]. However, according to our previous examinations on thiol depletion, these compounds have other biological targets besides alkylation [1]. In our previous report [1], quantitative structure–activity relationship (QSAR) study has demonstrated that maximum positive charge of the molecule (Max Q^+) was one of the most significant parameters to describe the efficacy of Mannich ketones against inhibiting growth of *Escherichia coli*. First order valence-connectivity index was correlated with the compound minimum inhibitory concentrations (MIC), albeit to a lesser extent than Max Q^+. In this present study, we sought QSAR models that would be valid to various fungal strains (*C. albicans*, *Candida krusei*, *Candida parapsilosis* and *S. cerevisae*).



No.	n	R	Ar
1	1	Pip	phenyl
2	1	Mor	phenyl
3	1	Pyr	phenyl
4	1	Thik	phenyl
5	1	Mor	4'-CH_3-C_6H_4
6	1	Pip	4'-OCH_3-C_6H_4
7	1	Mor	4'-OCH_3-C_6H_4
8	1	Pip	3'-OCH_3-C_6H_4
9	1	Mor	3'-OCH_3-C_6H_4
10	1	Mor	2'-OCH_3-C_6H_4
11	1	Pip	2'-OCH_3-C_6H_4
12	1	Pip	3',4',5'-$(OCH_3)_3$-C_6H_3
13	1	Mor	3',4'-OCH_2O-C_6H_3
14	2	Mor	phenyl
15	2	Pyr	phenyl
16	2	4-Pip	phenyl
17	2	Mor	4'-CH_3-C_6H_4
18	2	Mor	4'-OCH_3-C_6H_4
19	2	Mor	3',4'-$(OCH_3)_2$-C_6H_4
20	3	Mor	phenyl
21	3	Pip	phenyl
22	4	Mor	phenyl
23	4	Pip	phenyl

Pip: 1-Piperidinyl; Mor: 4-Morpholinyl; Pyr: 1-Pyrrolidinyl; Thik: 1,2,3,4-Tetrahydroisoquinolinyl; 4-Pip: 4-methyl-1-piperidinyl

Fig. 1. Structure of the α,β-unsaturated Mannich ketones.

2. Chemistry

2.1. Synthesis

The synthesis of unsaturated Mannich ketones (**1**–**23**, Fig. 1) involved two steps: a base catalyzed aldol condensation and a classical three-component Mannich reaction. Structure verification was based on FT-IR, ^1H NMR and ^{13}C NMR methods [1]. The preparation of the amino alcohols (**24**–**32**, Fig. 2) was performed mostly with sodium borohydride, and these reductions proved to be rather stereoselective yielding mainly one stereoisomer [2].

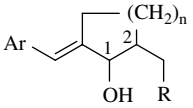
3. Results and discussion

3.1. Antifungal activity

The test compounds generally showed high activity against the different *Candida* and the *Saccharomyces* strains (Table 1). However, they exhibited much lower activity toward the *Aspergillus* spp. Compound **5** exerted the highest effect here (MIC = 25 μg/mL). Among the *Candida* strains studied, *Candida glabrata* proved to be a little less sensitive than the others. Considering Mannich ketones with five-membered rings, the aromatic substitution patterns did not influence the antifungal effect considerably; these ketones produced the maximal antifungal activity that decreased gradually for Mannich ketones with six-, seven-, and eight-membered rings.

Regarding the amino alcohols, the reduction of the keto group dramatically decreased their antifungal activity compared to the parent Mannich ketones (Table 2). Apparently, the conjugated keto function is required for the antifungal activity. Nevertheless, some amino alcohols retained certain antifungal activity (**24tr**, **30cis** and **31tr**). Moreover, **33cis** showed the maximal antifungal activity – much higher than that of the parent compound. These compounds were among the most hydrophobic ones in our study with seven- or eight-membered rings.

None of the test compounds exceeded the antifungal effect of amphotericin B used as reference agent with MIC of 0.2 μg/mL against *C. albicans*.



Comp.	n	R	Ar	Configuration
24 tr	1	Pip	Ph	tr
25 tr	1	Pip	4-OCH_3 - C_6H_4	tr
26 cis	2	Mor	Ph	cis
27 tr	2	Mor	Ph	tr
28 tr	2	4-Pip	Ph	tr
29 cis	3	Mor	Ph	cis
30 cis	3	Pip	Ph	cis
31 tr	3	Pip	Ph	tr
32 cis	4	Mor	Ph	cis
33 cis	4	Pip	Ph	cis

Pip: 1-Piperidyl; Mor: 4-Morpholinyl; 4-Pip: 4-methyl-1-piperidyl

Fig. 2. Structure of the α,β-unsaturated amino alcohols.

Table 1
In vitro antifungal data of Mannich ketones, expressed as minimum inhibitory concentration values (MIC, μg/mL).

