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**Biological activity and conformational analysis of nikkomycins
and their analogs**

Krainer, Eduardo, Ph.D.

City University of New York, 1990

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A

BIOLOGICAL ACTIVITY AND CONFORMATIONAL ANALYSIS

OF NIKKOMYCINS AND THEIR ANALOGS.

by

EDUARDO KRAINER

A dissertation submitted to the Graduate Faculty in
Chemistry in partial fulfillment of the requirements for
the degree of Doctor of Philosophy, The City University
of New York.

1990

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This manuscript has been read and accepted for the Graduate Faculty in Chemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

BIOLOGICAL ACTIVITY AND CONFORMATIONAL ANALYSIS OF
NIKKOMYCINS AND THEIR ANALOGS.

by

Eduardo Krainer

Adviser: Professor Fred Naider

Nikkomycins and polyoxins are peptidyl nucleoside antibiotics produced by *Streptomyces* species. They are potent inhibitors of chitin synthetases from pathogenic fungi, and polyoxins have been used in Japan for many years as agricultural fungicides and for insect control. Since chitin synthetase is not found in mammals and both families of antibiotics are not toxic, these inhibitors are potentially attractive for clinical use. Unfortunately these compounds seem to have only a fungistatic action, and they are not used clinically at present.

A procedure for efficiently obtaining nikkomycins X and Z of very high purity from crude antibiotic is described. These nikkomycins are very intractable to separation by conventional chromatography. Our protocol relies on the use of reversed-phase HPLC with a reactive mobile phase (aqueous sodium bisulfite). A polar bisulfite addition product is formed with nikkomycin X, but not

nikkomycin Z, within the column bed. This drastically changes the mobility of the former antibiotic and leads to the full resolution of both compounds. The bisulfite addition compound can be easily reverted to nikkomycin X. Recovery yields are in the order of 65-80 %.

Five tripeptide analogs (1-5, Table 7) and four dipeptide analogs (9-12, Table 8) of polyoxins and nikkomycins with expected enhanced toxicity toward *C. albicans* were synthesized, and their biological properties tested against various strains of this yeast. The dipeptides were designed as double-targeted drugs, able to inhibit chitin synthetase and/or release a toxic amino acid upon hydrolysis. One of the tripeptides, L-leucyl-nikkomycin X (3), behaved as a prodrug, releasing a toxic peptide upon intracellular hydrolysis. None of the synthetic antibiotics proved more toxic than nikkomycin.

The conformation of nikkomycin X in aqueous solution was examined by using nuclear Overhauser effects as well as by pH titration studies in which ^1H -nmr shifts were monitored. The antibiotic exhibits a very flexible structure, with coexisting *syn* and *anti* conformations of the nucleoside moiety. Conformational shifts of the pentose ring, and also along the C4'-C5'bond, occurred during the titration. The studies in aqueous solution were undertaken

as a preamble to studies of the antibiotic bound to chitin synthetase and perhaps to peptide permeases of *C. albicans*. NOe measurements were carried out on nikkomycin X in the presence of fungal membranes containing active enzymes, in an effort to obtain preliminary conformational information for the bound molecule.

Acknowledgments

I wish to thank first Professor Fred Naider for all the encouragement and guidance, for his patience, for ideas and suggestions, for spirited discussions, for help in writing papers, for creating an intellectually stimulating atmosphere in the lab, and for many demonstrations of fairness and intellectual honesty.

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I am grateful to Professor Jeffrey M. Becker for discussions, advice, and fruitful exchange of ideas. The biological assays were carried out in his lab by David Miller, who also prepared and assayed the membranes for the trnOe experiments.

I have enjoyed interacting personally and/or scientifically with members of this research group, as well as members of Professor Broido's research group, including Michael Tallon, Ariel Ewenson, Jaya Shrinivasan, Jose

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Thesis plan.

Chapter I: The introduction starts with a review of the current knowledge of the chemical structure of the cell-wall of *Candida albicans*. Chitin synthesis is discussed as a target for antifungal drugs. The known natural inhibitors of chitin synthetase, polyoxins and nikkomycins, are presented and their mechanism of action, as well as their limitations, are discussed. The current knowledge of the peptide transport system of *C. albicans* is discussed in some detail, since it is utilized by the chitin synthetase inhibitors for permeation into the cell. Finally, data from the literature as well as our own observations on the stability of the nikkomycins under hydrolytic conditions is examined.

Chapter II: The introduction to this chapter is a brief discussion on the methods in the literature for the purification and separation of the nikkomycins. It is shown how a mixture of nikkomycins X + Z was isolated from crude antibiotic by reversed-phase HPLC. Attempts at resolving this mixture are discussed, and the concepts behind chemochromatography are presented.

'Chemochromatography: its use for the separation of nikkomycins X and Z' describes the development of this novel mode of reversed-phase HPLC and its success in the

analytical and preparative separation of nikkomycins X and Z. This work was published in *Analytical Biochemistry*, 1987, with R. K. Khare, Fred Naider, and Jeffrey M. Becker.

Chapter III: The introduction to this chapter is a review of the polyoxin analogs that have been reported in the literature. This review is separated into two sections, dipeptide analogs and tripeptide analogs, in order to make the presentation more clear. The effects of different chemical modifications on the toxicity of the analogs against *C. albicans* are discussed. When there is no data available, the toxicity against phytopathogenic fungi is presented instead. 'Synthesis and biological evaluation of dipeptidyl and tripeptidyl polyoxin and nikkomycin analogs as anticandidal prodrugs' was submitted in a modified version to *Journal of Medicinal Chemistry* in 1990, with Fred Naider and Jeffrey M. Becker, and was accepted for publication. This work tries to extend previous concepts and introduces novel approaches to the problem of anticandidal drug design. Finally, the appendix presents data showing nikkomycin to be resistant to hydrolysis by peptidases from *C. albicans*.

Chapter IV: In the first section, the problems and advantages of different approaches for obtaining ^1H -nmr spectra of nikkomycin X in aqueous solution are discussed,

and the results of preliminary experiments are shown. The second section, 'Conformational studies of nikkomycin X in aqueous solution' is a work published in *Biopolymers* in April 1990 with Fred Naider and Jeffrey M. Becker. In it, the conformational behavior of nikkomycin X in solution is explored. Appendix B expands the conformational studies of the ribose ring from the previous section, presenting data used in the calculations, as well as drawings of the conformers. The next section introduces the concept of transferred nuclear Overhauser effect; finally, in the last section, preliminary results of nmr studies on nikkomycin in the presence of active enzymes from *C. albicans* membranes are presented.

Note: The references are grouped at the end of the last section, last Chapter. Sections that have also been submitted or published elsewhere have an independent system of references.

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List of Abbreviations

Ala	alanine
Arg	arginine
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
C ₁₈	silica gel with a 18 carbons hydrocarbon chain chemically attached
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DPPC	dipalmitoylphosphatidylcholine
DSS	sodium 3-(trimethylsilyl)-1-propane- sulfonate
DTE	dithioerythritol
[E]	concentration of enzyme or protein
EDTA	sodium ethylenediaminetetraacetate
FAB	fast atom bombardment
FID	free induction decay
FMDP	N ³ -(4-methoxyfumaroyl)-L-2,3- diaminopropanoic acid
FT	Fourier transformation
GlcNAc	N-acetyl glucosamine
Glu	glutamic acid
Gly	glycine
HOBT	hydroxybenzotriazole
HPLC	high-performance liquid chromatography

Hse	homoserine
ID ₅₀	concentration at which chitin synthetase is inhibited by 50 %.
J _{i,j}	coupling constant between protons i and j, in Hz
K _i	inhibition constant
K _m	Michaelis-Menten constant
k ₁	rate constant for ligand + protein forward reaction
k ₋₁	rate constant for ligand + protein backward reaction
Met	methionine
MIC	minimum inhibitory concentration
nik	nikkomycin
Nle	norleucine
NMM	N-methylmorpholine
nOe	nuclear Overhauser effect
(oct)Lys-	N-ε-(octanoyl)-lysyl
Oly	L-4-oxalysine
P	phase angle of pseudorotation
pD*	pH-meter reading in D ₂ O solutions, uncorrected for the isotope effect on the glass electrode.
Phe	phenylalanine
RD	relaxation delay
Ref.	reference

RP-8	silica gel with a 8 carbons hydrocarbon chain chemically attached
RT	retention time
STI	soy trypsin inhibitor
t	trans rotamer of the ribose exocyclic C4'-C5' bond. The dihedral angle defined by C3'-C4'-C5'-N5' is 180°
T _c	lipid phase transition temperature
TFA	trifluoroacetic acid
TMS	tetramethylsilane
trnOe	transferred nuclear Overhauser effect
UDP	uridine diphosphate
UDP-GlcNAc	uridine diphosphate N-acetylglucosamine
V _m	maximum initial rate in the Michaelis-Menten model. This rate is attained when the enzyme is saturated.
W(i)	chemical shift of proton i
W ₀	rate constant for zero quantum transition
W ₁ ⁱ	rate constant for single quantum transition of nucleus i
W ₂	rate constant for double quantum transition
X _N	mole fraction of type N ribose conformer
X _S	mole fraction of type S conformer

Δ^Z -Phe	2,3-dehydrophenylalanine
δ	chemical shift, in parts per million
$\eta_i(s)$	nOe at nucleus i when nucleus s is saturated
λ	wavelength
ρ_i	spin-lattice relaxation of nucleus i
ρ_{iF}	spin-lattice relaxation of nucleus i in free ligand
σ_{is}	net cross relaxation of nuclei i and s
τ_i	endocyclic torsion angle about the bond $C(i')-C((i+1)')$ ($i=1$ to 3) of the ribose ring
τ_m	puckering amplitude or maximum angle of torsion
τ_N	puckering amplitude of type N conformer
τ_S	puckering amplitude of type S conformer

Chapter I. Introduction and background.

Chitin synthesis and peptide transport in *Candida albicans*.

Candida albicans is an imperfect dimorphic yeast usually associated with warm-blooded animals as a harmless and asymptomatic commensal. In immunologically weakened hosts, however, *C. albicans* can manifest itself as a virulent pathogen. Despite its being the most common and most serious fungal pathogen of man, many important aspects of its biology are still not understood (1).

The cell-wall of *C. albicans*, as that of *Saccharomyces cerevisiae*, is composed mainly of β -glucans, mannoproteins, chitin and lipids (2,3). The main structural component of the *C. albicans* wall is β -glucan (48-60%) containing 1 $\rightarrow$ 3 and 1 $\rightarrow$ 6 linkages (3,4). Mannoprotein (20-23%) (3) is distributed throughout the cell-wall but also accumulates on the surface and next to the plasma membrane (1). β -(1 $\rightarrow$ 6) Glucan may be cross-linked to mannoprotein (5). Chitin (1-3 %) (3) is located mainly in the bud scar in *C. albicans* yeast cells, but smaller amounts are distributed throughout the inner wall (6,7). Chitin, a linear polysaccharide composed of β -(1 $\rightarrow$ 4)-linked N-acetylglucosamine units, is an essential component for maintenance of the structural stability of the cell-wall

and for the formation of primary septa in budding cells (8). The N-H and C=O groups in adjacent chains of chitin associate by hydrogen bonding. The very strong association between chains accounts for the formation of fibrils, as well as for the insolubility of chitin in water (9, 10). Covalent linkages between chitin and glucans have only been investigated recently (1, 11), and there is evidence for a glycosidic bond at position 6 of N-acetylglucosamine and position 1 of the glucose in the β -(1 $\rightarrow$ 6)-glucan.

