Structure–activity relationships of the nikkomycins

H. DECKER,1 H. ZÄHNER,1 H. HEITSCH,2 W. A. KÖNIG2 and H.-P. FIEDLER1*

1 Universität Tübingen, Biologisches Institut, LB Mikrobiologie/Antibiotika, D-7400 Tübingen, FRG
2 Universität Hamburg, Institut für Organische Chemie, D-2000 Hamburg 13, FRG

(Received 8 January 1991; revised 3 May 1991; accepted 15 May 1991)

The structure–activity relationships of different nikkomycins were studied to evaluate the structural requirements for a potent chitin synthase inhibitor. We investigated the transport of the nikkomycins via the peptide transport system of the yeast Yarrowia lipolytica and determined the kinetic parameters for nikkomycin Z uptake 

\[ K_m = 24 \mu M, \quad V_{\text{max}} = 2.2 \text{ nmol min}^{-1} \text{ (mg dry wt)}^{-1} \].

We demonstrated that the \( \beta \)-methyl group of the N-terminal amino acid of dipeptide nikkomycins protects the molecule against peptidase activity in crude cell-extracts of different fungi. Furthermore, the relationship between inhibition constants for chitin synthase, transport of the nikkomycins via the peptide transport system, susceptibility to degradation by cellular proteases and whole-cell activity of the nikkomycins are discussed.

Introduction

Nikkomycins (Table 1) and polyoxins are peptide-nucleoside antibiotics which competitively inhibit the chitin synthase of fungi and insects because of their structural similarity to UDP-N-acetylglucosamine (Isono & Suzuki, 1979; Müller et al., 1981; Fiedler et al., 1982; Cohen, 1987).

As the nikkomycins and polyoxins are non-toxic and easily degradable they could be used as insecticides in agriculture or as antifungal agents in human therapy (Becker et al., 1983; Zoebelien & Kniehase, 1985; Hector et al., 1990). Although nikkomycins in micromolar concentrations inhibit the chitin synthase of many organisms, the inhibitory activity against whole cells of Candida albicans or Saccharomyces cerevisiae is very low (McCarthy et al., 1985a). Only high concentrations of nikkomycin affect growth inhibition of these organisms, although chitin synthesis is essential for cell viability of, e.g. S. cerevisiae (Silverman et al., 1988). Furthermore, Furter & Rast (1985) demonstrated that both dipeptide and tripeptide nikkomycins exhibit a high \textit{in vitro} activity towards the chitin synthase of Mucor rouxii, but the antifungal activity of the tripeptide nikkomycins was two orders of magnitude lower than the antifungal activity of the dipeptide nikkomycins. Because a high affinity for chitin synthase does not guarantee antifungal activity, other factors must be important. In organisms where chitin synthesis is essential for cell wall morphogenesis, maintenance of the structural integrity of the cell wall or formation of the primary septum, the antifungal activity of the nikkomycins against whole cells depends on the following three factors: (a) efficiency of transport via the peptide transport system, (b) susceptibility to degradation, and (c) affinity for chitin synthase.

To facilitate the design of nikkomycins with altered biological properties we decided to examine the structure–activity relationships of this class of compounds. We determined the \( K_i \) values of natural and synthetic nikkomycins towards the chitin synthase of Coprinus cinereus, the stability of the nikkomycins against intracellular degradation by different yeasts and fungi, and we investigated the uptake of these antibiotics by Yarrowia lipolytica.

Methods

\textit{Culture conditions.} For peptidase studies, cultures of \textit{Saccharomyces cerevisiae} CBS 1369, \textit{Candida albicans} CBS 2718, \textit{Mucor rouxii} ATCC 29405, \textit{Aspergillus niger} ATCC 16404 and \textit{Yarrowia lipolytica} ATCC 8662 were incubated overnight at 27 °C on a rotary shaker in 500 ml Erlenmeyer flasks containing 100 ml medium 1 which contained (l⁻¹): yeast extract, 4 g; glucose, 4 g; malt extract, 10 g (pH 5.5). For transport studies \textit{Y. lipolytica} was cultivated at 27 °C to early exponential phase in 500 ml Erlenmeyer flasks containing 100 ml medium 2 which contained (l⁻¹): yeast nitrogen base without amino acids and NH₄, 1-7 g; leucine, 4-5 g; glucose, 20 g (Monetoun et al., 1986).