Compound	*Candida albicans* ATCC 90028	*C. glabrata* ATCC 3916	*C. krusei* ATCC 30068	*C. parapsilosis* ATCC 22019	*Saccharomyces cerevisiae*	*Aspergillus* spp.
1	6.25	12.5	12.5	6.25	1.56	100
2	3.125	6.25	3.125	3.125	0.8	50
3	6.25	12.5	6.25	6.25	3.125	50
4	12.5	6.25	12.5	12.5	1.56	200
5	6.25	3.125	12.5	6.25	1.56	25
6	12.5	6.25	12.5	6.25	3.125	100
7	3.125	6.25	12.5	6.25	1.56	200
8	12.5	12.5	12.5	6.25	6.25	100
9	3.125	3.125	3.125	3.125	1.56	50
10	3.125	12.5	3.125	12.5	6.25	>200
11	6.25	12.5	12.5	12.5	6.25	100
12	12.5	100	12.5	50	25	100
13	1.56	3.125	6.25	6.25	3.125	>200
14	25	>200	25	12.5	3.125	>200
15	25	25	25	12.5	12.5	100
16	12.5	100	100	25	12.5	100
17	100	>200	200	25	3.125	>200
18	25	>200	25	12.5	12.5	>200
19	25	100	50	50	25	>200
20	25	>200	100	100	50	200
21	50	100	100	200	100	100
22	>200	>200	>200	>200	200	>200
23	100	>200	200	200	100	100

Table 2
In vitro antifungal data of amino alcohols, expressed as minimum inhibitory concentration values (MIC, μg/mL).

Compound	*C. albicans* ATCC 90028	*C. glabrata* ATCC 3916	*C. krusei* ATCC 30068	*C. parapsilosis* ATCC 22019	*Saccharomyces cerevisiae*	*Aspergillus* spp.
24tr	100	>200	50	>200	50	>200
25tr	100	>200	100	>200	100	200
26cis	>200	>200	>200	>200	>200	>200
27tr	200	>200	200	>200	200	>200
28tr	>200	>200	>200	>200	100	>200
29cis	>200	>200	>200	>200	>200	>200
30cis	>200	200	200	>200	50	200
31tr	50	100	100	100	25	>200
32cis	200	200	200	200	100	>200
33cis	100	50	6.25	50	6.25	50

3.2. Study of the inhibition of fungal ergosterol synthesis

The mechanism of action of azoles as antifungal agents is based on the disruption of sterol biosynthesis yielding the reduction of the ergosterol production [7]. This blocks the functions of ergosterol in fungal membranes and disturbs both the structure and the functions of the membrane. Because ergosterol also plays a hormone-like (“sparking”) role in fungal cells, which stimulates growth [8], the net effect of azoles is the inhibition of the fungal growth [9].

The method described by Arthington-Skaggs et al. [10] uses the characteristic absorption spectrum of the fungal sterols between 240 and 300 nm. These sterols are composed of ergosterol and 24(28)-dehydroergosterol, a late sterol pathway intermediate. Both have an absorption maximum at 281.5 nm and the latter have another separate maximum at 230 nm; thus, the ergosterol content can be determined. The spectrum has a four-peaked profile (Fig. 3). We applied this method as a qualitative procedure to demonstrate the change in ergosterol content of the fungal membrane.

Some Mannich ketones, specifically as **9**, **10**, **13**, **20** and **21**, were selected for this study, and the effect of **9** (potent antifungal agent) and that of **21** (poor antifungal agent) was displayed in Fig. 3. The UV spectrophotometric sterol profiles of *C. albicans* isolates were practically identical in the control samples, with compound **9** having good antifungal activity and with compound **21** having poor antifungal efficacy. According to this observation, ergosterol synthesis was not inhibited by the Mannich ketones studied. However, the sterol profile of the isolate treated with fluconazole provided a flat line indicating the absence of the spectrophotometrically detectable ergosterol (Fig. 3). Of course, it has not been a direct proof that the biosynthesis was inhibited. Another aspect of this experimental result is that ergosterol is biosynthesized in the

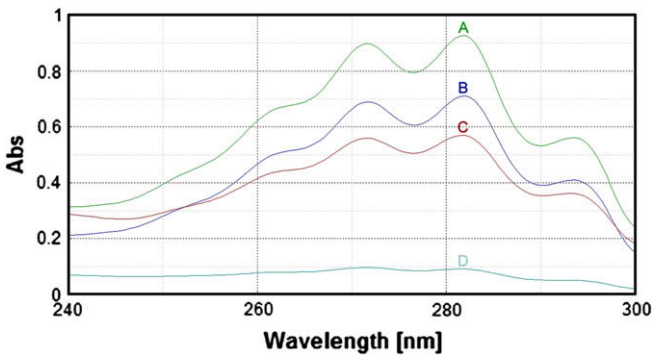


Fig. 3. Spectrophotometric sterol profile of a fluconazole-susceptible *C. albicans* isolate. (A) Control experiment (untreated). (B) Isolates were grown for 16 h in broth containing 1.56 μg/mL of **9**. (C) Isolates were grown for 16 h in broth containing 25 μg/mL of **21**. (D) Isolates were grown for 16 h in broth containing 1.56 μg fluconazole/mL (positive control). Test compounds were used in 0.5 × MIC concentrations.