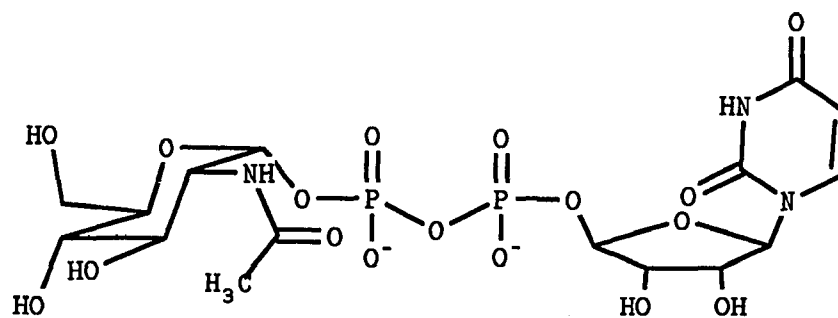
Chitin synthetase, the enzyme responsible for the last step in chitin biosynthesis, is located predominantly in the plasma membrane (12). Its substrate, UDP-GlcNAc, approaches the membrane from the cytoplasmic face and transfers the GlcNAc through the synthetase to a chain of chitin which, as it grows, is simultaneously extruded (9). No energy source other than that provided by the free energy of hydrolysis of the bond between the amino sugar and UDP appears to be necessary (9). Chitin synthetase is present in the cell as a zymogen and can be activated *in vitro* by proteolysis. Chitin synthetase from *S. cerevisiae* has been isolated in its active form by co-precipitation with its product, chitin (13). Interestingly, the chain length of chitins synthesized *in vivo*, with a membrane preparation, or with the purified enzyme, is the same in all cases (somewhat more than 100 GlcNAc units). This

suggests that chitin synthetase itself is capable of determining the length of the chitin chains (13).

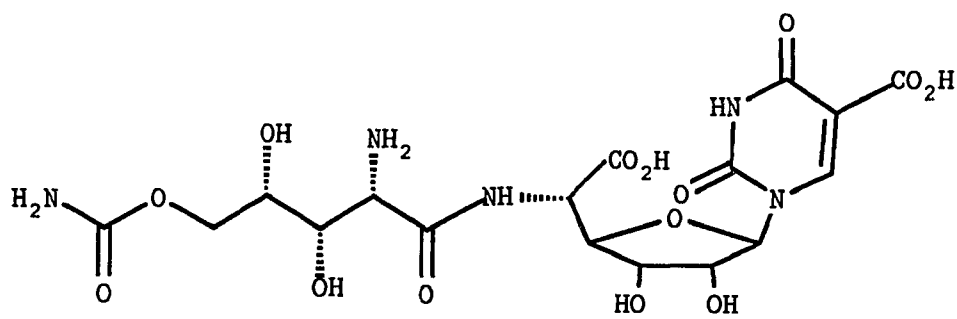
Chitinase is a soluble enzyme that shows maximum activity during *C. albicans* yeast growth (14). It is probably required for localized hydrolysis of wall chitin and for the softening of walls which appears to be necessary for growth (9).

It has been found recently (15, 16, 17) that there are two chitin synthetase activities in *S. cerevisiae*. Chitin synthetase I, which is not essential for cell growth and seems to be just a repair or auxiliary enzyme, is the main *in vitro* measurable activity. Chitin synthetase II, on the other hand, is essential for synthesis of chitin and septum formation. Very recently (18), evidence was also presented for the existence of at least two chitin synthetases in *C. albicans*. A gene coding for one of the chitin synthetases in *C. albicans* (chitin synthetase I, a protein of 776 amino acids) was cloned by expression in *S. cerevisiae*. This candidal gene is homologous (37% amino acid identity) to the gene coding for chitin synthetase I in *S. cerevisiae*. It is not known whether chitin synthetase I in *C. albicans* is also non-essential for growth.

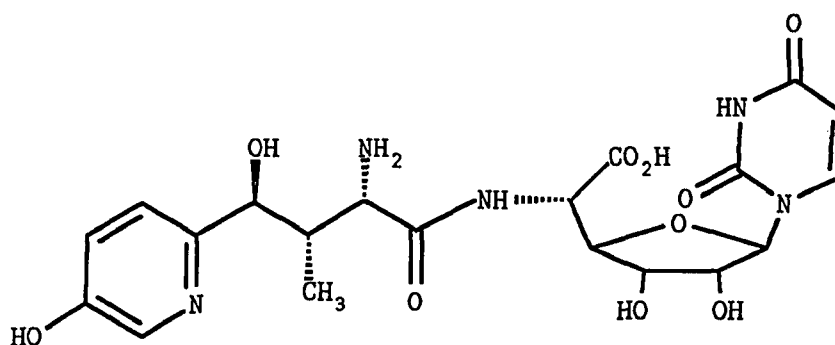
Chitin synthetase is a potential target for clinically



Uridine diphosphate N-acetyl glucosamine



Polyoxin D



Nikkomycin Z

Figure 1 Substrate and inhibitors of chitin synthetase.

important antifungal drugs, since the structural polysaccharide chitin is not present in mammalian cells. The most effective known inhibitors of fungal chitin synthetase, polyoxins (19) and nikkomycins (20) (Figure 1), are not used clinically at present. It has been shown, however, that a mixture of nikkomycins X and Z (see Table 2 for structures) is effective in prolonging the survival time of mice experimentally infected with *C. albicans* (21), but these antibiotics seem to have just a fungistatic action. This behavior correlates with the existence of large discrepancies (2-3 orders of magnitude) between reported MIC values for inhibition of fungal growth and K_i values for the inhibition of chitin synthetase *in vitro*. This discrepancy is due to the failure of the nikkomycins and polyoxins to reach their target within the cell. Translocation of these antibiotics into the cell takes place through peptide permeases (22,23). All polyoxins and nikkomycins have at least one peptide bond, and the membrane transport system of *C. albicans* recognizes these antibiotics as peptides (23). The cause of the failure of these inhibitors to accumulate intracellularly appears to be a low rate of permeation, and not their putative metabolism by the yeast (see below and Chapter III).

Peptide uptake in *C. albicans* takes place through an active peptide transport mechanism, with passage of the

intact peptide across the cell membrane. The possibility of hydrolysis in the membrane, with vectorial transport, cannot be ruled out in some strains of *C. albicans* (24). In general, separate amino acid and peptide transport systems seem to exist, but the evidence for this is not conclusive and the question will probably remain open until genetic studies of peptide transport are undertaken (24).

Independent dipeptide and oligopeptide permeases have been suggested to be present in *C. albicans* WD 18-4 (25), but this point is not very clear (24). A common transport system has been proposed for the uptake of both di- and tripeptides by *C. albicans* 6406 (26), and two distinct transport systems, one accepting both di- and tripeptides and the second more specific for trimethionine, were postulated in the case of *C. albicans* ATCC 26278 (27).

Although it is possible that different *C. albicans* strains may possess different peptide transport systems, evidence to date seems to support the presence of a general system which recognizes di- and tripeptides (and perhaps also tetrapeptides). A second system (or more than one) does not have an appreciable affinity for most dipeptides (24).

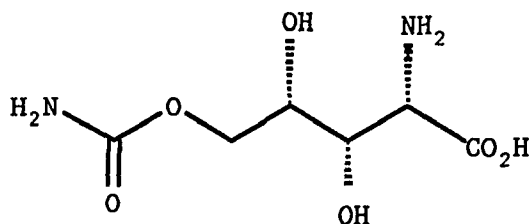
Knowledge of the side chain specificity of the different peptide permeases can be exploited in the design of anticandidal drugs. At present, there is not enough data to discuss the specificity of individual transport systems

in *C. albicans*. It is generally accepted that peptides containing lysine are not well transported into this yeast, whereas hydrophobic peptides are excellent substrates for the permeases (24) (nevertheless see Chapter III). On the other hand, α -N-acylated peptides are usually transported into *C. albicans*, whereas peptide esters are not (24). A dipeptide with a D residue at either the amine or carboxyl terminus is non transportable, but the situation with oligopeptides is less clear (24). It appears that some tripeptides containing D residues, such as D-Met-L-Met-L-Met (25), or Arg-D-X-Phe where X is an amino acid (28), are transported. The D residue cannot be located at the carboxyl terminus. On the other hand, at present, no peptide with more than one D-residue is known to be recognized by the transport system of *C. albicans* (24). The above information was incorporated into the design of modified polyoxins and nikkomycins with improved permeability (see Chapter III).

Polyoxins.

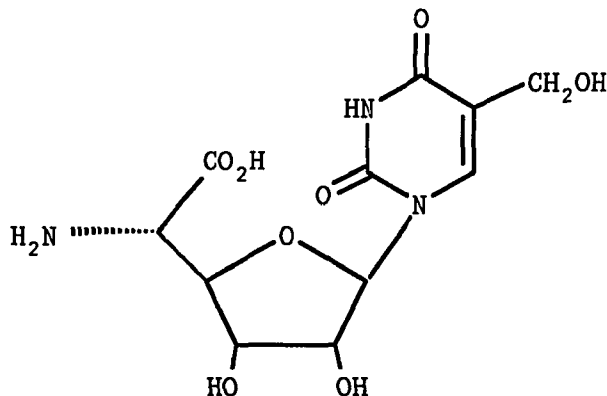
The polyoxins are a family of peptidyl nucleoside antibiotics produced by *Streptomyces cacaoi*. Their structures (see Table 1) were elucidated in the sixties by Isono's group (19). The unusual amino acids found in these compounds have the L configuration and are, from the amino terminus:

a) δ -Carbamoyl polyoxamic acid,



(3*S*,4*S*)-5-[(aminocarbonyl)oxy]-3,4-dihydroxy-L-norvaline, or in the case of polyoxin G, carbamoyl deoxypolyoxamic acid, where the hydroxyl group at the β position is absent.

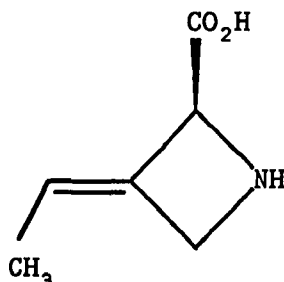
b) Polyoxin C, or



1-(5'-amino-5'-deoxy- β -D-allofuranosyluronic acid)-5-hydroxymethyluracil. The hydroxymethyl group in position 5 of the uracil ring can be replaced by a carboxyl

or a methyl group, or by an hydrogen atom (see Table 1).

c) Polyoximic acid,



(2*S*)-3-ethylideneazetidine-2-carboxylic acid, has an ethylidene group which is *trans* with respect to the carboxyl group. The allylic character of the α hydrogen accounts for the facile racemization of this compound when it is obtained by acidic hydrolysis of polyoxins (19).

Polyoxins D, E, and F have been converted (29) to polyoxins L, M, and K respectively, by decarboxylation of the 5-carboxyuracil moiety. This reaction was carried out in two steps: first, the nucleophilic addition of sodium bisulfite to the 5,6-double bond accompanied by decarboxylation, and then elimination of the sulfonate group by alkali.

Some of the constituent amino acids of the polyoxins have already been synthesized. The synthesis of carbamoyl polyoxamic acid is described in (30), and the synthesis of polyoxin C-type amino acids in (31-33). The preparation of a precursor of polyoximic acid is described in (34).

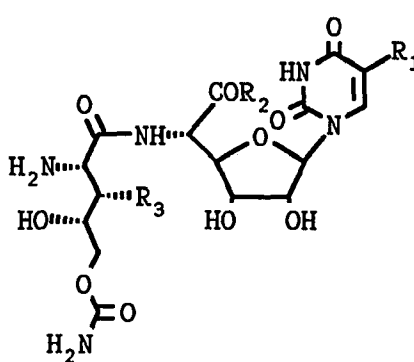
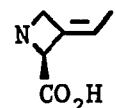
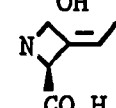
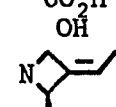
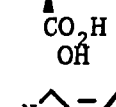
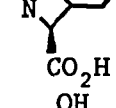
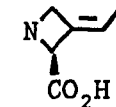
	<u>Polyoxin</u>	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>	<u>Comments</u>
	A	CH ₂ OH		OH	
	B	CH ₂ OH	OH	OH	
	D	CO ₂ H	OH	OH	
	E	CO ₂ H	OH	H	
	F	CO ₂ H		OH	
	G	CO ₂ H		H	
	H	CH ₃		OH	
	J	CH ₃	OH	OH	
	K	H		OH	
	L	H	OH	OH	
	M	H	OH	H	semisynthetic
	C	CH ₂ OH	OH		biologically inactive
	I	CH ₂ OH			biologically inactive

Table 1 Polyoxins (19, 29).