\textit{Fermentation and isolation of nikkomycins.} All procedures have been described in previous studies (Fiedler et al., 1988; Fiedler, 1989; Decker et al., 1989, 1990). Pure nikkomycin X could be isolated from the culture broth of \textit{Streptomyces tendar} TÜ 901/172, which is auxotrophic for uracil. This strain does not produce nikkomycin Z but...
Table 1. Structures of nikkomycins

All nikkomycins were isolated from Streptomyces tendae TÜ 901 or derived by chemical synthesis (labelled with †).

<table>
<thead>
<tr>
<th></th>
<th>R¹</th>
<th>R²</th>
<th>R³</th>
<th>R⁴</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>A</td>
<td>OH</td>
<td>CH₃</td>
<td>OH</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>Z</td>
<td>B</td>
<td>OH</td>
<td>CH₃</td>
<td>OH</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>Pseudo-Z</td>
<td>C</td>
<td>OH</td>
<td>CH₃</td>
<td>OH</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>Zᵦ</td>
<td>D</td>
<td>OH</td>
<td>CH₃</td>
<td>OH</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>Z₉</td>
<td>E</td>
<td>OH</td>
<td>CH₃</td>
<td>OH</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>I</td>
<td>A</td>
<td>Glu</td>
<td>CH₃</td>
<td>OH</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>J</td>
<td>B</td>
<td>Glu</td>
<td>CH₃</td>
<td>OH</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>Oₓ</td>
<td>A</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>(2S,4R)</td>
</tr>
<tr>
<td>Oₓ</td>
<td>A</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>(2R,4R)</td>
</tr>
<tr>
<td>O₂</td>
<td>B</td>
<td>OH</td>
<td>H</td>
<td>OH</td>
<td>(2S,4R)</td>
</tr>
<tr>
<td>Kₓ</td>
<td>A</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>(2S,4R)</td>
</tr>
<tr>
<td>K₂</td>
<td>B</td>
<td>OH</td>
<td>H</td>
<td>H</td>
<td>(2S,4R)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>R¹</th>
<th>R²</th>
<th>R⁴</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bₓ</td>
<td>A</td>
<td>CH₃</td>
<td>OH</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>B₂</td>
<td>B</td>
<td>CH₃</td>
<td>OH</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>MeOBₓ</td>
<td>A</td>
<td>CH₃</td>
<td>OCH₃</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>MeOB₂̅</td>
<td>B</td>
<td>CH₃</td>
<td>OCH₃</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>29t</td>
<td>B</td>
<td>CH₃</td>
<td>H</td>
<td>(2S,3S,4S)</td>
</tr>
<tr>
<td>30t</td>
<td>B</td>
<td>H</td>
<td>OCH₃</td>
<td>(2S,4S)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>R¹</th>
<th>R³</th>
<th>Configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wₓ</td>
<td>A</td>
<td>Tyr</td>
<td>(2S)</td>
</tr>
<tr>
<td>W₂</td>
<td>B</td>
<td>Tyr</td>
<td>(2S)</td>
</tr>
<tr>
<td>ArgC₂̅</td>
<td>B</td>
<td>Arg</td>
<td>(2S)</td>
</tr>
</tbody>
</table>
does produce nikkomycin X (150 mg l\(^{-1}\)) in a medium containing (l:\(^{-1}\)):
starch, 20 g; mannitol, 20 g; soybean meal, 20 g; yeast extract, 10 g;
uracil, 75 mg.

**Antifungal activity of the nikkomycins.** Methods for the determination of the minimal inhibitory concentration (MIC) values for nikkomycins have been described previously (Decker et al., 1989).

**Chitin synthase assay.** Chitin synthase from *Coprinus cinereus* TÜ 637 was prepared and assayed as described by Gooday & De Rousset-Hall (1975). K\(_i\) values were determined according to the method of Dixon (1953). The reaction mixture (20 µl total vol.) contained (final concentrations) 50 mM-HEPES (adjusted to pH 7.5 with NaOH), 10 mM-MgCl\(_2\), 1 mM-EDTA, 0.5 or 1.0 mM-UDP-[U-\(^{14}\)C]N-acetylgulcosamine (20000 or 40000 d.p.m., respectively), 0.8–1.0 µg protein from the chitin synthase preparation and various nikkomycin concentrations to give an inhibition of chitin synthase activity of 30–70%. The digitonin-solubilized chitin synthase preparation showed no loss of activity after 2 months at −20 °C.

**Preparation of crude cell-extracts and peptidase assay.** The cells were harvested at stationary phase by centrifugation and washed twice with 50 mM-KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) buffer (pH 5-5). The yeasts were homogenized as described by Logan (1987). Filamentous fungi were disrupted using a French pressure cell. Whole cells and, if present, glass beads were removed by centrifugation and filtration of the supernatants through a 0.22 µm sterile membrane filter. Glycerol was added to a final concentration of 20% (v/v) and the extracts were stored at −20 °C.

