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ORIGINAL PAPER

Antifungal Activity of Thionated Analogues of Nva-FMDP and Lys-Nva-FMDP

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Abstract

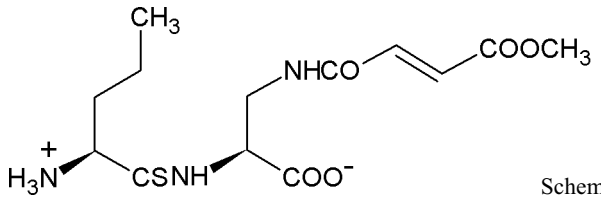
The antifungal activity of synthetic endothiopeptdides, *i.e.* NvaΨ[CSNH]-FMDP and LysΨ[CSNH]-NvaΨ[CSNH]-FMDP was studied in medium containing blood serum, against selected *Candida* strains; *Candida albicans* Gu4 (fluconazole sensitive), *C. albicans* Gu5 (fluconazole resistant), *C. albicans* ATCC 10231, *Candida krusei* DSM 6128 and *Candida parapsilosis* DSM 5784. Although thiopeptide bonds in the tested peptides increased their stability in blood serum, their antifungal activity, however, drastically decreased in comparison with the peptides containing non-modified peptide bonds. Moreover, the inhibitory activity towards glucosamine-6-phosphate synthase of thionated synthetic analogue of FMDP was performed. The thiopeptdide bond also influenced its inhibitory properties against enzyme from *C. albicans*.

K e y w o r d s: antifungal activity, antifungal compounds, endothiopeptides, peptides cleavage, GlcN-6-P synthase inhibitors

Introduction

Glucosamine-6-phosphate synthase (GlcN-6-P), an enzyme involved in the first step in the formation of the core aminosugar, N-acetyl glucosamine in the biosynthesis of microbial cell wall, is an attractive target in antifungal chemotherapy. There is a quite broad range of glutamine analogues, that inhibit the enzyme, *e.g.* 6-diazo-5-oxo-L-norleucine, azaserine and anticapsin, but they show lack of specificity and inhibit other enzymes that use glutamine as a substrate (Chmara *et al*., 1986; Badet *et al*., 1987). A rationally designed glutamine analogue, N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP) is a strong and selective inhibitor of GlcN-6-P *in vitro*, inhibiting the enzyme by forming a covalent linkage with the Cys residue located in the glutamine-binding domain (Andruszkiewicz *et al*., 1987; Kucharczyk *et al*., 1990). Unfortunately, FMDP is not transported by specific amino acid permeases (Milewski *et al*., 1991). In contrast to amino acid permeases, peptide permeases can transport peptides with modified side chains. It is therefore possible for FMDP and other amino acid mimetics to be absorbed into the microbial cell as peptides, which are then cleaved by intracellular peptidases (Milewski *et al.*, 1991; Payne, 1995a). The concept of utilizing a peptide transport system for delivery of toxic amino aids into microbial cells is known among others as the "smuggling" (Payne, 1995b) or "warhead delivery" approach (Ringrose, 1980).

Although biologically active peptides are not stable in blood serum, we have recently showed that endothiopeptides are more resistant towards enzymatic degradation by peptidases than natural peptides and, furthermore, are transported well enough *via* membranes of bacterial cells to be considered a drug delivery system (Nowak-Jary *et al*., 2008b). Therefore, we have decided to carry out studies on the activity of thionated antimicrobial peptides, *i.e.* NvaΨ[CSNH]-FMDP and LysΨ[CSNH]-NvaΨ[CSNH]-FMDP – di- and tripeptides containing one or two thioamide bonds respectively, without thionated FMDP (Scheme I and Scheme II).



Scheme I

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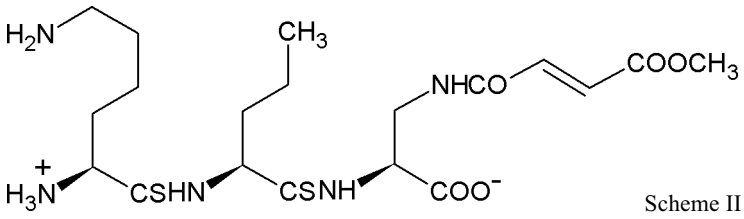
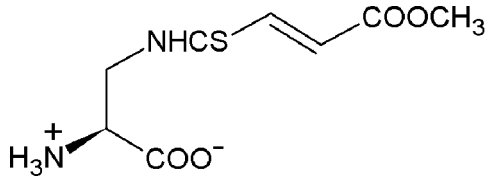


Scheme II



Scheme III

Moreover, we have also synthesised the thionated analogue of FMDP (FM[S]DP) (Scheme III) in order to evaluate its inhibitory activity against fungal glucosamine-6-phosphate synthase. If the activity of thionated inhibitor is comparable to that of FMDP itself, the synthesis of fully thionated peptide will be feasible. On the other hand, the lack of inhibitory activity of the thionated inhibitor precluded the synthesis of active antimicrobial peptide with that inhibitor.