Polyoxins are structurally similar to uridine diphosphate N-acetyl glucosamine (Figure 1), the substrate of chitin synthetase. They are competitive inhibitors of this enzyme, the inhibition constant K_i being remarkably constant for chitin synthetases from different sources. K_i

values are in the 10^{-6} - 10^{-7} M range (9). Polyoxins enter *C. albicans* through a peptide transport system (35, 23). Thus, di- and tripeptides present in the growth media antagonize the anti-*Candida* activity of polyoxins. Furthermore, polyoxin-resistant mutants of *C. albicans* exhibit cross-resistance to bacilysin and other toxic di- and tripeptides (23).

The free carboxyl group at C-5', as well as the free (positively charged) amino group are required for chitin synthetase inhibition (36-38). Thus acylation or alkylation of the C₂''-amino group, as well as amidation of the carboxyl group, results in inactive or poor enzyme inhibitors (38-40). Further aspects of the effects of chemically modifying the polyoxins will be examined in Chapter III.

Nikkomycins.

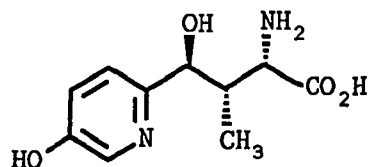
Nikkomycins are peptidyl nucleoside antibiotics structurally related to polyoxins (Table 2). Like the polyoxins, they are potent competitive inhibitors of chitin synthetase and use the peptide transport system for entry into yeast cells (41, 42). Their isolation from fermentation broths of *Streptomyces tendae*, as well as their chemical structures have been described in the late seventies by a German group (20, 43-46). In 1980 Isono's group reported the detailed structures of a new family of antibiotics which they called neopolyoxins A, B, and C (47-49). The neopolyoxins were produced by *Streptomyces cacaoi* subsp. *asoensis*. These antibiotics turned out to be identical to nikkomycin X, an oxidized form of nikkomycin X, and nikkomycin Z, respectively. Despite some initial confusion about whether or not there were stereochemical differences between the constituent amino acids of the nikkomycins and the neopolyoxins (48, 49), it is now clear that these antibiotics are identical (50). Neopolyoxins A and C will be referred to as nikkomycins X and Z.

The amino acids present in the nikkomycins have the L configuration, are biologically inactive in their free form, and are, from the amino terminus:

	<u>Nikkomycin</u>	<u>X</u>	<u>Y</u>	<u>W</u>	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
	B	CHCO	CH	CH	OH	CH ₃	OH
	B _X	CCHO	CH	CH	OH	CH ₃	OH
	Z	CHCO	N	CH	OH	CH ₃	OH
	Z _T	C(CH ₃)CO	N	CH	OH	CH ₃	OH
	Z _H	C(CH ₂ OH)CO	N	CH	OH	CH ₃	OH
	X	CCHO	N	CH	OH	CH ₃	OH
	J	CHCO	N	CH	OH	CH ₃	Glu
	J _T	C(CH ₃)CO	N	CH	OH	CH ₃	Glu
	J _H	C(CH ₂ OH)CO	N	CH	OH	CH ₃	Glu
	I	CCHO	N	CH	OH	CH ₃	OH
	K _Z	CHCO	N	CH	H	H	OH
	K _X	CCHO	N	CH	H	H	OH
	O _Z	CHCO	N	CH	OH	H	OH
	O _X	CCHO	N	CH	OH	H	OH
	Q _Z	CHCO	N	CH	OH	CH ₃	Hse
	Q _X	CCHO	N	CH	OH	CH ₃	Hse
	neopolyoxin B	CHCO ₂ H	N	CH	OH	CH ₃	OH
	P _X	CCHO	CH	N	H	CH ₃	OH
	R _Z	CHCO	CH	N	H	CH ₃	Glu
	N	CHCO					Glu
	M	CCHO					Glu
	C	CHCO					OH
	C _X	CCHO					OH
	D		N	CH	OH	CH ₃	
	E		N	CH	H	CH ₃	

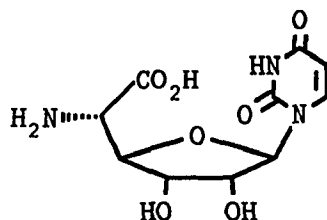
Table 2 Nikkomycins (47, 51-54).

a) nikkomycin D,

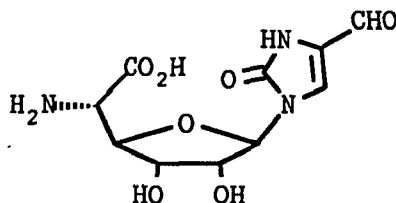
(2*S*,3*S*,4*S*)-2-amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)-3-

-methyl butyric acid, or variations in which the phenolic hydroxyl group or the methyl group are missing, or the nitrogen atom in the pyridine ring is in a different position or absent (see Table 2).

b) Nikkomycin C, 1-(5'-amino-5'-deoxy- β -D-allofuranosyluronic acid)uracil,



and nikkomycin C_x, 1-(5'-amino-5'-deoxy- β -D-allofuranosyluronic acid)4-formyl-4-imidazoline-2-one



It has been shown that in the biosynthesis of the polyoxins the pyrimidine nucleoside moiety is formed by condensation of uridine and phosphoenol pyruvate (55, 56). It is very likely that the nucleoside moiety of nikkomycin C is formed from uridine and that a subsequent ring contraction of the uracil to the imidazolinone ring then takes place (49). This kind of rearrangement has been previously described (57), and was used for the conversion of nikkomycin C into nikkomycin C_x (49).

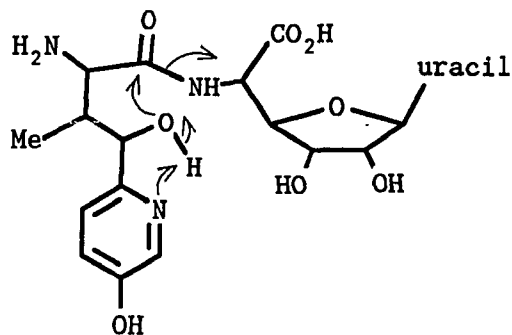
c) Glutamic acid or homoserine are present as carboxy terminal amino acids in some of the nikkomycins (see Table

2).

The synthesis of the N-terminal amino acid of the nikkomycins has been carried out by different groups. A non-stereoselective synthesis of the N-terminal amino acid of nikkomycin B, where the key step is a 1,3-dipolar cycloaddition, is described in (44). Further refinements of this synthesis leading to nikkomycin D are described in (58). Two stereoselective syntheses of the N-terminal amino acid of nikkomycin B have been achieved, one by Banks *et al.* (59), and another by Melnick *et al.* (60), who used an intramolecular [4 + 2]-cycloaddition reaction.

An important point to be considered when studying the biology of these antibiotics, or when one has to manipulate them, i.e. for purification, is their stability toward chemical and enzymatic hydrolysis. The peptide bond in polyoxin D is resistant to hydrolysis by peptidases from *C. albicans* (61). There is no previous report, to our knowledge, on the stability of nikkomycins toward degradation by fungal peptidases. This latter point will be further addressed in Chapter III. Nikkomycins X and Z are fairly stable in aqueous solution at below neutral pH, but are rapidly degraded under alkaline conditions. Nikkomycins K_X and O_X, in comparison, have been shown to be stable at pH 7 and 8 (52). It has been proposed that anchimeric

assistance is the reason for the unusual instability of nikkomycins in aqueous solution (48):



Data from the literature on the hydrolysis of nikkomycins, as well as our own results, are summarized in Table 3. Only dipeptide nikkomycins are considered. The reported products of hydrolysis are also shown.

Nikkomycin	Conditions			Products	Extent or		Ref.
	Solvent	Temp.	Time		Yield (%)	Comments	
X + Z	TFA 1N	100°C	1 hr	nik D	42	isolated	20
				nik C+C _x	29	isolated	
				nik X+Z	14	isolated	
Z (?)	HCl 6N	110°C	20 hr	nik D, <u>1</u> , <u>2</u> , uracil	100		43
	HCl 12N	37°C	48 hr		partial		
	TFA 1N	100°C	1 hr	nik D+C	partial		43
	pH 3-4- 5-6-7- 8-9. 1 mg/mL	20°C			0	biol. test	
B _x	TFA 1N	100°C	1 hr	nik C _x , <u>3</u> , <u>4</u> , <u>5</u> .			44
X	NaOH ½N	100°C	1 hr	nik D+C _x , base			49
	leucine- amino- pepti- dase. pH 6.4	37°C	6 day	nik D nik C _x	55 39	isolated isolated	
Z	NaOH ½N	100°C	1 hr	nik D uracil	16 17	isolated isolated	52
X	pH 5.5	20°C	8 day		18	HPLC	
	pH 7.0	20°C	4.7 day		50	HPLC	
	pH 8.0	20°C	4.6 day		50	HPLC	
K _x	pH 5.5	20°C	30 day		47	HPLC	
	pH 7.0	20°C	30 day		35	HPLC	
	pH 8.0	20°C	30 day		15	HPLC	

Table 3 Hydrolysis of nikkomycins (dipeptides).

Nikkomycin	Conditions			Products	Extent or		Ref.
	Solvent	Temp.	Time		Yield (%)	Comments	
O _x	pH 5.5	20°C	30 day		17	HPLC	52
	pH 7.0	20°C	30 day		50	HPLC	
	pH 8.0	20°C	30 day		55	HPLC	
X	pH 7.23	37°C	44 hr		50	HPLC	this work
Z	pH 7.23	37°C	44 hr		50	HPLC	this work
X + Z	TFA .05%	60°C	2.2hr		3.7	HPLC	this work

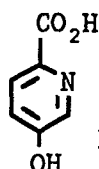
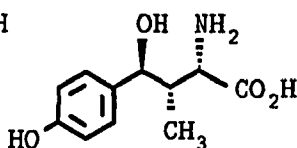
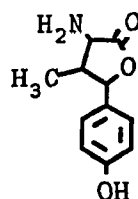
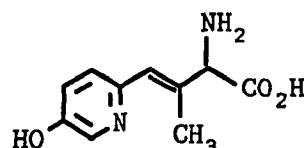
12345

Table 3 (continued).

Chapter II. Purification of nikkomycins X and Z.

Introduction.

Crude nikkomycin was obtained as a gift from Bayer Pflanzenschutz Leverkusen. This material contained a mixture of nikkomycins X and Z (~ 48%) and small amounts of other nikkomycins, as well as extraneous materials (salts, pigments, etc.). The purification of the crude material using reversed-phase HPLC (high-performance liquid chromatography) was attempted, in order to obtain 100 mg amounts of nikkomycins X and Z for biological and chemical work.

The original purification of nikkomycins X and Z by H. Hagenmaier *et al.*, and specifically the separation of these two major antibiotic components of *Streptomyces* fermentation broths, employed derivatization with dimedone and column chromatography on cellulose, as well as treatment with an alkylamine resin and further purification by HPLC using an RP-8 column (20). These methods sacrifice one component to obtain the other, and the reported yields are extremely low.

Another approach for the separation of nikkomycins (62, 63) has utilized reversed-phase HPLC with a RP-8

column, eluting with a combination of step and linear gradients with 30 mM ammonium formate buffer (pH 3.75) and 1 mM heptanesulfonic acid in water and in methanol. The heptane sulfonic acid acts as a lipophilic counter-ion. This was used as a quantitation method of nikkomycins in fermentation broths. Although nikkomycins X and Z are separated from other components, it would not be practical to scale up these conditions for the preparative purification of the nikkomycins because the counter-ion, heptanesulfonic acid, would be difficult to remove. Furthermore, baseline separation between nikkomycins X and Z was not achieved and the little resolution of these antibiotics would be lost in the scale-up. It is interesting that the authors (63) stated that chromatographic separation of nikkomycins that differ only in the nucleobase is difficult, almost impossible.

Purification of nikkomycins.

