A diffusible analogue of *N*³-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid with antifungal activity

Dorota Zgódk, Sławomir Milewski and Edward Borowski

Author for correspondence: Edward Borowski. Tel: +48 58 347 25 23. Fax: +48 58 347 26 94.
e-mail: borowski@altis.chem.pg.gda.pl

Department of
Pharmaceutical
Technology and
Biochemistry, Technical
University of Gdańsk,
11/12 Narutowicza St,
80-952 Gdańsk, Poland

***N*³-(4-Methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP), a specific and potent inactivator of glucosamine-6-phosphate (GlcN-6-P) synthase from *Candida albicans*, exhibits relatively poor anticandidal activity, with an MIC value amounting to 50 µg ml⁻¹ (200 µM). Uptake of FMDP into *C. albicans* cells follows saturation kinetics and is sensitive to the action of metabolic inhibitors, thus indicating the active transport mechanism. However, the acetoxymethyl ester of FMDP penetrates the fungal cell membrane by free diffusion and is rapidly hydrolysed by *C. albicans* cytoplasmic enzymes to release the free FMDP. This mechanism gives rise to continuous accumulation of the enzyme inhibitor and results in higher antifungal activity of the FMDP ester (MIC = 3.1 µg ml⁻¹, 10 µM). These results show that the 'pro-drug' approach can be successfully applied for the enhancement of antifungal activity of glutamine analogues that inhibit GlcN-6-P synthase.**

Keywords: glucosamine-6-phosphate synthase, glutamine analogues, pro-drugs, diffusion, antifungal compounds

INTRODUCTION

Systemic fungal infections, caused mainly by the opportunistic pathogen *Candida albicans*, are regarded as one of the most important problems to be solved in modern chemotherapy (Polak & Hartman, 1991; Ghannoum, 1997). None of the antimycotics used in clinical practice meets all the criteria expected for a good chemotherapeutic agent, so there is a strong need for novel compounds. Potentially interesting and valuable agents in chemotherapy are amino acid analogues. They act mainly as enzyme inhibitors. Unfortunately, their use is often limited by restrictions on their transport into cells. Amino acid permeases are the only means of transport for these compounds, but they are rather selective in their action and do not take up the more highly modified analogues. Consequently, only a limited number of such compounds exhibit activity against cells. Examples of such amino acid analogues include some antifungal agents: azoxybacillin, cispentacin, BAY 10-8888 and RI 331 (Aoki *et al.*, 1994; Konishi *et al.*, 1989; Capobianco *et al.*, 1993; Ziegelbauer *et al.*,

1998; Yamaki *et al.*, 1988). Bypassing the carrier transport by appropriate modification of the amino acid analogues may result in free diffusion of novel derivatives. Those modifications have become a challenge in the design of an active agent.

In our research programme, aimed at the development of novel antifungal agents, we have focused on a glutamine analogue, *N*³-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP), a potent and selective inhibitor of glucosamine-6-phosphate synthase (GlcN-6-P synthase) EC 2.6.1.16 from *C. albicans* (Andruskiewicz *et al.*, 1986, 1993; Milewski *et al.*, 1985). This enzyme, which plays a key role in the biosynthesis of glucosamine-containing microbial cell wall macromolecules, chitin, mannoprotein and peptidoglycan, is considered to be a target for potential antimicrobial drugs (Chmara *et al.*, 1986; Milewski *et al.*, 1986; Borowski, 2000).

In our previous studies it has been found that FMDP, an amino acid analogue, exhibits only moderate antifungal activity and the presence of some amino acids, especially glutamine and glutamic acid, strongly decreases its antifungal efficacy (Cybulska *et al.*, 1997). In order to overcome this problem, we have performed chemical modifications of the FMDP molecule aimed at the construction of latent lipophilic derivatives, which could

Abbreviations: FMDP, *N*³-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid; GlcN-6-P synthase, glucosamine-6-phosphate synthase; TNBS, 2,4,6-trinitrobenzenesulfonate.