Table 3
Formation of pseudohyphae in different *Candida* strains treated with 0.5 × MIC of some selected Mannich ketones.

		C. albicans ATCC 90028 (positive control)			*C. glabrata* ATCC 3916 (negative control)			*C. albicans* ATCC 90028 treated with **21**			*C. albicans* ATCC 90028 treated with **10**			*C. albicans* treated ATCC 90028 with amphotericin B		
		I	II	III	I	II	III	I	II	III	I	II	III	I[b]	II	III
t_0	[a]	0/100	0/100	0/100	1/99	0/100	3/97	0/100	0/100	0/100	0/100	0/100	0/100	0/100	0/100	0/100
$\Delta t_1 t_0 + 6$ h	[a]	58/42	52/48	63/37	3/97	7/93	5/95	53/47	61/39	47/53	9/81	13/87	11/89	18/82	20/80	23/77

[a] Fungal cells with pseudohyphae out of 100 cells/fungal cells without pseudohyphae out of 100 cells.
[b] I, II and III are three independent experiments.

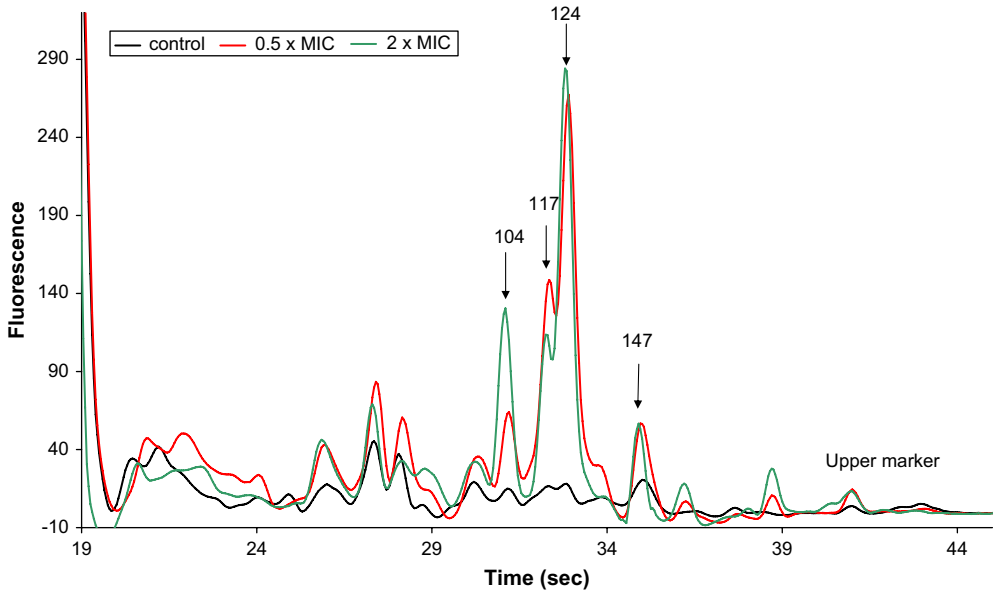


Fig. 4. Protein profile of *Candida albicans* ATCC 90028 by microchip electrophoresis without treatment and after treatment with compound **6**. Protein patterns obtained in untreated case were displayed with black curve. Effect of sub-inhibitory concentrations of Mannich ketones is shown in red, while that of supra-inhibitory concentrations are distinguished by green color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