Reversed-phase packings, as well as the use of lipophilic ion-pairing reagents, have been increasingly popular for the analytical and preparative HPLC of peptides (64-68). The reversed-phase packings consist of silica gel to which a hydrocarbon, usually a chain of 4, 8 or 18 carbon atoms, has been chemically attached. Partitioning of the solute occurs between the hydrophobic stationary phase

and a polar aqueous mobile phase. Being able to use aqueous solvents is very advantageous in processing polar non-solvent extractable compounds. Trifluoroacetic acid is widely used as an anionic ion-pairing reagent in the reversed-phase HPLC of peptides because its low boiling point allows for easy removal during freeze-drying. Furthermore, C₁₈ reversed-phase chromatography using trifluoroacetic acid / water as the mobile phase has been successfully applied to the purification of natural and synthetic polyoxins (69, 70).

Attempts to purify the nikkomycins from the crude fermentation product using a TFA 0.05% mobile phase and a μ Bondapak C₁₈ (Waters) stationary phase resulted in isolation of a mixture of nikkomycins X and Z. Figure 2 shows analytical chromatograms at different concentrations of TFA. The major peak corresponds to the mixture of nikkomycins X and Z. The resolution of these components could not be increased by varying the concentration of TFA (from 0 to 0.10%). Although the higher concentrations led to increased retention and marginal resolution (not seen in the figure), the high acidity is detrimental to the column (69). The increase in retention times appears to be due to ion pairing with protonated amino groups, as well as to an ion suppression effect on the carboxylic acid (65, 71) . A similar chromatographic behavior, namely a sharp decrease

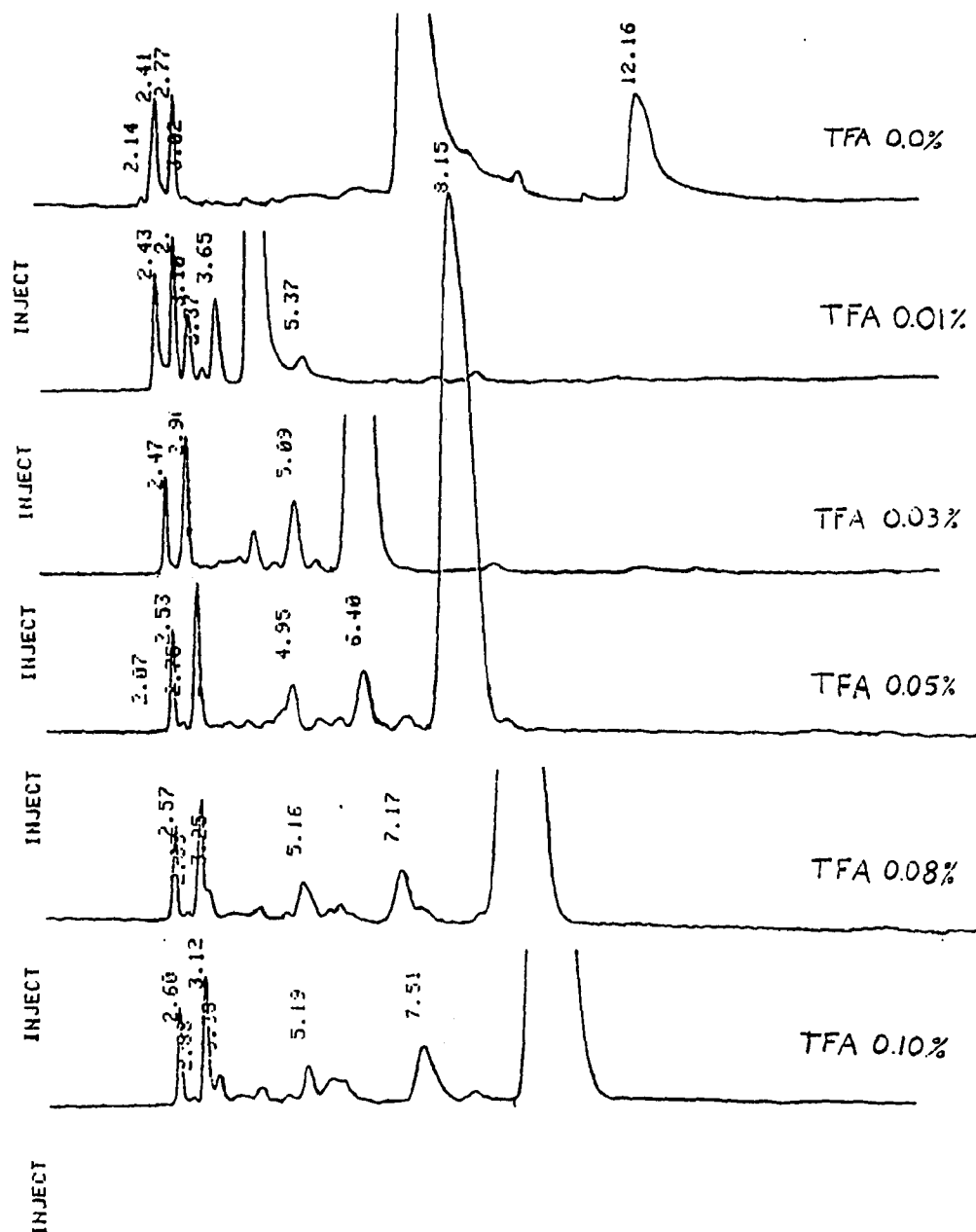


Figure 2 Analytical chromatogram of crude nikkomycin, with various amounts of TFA in the eluant. Support: μ -Bondapak C₁₈ (30cm $\times$ 3.9mm) (Waters). Flow rate: 1.40 mL/min. λ :254 nm. Retention times in minutes.

of retention times with the addition of small amounts of TFA and then increased retention as more TFA is added, has been described for small unrelated peptides (65).

257 mg of pure nikkomycins X + Z were recovered from 500 mg of crude antibiotic when the conditions shown in Figure 2, at 0.05% TFA, were scaled-up for preparative work. The ^1H -nmr spectrum of this mixture is shown in Figure 3. The mixture is 75:25 nikkomycin X / nikkomycin Z, as estimated from the relative areas of peaks such as the ones at δ 7.72 (s, H-5, nik X), 9.24 (s, CHO, nik X) and 5.68 (d, H-5, nik Z), 7.46 (d, H-6, nik Z).

Since nikkomycin X has an aldehyde group, it should in principle be possible to form a derivative which could be separated from nikkomycin Z and then reversed to nikkomycin X. Sodium bisulfite has been used extensively for the separation of carbonyl compounds from other materials and was the obvious reagent choice. However it should be noted that nikkomycins are non-extractable water-soluble materials, and a bisulfite derivative of nikkomycin X would also be water soluble and more polar.

First attempts at resolving the nikkomycin X + Z mixture consisted of dissolving it in a saturated solution of NaHSO_3 in H_2O and injecting onto the reversed-phase C_{18}

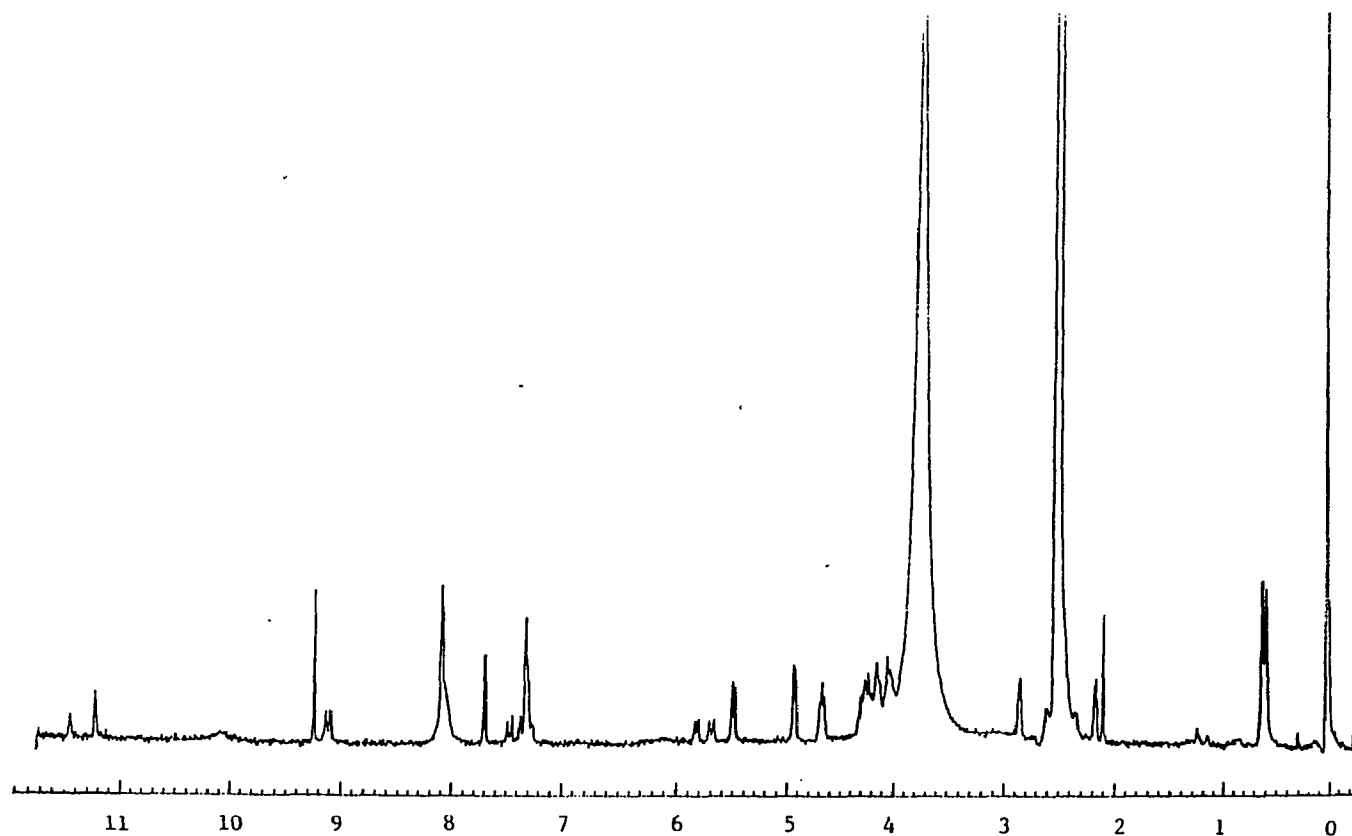
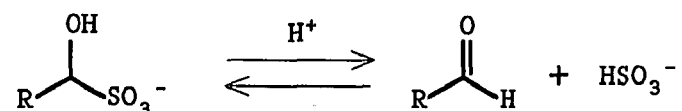


Figure 3 200 MHz ¹H-nmr spectrum of nikkomycin X + Z in DMSO-d₆.
Tip angle: 30°;Relaxation delay: 4 sec;Reference: internal TMS.

column, with H₂O/TFA as eluant. Interestingly, three different peaks could be clearly distinguished in the resulting chromatogram (see Figure 4). The first large peak, coming with the solvent front, is NaHSO₃. The second peak, with retention time of 3.52 min is presumably the nikkomycin X bisulfite addition compound. Some at first sight odd or unexpected features of this chromatogram are immediately apparent: a) the baseline between the second and third peak is somewhat above the rest of the baseline and has a positive slope, and b) the third peak, which would on first thought correspond to nikkomycin Z, the minor component (25%), is actually much larger than the second peak. This can be explained by noting that the bisulfite addition compound is in equilibrium with the free aldehyde,



and its decomposition, catalyzed by the acid present in the eluant, occurs in the column as the run proceeds. Thus, the third peak contains some nikkomycin X, and the elevation of the baseline observed between the second and the third peak is caused by nikkomycin X, which is formed gradually and migrates at a slower rate than the addition compound.