Fig. 1. Structures of FMDP and the FMDP acetoxymethyl ester.

be transported into cells by diffusion. Following uptake, the modifying group could be removed intracellularly. Very commonly, the development of pro-drugs, in particular in the penicillin group, involves the formation of esters or other easily hydrolysable derivatives of the active agent in order to increase penetration of compounds into the cell (Daehne *et al.*, 1970). Recently, we synthesized and examined the FMDP derivative acetoxymethyl ester. The structure of this compound is shown in Fig. 1. This novel derivative inhibits the activity of isolated pure GlcN-6-P synthase and its enzyme inhibitory potency is only a few-fold lower than that of the unsubstituted FMDP ($IC_{50} = 3.2 \mu M$ for FMDP and $11.5 \mu M$ for the FMDP ester; Zgódka *et al.*, 1999). In this paper, we present evidence that the FMDP acetoxymethyl ester penetrates into the fungal cells by free diffusion and, once inside, generates free FMDP upon enzymic cleavage. As a consequence, the compound inhibits growth of *C. albicans* cells. Our results show that the 'pro-drug' approach can be successfully applied to the design of derivatives of GlcN-6-P synthase inhibitors which bypass the amino acid carrier system and exhibit good antifungal activity.

METHODS

Chemicals. FMDP and the acetoxymethyl ester of FMDP were synthesized in our laboratory by methods described previously (Andruszkiewicz *et al.*, 1986; Zgódka *et al.*, 1999). 2,4,6-Trinitrobenzene sulfonic acid (TNBS) was from Sigma. Other chemicals were of the highest grade commercially available. Filter disks were manufactured by Whatman.

Organisms. *Candida albicans* ATCC 26278 cells were stored on Sabouraud Dextrose Agar slants (1% Bacto peptone, 1% yeast extract, 2% glucose, 2% agar) at 4 °C and transferred monthly.

Growth conditions. Minimal medium (YNBG) contained 1.7 g YNB (Yeast Nitrogen Base; Difco), 20 mg L-Trp, 20 mg L-Met, 10 mg L-His and 10 g glucose in 1000 ml water. Overnight cultures in Sabouraud Dextrose medium were diluted in fresh medium to a concentration of 10^6 cells ml^{-1} and incubated for about 2 h at 30 °C with shaking (200 r.p.m.) to obtain exponential-phase culture cells.

Growth experiments

(a) Antifungal susceptibility tests. To determine the 50% inhibitory concentrations (IC_{50}) and MICs of FMDP and its ester, *C. albicans* cells grown overnight in Sabouraud Dextrose

medium at 30 °C were inoculated at a cell density of 10^4 ml^{-1} in YNBG medium containing the test compounds at concentrations of 0–400 μM . Cultures were incubated for 48 h at 30 °C, and cell growth was quantified by measuring the OD_{660} to determine the IC_{50} values. The MIC was defined as the lowest drug concentration preventing visible growth.

(b) Determination of kinetics of growth inhibition. YNBG liquid medium was inoculated with 10^6 yeast cells ml^{-1} from the overnight culture in Sabouraud broth. After a 3 h pre-incubation at 30 °C with shaking (200 r.p.m.), the compounds were added to give a final concentration in the range of 0–160 μM . Incubation was continued for 8 h under the same conditions. Growth was determined spectrophotometrically at 660 nm at hourly intervals.

Uptake studies. For uptake studies, exponential-phase *C. albicans* cells grown in Sabouraud Dextrose medium were washed with 50 mM potassium phosphate buffer, pH 7.0, containing 1% (w/v) glucose and suspended in the same buffer to a cell density of 10^7 cells ml^{-1} (or 10^6 cells ml^{-1} to determine the effects of cell density on FMDP ester uptake). Cell suspensions were preincubated for 15 min at 30 °C and uptake was started by adding FMDP or its ester at final concentrations from 0.05 to 4 mM. At that moment, and at 5 min intervals thereafter, 2 ml samples of the cell suspensions were withdrawn, immediately collected on filter disks (GF/A Whatman filters, pore size 0.22 μm), and the filtrates were used to determine the concentration of the antifungal agents. Then, 1 ml samples of the filtrates were taken and combined with 1.25 ml of a solution containing 4% $Na_2B_4O_7 \cdot 10H_2O$ and 0.8 mg TNBS ml^{-1} . Reactions were carried out at 37 °C for 30 min. The A_{420} was measured and concentrations of FMDP or its ester were read from standard curves. Data were plotted as nmol amino acid (or amino acid ester) taken up by 1 mg (dry wt) cells versus time. The initial uptake velocities were determined from the slopes of the linear part of the curves in the 0–10 min region.