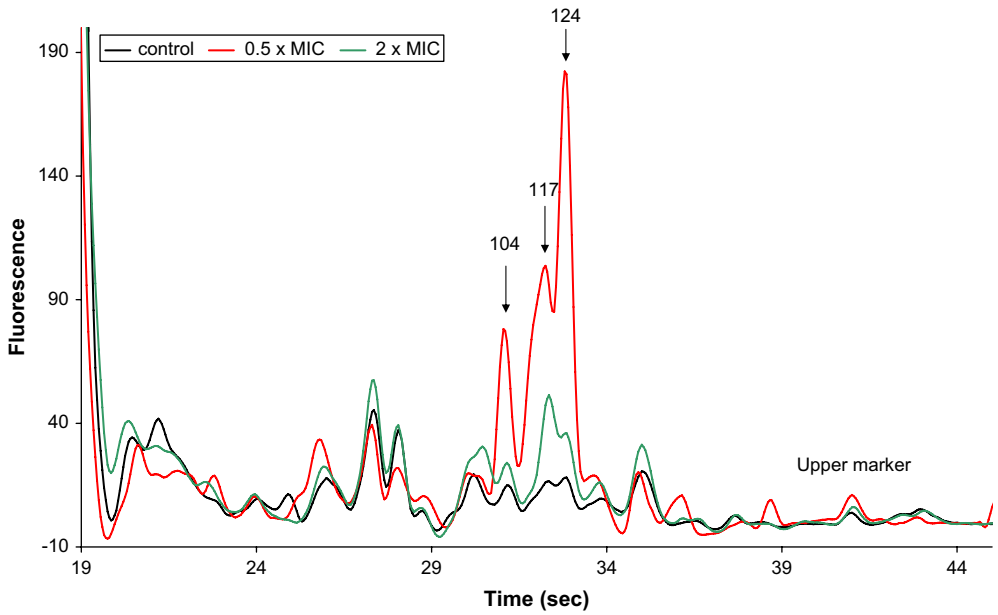


Fig. 5. Protein profile of *Candida albicans* ATCC 90028 by microchip electrophoresis without treatment and after treatment with compound **25tr**. Protein patterns obtained in untreated case were displayed with black curve. Effect of sub-inhibitory concentrations of Mannich ketones is shown in red, while that of supra-inhibitory concentrations is distinguished by green color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

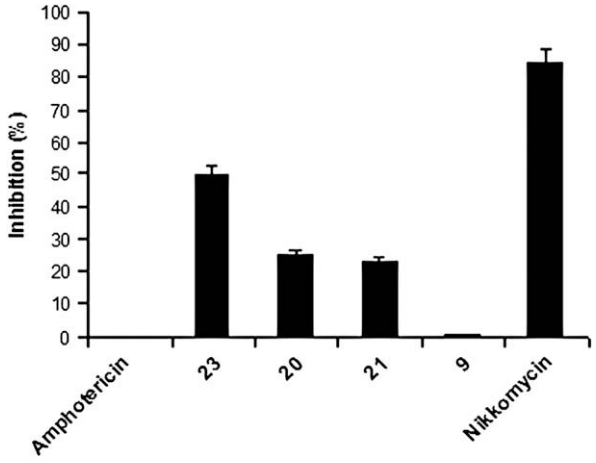


Fig. 6. Relative inhibition of chitin synthase of *C. albicans* (%). Compound (used in 1.5 × MIC concentrations) compared to total inhibition (10 M NaOH).

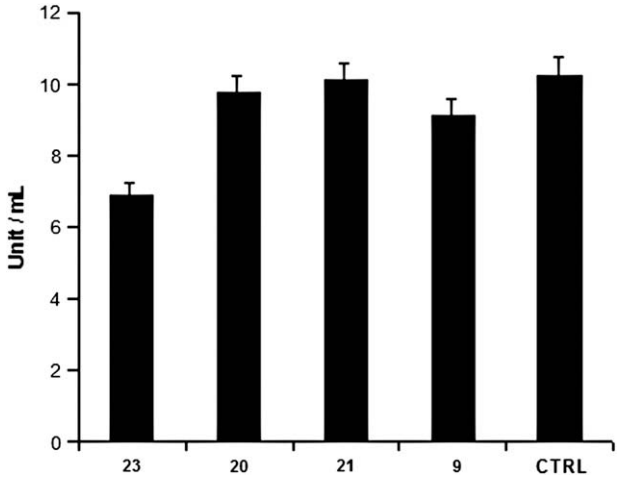


Fig. 7. Enzyme activity of LDH (U/mL). Compound (used in 1.5 × MIC concentrations). CTRL = control.

presence of fluconazole by the fungi, but it cannot remain inside the fungal cell.

3.3. *Investigation of pseudohypha formation of* C. albicans *and* C. glabrata *by light microscopy*

Mannich ketones **3**, **7**, **10**, **13** and **21** were chosen for these experiments. According to our experiments, compound **10** – (a potent antifungal agent) inhibited the development of pseudohyphae of *C. albicans* (see Table 3), similarly to amphotericin B. It means that this molecule could influence cell wall biosynthesis and stop the development of germ tube in *C. albicans*, while compound **21** (a poor antifungal agent) could not act on the test fungus.

3.4. *Effect of the Mannich ketones on protein composition*

Effect of compounds on protein composition was tested in the international standard *C. albicans* ATCC9028 strain. Protein composition of the untreated and Mannich ketone treated fungal cells was analysed by microchip technology as it is described in Section 5. Treatment of the *Candida* cells was made at supra-inhibitory (2 × MIC) and sub-inhibitory (0.5 × MIC) concentrations of the Mannich ketones.

