The Influence of Newly Synthesised Fenpropimorph Derivatives on Some Pathogen Yeasts

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The effect of minimum inhibitory concentrations (MICs) of six novel fenpropimorph derivatives on lipid and sterol composition of *Candida albicans*, *Cryptococcus neoformans*, *Malassezia pachydermatis* and *Malassezia furfur* was investigated. The MICs for the most effective derivatives were found in the range from 3.7 to 56.7 μM and were 2–3 times lower compared to the commercial fungicide bifonazol. The more efficient fenpropimorph derivatives were the piperidine derivative for *C. albicans* and the allylamine derivative for *Cr. neoformans, M. pachydermatis and M. furfur* . The inhibitor in the growth medium reduced the unsaturation index of the total lipid content in *M. furfur* and *C. albicans.*

Introduction

The increasing incidence of yeast infections has greatly stimulated the interest in the development of new antimycotic drugs affecting the sterol biosynthesis pathway. The most frequently used fungicides are azoles and morpholines. The target enzyme for azoles is lanosterol demethylase (Joseph-Horne and Hollomon, 1997), morpholines and piperidines inhibit the Δ^{8-7} isomerase and Δ^{14}-reductase (Marcireau *et al.*, 1992). Although attack of ergosterol biosynthesis is usually the primary reason of growth inhibition, unsaturated sterols can replace ergosterol in the membranes. So, a fungistatic effect can not only be due to accumulation of abnormal sterols in treated cells, but is linked with other adverse influences (Marcireau *et al.*,1990). In accordance with this suggestion Georgopapadakou *et al.* (1987) showed that changes in membrane fatty acids induced by fungicides rather than decreased ergosterol were responsible for growth inhibition of *Candida albicans.* All yeasts used belong to the most important opportunistic pathogens for immunocompromised humans or animal hosts (Groshek, 1998) *C. albicans* is the main cause of superficial and systematic mycoses, and *Cryptococcus neoformans* is an important pathogen for individuals with AIDS and other immunocompromising diseases (Kelly *et al.*, 1999; Bujdáková *et al.,* 1999). The lipophilic yeast *Malassezia pachydermatis* and lipid-dependent species *Malassezia furfur* are part of the normal cutaneous microflora of most warm-blooded vertebrates (Boerhout *et al.*, 1998; Guillot and Bond, 1999).

The objective of the present study was to compare the antifungal activity of six fenpropimorph derivatives including allylamines, piperazines and piperidines. Their inhibitory effect was compared with the azol fungicide bifonazol.

Materials and Methods

The following pathogen yeasts were used: *Candida albicans* CCY 29–3-101, CCY 29–3-102 , *Cryptococcus neoformans* CCY 17–1-2, *Malassezia furfur* CCY 85–2-1 and two strains of *Malassezia pachydermatis* (CCY 85–1-5, CCY 85–1-10). The strains were obtained from the Culture Collection of Yeasts (Institute of Chemistry, SAS, Bratislava, Slovakia).

The cultures were grown on malt agar at 37 °C, only *M. furfur* was cultivated on Dixon's modified medium (7 g malt extract, 1 ml Tween 40, 0.25 ml glycerol, 1 g peptone, 0.01 g yeast extract, 0.05 g stearic acid and 2 g agar in 100 ml medium). Inhi-

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bition effects of the newly synthesised fungicides (Fig. 1) prepared according to Veverka *et al.*, 1990, was compared with bifonazol (Bayer AG, Germany). Paper discs (0.5 cm in diameter) were impregnated with 10 µl of inhibitor solution in 96% ethanol at the final amounts of 250, 125 and 50 µg per disc. The discs were placed on agar surface covered with culture suspension. The yeast strains were incubated at 37 °C for 48–72 h. Zones of growth inhibition (mm in diameter) were measured for each amount of fungicide. For the most effective inhibitors the minimum inhibitory concentrations (MICs) were determined by a microdilution method according Carillo-Munoz and Tur-Tur (1997). All determinations were made in triplicate. Cells used for sterol and fatty acid analysis were grown on malt agar with a suspended fungicide concentration of 5 $\mu g \cdot ml^{-1}$ in the medium. Lipids were extracted from lyophylised yeasts with a chloroform / methanol mixture (2:1 v/v) (Folch *et al.*, 1957). Fatty acids were liberated by alkaline hydrolysis, methylated with diazomethane (Schlenk and Gellerman, 1960) and analysed according to Bohov *et al.* (1997). Sterols were determined by GC (Pesti *et al.*, 1997) and HPLC (Lamačka and Šajbidor, 1997; Steel *et al.*, 1989).