In some experiments, cells were preincubated with the inhibitor or ionophore for the indicated times at 30 °C, before the determination of uptake by the antifungal agents.

Analysis of FMDP ester metabolism. Cell-free extracts from *C. albicans* were prepared by the method described previously (Milewski *et al.*, 1991). FMDP ester was added to the extract, to give a final concentration of 100 μM . The mixture was incubated at 30 °C, and 1 ml samples were collected at 1 min intervals and deproteinized by addition of 1 ml ethanol. Precipitate was removed by filtration and the supernatant was analysed by TLC. TLC analyses were performed on Kieselgel 60 F 254 (Merck) and cellulose plates (Merck) in the following solvent systems: A: n-propanol: NH_3 : $CHCl_3$ (12:8:1, by vol.); B: n-propanol: H_2O (7:3, v/v). The filtrates obtained

after cell harvesting in uptake studies were also subjected to TLC analysis in the same systems.

RESULTS AND DISCUSSION

Growth inhibitory effect of FMDP and the FMDP acetoxymethyl ester

The acetoxymethyl ester of FMDP exhibited a stronger antifungal effect than FMDP. A 50% reduction of fungal growth was noticed upon the action of 3 μM FMDP ester and for 10 μM FMDP. In terms of the MIC values, the difference between both agents was even higher (3.1 $\mu\text{g ml}^{-1}$ /10 μM and 50 $\mu\text{g ml}^{-1}$ /200 μM , respectively). The growth inhibitory effect of both com-

pounds was inoculum size dependent. The MIC for the FMDP ester was enhanced to the value of 35 μM and that for FMDP to 400 μM , when the growth medium was inoculated with 10^6 cells ml^{-1} . The better antifungal potency of the FMDP ester was also confirmed in kinetic studies. As shown in Fig. 2, this compound in concentrations above 20 μM caused, after some delay, the complete growth inhibition of *C. albicans* cells, while FMDP only reduced the growth rates. Microscopic observations of *C. albicans* cells treated with FMDP or its ester revealed substantial morphological changes of fungal cells, such as swelling, clumping and inhibition of septum formation. These effects are characteristic for compounds inhibiting chitin biosynthesis and were previously observed in our earlier studies on the mechanism of action of FMDP-peptides (Milewski *et al.*, 1991). Growth inhibitory and morphological effects were totally reversed when *N*-acetyl-D-glucosamine, 5 mM, was added to the FMDP- or FMDP-ester-treated cultures (data not shown). These observations, taken together with the previously established high GlcN-6-P synthase inhibitory potency of both compounds (Zgódka *et al.*, 1999), confirm that this enzyme is the only target crucial for growth of *C. albicans* cells which is inhibited upon the action of FMDP and its ester.

Kinetics of uptake of FMDP and the FMDP acetoxymethyl ester

The uptake of low-molecular-mass compounds by microbial cells is usually measured using radioisotopic derivatives. Since such derivatives were not available, we applied a colorimetric method, based on the formation of yellow products upon the reaction of TNBS with the amino group of organic compounds. Since either FMDP or its ester was the only amino-containing compound added to the incubation medium, the decrease in concentration of TNBS-positive substance in filtrates remaining after removal of fungal cells allowed determination of the initial uptake rates of these compounds. The uptake rates were found to be dependent on the concentrations of FMDP or the FMDP ester in the medium. At low concentrations, the initial rates of FMDP uptake were higher than those of the ester [1.49 nmol min^{-1} (mg dry wt) $^{-1}$ vs 0.56 nmol min^{-1} (mg dry wt) $^{-1}$, respectively, at 100 μM]. At concentrations higher than 1 mM, the FMDP ester was taken up much faster than its unsubstituted counterpart. The dependence of the initial velocity of FMDP acetoxymethyl ester uptake on the initial concentration of this compound in the medium showed a linear fit; coefficient of determination, $r^2 = 0.9976$ (Fig. 3). This dependence was maintained up to an FMDP ester concentration of 4 mM. The initial velocity of FMDP uptake exhibited saturation kinetics with a $V_{\text{max}} = 5 \text{ nmol min}^{-1}$ (mg dry wt) $^{-1}$. This behaviour is characteristic for carrier-mediated transport, while the kinetics of FMDP ester uptake follows the pattern characteristic for passive diffusion.