The rate at which the chemical reaction occurs in the column has to have a large impact in the appearance of the

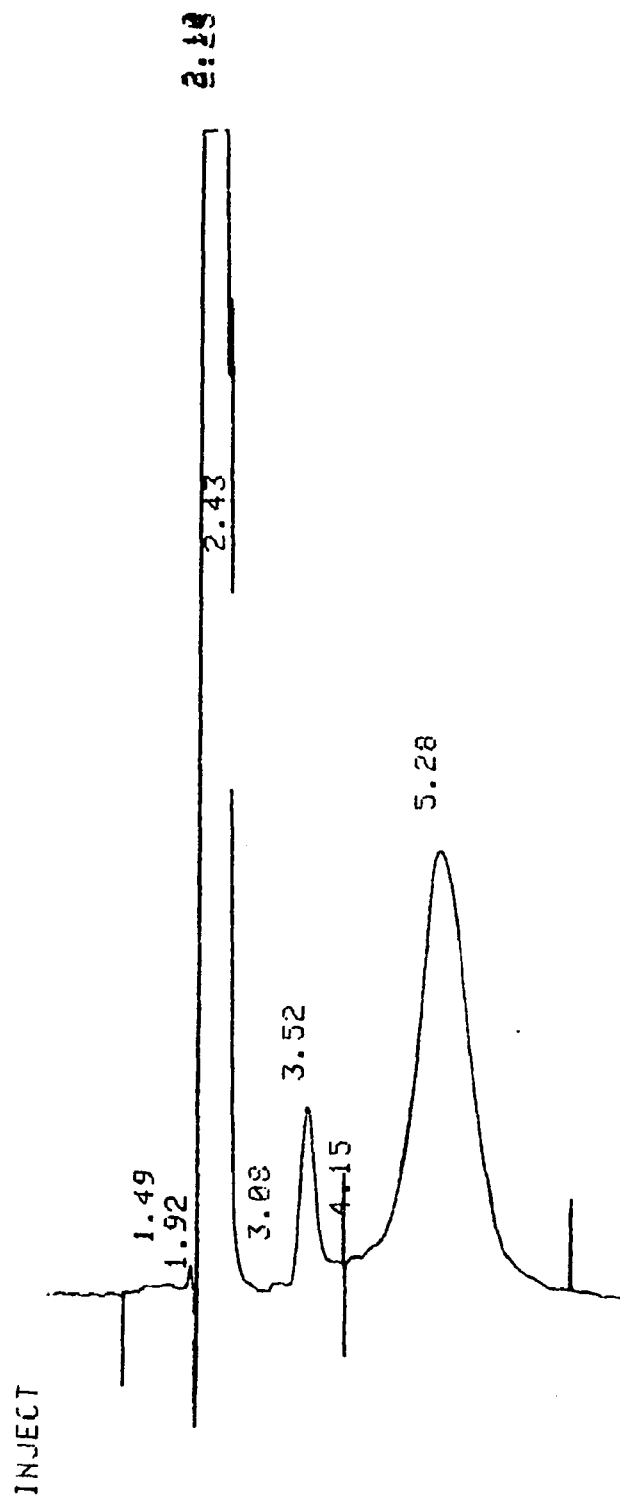


Figure 4

Analytical chromatogram of nikkomycin X + Z dissolved in aqueous NaHSO_3 . Eluant: TFA 0.005%. Support: μ -Bondapak C_{18} (30cm $\times$ 3.9mm) (Waters). Flow rate: 1.40 mL/min. λ :254 nm. Retention times in minutes.

final chromatogram. If the rate of decomposition of the bisulfite addition compound were very slow as compared to the rate at which the solutes move in the column, good separation would be achieved: the third peak would only consist of nikkomycin Z, and the baseline between the second and third peak would be flat. Conversely, one can imagine that if the rate of decomposition were very fast, the second peak ($RT = 3.52$ min) would completely disappear and the third peak would consist of the original mixture of nikkomycins X and Z. The actual chromatogram seen (Figure 4) corresponds to an intermediate case, where the rates of reaction and of migration are comparable.

In actuality the rate of formation of the bisulfite adduct under conditions compatible with the chromatographic run is much faster than the rate of decomposition; taking advantage of this, the separation of the two nikkomycins can be achieved. This will be shown in the manuscript which follows.

Chemochromatography: its use for the separation of
nikkomycins X and Z.

Chemochromatography: Its Use for the Separation of Nikkomycins X and Z¹

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A novel mode of reversed-phase high-performance liquid chromatography in which the mobile phase reacts chemically with the compounds to be separated was developed. Nikkomycin X and nikkomycin Z, two natural isomeric nucleoside peptide antibiotics, move as a single peak on a C₁₈ reversed-phase column using an aqueous trifluoroacetic acid mobile phase. Addition of sodium bisulfite (1.0%) to the mobile phase results in the formation of a polar bisulfite addition product with nikkomycin X, but not with nikkomycin Z, inside the HPLC column. This type of reactive chromatography, or chemochromatography, led to the analytical and preparative separation of nikkomycins X and Z which are normally very intractable to separation by conventional chromatographic techniques. © 1987 Academic Press, Inc.

KEY WORDS: chemochromatography; nikkomycin; nucleoside peptide antibiotics; reversed-phase HPLC techniques; NMR.

Nikkomycins, nucleoside peptide antibiotics produced by *Streptomyces tendae*, have potential value as therapeutic agents and pesticides (1-6). As a result of the structural resemblance of the nikkomycins to uridine diphosphate-*N*-acetylglucosamine, the natural substrate of chitin synthetase, these antibiotics are potent inhibitors of this enzyme in both fungi and arthropods (7-10).

Previous attempts to separate nikkomycin X (1) and nikkomycin Z (2) (two major biologically active components of *Streptomyces* fermentation broths) (Fig. 1) have utilized column chromatography on cellulose (3) and reversed-phase chromatography on an RP-8 column (9,11). In all cases it was extremely difficult to separate 1 from 2 without sacrificing one of the components. Some resolution of nikkomycins X and Z was obtained using analytical HPLC (3,11), but these conditions

could not be scaled up for the preparative separation of these antibiotics since baseline separation was not achieved and the counterion, heptanesulfonic acid, would prove difficult to remove. Indeed investigators stated that separation of nikkomycins which differ only in the nucleobase is difficult, almost impossible (9). With trifluoroacetic acid as the ion-pairing agent, we found that 1 and 2 moved as a single unresolved peak.

The chromatographic modes which use a chemical reaction to selectively influence the mobility of the sample, and which we call collectively chemochromatography, traditionally use a reactive stationary phase. Such is the case for the AgNO₃-impregnated silica gel plates for olefinic samples, or the borate-impregnated silica gel for complexing glycol compounds (12). To separate 1 and 2 we decided to use a reactive mobile phase. Specifically, we used an additive in the mobile phase of our reversed-phase HPLC system which would react with 1 (an aldehyde) but not with 2, reasoning that mobility changes accompanying such a reaction might make the separation possible.

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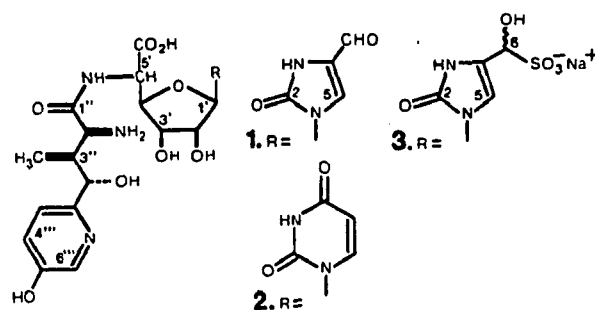


FIG. 1. Structure of nikkomycins. Compounds 1, 2, and 3 are nikkomycin X, nikkomycin Z, and the bisulfite addition compound of nikkomycin X, respectively.

The desirable characteristics for this reaction "in the column" are: (i) The reaction should be quantitative or nearly quantitative. (ii) Its rate has to be very fast as compared to the rate at which the compounds of interest move in the column so that the reaction is complete in the very first portion of the column bed. (iii) The chromatographic mobility of the derivative formed has to be different from the mobility of the unreacted compound. (iv) From a preparative point of view, the reaction should be reversible, so that the original material can be recovered easily.

In this communication we report the successful analytical and preparative separation of 1 and 2 using chemochromatography. Our procedure employs sodium bisulfite as the component of the mobile phase which rapidly reacts with 1 forming a derivative with modified chromatographic properties.

MATERIALS AND METHODS

A Waters Associates (Milford, MA) HPLC system consisting of an M-45 solvent delivery unit, a U6K universal liquid chromatograph injector, a Waters Lambda-Max Model 481 spectrometer, and a Waters M730 data module was used for analytical separations. All separations were accomplished using a μ Bondapak C_{18} column (Waters) (particle size, 10 μ m; length, 30 cm; internal diameter, 3.9 mm).

Preparative separations were carried out

on a Waters Prep LC/system 500 instrument equipped with a refractive index detector. Either a Waters One-Inch Semiprep column (1 in. $\times$ 1 ft) packed with Waters preparative C_{18} -column packing material (particle size, 55–105 μ m) or a Waters Prep PAK-500/ C_{18} cartridge (80- μ m mean particle size, 30 $\times$ 5.7 cm) was used for purifications.

TFA² (Aldrich, Milwaukee, WI) was of reagent grade and was used without purification. The sodium bisulfite used (Fisher Scientific Co., Fair Lawn, NJ) is a mixture of sodium bisulfite (NaHSO_3) and sodium *meta*-bisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) (assay as SO_2 not less than 58.5%). The solutions were prepared with glass-distilled water, filtered (Milipore, 0.45 μ m), and deaerated under vacuum before acidification. All concentration percentages are expressed as weight per volume. Activated carbon (Aldrich) used for desalting was of nucleotide desalting grade and was slurry-packed after degassing the slurry under vacuum. Crude nikkomycin (~48% 1 + 2) was obtained from Bayer Pflanzenschutz Leverkusen.

¹H NMR spectra at 200 MHz were obtained on a Bruker spectrometer and were run at 25°C. Reported chemical shifts are relative to TMS or DSS³ as internal refer-

² Abbreviations used: TFA, trifluoroacetic acid; TMS, tetramethylsilane; DSS, sodium 3-(trimethylsilyl)-1-propanesulfonate; RT, retention time; MIC, minimum inhibitory concentration.

³ Used for samples run in D_2O .

CHEMOCHROMATOGRAPHY OF NIKKOMYCINS X AND Z

ences. Optical rotations were determined on a Perkin-Elmer Model 141 polarimeter. For elemental analyses, freeze-dried samples were further dried under vacuum over P_2O_5 at $65^\circ C$, for 44 h. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tennessee.

Purification of nikkomycin. In order to obtain a mixture of pure **1** and **2** from commercial nikkomycin, 500 mg of crude antibiotic was dissolved in 15 ml of aqueous TFA (0.25%), injected onto the Prepak-500/ C_{18} preparative column, and eluted with TFA (0.05%). Fractions were monitored using analytical HPLC (Fig. 2). The fractions containing the peak at RT 8.15 min were pooled and freeze-dried. A TFA salt (257 mg) was obtained in this way. ^1H NMR analysis showed this material to be a 75:25 mixture of **1** and **2**. Specifically in $DMSO-d_6$ **1** exhibited singlets at 7.72 ppm (H-5) and 9.24 ppm (CHO) whereas **2** exhibited doublets at 5.68 and 7.46 ppm for H-5 and H-6, respectively. The relative areas under these resonances were used to estimate the content of isomers **1** and **2**.

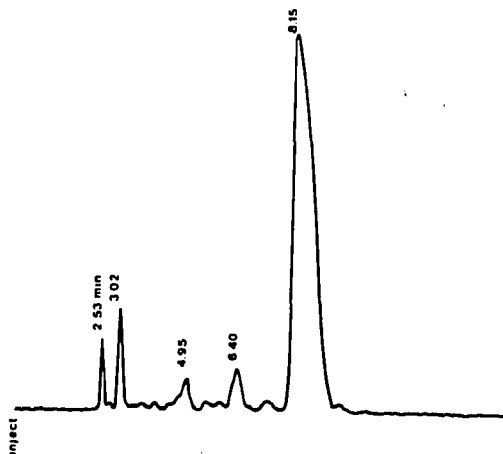


FIG. 2. Analytical chromatogram of crude nikkomycin, using a μ Bondapak C_{18} support. The eluant is CF_3CO_2H (0.05%), the flow rate was set to 1.40 ml min^{-1} , and the detector was adjusted in the absorbance units mode at 254 nm. Retention times are in minutes.