Peptidase activity was assayed by mixing 50 µl cell extract (1−5 mg protein ml\(^{-1}\)), 75 µ1 50 mM-KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) buffer (pH 6.5) and 25 µl nikkomycin (1 mg ml\(^{-1}\)), and incubating at 27 °C. Samples (50 µl) were removed at various times and added to 10 µ1 acetic acid. The samples were centrifuged (13000 g) and nikkomycin concentrations were analysed by HPLC (Fiedler, 1984).

**Transport studies.** *Y. lipolytica* was grown to early exponential phase (OD\(_{578}\) 0.6–0.8), harvested by centrifugation and washed twice with 50 mM-KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) buffer (pH 5-5). The cells were resuspended in buffer supplemented with 2% (w/v) glucose (pH 5.5) to give a final concentration of 5 x 10\(^5\) cells (corresponding to an OD\(_{578}\) of 3.0 and a dry wt of 1 mg ml\(^{-1}\)). The cell suspension could be stored on ice for 3 h prior to use without affecting the result. The cell suspensions (5 ml) were pre-incubated (10 min) on a gyratory shaker at 37 °C in a 25 ml Erlenmeyer flask. After addition of nikkomycin, 0.4 ml samples were taken at various intervals and immediately centrifuged to remove the cells. The supernatant (10 or 20 µl) was analysed by HPLC for the presence of nikkomycins. The uptake of nikkomycin Z was measured during the first 3–6 min of the incubation period.

In competition studies the influence of nikkomycin Z at 40 µM on the uptake of another nikkomycin, also at 40 µM, was tested.

The wet volume of cells of *Y. lipolytica* grown in liquid culture was determined by harvesting the cells in a graduated centrifuge tube.

**Analysis of nikkomycins by HPLC using UV- and fluorescence-detection.** The quantitation and characterization of nikkomycins at concentrations up to 20 µM was performed by HPLC and photodiode array detection (HP 1090M, Hewlett-Packard) as described by Fiedler (1984). To determine nikkomycin Z at concentrations up to 25 µM we used the same chromatographic HPLC system equipped with a fluorescence detector (HP 1046A, Hewlett-Packard). The hydroxypyridyl moiety of the nikkomycin Z molecule shows a characteristic absorption maximum at 285 nm and an emission maximum at 420 nm, which is useful for sensitive quantification of nikkomycins Z, X, I, J, O\(_2\) and O\(_3\) by fluorescence detection.

**Isolation of peptide-transport-deficient mutants.** Paper disks (diam. 6 mm) containing 10 µg nikkomycin Z were placed on agar plates (medium 1 containing 2% agar) seeded with *Y. lipolytica*. Spontaneous mutants of *Y. lipolytica*, which grew within the zone of inhibition surrounding the paper disc, were isolated and characterized.

**Results**

**Inhibition of chitin synthase of *Coprinus cinereus***

The strength of binding of the nikkomycins to chitin synthase is related to the K\(_i\) values, because nikkomycins with a low K\(_i\) value exhibit a higher affinity for the enzyme and vice versa (Table 2). The K\(_i\) values of the chitin synthase of *Coprinus cinereus* for the different nikkomycins were determined according to the method of Dixon (1953).

We demonstrated that a complete peptide-nucleoside structure is essential for the inhibitory activity towards chitin synthase, since the nucleoside nikkomycins C\(_Z\) and C\(_X\) exhibited only a low inhibitory activity. Nikkomycins C\(_Z\) and C\(_X\) showed 20 and 40% inhibition, respectively, at a concentration of 500 µM and a UDP-N-acetylgulcosamine concentration of 500 µM, while 5 µM-nikkomycin Z gave 50% inhibition at the same UDP-N-acetylgulcosamine concentration. This is in accordance with studies on the mode of action of the polyoxins which suggest that chitin synthase has a nucleoside binding site and an amino acid binding site (Hori et al., 1971).

As shown in Table 1 the binding affinity of nikkomycins for the chitin synthase of *Coprinus cinereus* depends on the structure of the nucleoside and the amino acid moiety of the molecule.

