Structure–activity relationships of the nikkomycins

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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 [$K_m = 24 \mu\text{M}$, $V_{\max} = 2.2 \text{ nmol min}^{-1} (\text{mg dry wt})^{-1}$]. We demonstrated that the β -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; Zoebelein & 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 *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

Culture conditions. For peptidase studies, cultures of *Saccharomyces cerevisiae* CBS 1369, *Candida albicans* CBS 2718, *Mucor rouxii* ATCC 24905, *Aspergillus niger* ATCC 16404 and *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 *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 (Moneton *et al.*, 1986).

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 *Streptomyces tendae* Tü 901/172, which is auxotrophic for uracil. This strain does not produce nikkomycin Z but

Abbreviation: PTT, phosphinothricyl-alanyl-alanine.

Table 1. Structures of nikkomycins

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

	R ¹	R ²	R ³	R ⁴	Configuration
X	A	OH	CH ₃	OH	(2S,3S,4S)
Z	B	OH	CH ₃	OH	(2S,3S,4S)
Pseudo-Z	C	OH	CH ₃	OH	(2S,3S,4S)
Z _T	D	OH	CH ₃	OH	(2S,3S,4S)
Z _H	E	OH	CH ₃	OH	(2S,3S,4S)
I	A	Glu	CH ₃	OH	(2S,3S,4S)
J	B	Glu	CH ₃	OH	(2S,3S,4S)
O _x	A	OH	H	OH	(2S,4R)
O _x †	A	OH	H	OH	(2R,4R)
O _z	B	OH	H	OH	(2S,4R)
K _x	A	OH	H	H	(2S,4R)
K _z	B	OH	H	H	(2S,4R)

	R ¹	R ³	R ⁴	Configuration
B _x	A	CH ₃	OH	(2S,3S,4S)
B _z	B	CH ₃	OH	(2S,3S,4S)
MeOB ₁ †	A	CH ₃	OCH ₃	(2S,3S,4S)
MeOB ₂ †	B	CH ₃	OCH ₃	(2S,3S,4S)
29†	B	CH ₃	H	(2S,3S,4S)
30†	B	H	OCH ₃	(2S,4S)

	R ¹	R ⁵	Configuration
W _x	A	Tyr	(2S)
W _z	B	Tyr	(2S)
ArgC _z †	B	Arg	(2S)

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* T $\ddot{U}$ 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-acetylglucosamine (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 μl 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 μl 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 5×10^7 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 quantification 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 2.5 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 $_x$ and O $_z$ 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-acetylglucosamine concentration of 500 μM , while 5 μM -nikkomycin Z gave 50% inhibition at the same UDP-N-acetylglucosamine 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*

Nikkomycin	K_i (μM)	Nikkomycin	K_i (μM)
Z	3.0	X	1.1
K $_z$	14.0	K $_x$	5.35
O $_z$	8.2	O $_x$	2.8
W $_z$	11.9	W $_x$	4.5
Z $_H$	1.1	Z $_T$	1.1
Pseudo-Z	9.25	J	8.5
B $_z$	2.4	B $_x$	0.81
MeOB $_z$	1.25	MeOB $_x$	0.43
ArgC $_z$	11.3	O $_x^*$	36.0
29	11.0	30	2.3

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

+++ , 100% degradation within 1 h; ++ , at least 50% degradation within 6 h; + , at least 25% degradation within 6 h; - , no detectable or less than 10% degradation within 6 h; ND, not determined.

Nikkomycin	Degradation by cellular peptidases				
	<i>Yarrowia lipolytica</i>	<i>Candida albicans</i>	<i>Saccharomyces cerevisiae</i>	<i>Mucor rouxii</i>	<i>Aspergillus niger</i>
Z	-	-	-	-	-
B _x	-	ND	ND	ND	ND
29	-	ND	ND	ND	ND
30	++	ND	ND	ND	ND
W _x	+++	+++	+++	+++	+++
O _x	++	++	+	-	+
K _x	++	++	++	-	-
J	++	++	++	-	-

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 nikkomycin Z, since nikkomycin J, an aminoacyl derivative of nikkomycin Z, exhibits a higher K_i value than nikkomycin 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 nikkomycins. The inhibitory activity of nikkomycins is increased by the presence of a methyl group at the β -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 nikkomycin 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, nikkomycin MeOB_x is the most effective chitin synthase inhibitor (Table 2).

The substitution of the nitrogen of the hydroxypyridyl ring of nikkomycin Z by carbon in nikkomycin 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 α -amino group of the N-terminal amino acid is another factor which has to be taken into account, since only the α -L-derivatives are effective inhibitors. For example, nikkomycin O_x^{*}, the α -D-derivative of nikkomycin O_x (Heitsch, 1988) is an inefficient inhibitor of chitin synthase, while nikkomycin 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 nikkomycin W_z and ArgC_z are about four times higher than for nikkomycin Z (Table 2).