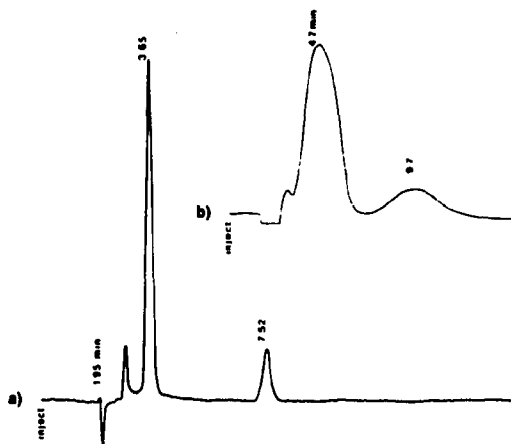


FIG. 3. (a) Analytical and (b) preparative chemochromatograms for a **1** + **2** mixture, using a C_{18} reversed-phase support. The eluant is water containing $NaHSO_3$ (1.0%) and CF_3CO_2H (0.005%). In (a) the flow rate was set to 1.40 ml min^{-1} and the detector was adjusted in the absorbance units mode at 290 nm. In (b) the flow rate was 50 ml min^{-1} and 50 mg of nikkomycin were injected. Detection of refractive indexes relative to the solvent. Retention times are in minutes.

Pure nikkomycin X and Z were recovered by dissolving 50 mg of the mixture of **1** and **2** in 1.5 ml of distilled water and injecting it onto the 1-in. C_{18} reversed-phase column, using a freshly prepared solution of $NaHSO_3$ (1.0%) and TFA (0.005%) (w/v) in water as the eluant. The column was previously equilibrated with the same mobile phase. Figure 3 shows both the analytical and the preparative chromatograms.

The fractions corresponding to **2** (peak with RT 7.52 min, Fig. 3a, and RT 9.7 min, Fig. 3b) were pooled and immediately desalted by percolating the solution through a column of activated carbon. Approximately 1 ml bed/mg of nikkomycin Z was used. The column was washed with water until no $NaHSO_3$ was present in the effluent. An attempt to recover **2** by eluting with acetone:water (40:60) was unsuccessful, but, after rewashing the column with water, **2** could be eluted with methanol:water:concentrated ammonium hydroxide (80:20:0.1). After concentration and freeze-drying 6.2 mg of **2** was obtained.

NMR. $\delta_{\text{H}}^{\text{D}_2\text{O}}$ 0.78 (3H, d, $J = 7.1$ Hz, Me-3"), 2.72 (1H, m, H-3"), 4.2–4.6 (H-2',3',4',5',2"), 5.13 (1H, d, $J = 2.9$ Hz, H-4"), 5.86 (1H, d, $J = 5.2$ Hz, H-1'), 5.87 (1H, d, $J = 8.0$ Hz, H-5), 7.45 (2H, m, H-3",4"), 7.69 (1H, d, $J = 8.0$ Hz, H-6), 8.08 (1H, broad, H-6").

Elemental analysis. Found: C, 41.54; H, 5.36; N, 12.30%. Calculated for $\text{C}_{20}\text{H}_{25}\text{O}_{10}\text{N}_5 \cdot 4.5 \text{H}_2\text{O}$: C, 41.67; H, 5.94; N, 12.15% $[\alpha]_{\text{D}}^{25^\circ\text{C}} = +25.0$ (c 0.1, H_2O)

The fractions corresponding to the bisulfite addition compound of nikkomycin X (3) (large peak with RT 3.65 min, Fig. 3a, and RT 4.7 min, Fig. 3b) were pooled, acidified with dilute HCl to pH 2.5, degassed under vacuum, reacidified, and concentrated under vacuum, maintaining the pH constant at 2.5 with dilute HCl. In order to regenerate free 1 from 3 the solution was diluted and concentrated several times under vacuum at pH 2.5. Alternatively 1 could be recovered from 3 by bubbling nitrogen through the solution for a few hours, while keeping the pH at 2.5. This transformation was followed by HPLC (TFA (0.10%) as the eluant) and stopped when complete. The concentrated solution was finally desalted by injection in the 1-in. C_{18} reversed-phase column, using TFA (0.10%) in H_2O as the eluant. Pure nikkomycin X (29.5 mg) was obtained after concentration and freeze-drying.

NMR. $\delta_{\text{H}}^{\text{DMSO-}d_6}$ 0.60 (3H, d, $J = 7.0$ Hz, Me-3"), 2.57 (1H, m, H-3") 3.9–4.4 (H-2',3',4',2"), 4.65 (1H, dd, H-5'), 4.95 (1H, d, $J = 2.7$ Hz, H-4"), 5.48 (1H, d, $J = 5.0$ Hz, H-1'), 7.31 (1H, dd, $J = 8.5$ and 2.2 Hz, H-4"), 7.36 (1H, d, $J = 8.5$ Hz, H-3"), 7.72 (1H, s, H-5), 8.09 (1H, dd, $J = 2.2$ and 0.8 Hz, H-6"), 8.0–8.2 (3H, NH_3^+), 9.13 (1H, d, $J = 7.8$ Hz, CONH), 9.24 (1H, s, CHO), 10.2 (broad, ArOH), 11.22 (1H, s, NH-3).

Elemental analysis. Found: C, 36.87; H, 3.72; N, 8.85; F, 14.71%. Calculated for $\text{C}_{20}\text{H}_{25}\text{O}_{10}\text{N}_5 \cdot 2\text{TFA} \cdot 3\text{H}_2\text{O}$: C, 37.07; H, 4.28; N, 9.01; F, 14.66%. $[\alpha]_{\text{D}}^{25^\circ\text{C}} = +16.0$ (c 0.1, H_2O).

RESULTS AND DISCUSSION

Trifluoroacetic acid has been shown to be a very useful anionic ion-pairing reagent in the reversed-phase HPLC of peptides (13–15). The increase in retention times observed at low concentrations of TFA in water is apparently due to ion-pairing with protonated amino groups and/or to an ion suppression effect in the carboxylic acid functionalities (13,16). The low boiling point of TFA allows its easy removal during freeze-drying.

Attempts to purify 1 and 2 from a crude fermentation product using a trifluoroacetic acid 0.05% mobile phase resulted in isolation of a mixture of these antibiotics. As seen in Fig. 2, 1 and 2 move as an asymmetric unresolved peak, RT 8.15 min. The resolution of these components could not be further increased by varying the trifluoroacetic acid concentration (0–0.10%, w/v). Although higher concentrations of the acid (0.10%) led to increased retention on the column and some additional resolution (data not shown), this concentration of acid slowly deactivated the reversed-phase column. Sodium bisulfite is also not stable in high concentrations of acid. A minimum retention time (4.28 min) was observed at a trifluoroacetic acid concentration of approximately 0.01%; below this concentration the mobility of the peptidyl-nucleoside antibiotics decreases and in pure water 1 and 2 move as an unresolved peak which tails badly. The trifluoroacetic acid concentration used for further separations was chosen to maximize the retention time of the antibiotics without destroying either the column or the sodium bisulfite. For this purpose the concentration region between 0 and 0.01% trifluoroacetic acid was most appropriate and 0.005% acid worked very well.

Separation of 1 from 2 was carried out by reversed-phase chromatography in the presence of sodium bisulfite. The analytical and preparative chemochromatograms for the 1 + 2 mixture are shown in Fig. 3. After a

CHEMOCHROMATOGRAPHY OF NIKKOMYCINS X AND Z

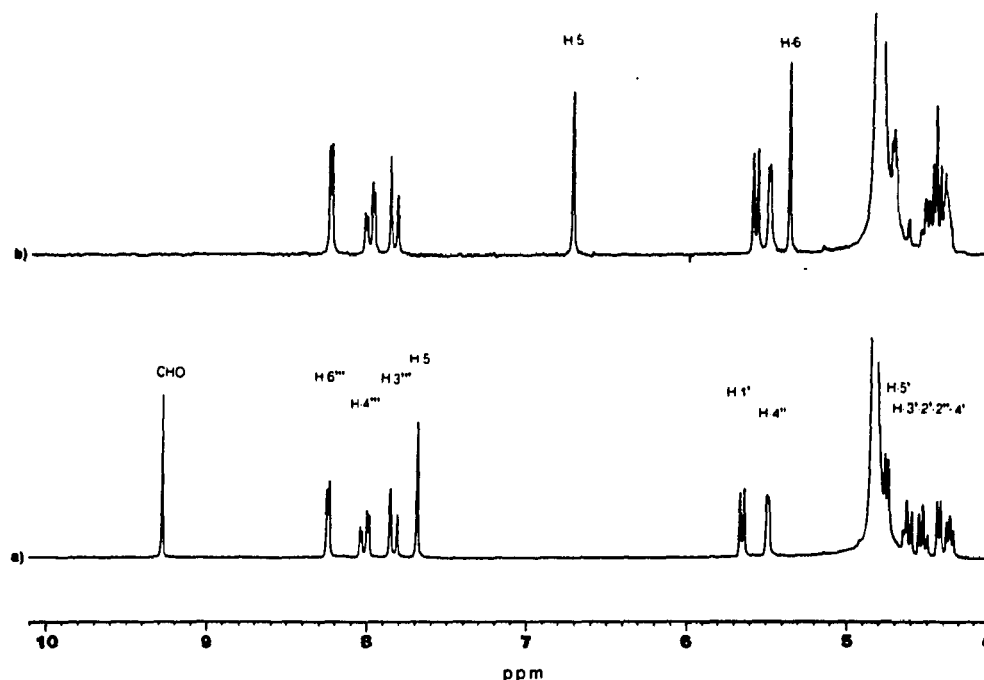


FIG. 4. Partial FT ^1H NMR spectrum of nikkomycin X at 200 MHz. (a) In D_2O . (b) With sodium bisulfite added (1.0%). Shifts from DSS (internal reference) in ppm.

negative peak at the origin, due to water, the peaks of interest appear. As expected from its polarity, the bisulfite addition compound 3 has a very low RT (3.65 min) and can be easily separated from 2 (RT 7.52 min). The preparative chemochromatogram corresponds to 50 mg of mixture and shows baseline separation between the peaks corresponding to 3 and 2 (Fig. 3b). The reaction of NaHSO_3 with 1 has the four characteristics mentioned in the introduction. Reinjection of purified 1 in this chromatographic system showed that the conversion of 1 to 3 is quantitative, since a single peak was observed at RT 3.65 min. Furthermore, no trace of 1 was observed in isolated 2.

Figure 4a shows the partial ^1H NMR spectrum of purified 1 in D_2O . When sodium bisulfite is added (1.0%), the result represented in Fig. 4b is obtained. Conversion of 1 to 3 in the NMR tube is indicated by loss of the aldehyde proton signal at 9.30 ppm (Fig.

4b) and the appearance of a new singlet at 5.35 ppm that corresponds to $-\text{CHODSO}_3^-$.⁴ The singlet at 7.70 ppm in Fig. 4a, corresponding to H-5 (1), is strongly shifted upfield in Fig. 4b (6.75 ppm) due to its proximity to the sulfonate moiety. These NMR results show that the aldehyde group in nikkomycin X is rapidly and quantitatively converted to an addition compound under conditions simulating those in the chromatography column.

After reversal of the addition compound 3 using the procedure described under Materials and Methods, nikkomycin X was desalted on a C_{18} reversed-phase column. In this manner we were able to recover 29.5 mg

⁴ The resonances of aldehyde protons in sodium bisulfite addition compounds fall in the range 4.5–5.0 ppm for aliphatic aldehydes listed in (17). For the bisulfite addition product of benzaldehyde, that resonance is at 5.51 ppm (in D_2O) (unpublished results).

of pure nikkomycin X. This represents a 78% yield assuming that 1 is present as the double trifluoroacetic acid salt in the mixture (1 + 2) as it is in purified 1.