Experimental

Materials and Methods

Chemicals. Compounds with thionated peptide bonds, *i.e.* NvaΨ[CSNH]-FMDP, LysΨ[CSNH]-NvaΨ [CSNH]-FMDP and FM[S]DP used in the study were obtained by the previously described methods (Nowak-Jary *et al*., 2008a).

Preparation of the crude enzyme (cell free extract) and the determination of glucosamine-6-phosphate synthase activity. *Candida albicans* glucosamine-6-phosphate synthase cell free extract was prepared by the previously described procedure (Milewski *et al.*, 1991). The concentration of glucosamine-6-phosphate was determined by the modified Elson-Morgan procedure (Kenig *et al.*, 1976). Reactions were carried out in 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA. L-Glutamine and D-fructose-6-phosphate concentrations were either fixed (10 mM and 7.5 mM respectively, for IC_{50} determinations) or variable (2–5 mM and 0.6–3 mM respectively, for K_i determinations).

Inhibition of glucosamine-6-phosphate synthase. Standard incubation mixtures containing 20 ml of a cell free extract of GlcN-6-P synthase (0.41 mg/ml of protein), 260 µl of 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 40 µl of 7.5 mM D-Fru-6-P, 40 l of 10 mM L-Gln and 40 µl of inhibitor in various concentrations in a total volume of 0.4 ml were incubated at 37°C for 30 min. Then the IC_{50} assays were performed as described previously (Milewski *et al.*, 1991).

The measurements were done in triplicate. Inhibitory constants were determined from the secondary plots of k_{app} versus inhibitor concentration, derived from Lineweaver-Burk plots.

Antifungal susceptibility tests. Minimal inhibitory concentrations (MIC's) of the examined compounds were determined by the serial twofold dilution microtiter plate method, in the minimal liquid Yeast Nitrogen Base (YNB) medium without amino acids and ammonium sulphate containing 2% glucose and L-proline (4 mg/ml). Wells containing serially diluted test compounds and control were inoculated with 10^4 cells/ml of an overnight culture of fungal cells and the microtiter plates were incubated for 24 and 48 h at 30°C. Fungal growth was measured using the microplate reader (Wallac 1420 Multilabel Counter, PerkinElmer) at $\lambda = 595$ nm. The MIC was defined as the inhibitor concentration preventing at least 80% of fungal growth, as compared to the inhibitor-free control.

The following fungal strains were used: *C. albicans* Gu4 (fluconazole sensitive), *C. albicans* Gu5 (fluconazole resistant, a gift from prof. J. Morschhauser, University of Wurzburg, Germany), *C. albicans* ATCC 10231, *Candida krusei* DSM 6128, *Candida parapsilosis* DSM 5784.

Results and Discussion

Inhibition studies. N^3-(4-Methoxyfumaroyl)-Ψ[CSN^3H]-L-2,3-diaminopropanoic acid hydrochloride (FM[S]DP×HCl) was tested as an inhibitor of *C. albicans* glucosamine-6-phosphate synthase. The compound inhibits the enzyme competitively with respect to L-glutamine (L-Gln, one of the substrates of the GlcN-6-P synthase) and, in contrary to FMDP (mixed inhibition), uncompetitive with respect to D-fructose-6-phosphate (D-Fru-6-P, the second substrate) (Fig. 1, 2). It indicates that the binding place of FM[S]DP to GlcN-6-P synthase is different than the binding place of D-Fru-6-P. The thionated FMDP inhibited enzyme with $IC_{50} = 388$ µM, $K_M = 1.45$ mM and $K_I = 0.13$ mM for L-glutamine; $K_I/K_M = 8.97\times10^{-2}$ (for comparison $IC_{50} = 4$ µM, $K_I = 0.1$ µM, $K_I/K_M = 9.20\times10^{-5}$ for FMDP (Milewski *et al.*, 1992). The relation K_I/K_M, which is the measure of the affinity of the inhibitor to the active centre, indicates that GlcN-6-P synthase binds FM[S]DP about 10^3 times weaker than FMDP itself. Therefore, we decided to undertake a multi-step synthesis of thionated peptides in order to obtain a more resistant compounds towards