Fungal cultures used in uptake kinetics studies contained 10^7 cells ml^{-1} . At this cell density, the amount of FMDP

Fig. 2. Growth inhibitory effect of FMDP and FMDP acetoxymethyl ester. Cells from an overnight culture in YNBG were diluted with YNBG medium to a cell density of 10^6 cells ml^{-1} . The FMDP or its ester was added to give a final concentration of 0–160 μM . All suspensions were incubated at 30 °C with shaking for 8 h. The rate of growth was monitored by determining the OD_{660} at hourly intervals. (a) FMDP; (b) FMDP acetoxymethyl ester. ○, 0; ●, 5 μM ; ▲, 10 μM ; ▼, 20 μM ; ◆, 40 μM ; ■, 80 μM ; ×, 160 μM .

Fig. 3. Kinetics of uptake of FMDP and the FMDP acetoxy-methyl ester. Exponential-phase *C. albicans* cells grown in modified Sabouraud medium were harvested, washed and suspended in potassium phosphate buffer, pH 7.0, containing 1% glucose (10^7 cells ml^{-1}). Cell suspensions were preincubated for 15 min at 30 °C, FMDP or its ester was added to give final concentrations of 0.05–4 mM, and the incubation was continued. Uptake of compounds was measured at 30 °C over a period of 30 min. Samples of the cell suspensions were withdrawn at time intervals (5 min), cells were removed by filtration and the concentration of tested compounds remaining in filtrates was determined by the TNBS method and then plotted against time. The initial uptake rates were determined from the slopes of the linear part of the uptake curves in the 0–10 min regions. The initial uptake velocities were then plotted against initial compound concentration to give the presented graphs. ■, FMDP; ●, FMDP ester. Bars represent SD.

Table 1. Effects of metabolic inhibitors and ionophores on uptake of FMDP and its ester

Initial uptake velocities were determined exactly as described in Methods and in the legend to Fig. 3. The initial concentration of FMDP or its ester was 100 μM . Metabolic inhibitors were added to cell suspensions at the indicated concentrations 10 min prior to the addition of FMDP/FMDP ester. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NEM, *N*-ethylmaleimide.

Metabolic inhibitor	Uptake rate $\pm$ SD [nmol min^{-1} (mg dry wt) $^{-1}$]	
	FMDP	FMDP ester
None	1.49 ± 0.18	0.56 ± 0.07
NaN_3 (0.01%, w/v)	< 0.02	0.49 ± 0.11
Sodium arsenate (1 mM)	0.20 ± 0.03	0.61 ± 0.14
CCCP (0.1 mM)	< 0.02	0.79 ± 0.08
NEM (1 mM)	< 0.02	0.70 ± 0.10

acetoxy-methyl ester which was accumulated by *C. albicans* cells increased from a value of $1.6 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$ at an initial concentration of 0.2 mM to $10.45 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$ at an initial concentration of 2 mM. The decrease in cell density to 10^6 ml^{-1} resulted in a proportional decrease in the amount of FMDP ester accumulated by cells to $0.121 \text{ nmol min}^{-1}$

$(\text{mg dry wt})^{-1}$ at an initial concentration of 0.2 mM and $1.31 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$ at initial concentration of 2 mM. These results also indicate that the acetoxy-methyl ester of FMDP is transported across biological membranes by diffusion.

The TLC analysis of the spent medium filtrates collected during uptake determination did not reveal any ninhydrin-positive substances, except the FMDP ester, for 25 min. After that time, the appearance of another ninhydrin-positive but UV-negative substance was observed. Its R_F value amounted to 0.03 and was lower than that of the diaminopropanoic acid ($R_F = 0.37$) and FMDP ($R_F = 0.93$), but we were not able to identify this compound. We suppose that this unidentified substance could be a product of FMDP intracellular metabolism, extruded by *C. albicans* cells.