In contrast to the desalting of nikkomycin X which was carried out on a C_{18} column, a different approach was taken to desalt nikkomycin Z. This change was necessitated by our observation that NaHSO_3 reacts slowly with nikkomycin Z under the conditions used to concentrate the HPLC fractions prior to desalting. We believe that the bisulfite adds to the double bond of the uracil ring. A similar reaction of bisulfite with a pyrimidyl double bond was previously observed during conversion of polyoxin D to polyoxin L (18). Although we could desalt 2 by degassing the concentrated solution at low pH, raising the pH with NaOH, and injecting onto a reversed-phase HPLC column, it proved more convenient to desalt using activated carbon. The total recovery of nikkomycin Z was 6.2 mg, which represents a 67% yield if one takes into account that the hydrated free peptide is obtained, and assuming a $3\text{H}_2\text{O} \cdot 2\text{TFA}$ form in the starting material, as in the case of nikkomycin X. Significant losses were taken during the desalting step and are probably due to irreversible adsorption to the activated carbon column. No attempt was made to optimize this step.

Inhibition of chitin synthetase was assayed for isolated 1 and 2 using a mixed membrane fraction from *Candida albicans* H-317 (19,20). For nikkomycin X an ID_{50}^5 value of 0.1 $\mu\text{g}/\text{ml}$ was obtained and for nikkomycin Z the ID_{50} value was 0.24 $\mu\text{g}/\text{ml}$. Under virtually identical conditions polyoxin D was 4- to 10-fold less active (20). The MIC^6 for nikkomycin X was determined to be $\sim 4 \mu\text{g}/\text{ml}$ when tested with *C. albicans* 124. These re-

sults are comparable to findings reported in the literature using other test organisms. Specifically using chitin synthetase from *Coprinus cinereus* nikkomycin was a 2- to 6-fold more effective inhibitor than polyoxin D (7). Using strain *C. albicans* 124, McCarthy *et al.* (21) report an MIC of 25 $\mu\text{g}/\text{ml}$ for a mixture of nikkomycins X and Z (80% pure). *C. albicans* Tü 164 yielded an MIC of 1 $\mu\text{g}/\text{ml}$ when tested against nikkomycin X (22). Given the known dependence of MIC values on media and laboratory protocol all of these findings are in reasonable agreement.

CONCLUSION

The methods described herein allow the rapid and efficient separation of nikkomycin X and nikkomycin Z. In addition to the preparative potential of the procedure it is obvious that chemochromatography with a reactive mobile phase is an excellent procedure for the analysis of mixtures of these compounds. The extension of this approach to other isomeric molecules differing in their functional groups should add a significant tool to the arsenal of the chromatographer.

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⁵ The concentration of compound that inhibited by 50% the activity of chitin synthetase as measured by the incorporation of *N*-acetylglucosamine into chitin.

⁶ The lowest concentration of antibiotic that clearly inhibits growth.

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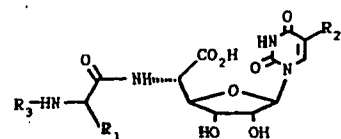
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Chapter III. Nikkomycin and polyoxin analogs.

Dipeptide polyoxin analogs.

In 1971 Isono *et al.* prepared dipeptide analogs of polyoxins and tested their toxicity against some phytopathogenic fungi (72, 73). Representative results for these and further experiments carried out by other investigators (see below) are shown in Table 4. The compounds at entries 2 and 3, showed a toxicity comparable to that of Polyoxin J (entry 1), indicating that the hydroxyl groups in the carbamoyl polyoxamic acid are not necessary for activity. The studies of these investigators also showed that compounds containing a second amino group in the amine terminal residue (entries 9 and 10, Table 4), as well as compounds where this residue has the D configuration (entries 8 and 12), have very weak or no toxicity.

In 1974 Hori *et al.* took these studies one step further and assayed the dipeptide analogues as inhibitors of a chitin synthetase preparation from *Piricularia oryzae* (74). They assessed the partial binding affinities to chitin synthetase of different atomic groups or moieties of the polyoxin molecule. Their results complement the findings by Isono. The investigators showed that the change

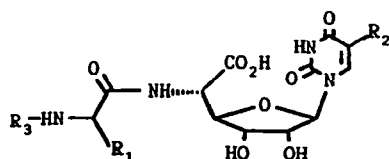


Entry	Compound or Residue	R ₁	R ₂	R ₃	MIC ^a		Chitin Synthetase Inhibition (ID ₅₀ ^b , μM)	Hydrolysis ^c	Transport inhibition ^d	Ref.
					(<i>C. albicans</i> H-317)	(<i>P. oryzae</i>) μg/mL				
1	Polyoxin J	L-CH(OH)CH(OH)CH ₂ OCONH ₂	CH ₃	H		25				72
2		DL-CH ₂ CH ₂ CH ₂ OCONH ₂	CH ₃	H		12.5				72
3	Citrullinyl	L-CH ₂ CH ₂ CH ₂ NHCONH ₂	CH ₃	H		12.5				72
4		L-CH ₂ CH ₂ CH ₂ NHCOCH ₃	CH ₃	H		12.5				72
5	Glutaminyl	L-CH ₂ CH ₂ CONH ₂	CH ₃	H		50				72
6	Glutamyl	L-CH ₂ CH ₂ CO ₂ H	CH ₃	H		>200				73
7	Norvalinyl	L-CH ₂ CH ₂ CH ₃	CH ₃	H		25				72
8	D-Norvalinyl	D-CH ₂ CH ₂ CH ₃	CH ₃	H		>200				73
9	Ornithinyl	L-CH ₂ CH ₂ CH ₂ NH ₂	CH ₂ OH	H		200				72
10	Lysyl	L-CH ₂ CH ₂ CH ₂ CH ₂ NH ₂	CH ₂ OH	H		>200				72
11	Alanyl	L-CH ₃	CH ₃	H		50				72
12	D-Alanyl	D-CH ₃	CH ₃	H		>200				72
13	Glycyl	H	CH ₃	H		>200				72
14	Phenylalanyl	L-CH ₂ - 	CH ₂ OH	H		200				73
15		L-CH ₂ CH ₂ CH ₂ NHCO- 	CH ₂ OH	H		>200				73
16		L-CH ₂ CH ₂ CH ₂ NHCOC ₁₁ H ₂₃	CH ₂ OH	H		>200				73
17	Polyoxin D	L-CH(OH)CH(OH)CH ₂ OCONH ₂	CO ₂ H	H	0.02		1.8	-	<10	77
18	Homophenylalanyl	L-CH ₂ CH ₂ - 	H	H	0.06		3.8	+	<10	77
19	Norleucyl	L-CH ₂ CH ₂ CH ₂ CH ₃	H	H	0.17		14	+	<10	77
20	Citrullinyl	L-CH ₂ CH ₂ CH ₂ NHCONH ₂	H	H	0.50		68		<10	77
21	Tyrosyl	L-CH ₂ - 	H	H	8.00		4.9	+	22	77
22	Tryptophanyl	L-CH ₂ - 	H	H	0.13		1.7	+	<10	77
23		L-CH ₂ - 	H	H	2.00		16		<10	77

^aMinimum inhibitory concentration. ^bConcentration at which chitin synthetase is inhibited by 50%.

^c(+) compound was and (-) compound was not hydrolyzed by *C. albicans* extract. ^dPercent inhibition of transport of (Met)₃ into *C. albicans*. A 10-fold molar excess of analogue was used. ^e[Percent inhibition of (Leu)₂]/[percent inhibition of (Met)₃].

Table 4 Dipeptide Polyoxins



Entry	Compound or Residue	R ₁	R ₂	R ₃	MIC ^a		Chitin Synthetase Inhibition (ID ₅₀ ^b , uM)	Hydrolysis ^c	Transport inhibition ^d	Ref.
					(<i>C. albicans</i>) H317 mM	(<i>P. oryzae</i>) ug/mL				
24		L-CH ₂ CH ₂ CH ₂ NHCONH ₂	H	C ₆ H ₅ CH ₂ OCO			>100			77
25		L-CH ₂ - 	H	C ₆ H ₅ CH ₂ OCO			>100			77
26		L-CH ₂ CH ₂ CH ₂ CH ₃	H	C ₆ H ₅ CH ₂ OCO			>100			77
27		L-CH ₂ CH ₂ CH ₂ CH ₃	H	CH ₃	>1.0		120	-	no	69
28		L-CH ₂ CH(CH ₃) ₂	H	CH ₃ CH ₂ CH ₂	2.5		>100	-	no	69
29	D-Tryptophanyl	D-CH ₂ - 	H	H	>1.0		>100	-	no	69
30	Octanoyllysyl	L-(CH ₂) ₄ NH-CO(CH ₂) ₆ CH ₃	H	H	>1.0		0.75	-	no	78
31		L-(CH ₂) ₂ -CONH-(CH ₂) ₇ CH ₃	H	H	>1.0		1.25	-	no	78
32	Threonyl	L-CHOH-CH ₃	H	H			90			80
33		L-CHOH-CH ₂ - 	H	H			3			80
34		L-(CH ₂) ₅ -CH ₃	H	H	0.37		4.7	+	71 / 6*	79
35		D-(CH ₂) ₅ -CH ₃	H	H	>0.75		>120	-	14 / 10*	79
36		(D,L)-(CH ₂) ₉ -CH ₃	H	H	0.15		0.56	-	87 / 0*	79
37		L-(CH ₂) ₁₃ -CH ₃	H	H	>0.6		18.5	-	83 / 10*	79
38		D-(CH ₂) ₁₃ -CH ₃	H	H	>0.6		>185	-	56 / 10*	79

Table 4 (continued)

of configuration at position α of the amino acyl residue from L to D, as well as the shift of the amino group position from α to β , reduces considerably the binding to the enzyme. Interestingly, they found a high inhibitory activity for phenylalanyl polyoxin C, a compound that Isono *et al.* (73) showed to be non-toxic (Table 4, entry 14). These results indicate that this analog does not reach its target enzyme within the cytoplasm: it is either unable to permeate efficiently the cell wall, or is rapidly degraded (by peptidases) inside the cell.

The authors also demonstrated that the amino group at C-2'' is crucial for binding to the enzyme (74). Furthermore, they showed that in order to have effective inhibition, the pH of the medium has to be such that the amino group at C-2'' is protonated (75).

It had been shown (76) that dipeptides antagonize the inhibitory action of polyoxins against phytopatogenic fungi. In 1983, studies from this laboratory demonstrated that polyoxin D greatly affects the morphology of the zoopathogenic yeast *C. albicans* and is fungicidal at millimolar concentrations. It was proposed that transport of polyoxin into *C. albicans* is mediated by peptide permeases (22). In 1984, Mehta *et al.* showed this to be indeed the case (23).

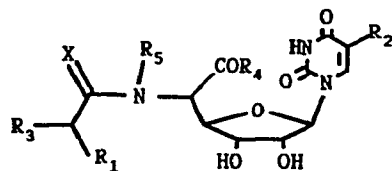
Thus, logical evaluation of polyoxin analogs should include not only the usual toxicity studies, but also assays of chitin synthesis inhibition and transport into the cell. Furthermore, since the antibiotics can be degraded by peptidases in the cytoplasm before reaching their target enzyme, stability in the presence of cell extracts should also be evaluated (70).

Shenbagamurthi *et al.* prepared a series of polyoxin L analogs specifically designed as anticandidal agents (Table 4, entries 18 to 23) (70). These compounds were good chitin synthetase inhibitors, but high doses were necessary in order to inhibit *C. albicans* growth. This was shown to be due to poor transport and/or degradation to inactive products by intracellular enzymes. If polyoxins could be chemically modified, in such a way that they would penetrate *C. albicans*, resist enzymatic degradation, and inhibit chitin synthetase, efficient killing of this yeast would result. Novel dipeptide chitin synthetase inhibitors resistant to intracellular degradation were prepared in this laboratory (70, 78) (Table 4, entries 27 to 31). The resistance to enzymatic hydrolysis was achieved by utilizing a D-amino acid (entry 29), or by N-alkylation of the terminal amine (entries 27 and 28). Resistance to peptidases also resulted from incorporation into the

polyoxin of an amino acid with a bulky hydrophobic residue (entries 30 and 31). An important finding was that although the first two strategies resulted in poor chitin synthetase inhibitors (entries 27 to 29), the hydrophobic polyoxins (entries 30 and 31) were excellent inhibitors of this enzyme. Nevertheless, these latter two antibiotics did not seem to utilize the peptide transport system to reach the cytoplasm and were non-toxic (78).