All nikkomycins which contain uracil as the base exhibited a lower binding affinity for chitin synthase than nikkomycins with the 4-formyl-4-imidazolin-2-one base (Table 2). Furthermore, substituents at the C-5 position of uracil in nikkomycin Z\(_H\) and Z\(_T\) have a positive effect on binding affinity to chitin synthase (Table 2). In contrast, if uracil is bound to the amino-hexuronic acid as a C-glycoside, as in the case of

**Table 2. Inhibition constant K\(_i\) of nikkomycins for the chitin synthase of *Coprinus cinereus***

<table>
<thead>
<tr>
<th>Nikkomycin</th>
<th>K(_i) (µM)</th>
<th>Nikkomycin</th>
<th>K(_i) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z</td>
<td>3.0</td>
<td>X</td>
<td>1.1</td>
</tr>
<tr>
<td>K(_Z)</td>
<td>14.0</td>
<td>K(_X)</td>
<td>5.35</td>
</tr>
<tr>
<td>O(_Z)</td>
<td>8.2</td>
<td>O(_X)</td>
<td>2.8</td>
</tr>
<tr>
<td>W(_Z)</td>
<td>11.9</td>
<td>W(_X)</td>
<td>4.5</td>
</tr>
<tr>
<td>Z(_H)</td>
<td>1.1</td>
<td>Z(_T)</td>
<td>1.1</td>
</tr>
<tr>
<td>Pseudo-Z</td>
<td>9-25</td>
<td>J</td>
<td>8.5</td>
</tr>
<tr>
<td>B(_Z)</td>
<td>2-4</td>
<td>B(_X)</td>
<td>0.61</td>
</tr>
<tr>
<td>MeOB(_Z)</td>
<td>1-25</td>
<td>MeOB(_X)</td>
<td>0.43</td>
</tr>
<tr>
<td>ArgC(_Z)</td>
<td>11-3</td>
<td>O(_Z)</td>
<td>36.0</td>
</tr>
<tr>
<td>29</td>
<td>11-0</td>
<td>30</td>
<td>2.3</td>
</tr>
</tbody>
</table>
nikkomycin pseudo-Z (Heitsch et al., 1989), the binding affinity towards chitin synthase is lowered (Table 2). The carboxyl group in position C-5' of the aminohexuronic acid also affects the activity of nikkomyacin Z, since nikkomyacin J, an aminoacyl derivative of nikkomyacin Z, exhibits a higher $K_i$ value than nikkomyacin Z (Table 2).

The structure of the N-terminal amino acid, which is linked to the nucleoside moiety by a peptide bond, is another structural element that influences the inhibition constants of the nikkomyacins. The inhibitory activity of nikkomyacins is increased by the presence of a methyl group at the $\beta$-position of the amino acid as well as by the presence of the para-hydroxy group at the pyridyl and phenyl ring (Tables 1 and 2). Furthermore, methylation of the para-hydroxy group of nikkomyacin B_x causes an increase in hydrophobicity, which leads to an enhanced binding affinity towards the chitin synthase of Coprinus cinereus. According to our data, nikkomyacin MeOB_x is the most effective chitin synthase inhibitor (Table 2).

The substitution of the nitrogen of the hydroxypyridyl ring of nikkomyacin Z by carbon in nikkomyacin B_x has only a slight positive effect on binding affinity to chitin synthase, but increases the stability of the peptide bond at neutral and alkaline pH (Rathmann, 1984).

The configuration of the $\alpha$-amino group of the N-terminal amino acid is another factor which has to be taken into account, since only the $\alpha$-L-derivatives are effective inhibitors. For example, nikkomyacin O_x, the $\alpha$-D-derivative of nikkomyacin O_X (Heitsch, 1988) is an inefficient inhibitor of chitin synthase, while nikkomyacin O_X itself is a potent inhibitor (Table 2). Furthermore, introduction of the natural amino acids L-tyrosine and L-arginine reduces the binding affinity to the chitin synthase of Coprinus cinereus. The $K_i$ values of nikkomyacin W_Z and ArgC_Z are about four times higher than for nikkomyacin Z (Table 2).

### Table 3. Enzymic degradation of nikkomyacins by cell extracts of different fungi and yeasts

<table>
<thead>
<tr>
<th>Degradation by cell extracts of fungi and yeasts</th>
<th>Yarrowia lipolytica</th>
<th>Candida albicans</th>
<th>Saccharomyces cerevisiae</th>
<th>Mucor rouxii</th>
<th>Aspergillus niger</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z</td>
<td>+ + +</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
<td>+ + +</td>
</tr>
<tr>
<td>B_x</td>
<td>+ + +</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>29</td>
<td>+ + +</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>+ + +</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>W_x</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>O_x</td>
<td>+ + +</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>K_x</td>
<td>+ + +</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>J</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+</td>
<td>+</td>
<td>+ + +</td>
</tr>
</tbody>
</table>

Cell-free crude extracts of all organisms investigated (Table 3) were able to cleave an N-terminal proteinogenic amino acid (L-tyrosine, L-arginine) from the nikkomyacin molecule. Nikkomyacins O_X, O_Z, K_X and K_Z were also degraded into the biologically inactive amino acid and nucleoside moieties by most of the cell extracts, whereas nikkomyacins Z, X, and B_x were resistant to enzymatic hydrolysis. Nikkomyacins O_X, O_Z, K_X, K_Z, and 30 were slightly more resistant to degradation than nikkomyacins W_X, W_Z, and ArgC_Z (Table 3). Nikkomyacin O_x was not susceptible to degradation at all, presumably because of the $\alpha$-D-configuration of the amino group at C-2'.