Effects of metabolic inhibitors on FMDP and FMDP acetoxy-methyl ester uptake

The influence of several metabolic inhibitors and ionophores on the uptake of these compounds is shown in Table 1. Metabolic inhibitors such as sodium azide and sodium arsenate reduced the initial velocity of FMDP uptake to 2 and 12% of the control value, respectively, when the cells were preincubated with the appropriate inhibitor for 10 min. These inhibitors and the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone did not affect the rate of FMDP acetoxy-methyl ester uptake into *C. albicans*. This indicates that uptake of this compound is neither energy-dependent nor proton-linked. *N*-Ethylmaleimide, an agent known to covalently modify cysteine side chains, did not reduce FMDP ester uptake.

Summarizing the results of the uptake studies, we believe that the linear kinetics, dependence of accumulation rate on cell density and lack of effects of metabolic inhibitors, proton ionophores and thiol group blockers on the rate of the FMDP acetoxy-methyl ester transport into fungal cells clearly indicate that this compound enters the candidal cells by free diffusion. On the other hand, the saturation kinetics and complete inhibition of uptake by metabolic inhibitors indicate that FMDP is taken up by an active transport mechanism, most likely by an amino acid permease(s). Since these carrier proteins are unlikely to accept amino acid esters (Horak, 1986; Prasad, 1987), their apparent reluctance towards the acetoxy-methyl derivative of FMDP cannot be surprising.

Metabolism of the FMDP ester

FMDP acetoxy-methyl ester was added to a crude cell-free extract from *C. albicans*, and the mixture was incubated at 30 °C. TLC analysis of deproteinized samples taken from this mixture at 1 min intervals revealed the immediate formation of FMDP. Traces of the FMDP ester were detected for 5 min, but totally absent from samples taken later. When proteins present in the cell-free extract were denatured by heating for 3 min at 100 °C prior to the addition of the FMDP ester,

decomposition of this compound was not observed. The ester was also stable in 50 mM potassium phosphate buffer (pH 7.0) as well as in the chromatographic solvent system used in TLC analysis. We can therefore conclude that the FMDP acetoxymethyl ester is rapidly hydrolysed to FMDP by unidentified enzymes present in the cytoplasm of *C. albicans* cells. It is very likely that this process also takes place inside intact fungal cells treated with the agent. Intracellular fast hydrolysis of the FMDP ester must lower the intracellular concentration of this agent to nearly negligible amounts, thus permitting its continuous influx. Moreover, the generated free FMDP is expected to be eliminated from the cytoplasmic environment due to the binding to its target, GlcN-6-P synthase. If the hydrolysis did not occur, the FMDP ester could be accumulated by free diffusion only until its intracellular concentration equilibrates with the extracellular one. According to Ziegelbauer *et al.* (1998), the intracellular volume of 10^7 *C. albicans* cells is roughly 5 μ l. Therefore, such an amount of fungal cells, present in 1 ml of suspension used in our uptake studies, would have been able to accumulate only 0.5% of the antifungal agent present in the medium. Our results clearly show that the accumulation rate is much higher. One may therefore assume that the intracellular hydrolysis of the FMDP ester keeps the concentration gradient of this compound as a driving force for continuous free diffusion.

Our earlier studies on physico-chemical properties of the acetoxymethyl ester of FMDP showed that the apparent lipophilicity of this compound is higher than that of FMDP (Zgódko *et al.*, 1999). Results of the studies presented in this paper suggest that the formation of acetoxymethyl esters can be a way of obtaining amino acid analogues lipophilic enough to diffuse through the fungal cell membrane. The application of the lipophilic 'pro-drug' approach for the design of derivatives of GlcN-6-P synthase inhibitors was shown to be a possible way of obtaining novel compounds exhibiting better antifungal properties than the parent compound.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support of these studies by the State Committee for Scientific Research (Warsaw), and in part by the Chemical Faculty of the Technical University of Gdańsk.