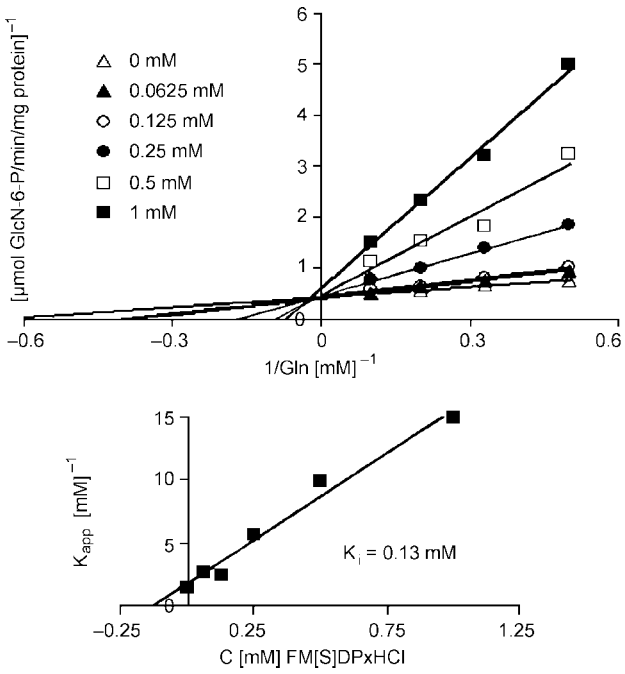


Fig. 1. Competitive inhibition of GlcN-6-P synthase by FM[S]DP in respect to L-glutamine.

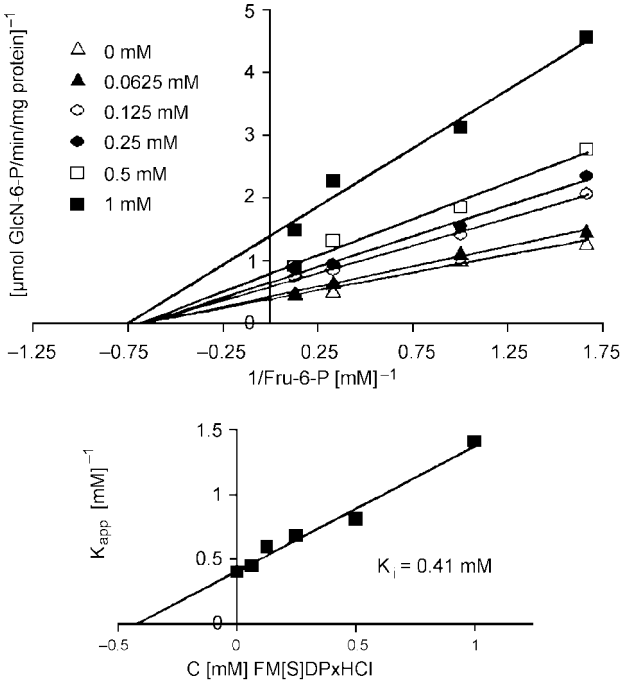


Fig. 2. Uncompetitive inhibition of GlcN-6-P synthase by FM[S]DP in respect to D-Fru-6-P.

enzymatic degradation, namely, antimicrobial peptides containing a thioamide backbone only between amino acids and FMDP.

Anticandidal activity. Previous research indicated that thiopeptides, which are characterized by high stability in blood serum, may be good carriers for amino acid analogues into microorganism cells (Nowak-Jary *et al.*, 2008b). Although rates of transport of thiopeptides through the cytoplasmic membrane were lower than rates of transport of natural peptides, intracellular concentration of the former after 1 minute of transport was very high. Moreover, thiopeptide hydrolysis inside cells seemed to be sufficient for the release of the potential inhibitors. With these facts in mind, we decided to carry out the synthesis of thionated analogues of two known antimicrobial peptides: NvaFMDP and LysNvaFMDP.