Interestingly, nikkomyacins J and I, the tripeptide analogues of nikkomyacins Z and X, were also metabolized and inactivated by yeast cell extracts by cleavage of the N-terminal amino acid from the nikkomyacin molecule (Table 3).

Control studies using heat-inactivated crude extracts showed that all nikkomyacins were stable under the experimental conditions during the incubation period. Independent from enzymic degradation, the peptide bond of nikkomyacins Z, X, and J was unstable at neutral and alkaline pH. This effect has already been observed in previous studies on the structure of the neopolyoxins, which exhibit the same N-terminal amino acid as nikkomyacins Z and X (Uramoto et al., 1980). It was suggested that the instability of the peptide bond of the neopolyoxins is caused by the position of the adjacent $\gamma$-hydroxyl group and nitrogen of the hydroxypyridine ring (Uramoto et al., 1980). Nikkomyacins W_X, W_Z, B_X and B_Z, which contain a hydroxyphenyl ring instead of a hydroxypyridyl residue, are stable at neutral and alkaline pH.
Transport studies with *Y. lipolytica*

Several methods are available for studying nikkomycin uptake using radioactively labelled substrates or fluorescamine for the continuous monitoring of substrate uptake (Yadan et al., 1984; McCarthy et al., 1985b). We determined the kinetic parameters of nikkomycin Z transport by monitoring the nikkomycins remaining in the incubation buffer by HPLC analysis using a photodiode array or a fluorescence detector.

In order to compare our results with other studies (McCarthy et al., 1985b; Moneton et al., 1986), the kinetic parameters of nikkomycin Z transport were determined from the initial rates of uptake. As reported for *S. cerevisiae* (Payne & Nisbet, 1981) we found that the transport of nikkomycin Z is also energy dependent in *Y. lipolytica* and can be inhibited by NaN₃. The rate of uptake was dependent on the initial nikkomycin Z concentration and followed Michaelis-Menten kinetics. At an initial extracellular concentration of 140 μM, uptake of nikkomycin Z resulted in a 450-fold accumulation after 2 h as approximated from the cell wet volume. Taking into account the fact that the cytoplasmic volume is smaller than the cell wet volume, the measurement is likely to be an underestimate of the intracellular concentration. Time course experiments revealed that nikkomycin Z uptake decreased after an initial linear uptake. The apparent maximal velocity ($V_{max}$) for nikkomycin Z transport evaluated from a Hanes' plot was determined to be 2-2 nm min⁻¹ (mg dry wt⁻¹) and the $K_m$ value was 24 μM.

Because mutants of *Y. lipolytica* resistant to nikkomycin Z are cross-resistant to the tripeptide nikkomycin J and the tripeptide antibiotic phosphinothricyl-alanylanaline (PTT), we conclude that *Y. lipolytica* transports di- and tripeptide nikkomycins as well as PTT via a single peptide transport system.

The transport rates of different nikkomycins were determined at an initial concentration of 40 μM (Table 4).

Competition experiments were performed to investigate the influence of different nikkomycins on the transport of nikkomycin Z and *vice versa*. This was done to evaluate differences in the affinity of the nikkomycins for the peptide transport system. The initial concentration for each nikkomycin was 40 μM and uptake was measured by HPLC analysis (see Table 4).

All nikkomycins investigated except the nucleoside C₂ and nikkomycin O₁, containing an N-terminal α-D-amino acid, were transported via the peptide transport system of *Y. lipolytica*. The affinity of the peptide transport system was significantly lower (18-fold) for the tripeptide nikkomycin J than for the dipeptide nikkomycin Z (Table 4). In contrast, the dipeptide nikkomycins Kₓ, Oₓ and Wₓ exhibited a transport rate only about twofold lower than nikkomycin Z (Table 4). While the substitution of a nitrogen atom in the hydroxypyridyl ring of nikkomycin X by a carbon atom, as in nikkomycin Bₓ, did not affect the transport, the methylation of the para-hydroxy group in the hydroxyphenyl residue of nikkomycin Bₓ decreased the rate of transport about twofold (Table 4).