Most of the Mannich ketones examined in this study including **1**, **3**, **7**, **11** and **21** were not able to induce any changes in the expression of fungal proteins. Two compounds, specifically **4** and **6**, induced significant changes in the profile of *C. albicans*. Protein pattern of *C. albicans* ATCC 90028 strain after chip based separation is shown in Fig. 4. Electrophoretic separation of proteins was completed within 45 s. Profile without treatment was given in black line in Fig. 4. This profile could be characterised by the presence of several protein peaks, and the dominating peaks were in the 40–50 kDa molecular weight region. Compounds **4** and **6** induced remarkable changes in the high molecular weight region. Quantities of at least four fungal proteins with molecular weight of 104, 117, 124 and 147 kDa, respectively, were increased significantly after the administration of these Mannich ketones. These protein peaks were indicated by arrowheads in Fig. 4. Both sub-inhibitory and supra-inhibitory concentrations of these compounds (**4** and **6**) were able to induce these changes.

Compound **25tr** is an amino alcohol, the reduction product of **6**. This agent was able to induce similar changes in the protein profile of *C. albicans* as compound **6**, which is demonstrated in Fig. 5. Quantity of the 104, 117 and 124 kDa proteins increased remarkably after the administration of this compound. In the meantime, expression of the 147 kDa protein was not modified by **25tr** treatment. As it can be observed in Fig. 5, this compound was able to induce these changes only at sub-inhibitory concentrations. Administration of supra-inhibitory concentration of compound **25tr** induced only mild increase in the expression of the 117 and 124 kDa proteins, while the quantity of the 104 and 147 kDa proteins remained unchanged.

3.5. *Chitin synthase assay*

The following test compounds were selected for these experiments: **1**, **9**, **20**–**23**, **32cis** and **33cis**. The chitin synthase assay published by Lefebvre et al. was applied [12]. Chitin synthase inhibitory effect of compounds **9**, **20**, **21** and **23** was demonstrated here in detail for the pathogenic fungus *C. albicans* ATCC9028. *C. albicans* has four genes, *CHS1*, *CHS2*, *CHS3* and *CHS8*, which encode chitin synthase isoenzymes with different biochemical properties and physiological functions [11]. We calibrated the assay for nikkomycin as positive control, because *C. albicans* expressed four kinds of chitin synthase. Nikkomycin showed significant inhibitory effect at 1.5 times of the MIC compared to a total inhibition of assay with 10 M NaOH. We measured the relative inhibitory effect of the Mannich ketones relative to nikkomycin; therefore, we used all antifungal chemicals in 1.5 times of their respective MIC. The negative control was amphotericin B. The relative inhibitory percentage of Mannich ketones was examined at 1 mM of UDP-GlcNAc (substrate), and the results are displayed in Fig. 6. The data indicated that increased lipophilicity correlated with the inhibitory effect. Mannich ketone **9** had no inhibitory effect on chitin synthases, while compounds **20** and **21** antagonized chitin synthase by approximately 24%. The best inhibitor was **23** (50% of inhibition) having the most non-polar feature. Regarding the other compounds investigated, **1** did not show any inhibitory effect, while **22** proved to be an effective inhibitor. From the amino alcohols, **32cis** exerted a significant inhibition, while the **33cis** was inactive.

The compounds discussed above in this section were investigated in an independent enzyme assay for lactate dehydrogenase (LDH) [13]. However, Mannich ketones **20** and **21** proved to be inhibitors of chitin synthase did not inhibit LDH related to the control (Fig. 7). While compound **23** revealed as a general inhibitor of the cell showing 30% inhibition in the LDH assay, compound **9** inhibited neither chitin synthase nor LDH. This latter substance had, therefore, specific inhibition due to a yet unknown

Table 4
Best QSAR models for antifungal potency of Mannich ketones.[a]

Fungal strain	Best 3-descriptor equation[a]	Best 2-descriptor equation[b]
C.krusei	pMIC −0.65 elect −0.08 sol + 1.4 valence + 1.4, $R^2 = 0.68$	pMIC −0.09 sol + 1.69 valence + 9.03, $R^2 = 0.64$
C. albicans	pMIC −0.36 polar + 0.24 atom + 1.42 arom + 0.20, $R^2 = 0.81$	pMIC −0.42 polar + 0.16 MR + 2.82, $R^2 = 0.75$
C. parapsilosis	pMIC 0.45 log *P* −0.49 polar + 1.02 conn (order 2, standard) + 2.68, $R^2 = 0.81$	pMIC −0.1 sol + 1.9 valence + 10.38, $R^2 = 0.73$
C. glabrata	pMIC −0.24 polar −0.57 solvent + 1.73 valence + 6.22, $R^2 = 0.64$	pMIC −0.40 polar + 1.06 valence + 2.61, $R^2 = 0.58$
S. cerevisiae	pMIC −0.50 polar + 0.78 conn (order 0, standard) + 0.73 valence (order 2, standard) + 1.12, $R^2 = 0.73$	pMIC −0.44 polar + 1.11 valence + 2.67, $R^2 = 0.68$

[a] Includes data for 33 additional compounds reported earlier [17].
[b] $R^2 > 0.5$ was considered acceptable fit.

biochemical pathway, which could also be implicated based on its low MIC value.