Minimum inhibitory concentration (MIC's) were determined in YNB medium and in YNB medium containing 5% blood serum. Tables I and II present

Table I
Antifungal activity of NvaFMDP and Nva-Ψ[CSN^{2}H]-FMDP

medium	YNB		YNB + 5% blood serum			YNB		YNB + 5% blood serum		MIC (µg/ml) or % of inhibition of growth at C = 500 µg/ml
time of incubation	24 h	48 h	24 h	48 h		24 h	48 h	24 h	48 h	
***Candida albicans* ATTC 10231**										
NvaFMDP	0.12–31.3	0.24– >100	0.24–>100	0.49–>100	Nva-Ψ[CSN^{2}H]--FMDP	24–35%	25–37%	23–25%	19–26%	
***Candida krusei* DSM 6128**										
NvaFMDP	0.98–1.95	3.9–>100	31.3–>100	>100	Nva-Ψ[CSN^{2}H]--FMDP	18–33%	16–35%	20–30%	23–40%	
***Candida parapsilosis* DSM 5784**										
NvaFMDP	0.98–>100	3.91–>100	31.3–>100	62.5–>100	Nva-Ψ[CSN^{2}H]--FMDP	7–40%	4–46%	8–30%	8–28%	
***Candida albicans* Gu4**										
NvaFMDP	0.03–0.12	0.24–0.49	0.03–0.24	0.24–0.98	Nva-Ψ[CSN^{2}H]--FMDP	14–35%	14–23%	25–31%	21–24%	
***Candida albicans* Gu5**										
NvaFMDP	0.06–0.12	0.12–0.49	0.06–0.24	0.24–0.98	Nva-Ψ[CSN^{2}H]--FMDP	27–50%	10–30%	31–45%	30–35%	

Table II
Antifungal activity of LysNvaFMDP and Lys-Ψ[CSNH]-Nva-Ψ[CSN^{2}H]-FMDP

medium	YNB		YNB + 5% blood serum			YNB		YNB + 5% blood serum		MIC (μg/ml) or % of inhibition of growth at C = 500 μg/ml
time of incubation	24 h	48 h	24 h	48 h		24 h	48 h	24 h	48 h	
***Candida albicans* ATTC 10231**										
Lys--NvaFMDP	0.12–0.24	0.24–0.48	0.24–0.48	0.49–0.98	Lys-Ψ-[CSNH]--Nva-Ψ[CSN^{2}H]--FMDP	26–30%	25–28%	23–25%	21–24%	
***Candida krusei* DSM 6128**										
Lys--NvaFMDP	0.98	3.9	31.3	62.6	Lys-Ψ[CSNH]--Nva-Ψ[CSN^{2}H]--FMDP	30–36%	20–35%	27–32%	23–30%	
***Candida parapsilosis* DSM 5784**										
Lys--NvaFMDP	>100	>100	>100	>100	Lys-Ψ[CSNH]--Nva-Ψ[CSN^{2}H]--FMDP	4–23%	5–16%	4–21%	6–16%	
***Candida albicans* Gu4**										
Lys--NvaFMDP	0.03	0.24–0.49	0.03–0.24	0.48–0.98	Lys-Ψ[CSNH]--Nva-Ψ[CSN^{2}H]--FMDP	14–16%	16–23%	18–25%	15–19%	
***Candida albicans* Gu5**										
Lys--NvaFMDP	0.12	0.12	0.06	0.24	Lys-Ψ[CSNH]--Nva-Ψ[CSN^{2}H]--FMDP	22–27%	9–15%	12–17%	11–19%	

antimicrobial activity against a few species. The two synthesized potential antimicrobial thiopeptides: Nva-Ψ[CSN^{2}H]FMDP and Lys-Ψ[CSNH]-Nva-Ψ[CSN^{2}H]-FMDP, exhibited poor antifungal activity with MIC's higher than 100 μg/ml. However, we decided to estimate the degree of inhibition of growth at concentration 500 μg/ml of the compound to show how their activity was changed depending on time of incubation and presence or lack the blood serum in medium in comparison to the activity of the known antifungal peptides: NvaFMDP and LysNvaFMDP. Antifungal activity of NvaFMDP and LysNvaFMDP in medium containing blood serum decreased significantly after 48 h of incubation, (Table I) but activity of their thionated analogues remained at about the same level. Suppression of the activity of NvaFMDP and LysNvaFMDP was caused by formation of spontaneous resistant mutants. Such a decrease in the case of thiopeptide was not observed. Probably the activity of the compound is too low to observe this phenomenon. Moreover, comparing the activity of NvaFMDP in both types of medium (YNB and YNB+blood serum), it can be seen that it is much lower in medium containing blood serum, especially after 48 h of incubation. We doubt that it was caused by the cleavage of the compound by hydrolases, whereas the activity of Nva-Ψ[CSN^{2}H]-FMDP did not change in a significant degree. It is caused by much higher resistance of thiopeptides towards hydrolases, as we showed previously (Nowak-Jary *et al.*, 2008). Analogous relationships were observed in case of LysNvaFMDP and Lys-Ψ[CSNH]-Nva-Ψ[CSN^{2}H]-FMDP (Table II). However, the antifungal activity of Lys-Ψ[CSNH]-Nva-Ψ[CSN^{2}H]-FMDP was little lower than the activity of Nva-Ψ[CSN^{2}H]-FMDP. One can suspect that the intracellular hydrolysis of the trithiopeptide is more difficult than the cleavage of dithiopeptide.