The rates of transport of the nikkomycins were, in general, only slightly affected by the chemical structure of the base. However, alteration of the N-glycosidic bond in nikkomycin Z to a C-glycosidic bond in nikkomycin pseudo-Z lowered the rate of transport (Table 4).

### Table 4. Transport of nikkomycins and competition between nikkomycin Z and other nikkomycins for the peptide transport system of *Y. lipolytica*

<table>
<thead>
<tr>
<th>Nikkomycin</th>
<th>Rate of uptake* [nmol min⁻¹ (mg dry wt⁻¹)]</th>
<th>Rate of transport†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z</td>
<td>1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>X</td>
<td>1.84</td>
<td>0.87</td>
</tr>
<tr>
<td>Wₓ</td>
<td>1.3</td>
<td>2.21</td>
</tr>
<tr>
<td>Kₓ</td>
<td>1.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Oₓ</td>
<td>1.4</td>
<td>2.4</td>
</tr>
<tr>
<td>30</td>
<td>1.14</td>
<td>3.8</td>
</tr>
<tr>
<td>Bₓ</td>
<td>1.69</td>
<td>1.09</td>
</tr>
<tr>
<td>MeOBₓ</td>
<td>0.84</td>
<td>2.9</td>
</tr>
<tr>
<td>Pseudo-Z</td>
<td>0.53</td>
<td>4.4</td>
</tr>
<tr>
<td>J</td>
<td>0.22</td>
<td>18.0</td>
</tr>
<tr>
<td>C₂</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>O₁</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

* Rate of uptake of nikkomycins.
† Ratio of the rate of transport of nikkomycin Z to the investigated nikkomycin.
Table 5. Antifungal activity of various nikkomycins compared with nikkomycin Z in the agar disk diffusion assay

<table>
<thead>
<tr>
<th>Nikkomycin</th>
<th>Yarrowia lipolytica</th>
<th>Candida albicans</th>
<th>Paeoctomyces variotii</th>
<th>Mucor hiemalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>X</td>
<td>147</td>
<td>243</td>
<td>239</td>
<td>129</td>
</tr>
<tr>
<td>Bx</td>
<td>152</td>
<td>380</td>
<td>183</td>
<td>92</td>
</tr>
<tr>
<td>MeOBx</td>
<td>8.7</td>
<td>177</td>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>Pseudo-Z</td>
<td>7.5</td>
<td>0</td>
<td>10</td>
<td>46</td>
</tr>
<tr>
<td>O5</td>
<td>7.2</td>
<td>20</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>O6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O7</td>
<td>1.8</td>
<td>0</td>
<td>1.4</td>
<td>15</td>
</tr>
<tr>
<td>Kx</td>
<td>5.4</td>
<td>18</td>
<td>3</td>
<td>69</td>
</tr>
<tr>
<td>Kz</td>
<td>2.1</td>
<td>0</td>
<td>1.4</td>
<td>31</td>
</tr>
<tr>
<td>W1</td>
<td>0.05</td>
<td>0</td>
<td>0.16</td>
<td>0.02</td>
</tr>
<tr>
<td>W2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Z0</td>
<td>117</td>
<td>36</td>
<td>63</td>
<td>77</td>
</tr>
<tr>
<td>Z5</td>
<td>123</td>
<td>41</td>
<td>91</td>
<td>78</td>
</tr>
<tr>
<td>29</td>
<td>2.2</td>
<td>9</td>
<td>0.08</td>
<td>0.24</td>
</tr>
<tr>
<td>30</td>
<td>0.04</td>
<td>0</td>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>1</td>
<td>3.4</td>
<td>9.5</td>
<td>7.2</td>
<td>6.9</td>
</tr>
<tr>
<td>J</td>
<td>0.1</td>
<td>0</td>
<td>3.8</td>
<td>0</td>
</tr>
<tr>
<td>ArgC5</td>
<td>0.080</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Antifungal activity of the nikkomycins

The effect of nikkomycins on the growth of various micro-organisms was examined by the disk diffusion assay. We determined the MIC for nikkomycin Z (Table 5) and compared the activity of the other nikkomycins in relation to nikkomycin Z. The MIC of nikkomycin Z varied for different micro-organisms over a wide range (Table 5). However, different nikkomycins showed a similar pattern of inhibitory activity against the test organisms (Table 5). S. cerevisiae showed no susceptibility to nikkomycin Z under the assay conditions used here.