Degradation of the nikkomycins by cell extracts

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 nikkomycin molecule. Nikkomycins 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 nikkomycin Z, X and B_x were resistant to enzymic hydrolysis. Nikkomycins O_x, O_z, K_x, K_z and 30 were slightly more resistant to degradation than nikkomycins W_x, W_z and ArgC_z (Table 3). Nikkomycin O_x^{*} was not susceptible to degradation at all, presumably because of the α -D-configuration of the amino group at C-2'.

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

Control studies using heat-inactivated crude extracts showed that all nikkomycins were stable under the experimental conditions during the incubation period.

Independent from enzymic degradation, the peptide bond of nikkomycins 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 nikkomycins Z and X (Uramato *et al.*, 1980). It was suggested that the instability of the peptide bond of the neopolyoxins is caused by the position of the adjacent γ -hydroxyl group and nitrogen of the hydroxypyridine ring (Uramato *et al.*, 1980). Nikkomycins 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.*, 1985*b*). 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.*, 1985*b*; 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 NaN_3 . The rate of uptake was dependent on the initial nikkomycin Z concentration and followed Michaelis-Menten kinetics. At an initial extracellular concentration of $140\text{ }\mu\text{M}$, 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 $2.2\text{ nm min}^{-1}\text{ (mg dry wt)}^{-1}$ and the K_m value was $24\text{ }\mu\text{M}$.

Because mutants of *Y. lipolytica* resistant to nikkomycin Z are cross-resistant to the tripeptide nikkomycin J

and the tripeptide antibiotic phosphinothricyl-alanyl-alanine (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 $40\text{ }\mu\text{M}$ (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 $40\text{ }\mu\text{M}$ and uptake was measured by HPLC analysis (see Table 4).

All nikkomycins investigated except the nucleoside C_Z and nikkomycin O_X^* , 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 K_X , O_X and W_X 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 B_X , did not affect the transport, the methylation of the *para*-hydroxy group in the hydroxyphenyl residue of nikkomycin B_X 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*

Initial nikkomycin concentration in each case was $40\text{ }\mu\text{M}$.

Nikkomycin	Rate of uptake*	
	[nmol min ⁻¹ (mg dry wt) ⁻¹]	
		Rate of transport†
Z	1.7	1.0
X	1.84	0.87
W_Z	1.3	2.21
K_Z	1.9	1.6
O_Z	1.4	2.4
30	1.14	3.8
B_X	1.69	1.09
MeOB_X	0.84	2.9
Pseudo-Z	0.53	4.4
J	0.22	18.0
C_Z	0	—
O_X^*	0	—

* 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

MIC values for nikkomycin Z: *Yarrowia lipolytica* ATCC 8662, 0.15 µg ml⁻¹; *Candida albicans* CBS 2718, 14.0 µg ml⁻¹; *Paecilomyces variotii* TÛ 136, 0.27 µg ml⁻¹; *Mucor hiemalis* TÛ 179, 0.00027 µg ml⁻¹.

Nikkomycin	Relative biological activity (%)			
	<i>Yarrowia lipolytica</i>	<i>Candida albicans</i>	<i>Paecilomyces variotii</i>	<i>Mucor hiemalis</i>
Z	100	100	100	100
X	147	243	239	129
B _X	152	380	183	92
MeOB _X	8.7	177	0	0.03
Pseudo-Z	7.5	0	10	46
O _X	7.2	20	5	22
O _X [*]	0	0	0	0
O _Z	1.8	0	1.4	15
K _X	5.4	18	3	6.9
K _Z	2.1	0	1.4	3.1
W _X	0.05	0	0.16	0.02
W _Z	0	0	0	0
Z _H	117	36	63	77
Z _T	123	41	91	78
29	2.2	9	0.08	0.24
30	0.04	0	0	0.01
I	3.4	9.5	7.2	6.9
J	0.1	0	0	3.8
ArgC _Z	0.080	0	0	0

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 O_X^{*}, 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 MeOB_X, the most potent inhibitor of the chitin synthase of *Coprinus cinereus*, exhibits a lower antifungal activity in the agar disk diffusion assay than nikkomycin B_X.

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; Shenbagamurthi *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 B_X, B_Z, W_X and W_Z,

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 sensitive 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 *Mucor* 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 comparable. 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 μM , similar to *C. albicans* (5.7 μM ; McCarthy *et al.*, 1985a) and *S. cerevisiae* (20 μM ; 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 nikkomycin 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 hydrophobic 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 μM , Gow & Selitrennikoff, 1984; *Mucor rouxii*, 3.5 μM , Müller *et al.*, 1981; *Coprinus cinereus*, 3 μM). 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 Rousset-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.

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