REFERENCES

- Andruszkiewicz, R., Chmara, H. & Borowski, E. (1986). Synthesis of N³-fumaramoyl-L-2,3-diaminopropanoic acid analogues, the irreversible inhibitors of glucosamine synthetase. *Int J Pept Protein Res* **27**, 449–453.
- Andruszkiewicz, R., Chmara, H., Milewski, S., Kasprzak, L. & Borowski, E. (1993). Structural determinants of inhibitory activity of N³-(4-methoxyfumaryl)-L-2,3-diaminopropanoic acid towards glucosamine-6-phosphate synthase. *Pol J Chem* **67**, 673–683.
- Aoki, Y., Kondoh, M., Nakamura, M., Fujii, T., Yamazaki, T., Shimada, H. & Arisawa, M. (1994). A new methionine antagonist that has antifungal activity: mode of action. *J Antibiot* **47**, 909–916.
- Borowski, E. (2000). Novel approaches in the rational design of antifungal agents of low toxicity. *Farmaco* **55**, 206–208.
- Capobianco, J., Zakula, D. Z., Coen, M. L. & Goldman, R. C. (1993). Anti-*Candida* activity of cispentacin: the active transport by amino acid permeases and possible mechanism of action. *Biochem Biophys Res Commun* **190**, 1037–1044.
- Chmara, H., Andruszkiewicz, R. & Borowski, E. (1986). Inactivation of glucosamine-6-phosphate synthetase from *Salmonella typhimurium* LT2 by fumaryl diaminopropanoic acid derivatives, a novel group of glutamine analogs. *Biochim Biophys Acta* **870**, 357–366.
- Cybulska, B., Milewski, S. & Andruszkiewicz, R. (1997). Antifungal effect of FMDP. In *Abstracts of the 6th International Symposium on Molecular Aspects of Chemotherapy*, Gdańsk 9–12 July 1997, Poland, p. 148.
- Daehne, W., Frederiksen, E., Gundersen, E., Lund, F. & Morch, P. (1970). Acyloxymethyl esters of ampicillin. *J Med Chem* **13**, 607–612.
- Ghannoum, M. A. (1997). Future of antimycotic chemotherapy. *Dermatol Ther* **3**, 104–111.
- Horak, J. (1986). Amino acid transport in eucaryotic microorganisms. *Biochim Biophys Acta* **864**, 223–256.
- Konishi, M., Nishio, M., Saitoh, K., Miyaki, T., Oki, T. & Kawaguchi, K. (1989). Cispentacin, a new antifungal antibiotic. I. Production, isolation, physico-chemical properties and structure. *J Antibiot* **42**, 1749–1755.
- Milewski, S., Chmara, H., Andruszkiewicz, R. & Borowski, E. (1985). Synthetic derivatives of N³-fumaryl-L-2,3-diaminopropanoic acid inactivate glucosamine synthetase from *Candida albicans*. *Biochim Biophys Acta* **828**, 247–254.
- Milewski, S., Chmara, H. & Borowski, E. (1986). Anticapsin: an active site directed inhibitor of glucosamine-6-phosphate synthase from *Candida albicans*. *Drugs Exp Clin Res* **12**, 577–583.
- Milewski, S., Andruszkiewicz, R., Kasprzak, L., Mazerski, J., Mignini, F. & Borowski, E. (1991). Mechanism of action of anticandidal dipeptides containing inhibitors of glucosamine-6-phosphate synthase. *Antimicrob Agents Chemother* **35**, 36–43.
- Polak, A. & Hartman, P. G. (1991). Antifungal chemotherapy – are we winning? *Prog Drug Res* **37**, 181–269.
- Prasad, R. (1987). Nutrient transport in *Candida albicans*, a pathogenic yeast. *Yeast* **3**, 209–221.
- Yamaki, H., Yamaguchi, M., Nishimura, T., Shinoda, T. & Yamaguchi, H. (1988). Unique mechanism of action of an antifungal antibiotic RI-331. *Drugs Exp Clin Res* **14**, 467–472.
- Zgódko, D., Cybulska, B., Milewski, S. & Borowski, E. (1999). Acyloxymethyl esters of N³-(4-methoxyfumaryl)-L-2,3-diaminopropanoic acid ('pro-drugs'). In *Abstracts of The Multi-disciplinary Conference on Drug Research*, Muszyna, 3–5 March 1999, Poland, p. P-76.
- Ziegelbauer, K., Babczinski, P. & Schonfeld, W. (1998). Molecular mode of action of the antifungal amino acid BAY 10-8888. *Antimicrob Agents Chemother* **42**, 2197–2205.

Received 21 July 2000; revised 23 February 2001; accepted 19 March 2001.