Nikkomycins with a proteinogenic N-terminal amino acid and nikkomycins without a β-methyl group in the N-terminal amino acid side chain were degraded by peptidases and, consequently, had no or only a low level of inhibitory activity, despite a high affinity for chitin synthase. Nikkomycin O6, a poor inhibitor of chitin synthase with weak or no affinity for the peptide transport system, is biologically inactive.

The low toxicities of the tripeptide nikkomycins I and J can be attributed to their inefficient uptake by the peptide transport system, their degradation by peptidases and their low affinity for chitin synthase in comparison to the dipeptide nikkomycins Z and X.

Nikkomycin MeOBX, the most potent inhibitor of the chitin synthase of Coprinus cinereus, exhibits a lower antifungal activity in the agar disk diffusion assay than nikkomycin Bx.

Discussion

The comparison of different nikkomycins provides new insights into the structure–activity relationships of these antibiotics. Previous studies on synthetic polyoxins have shown that many structural variations of the N-terminal amino acid and base residues are tolerated without loss of activity towards chitin synthase (Hori et al., 1974; Shenbergamurti et al., 1983, 1986; Smith et al., 1986; Khare et al., 1988). However, in many cases uptake of the compounds was shown to be inefficient or intracellular degradation occurred, so that no inhibitory activity to whole cells was exhibited. In some reports, transport and degradation of the chemically synthesized substances were not assayed (Hori et al., 1974; Azuma et al., 1977), but it is very likely that compounds containing a proteinogenic amino acid will be degraded by peptidases, and compounds with an N-terminal α-D-amino acid will not be internalized by the peptide transport system.

The essential structural features of a biologically active chitin synthase inhibitor are the same for the nikkomycins and polyoxins and can be summarized as follows: (1) complete nucleoside peptide structure, (2) N-terminal α-L-amino acid with a free α-amino group, (3) free carboxyl group in position C-5', and (4) polar and hydrophobic substituents at the N-terminal amino acid.

Specific characteristics relating to the antifungal activity of the nikkomycins were determined. The resistance of dipeptide nikkomycins, but not of tripeptide nikkomycins, towards degradation by peptidases is caused by the β-methyl substituent of the N-terminal amino acid side chain. Yeasts possess many peptidases in various cellular locations with different substrate specificity (Rendueles & Wolf, 1988), which probably accept dipeptide nikkomycins without a β-methyl group, and tripeptide nikkomycins as substrates, but not nikkomycin Z or X. It must be considered that the peptidase activity in the crude cell-extracts probably does not account for every cellular peptidase because not all will be active under the assay conditions used. Furthermore, we cannot distinguish whether degradation of the nikkomycins was catalysed by cytoplasmic, vacuolar, periplasmic or membrane-bound peptidases.

Non-enzymic degradation of nikkomycin Z or X at neutral and alkaline pH can be prevented by replacing the nitrogen atom of the hydroxypyridyl ring by a carbon atom. For example, nikkomycins Bx, Bz, Wx and Wz,
which have a hydroxyphenyl ring, are much more stable
at neutral and alkaline pH than nikkomycins Z and X.

In previous studies it was demonstrated that nikkomycins
enter the cells of Candida albicans and S. cerevisiae
via peptide transport systems (McCarthy et al., 1985a, b; Moneton et al., 1986). We investigated nikkomycin
uptake by the yeast Y. lipolytica, because this organism,
in contrast to C. albicans and S. cerevisiae, is very sensi
tive to most of the nikkomycins. In Y. lipolytica, as in S.
cerevisiae, chitin is mainly located in the bud scars,
although the chitin content of Y. lipolytica is six-fold
higher than in S. cerevisiae (Vega & Dominguez, 1986).
However, Bartnicki-Garcia & Lippman (1972) reported
that there is no correlation between chitin content of
the cell wall and susceptibility to polyoxin in closely related
Moneton spp.

Y. lipolytica transported di- and tripeptide nikkomycins
via a single transport system as previously shown for S.
cerevisiae (Moneton et al., 1986). All nikkomycins with
a complete nucleoside peptide structure and an N-
terminal L-amino acid were transported by Y. lipolytica.
Except for the tripeptide nikkomycin J, the rate of
translocation of the various nikkomycins was compar-
able. The competition studies provided additional
information about the affinity of the nikkomycins for the
peptide transport system of Y. lipolytica in relation to
nikkomycin Z.