Results and Discussion

The tested fenpropimorph derivatives (Fig. 1) showed differences between basidiomycete species *M. pachydermatis*, *M. furfur*, and *Cr. neoformans* and the ascomycete *C. albicans* (Table I). Allylamine R_1 and piperazine R_2 derivatives are weak inhibitors for all tested strains. The piperidine derivative R_3 was effective against *C. albicans* and *Malassezia* sp. Similarly, R_4 was inhibitory for two isolates of *M. pachydermatis*, but an effect on *M. furfur* was not detected. R_5 and R_6 strongly influenced the growth of *Malassezia* and *Cryptococcus*, but not *C. albicans*. Comparison of the MIC showed, that the allylamine R_5 derivative was the effective fungicide for *M. pachydermatis*, *M. furfur* and *Cr. neoformans*. Similarly, R_3 was more potent than the reference for both *Candida* strains (Table II).

As the target of all tested fungicides are enzymes of the sterol pathway, changes in the content of ergosterol were investigated. It is evident that the presence of inhibitor caused its decrease in all samples, but a direct correlation between the extent of ergosterol reduction and MIC was not observed. The decline in ergosterol, di-

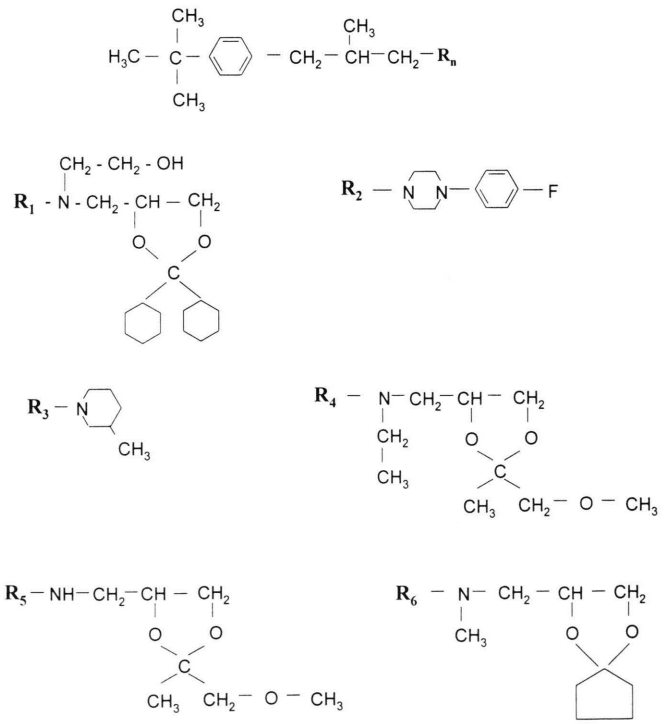

Fig. 1. Chemical structures of newly synthesised inhibitors.

Table I. Influence of three concentrations of inhibitors on growth of some pathogenic yeasts.

Inhibitor	c μg/disc	Yeast strain CCY					
		M. pachydermatis 85-1-5	*M. pachydermatis* 85-1-10	M. furfur 85-2-1	*Cr. neoformans* 17-1-2	*C.* albicans 29-3-101	*C.* albicans 29-3-102
		Inhibition[a]					
	50	–	–	–	–	–	–
R_1	125	+	–	–	–	–	–
(allylamine)	250	+	–	–	–	–	–
	50	–	–	–	–	–	–
R_2	125	+	–	–	–	–	–
(piperazine)	250	+	–	–	–	–	–
	50	–	–	–	–	–	+
R_3	125	++	++	–	–	+	+
(piperidine)	250	++	++	–	–	++	++
	50	–	–	–	–	–	+
R_4	125	++	++	–	–	–	+
(allylamine)	250	++	++	–	–	+	+
	50	++	++	++	++	–	–
R_5	125	+++	+++	++	++	–	–
(allylamine)	250	+++	+++	+++	++	–	+
	50	++	++	+	+	–	–
R_6	125	++	++	++	+	–	–
(allylamine)	250	++	++	++	+	–	+
	50	++	++	+	+	–	–
bifonazol	125	++	++	++	+	–	–
	250	++	++	++	++	–	+

c – concentration of inhibitor per disc; [a] – zone of inhibition (mm in diameter): 0 (–), 0–10 (+), 10–20 (++), 20–30 (+++).

Table II. Ergosterol content of yeast cultivated without (–) and with (+) inhibitors at the minimal inhibition concentration (MIC) of the most effective inhibitors and bifonazol.