3.6. QSAR

In the QSAR study, we sought to correlate potency to inhibit growth of various fungus strains (*C. krusei*, *C. albicans* and *C. parapsilosis*, *C. glabrata, Aspergillus* sp. and *Saccharomyces cerevisiae*) with molecular properties to include Mannich ketones previously synthesized [1], and reported above (**1**–**33**). Our analysis also increased the number of descriptors compared to the previous effort focusing on antibacterial activity of these compounds [17], and meaningful three- and two-parameter QSAR equations were obtained for all fungal strains except *Aspergillus* sp. Descriptors found to correlate with pMIC for *C. krusei* involved electron affinity (elect, in eV), solvent accessible surface area (sol, Å^2) and valence-connectivity index (valence, order 0, standard) [14], as shown in Table 4. Polarizability (polar, Å^3), atom count (atom), aromatic ring count (arom) and molar refractivity (MR) influenced pMIC of Mannich ketones against *C. albicans* (Fig. 8).

Polarizability (polar), valence-connectivity index (valence, order 0, standard) and solvent accessible surface area (sol) of these compounds were important regarding inhibiting the growth of *C. glabrata*. Descriptors found to give the best QSAR model for pMIC of *C. parapsilosis* involved log *P* (logarithm of octanol–water partition coefficient, the measure of lipophilicity), polarizability (polar), solvent accessible surface area, valence-connectivity index and connectivity index (connect, order 0, standard). Descriptors correlating with antifungal potency against *S. cerevisiae* included polarizability (polar), valence-connectivity index and connectivity index (connect, order 0, standard). Except for *C. krusei*, polarizability was a descriptor universally correlating with the antifungal activity of Mannich ketones.

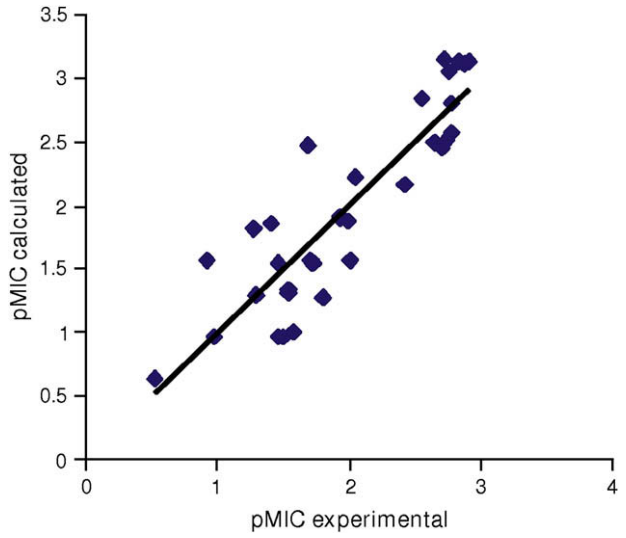


Fig. 8. Experimental versus calculated pMIC values for *C. albicans* based on the equation. pMIC = 0.42 polar + 0.16 MR + 2.82 ($R^2 = 0.75$).

4. Conclusion

Based on our studies, the antifungal activity of the Mannich ketones investigated is not connected to the inhibition of the ergosterol synthesis; however, these compounds can influence the development of pseudohyphae of *C. albicans* strains. In addition, they are able to induce significant changes in the protein composition of *C. albicans*. Up-regulation of the discussed proteins after antifungal treatment indicates their involvement in stress functions of the cells. Fluconazole and amphotericin B (the most frequently used antimycotic drugs) can induce similar alterations in the protein pattern of *C. albicans* as our experiments demonstrated. In addition, the title compounds show a weak or medium inhibition of the chitin synthase enzyme.

5. Experimental

5.1. Microbiological methods

5.1.1. Determination of MIC values by macro-tube dilution method [15]

The test materials were diluted twofold in RPMI-1640 medium (Sigma–Aldrich, Hungary) buffered to pH 7.0 with 0.165 M MOPS, the inoculum concentration was 10^4 cells/mL, and we read the lowest concentration of test compounds which could stop the growth of fungi (MIC) after incubation at 30 °C for 48 h.

5.1.2. Study of the inhibition of fungal ergosterol synthesis by UV spectrophotometry

C. albicans ATCC 90028 was cultivated in the presence of 0.5 × MIC of fluconazol (Diflucan, Pfizer Pharmaceuticals, USA) or Mannich ketones **9** or **21** for 16 h at 30 °C, cells were harvested by centrifugation. Alcoholic KOH (25% w/v, 3 mL) was added to suspend the pelleted cells, and the suspension was incubated in an 85 °C water bath for 1 h. After cooling down to room temperature, sterols were extracted by addition of a mixture of 1 mL distilled water and 3 mL *n*-heptane. Then, the mixture was vigorously vortexed for 3 min. The heptane layer was collected and diluted with ethanol to scan spectrophotometrically between 240 and 300 nm [10].