The $K_m$ value for nikkomycin Z transport was 24 $\mu$m,
similar to C. albicans (5-7 $\mu$m; McCarthy et al., 1985a)
and S. cerevisiae (20 $\mu$m; Moneton et al., 1986). In
contrast, the apparent maximal velocity was 35- and 9-
fold higher in Y. lipolytica than for C. albicans and S.
cerevisiae, respectively, and in the same range as the rate
of uptake of peptides in S. cerevisiae [1-5 nmol min$^{-1}$
(mg dry wt)$^{-1}$; Nisbet & Payne, 1979]. The comparative
sensitivity of Y. lipolytica to nikkomycin Z could be
attributed to the elevated rate of uptake of nikkomycin Z
in this species leading to a higher intracellular nikkomy-
cin concentration sufficient to inhibit chitin synthase
activity past the point required for viability. This
inhibitory concentration may be reached less rapidly and
only at higher nikkomycin concentrations in C. albicans
and not at all in S. cerevisiae. However, an alternative
explanation involving differential sensitivity of chitin
synthases in Y. lipolytica, C. albicans and S. cerevisiae
cannot be ruled out.

Another factor that influences in vitro activity of the
nikkomycins is the hydrophobicity of the aromatic ring
of the N-terminal amino acid of nikkomycin B$_X$.
Methylation of the para-hydroxy group of nikkomycin
B$_X$ increases the in vitro activity of this compound but
drastically decreases in vivo antifungal activity (Hahn et
al., 1987). This is supported by Khare et al. (1988), who
reported that hydrophobic synthetic chitin synthase
inhibitors were effective in vitro but exhibited only a low
activity towards whole cells. They concluded that the
hydrophobic chitin synthase inhibitors had a high
affinity for the peptide transport system, but were not
translocated into the cells. In accordance with this
hypothesis we have observed the uptake of the hydro-
phobic nikkomycin MeOB$_X$, by Y. lipolytica to be reduced
by a factor of two- to threefold, and the antifungal
activity by a factor of ten, in comparison to nikkomycin
Z.

The $K_i$ constants of nikkomycin Z for chitin synthases
from a wide taxonomic range of fungi are remarkably
similar ($K_i$ constants for chitin synthase for nikkomycin
Z: Neurospora crassa, 2 $\mu$m; Gow & Selitrennikoff, 1984;
Mucor rouxii, 3-5 $\mu$m, Müller et al., 1981; Coprinus
cinereus, 3 $\mu$m). Gooday & Trinci (1980) suggested a high
degree of genetic conservation of chitin synthase during
evolution, based on the similar properties of chitin
synthases from various organisms. We used chitin
synthase prepared from Coprinus cinereus (Gooday & De
Rouset-Hall, 1975) as a test model, assuming that chitin
synthases from yeast and filamentous fungi would share
similar properties. We expected that we could then
extrapolate our results obtained for chitin synthase of
Coprinus cinereus to other systems. The similar pattern of
inhibitory activity of the nikkomycins towards whole
cells of different organisms supported this hypothesis.

However, studies on chitin synthesis in S. cerevisiae
(Bulawa et al., 1986; Sburlati & Cabib, 1986; Orlean,
1987) demonstrated that S. cerevisiae has at least two
different chitin synthases (Chs1 and Chs2) which exhibit
different enzymic properties (e.g. Chs2 is less sensitive
to polyoxin D than Chs1). It was shown that disruption of
the CHS2 gene, but not the CHS1 gene, was lethal
(Silverman et al., 1988). Although Chs1 represents the
major chitin synthase activity of S. cerevisiae, it is only an
auxiliary or emergency enzyme (Cabib et al., 1989). The
low antifungal activity of the polyoxins, and also of the
structurally related nikkomycins, towards whole cells of
S. cerevisiae can probably be attributed to the fact that
the various chitin synthases have different properties
and that the inhibitory activity of the nikkomycins and
polyoxins towards these enzymes is not sufficient to
obtain anti-fungal activity. A better understanding of
chitin synthesis may therefore be required in order to
successfully design new chitin synthase inhibitors.

Nevertheless, it may be supposed that many different
factors influence the antifungal activity of the polyoxins
and nikkomycins towards whole cells.

We suggest that the low activity of the nikkomycins
towards many organisms can be explained by one or
more of the following reasons: (1) different chitin
synthases with differing properties; (2) differences in the
importance of chitin synthesis for cell viability of the
organisms; (3) inactivation of nikkomycins by peptidases; and (4) low uptake rate and low affinity for the peptide transport system resulting in insufficient intracellular accumulation of the nikkomycins, such that chitin synthesis is not completely inhibited.

According to our results, nikkomycins can be designed and synthesized to create efficient chitin synthase inhibitors that are resistant to degradation by cellular peptidases and are good substrates for peptide transport systems.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 323). The authors thank K. J. Linton and J. Anderson for critical reading of the manuscript.

This paper is no. 259 in the series Metabolic products of microorganisms.

References