Yeast strain CCY	MIC (μM)		Ergosterol content (μg·mg^{-1} DC)	
	Bifonazol	inhibitor[a]	–	+
M. pachydermatis 85-1-5	26.5	9.2	1.5	0.3
M. pachydermatis 85-1-10	28.7	7.7	1.2	0.5
M. furfur 85-2-1	8.6	3.7	0.5	0.03
Cr. neoformans 17-1-2	57.1	17.5	0.4	0.3
C. albicans 29-3-101	115.3	56.7	2.2	0.09
C. albicans 29-3-102	98.9	43.1	2.2	0.4

[a] Inhibitor R_5 was used for 85-1-5, 85-1-10, 85-2-1 and 17-1-2, inhibitor R_3 was tested against 29-3-101 and 29-3-102 strains; DC – dry cell weight; (–) absence of inhibitors; (+) presence of inhibitors.

hydroergosterol and episterol in *C. albicans* was accompanied with the increasing of zymosterol, ignosterol, and fecosterol (Table III). Accumulation of zymosterol could be related to the reduction of C24 sterol methyl transferase activity (Barrett-Bee and Dixon, 1995), probably the high

Table III. The influence fenpropimorph derivative R_3 on the profile of sterols using the more resistant strain *Candida albicans* CCY 29-3-102.

Sterol	Sterol content (μg·g^{-1} DC)	
	–	+
Ergosterol	802	162
Zymosterol	10	258
Dihydroergosterol	93	2
Ignosterol	6	478
Lanosterol	12	38
Fecosterol	4	59
Episterol	75	3

DC – dry cell weight; (–) absence of inhibitors, (+) presence of inhibitors.

Table IV. Fatty acid composition of *M. furfur* and *C. albicans* cultivated without (–) and with (+) the most effective inhibitors (R_5 for *M. furfur* and R_3 for *C. albicans*).

Fatty acids	***Malassezia furfur*** CCY 85–2-1		***Candida albicans*** CCY 29–3-102	
	–	+	–	+
	Fatty acid content % DC			
12:0	0.5	9.6	0.1	0.1
13:0	ND	0.5	ND	ND
14:0	2.8	11.2	0.7	0.7
15:0 ai	8.6	ND	ND	ND
14:1 n-5	ND	0.4	0.1	ND
15:0	0.8	2.4	0.2	0.3
16:0 i	2.6	ND	ND	ND
16:0	44.0	54.6	16.9	12.0
16:1 n-7t	0.3	0.5	0.1	0.2
16:1 n-9c	1.7	0.2	0.2	0.3
16:1 n-7c	1.9	3.7	5.7	9.9
17:0 ai	2.2	0.2	0.6	0.2
17:0	0.9	2.0	1.1	0.7
17:1 n-8c	0.3	0.2	1.4	1.8
18:0	15.9	5.8	7.0	6.3
18:1 n-9t	0.4	ND	0.2	0.3
18:1 n-7t	ND	0.2	0.1	0.2
18:1 n-9 c	10.4	0.6	43.6	41.8
18:1 n-7c	0.6	2.9	0.7	0.8
18:2 n-6c	4.3	3.7	15.3	19.0
20:0	1.1	0.1	0.3	0.3
18:3 n-3c	ND	ND	1.7	2.3
20:2 n-6c	0.2	1.3	0.1	0.1
22:0	0.1	ND	0.2	0.2
24:0	0.2	ND	2.7	1.8
25:0	ND	ND	0.3	0.4
26:0	0.2	ND	0.6	0.4
I.U	**0.2464**	**0.1866**	**0.8812**	**1.0024**

DC – dry cell weight, ai – ante-iso, i – iso, I.U – index of unsaturation calculated according Kates and Baxter, 1962, ND – not determined.

content of ignosterol was the consequence of Δ^{14}-reductase inhibition (Marcireau *et al.,* 1992). Increase of fecosterol was always accompanied with Δ^{8-7} isomerase inhibition (Kelly *et al.*, 1994).

Cultivation in the presence of fungicide induced adaptive changes not only in the sterol profile, but also in fatty acid content and composition (Table IV). It is interesting that fungicides caused only a slight decrease of the total fatty acid level amount in both tested strains. Although growth inhibition is usually accompanied with lipid reduction (Barrett-Beeand Dixon, 1995), the increase of the neutral lipid level can also be a consequence of ergosterol disappearance (Hitchcock *et al.*, 1987). GC analyses showed that the content of individual fatty acids in *Malassezia furfur* and *Candida albicans* were quite different. The main fatty acid in *M. furfur* was palmitic in contrary with *C. albicans*, in which oleic acid was detected as the most abundant structure. *M. furfur* shows the inhibitory effect by stearic and oleic acid reduction with concomitant increase of palmitic acid. On the other hand, cultivation of *C. albicans* in the presence of the R_3 derivative decreased both, palmitic and oleic acid as well. Decline of these was compensated by accumulation of palmitoleic, linoleic and linolenic acid. Generally, the adaptation response of *M. furfur* included a slight reduction of lipid unsaturation unlike *C. albicans* where the opposite trend was observed.

The results demonstrate that fenpropimorph derivatives exhibit potent mycostatic and mycocidal activity against *Cr. neoformans, Malassezia pachydermatis and M. furfur* with the R_5 - allylamine derivative and against *C. albicans* with the R_3 – piperidine derivative.

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