5.1.3. Investigation of pseudohypha formation of C. albicans ATCC 90028 by light microscopy

A small portion of an isolate colony of *C. albicans* ATCC 90028 and *C. glabrata* ATCC 3916 strains was suspended in 0.5 mL of human plasma. The control tubes without antimycotics and the test tubes with 0.5 × MIC final concentration of amphotericin B (Fungizone, Bristol-Myers Squibb, Epernon, France), or Mannich ketones **10** and **21** were kept at 30 °C. After 0, 3 and 6 h of incubation, one drop of the tube contents was Gram-stained and checked for pseudohypha by light microscope (1400×). We determined the fungal cell numbers with and without pseudohyphae out of 100 cells [7]. All experiments were carried out in triplicate (see Table 3).

5.1.4. Effect of the Mannich ketones on protein composition

5.1.4.1. Sample preparation for protein analysis. The extraction of fungal proteins was done according to a procedure reported previously [16]. Briefly, *C. albicans* ATCC 90028 strain was cultivated in Mueller–Hinton broth containing 5% glucose for 24 h at 30 °C with 200 rpm shaking. 0.5 × MIC or 2 × MIC of drugs were administered and incubated for one more hour. An untreated culture was used as a control. The fungal cells were collected by centrifugation, washed, frozen and kept at −20 °C.

Two grams of fungal cells were treated with liquid nitrogen, incubated with 2 mL of extraction buffer (45 min at 4 °C) and lysed. The extraction buffer consisted of 0.37 M Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM β-mercapthoethanol, 1 mM PMSF (phenylmethylsulfonyl-fluoride) and 1% Triton X-100. After centrifugation (4 °C), 100 μL of the supernatant was mixed with 100 μL of buffer (0.175 M Tris–HCl, pH 6.8; 4% SDS, 10% β-mercapthoethanol) and the resultant sample was analysed electrophoretically for protein content.

5.1.4.2. Chip technology. Analysis of fungal cell protein compositions were performed by the Protein 230 Plus LabChip Kits (catalogue number: 5067-1517) of a model 2100 Bioanalyzer System (Agilent Technologies, PaloAlto, CA, USA). The microcapillary system of the protein chips was filled with a gel supplied with the kit prior to electrophoretic analysis. This gel contained a linear polymer as sieving agent that ensured separation of proteins based on their molecular weight. A fluorescent dye was mixed into this gel before injection to label the fungal proteins on the chip after entering into the separation channel.

Preparation of fungal samples included the addition of 2 μL denaturing solution containing β-mercaptoethanol to 4 μL of each sample, boiling for 5 min and diluting with 14 μL of distilled water. Six microlitres of the samples were loaded on the proper sample well of the chip. Laser induced fluorescence was used for detection.

Interpretation and evaluation of data were performed by the Protein 230 assay software. Molecular weight of the fungal proteins was determined by application of molecular weight markers (ladder) supplied by the kit. Concentration of the proteins could also be determined by a one-point calibration. All measurements were repeated at least 3 times.

5.1.5. Chitin synthase assay

5.1.5.1. Permeabilization procedure. To each gram (wet weight) of *C. albicans* ATCC 90028 cells were added 1.4 mL of 0.1 M EDTA, 24 μL of 2-mercaptoethanol, and water to a final volume of 3.5 mL. The suspension was incubated with shaking at 30 °C for 30 min, followed by centrifugation at 1200 *g* for 10 min. The cells were washed by centrifugation with 5 mL of 0.8 M sorbitol and suspended in the following medium: 0.57 mL of citrate phosphate buffer, pH 6.3, 67 mL of 0.1 M EDTA, 0.64 mL of 1.6 M sorbitol, and 0.8 M sorbitol to a final volume of 6.7 mL. The suspension was incubated for 30 min at 30 °C with shaking and centrifugation as above. The cells were suspended in 30 mL of cold 0.05 M Tris chloride, pH 7.5 (osmotic shock), kept on ice for 5 min, and centrifuged again. The cell concentration was adjusted to 1.5 g wet weight/mL with 0.05 M Tris chloride pH 7.5.

5.1.5.2. Chitin synthase activation. Thus, an aliquot of permeabilized cell suspension was incubated for 15 min in 30 mM Tris–HCl (pH 6.5) containing 55 mM GlcNAc, digitonin (5.2 mg/mL) and trypsin (1.0 mg/mL). Activation was stopped by adding soybean trypsin inhibitor (1.5 mg/mL). The resulting preparation was kept at 0 °C for assay.