In case of species *C. albicans* ATTC 10231, Gu4 and Gu5 the degree of inhibition of growth was close correlated with concentration of the thiopeptide. It was surprising, that inhibition of growth of *C. krusei* and *C. parapsilosis* by thiopeptides was very variable and did not depend on the concentration of the compound. These thiopeptides turned out to be compounds with low antifungal activity. Without doubt, neither rate of transport of these compounds into fungal cells nor their intracellular hydrolysis is sufficient to accept them as good antifungal peptides.

Acknowledgement

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Literature

Andruszkiewicz R., H. Chmara, S. Milewski and E. Borowski. 1987. Synthesis and biological properties of *N*-3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid dipeptides, a novel group of antimicrobial agents. *J. Med. Chem.* 30: 1715–1719.

Badet B., P. Vermoote, P.Y. Haumont, F.Lederer and F. Le Goffic. 1987. Glucosamine synthetase from *Escherichia coli*: purification, properties and glutamine-utilizing site location. *Biochemistry* 26: 1940–1948.

Chmara H., R. Andruszkiewicz and E. Borowski. 1986. Inactivation of glucosamine-6-phosphate synthetase from *Salmonella typhimurium* Lt2 by fumaroyl diaminopropanoic acid derivatives, a novel group of glutamine analogs. *Biochim. Biophys. Acta* 870: 357–366.

Kenig M., E. Vandamme and E.P. Abraham. 1976. The mode of action of bacilysin and anticapsin and biochemical properties of bacilysin – resistant mutants. *J. Gen. Microbiol.* 94: 46–54.

Kucharczyk N., M.A. Denisot, F. Le Goffic and B. Badet. 1990. Glucosamine-6-phosphate synthase from *Escherichia coli* – determination of the mechanism of inactivation by *N*-3-fumaroyl-L-2,3-diaminopropionic derivatives. *Biochemistry* 29: 3668–3676.

Milewski S., R. Andruszkiewicz, L. Kasprzak, J. Mazerski, F. Mignini and E. Borowski. 1991. Mechanism of action of anticandidal dipeptides containing inhibitors of glucosamine-6-phosphate synthase. *Antimicrob. Agents Chemother.* 35: 36–43.

Milewski S., H. Chmara, R. Andruszkiewicz and E. Borowski. 1992. N^3-haloacetyl derivatives of L-2,3-diaminopropanoic acid: novel inactivators of glucosamine-6-phoshate synthase. *Biochim. Biophys. Acta* 1115: 225–229.

Nowak-Jary J. and R. Andruszkiewicz. 2008a. Synthesis and antimicrobial activity of Nva[CSNH]-FMDP. *J. Peptide Sci.* Suppl. 14: 118–119

Nowak-Jary J., R. Andruszkiewicz and J.W. Payne. 2008b. Enzymatic degradation and transport of endothiopeptides into *Escherichia coli* K12 mutant strains. *FEMS Microbiol. Lett.* 285: 291–297.

Payne J.W. 1995a. Bacterial peptide permeases as a drug delivery target, pp. 341–367. In: M.D. Taylor and G.L. Amidon (eds). Peptide Based Drug Design: Controlling Transport and Metabolism ACS, Washington, D.C.

Payne J.W. 1995b. Peptides and microorganisms. *Adv. Microbiol. Physiology* 13: 55–113.

Ringrose P.S. 1980. Peptides as antimicrobial agents, pp. 641–657. In: Payne J. W.(ed) Microorganisms and Nitrogen Sources. John Wiley and Sons Ltd., Chichester, United Kingdom.