5.1.5.3. Chitin synthase assay. Digitonin, trypsin, soybean trypsin inhibitor, UDP-GlcNAc were obtained from Sigma. UDP trisodium salt hydrate was from Aldrich and *N*-acetyl-D-glucosamine from Acros. [UDP^{14}C]-GlcNAc (289 mCi/mmol) came from Perkin–Elmer Life Sciences. Assays were carried out at 30 °C in a volume of 60 μL which contained, in addition to the activated permeabilized cell preparations (40 μL), the following final concentrations of components: 30 mM Tris–HCl (pH 6.5), 35 mM GlcNAc, 3 mM $Mg(Ac)_2$, digitonin (3.5 mg/mL), cell suspension (52 mg/mL) and 1 mM UDP-[^{14}C]-GlcNAc (20,000 cpm). Reaction was initiated by addition of the cell suspension. After 30 min incubation the reaction was stopped by adding 1 mL of 10% trichloroacetic acid and 950 μL of the resulting suspension were filtered through a glass-fiber filter (Whatman GF-C). The filter was washed 3 times with 1 mL 60% aqueous ethanol and dried. The ^{14}C-chitin formed was quantitated by liquid scintillation analyzer (Packard).

5.1.6. QSAR

QSAR analysis was done using the BioMedCAChe® 6.1 program for Windows (Fujitsu, Beaverton, OR). Structures were preoptimized using augmented MM3 parameters followed by semiempirical PM/3 optimization. A total of 32 descriptors were calculated for each compound. The empirical MICs were expressed in molality and converted to pMIC (negative logarithm of MIC) values. Only compounds with an MIC < 200 mg/L were used in the QSAR analysis. The best QSAR equations with three and two descriptors were selected by multiple linear regressions via the Project Leader module of BioMedCAChe. Model validations were done by randomization of pMIC and leaving out 10% of the compounds.

Acknowledgements

The work was supported by the grants GVOP-3.2.1-0168 and RET 008-2005. László Prókai is the Robert A. Welch Professor of the University of North Texas Health Science Center (grant number BK-0031). The authors thank Mrs. Erika Kocsis and Mrs. Krisztina Sajti for their technical assistance.

References

[1] T. Lóránd, B. Kocsis, P. Sohár, G. Nagy, Gy. Kispál, H.G. Krane, H. Schmitt, E. Weckert, Eur. J. Med. Chem. 36 (2001) 705–717.
[2] T. Lóránd, E. ősz, Gy. Kispál, G. Nagy, E. Weckert, D. Luebbert, A. Meents, B. Kocsis, L. Prókai, Arkivoc vii (2004) 34–52.
[3] B. Mutus, J.D. Wagner, C.J. Talpas, J.R. Dimmock, O.A. Phillips, R.S. Reid, Anal. Biochem. 177 (1989) 237–243.
[4] E.K. Manavathu, S.C. Vashishtha, G.J. Alangaden, J.R. Dimmock, Can. J. Microbiol. 44 (1998) 74–79.
[5] E.K. Manavathu, J.R. Dimmock, S.C. Vashishtha, P.H. Chandrasekar, Antimicrob. Agents Chemother. 43 (1999) 2950–2959.
[6] J.R. Dimmock, K.K. Sidhu, M. Chen, R.S. Reid, T.M. Allen, G.Y. Kao, G.A. Truitt, Eur. J. Med. Chem. 28 (1993) 313–322.
[7] W.C. Winn Jr., S.D. Allen, W.M. Janda, E.W. Koneman, G.W. Procop, P.C. Schreckenberger, G.L. Woods, Koneman's Color Atlas and Textbook of Diagnostic Microbiology, in: Mycology, sixth ed. Lippincott Willams & Wilkins, 2006, pp. 1151–1243 [chapter 21].
[8] T.C. White, K.A. Marr, R.A. Bowden, Clin. Microbiol. Rev. 11 (1998) 382–402.
[9] Y. Koltin, C.A. Hitchcock, Curr. Opin. Chem. Biol. 1 (1997) 176–182.
[10] B.A. Arthington-Skaggs, H. Adi Jr., T. Desai, C.J. Morrison, J. Clin. Microbiol. 37 (1999) 3332–3337.
[11] M.D. Lenardon, R.K. Whitton, C.A. Munro, D. Marshall, N.A. Gow, Mol. Microbiol. 66 (2007) 1164–1173.
[12] I.G. Lefebvre, J.B. Behr, G. Guillerm, M. Muzard, Eur. J. Med. Chem. 40 (2005) 1255–1261.
[13] J. Kazimierczak, P. Chavaz, R. Krstić, O. Bucher, Histochemistry 46 (1976) 107–120.
[14] L.H. Hall, L.B. Kier, J. Mol. Graphics Model. 20 (2001) 4–18.
[15] National Committee for Clinical Laboratory Standard. Reference Method for Broth Dilution Susceptibility Testing of Yeast; Approved Standard M27A. NCCLS Villanova, 1997.
[16] I. Kustos, A. Nyul, T. Lóránd, B. Kocsis, F. Kilár, J. Biochem. Biophys. Methods 69 (2006) 57–65.
[17] T. Lóránd, B. Kocsis, P. Sohár, G. Nagy, J. Pál, Gy. Kispál, R. László, L. Prókai, Eur. J. Med. Chem. 37 (2002) 803–812.