Recently, hydrolysis resistant and transportable inhibitors were prepared in this laboratory (79) (i.e. entry 36, Table 4). The MIC values were perhaps higher than one would expect, and probably not low enough for clinical applications. It should be pointed out that extreme care has to be exercised when interpreting the biological assays. In particular, the transport competition results might very well be reflecting affinity of the inhibitor but not translocation into the cytoplasm. It has been shown, for instance, that nikkomycin has a high K_m for the peptide permeases but its V_m is much lower (~5%) than that of dimethionine (27). Further limitations of the biological assays will be discussed later.

Table 5 lists other dipeptide polyoxin analogs found in the literature, with structural modifications such as a N-methyl group at the amide (entry 2), or a D configuration



Entry	Residue	R ₁	R ₂	R ₃	R ₄	R ₅	X	Configuration		Chitin Synthetase Inhibition		Hydro-lysis	Ref.
								at H-5'	at H-2''	<i>C. albicans</i>	<i>P. oryzae</i>		
1		CH ₃	H	H ₂ N	OH	H	H ₂	L		no			80
2		CH ₃	H	H ₂ N	OH	CH ₃	O	L	L	no			80
3	Alanyl	CH ₃	H	H ₂ N	OH	H	O	D	L	no			80
4	Citrullyl	CH ₂ CH ₂ CH ₂ NHCONH ₂	H	H ₂ N	OH	H	O	D	L	no			80
5	Acetyl-UPOC	H	H	H	OH	H	O	L			K _i -1100uM		74
6	β-alanyl-UPOC	CH ₂ NH ₂	H	H	OH	H	O	L			K _i -270 uM		74
7	Polyoxin B	CH(OH)CH(OH)CH ₂ OCONH ₂	CH ₂ OH	H ₂ N	OH	H	O	L	L		K _i -2.95uM		74
8		CH ₂ - 	H	H ₂ N-O-	OH	H	O	L	L	ID ₅₀ >100uM		-	69
9		CH ₂ CH ₂ CH ₂ CH ₃	H	H ₂ N	NH ₂	H	O	L	L	ID ₅₀ ~200uM		+	69

Table 5 Modified dipeptide polyoxins

at C-5' (entries 3 and 4). The mentioned compounds were intended to be hydrolysis resistant (80). Analogues with a β -amino group, or a α -aminooxy group (entries 6 and 8) have also been prepared (70, 74). None of these analogues proved to be a good chitin synthetase inhibitor.

Tripeptide polyoxin analogs.

Hoping to obtain analogs with greater permeability, Isono's group prepared some tripeptidyl polyoxins (Table 6, entries 1 to 7) along with the dipeptides mentioned above (73). Inclusion of an adamantoyl group in the δ -amino group of an amine-terminal ornithine (entry 6) resulted in a considerable decrease of activity. The toxicity of the other tripeptides toward *P. oryzae* was not significantly higher than that of Polyoxin J (Table 4, entry 1), the best drug being Leucyl-Polyoxin L (Table 6, entry 4).

Naider *et al.* (81) synthesized tripeptidyl polyoxins (Table 6, entries 8 to 10), some of which behaved as prodrugs. Although none of these compounds were chitin synthetase inhibitors, Leu-Polyoxin D and Leu-Nle-UPOC (entries 8 and 9) were shown to deliver toxic species (Polyoxin D and Nle-UPOC respectively) after hydrolysis inside *C. albicans*. Nle-UPOC was hydrolyzed further, to Nle and UPOC, both inactive species, a fact that greatly

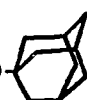
Entry	Compound	Chitin Synthetase Inhibition (ID ₅₀ ^b , uM)	MIC ^a		Hydro- lysis ^c	Transport inhibition % ^d	Ref
			(<i>C. albicans</i> H-317)	mM	(<i>P. oryzae</i>) ug/mL		
1	Gly-Polyoxin L				50		73
2	Ala-Polyoxin L				12.5		73
3	(D)Ala-Polyoxin L				12.5		73
4	Leu-Polyoxin L				6.25		73
5	Phe-Polyoxin L				50		73
6	H ₂ N-CHCO-Polyoxin L (CH ₂) ₂ CH ₂ NHCO- 				100		73
7	H ₂ N-CHCO-Polyoxin L (CH ₂) ₂ CH ₂ -NH-CO-(CH ₂) ₉ CH ₃				25		73
8	Leu-Polyoxin D	>100	0.25		+	11	81
9	Leu-Nle-UPOC	>100	2		+	59	81
10	Nle-UPOC-Leu	>100	4		+	7	81
11	Phe-Δ ² Phe-UPOC	40	>2		-	no	69
12	CH ₃ (CH ₂) ₆ CO-NH-Phe-Polyoxin D	47.5	1		-	no	69
13	Polyoxin D		0.03		-	10	81

Table 6 Tripeptide Polyoxins

diminished the efficacy of the drug.

Other tripeptidyl polyoxins prepared in this laboratory (70) (entries 11 and 12) inhibited chitin synthetase to some extent, a fact that was unexpected. These compounds are the first known tripeptide inhibitors of chitin synthetase. They resisted degradation and had low toxicity against *C. albicans*.

New tripeptidyl polyoxins and nikkomycins, designed to be degraded by peptidases at specific bonds, will be examined in the next section. Dipeptide analogs designed as double targeted drugs will also be considered.

Synthesis and biological evaluation of dipeptidyl and tripeptidyl polyoxin and nikkomycin analogs as anticandidal prodrugs.

Synthesis and Biological Evaluation of dipeptidyl and
tripeptidyl Polyoxin and Nikkomycin analogs as Anticandidal
Prodrugs

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ABSTRACT

Nine analogs (1-5, 9-12) of the peptidyl nucleoside antibiotics nikkomycin and polyoxin were synthesized and tested for their biological properties against different strains of the pathogenic yeast *Candida albicans*. The tripeptidyl series of analogs (1-5) was designed to behave as prodrugs, releasing a toxic moiety upon enzymatic hydrolysis inside the cell. The dipeptidyl series (9-12) was designed as double-targeted drugs, being themselves toxic and releasing a toxic amino acid upon hydrolysis. All the analogs were prepared by coupling suitably protected aminoacid *p*-nitrophenyl esters to 1-(5'-amino-5'-deoxy- α -D-allofuranosyluronic acid) uracil (UPOC) or the corresponding polyoxins and nikkomycins, with subsequent removal of the protecting group. Improved coupling yields were observed when DMSO was used as the solvent. Products were purified using reversed-phase HPLC and, in one case, diastereomeric products (compound 11) were resolved using this procedure. One of the tripeptidyl nikkomycins behaved as a prodrug but none of the compounds, as measured by *in vitro* testing, proved more effective than nikkomycin as an anticandidal agent.

INTRODUCTION

Opportunistic infections by *Candida albicans* are major contributors to patient morbidity and mortality in immunocompromised hosts^{1,2}. This has become particularly significant in recent years with increased use of immunosuppressive therapies and with the emergence of acquired immunodeficiency syndrome. Since the drugs currently in use for the treatment of candidiasis suffer from significant clinical limitations, a clear need exists for the development of effective anti-candidal drugs.

Polyoxins and nikkomycins are closely related families of peptidyl nucleoside antibiotics produced by species of *Streptomyces*. These compounds are toxic to phytopathogenic fungi³⁻⁵ and, to a lesser degree, to some zoopathogenic fungi such as *Candida albicans*^{6,7}. The intracellular target for both families of antibiotics is chitin synthetase. This inner membrane enzyme catalyzes the synthesis of chitin, a polysaccharide of N-acetylglucosamine and an important structural component of fungal cell walls. Since no similar macromolecular constituent is present in mammalian cells, chitin synthetase is an obvious target for an anticandidal agent.

The polyoxins and nikkomycins are potent competitive

inhibitors of *C. albicans* chitin synthetase. Values of ID₅₀ for nikkomycins X or Z fall in the 0.1 - 0.2 ug/mL range⁸ when enzyme inhibition is measured in a membrane preparation from disrupted *C. albicans* cells. The MIC values, however, are significantly higher ($\approx$ 10 ug/mL for the nikkomycins, see below) when measuring the effect of the inhibitors on growth of *C. albicans* in culture. These findings could be the result of the failure of these antibiotics to accumulate intracellularly or to their metabolism by the yeast. Degradation inside the cell does not seem to be the problem, since polyoxins have been shown to resist *Candida* peptidases⁹, and nikkomycins, on the basis of their structural relation to polyoxins, would be expected to behave similarly in that respect. In contrast, polyoxin and nikkomycin permeation into the cell takes place through peptide permeases and this is the step that appears to be rate limiting in the case of *C. albicans*^{7, 10}.

We have prepared polyoxins that are very good competitors against dileucine uptake in *C. albicans*, indicating high affinity for the dipeptide transport system¹¹ yet these compounds were not effective in the inhibition of cell growth. It is possible, however, that even antibiotics with high affinity for the peptide transport system are not efficiently translocated into *C. albicans*. Nikkomycin Z has been shown to have the same K_m

for transport as dimethionine but the V_m was only five percent of that of the dipeptide¹⁰. Furthermore, α -aminolauryl UPOC, a synthetic polyoxin analog recently prepared by us¹¹ is an excellent chitin synthetase inhibitor, is stable to intracellular degradation and has high affinity for the dipeptide transport system. However, it fails to kill *C. albicans* possibly because it is not translocated efficiently into the yeast.

Some strains of *C. albicans* handle peptides through at least two distinct transport systems with different selectivities for dipeptides and tripeptides^{7,10}. It is possible that the efficacy of the polyoxins and nikkomycins might be increased if these dipeptidyl nucleosides could also enter *C. albicans* through the tripeptide transport system. In addition even if nikkomycin or polyoxin were degraded inside the yeast, killing would still result if at least one of the degradation products remained toxic. To explore the above strategies, we have prepared a number of tripeptidyl nikkomycins and polyoxins using amino acid residues expected to improve recognition by the tripeptide transport system (Table 7). These tripeptides should not be inhibitors of chitin synthetase but should be hydrolyzed to toxic dipeptides. Thus they are putative prodrugs. We have also synthesized a variety of polyoxin analogs containing a known antimetabolite (Table 8, 10-12). These multi-targeted

drugs have the potential to inhibit chitin synthetase and release the toxic amino acids oxalysine¹², *meta*-fluorophenylalanine¹³ and N³-(4-methoxyfumaroyl)- γ -L-2,3-diaminopropanoic acid (FM DP)¹⁴. In this communication we report the synthesis of novel polyoxin and nikkomycin analogs and the evaluation of their intracellular stability, transportability, inhibition of chitin synthetase, and anticandidal activity.

CHEMISTRY

1-(5'-amino-5'-deoxy- α -D-allofuranosyluronic acid) uracil, which we have previously designated UPOC (uracil polyoxin C)), is the carboxyterminus amino acid of the synthetic dipeptides 9, 10, 11, 12, and of tripeptides 1 and 2. It was synthesized from uridine, according to Damodaran *et al*¹⁵. All analogs were prepared by coupling suitably protected amino acid *p*-nitrophenyl esters with UPOC or with polyoxins and nikkomycins (compounds 6 to 9) and removing the protecting group. Compound 9 has previously been synthesized in our laboratory¹⁶. In the former report, Z- ϵ -(oct)Lys-ONp was coupled to UPOC in DMF/H₂O, in the presence of NMM. The overall yield (coupling and deprotection) was only 29%. This was attributed to the low solubility of UPOC in DMF. Normally, this can be improved by addition of water but in this case

the amount of water had to be kept low to avoid precipitation of Z-(oct)Lys-ONp. Dramatic improvement in yield and product purity was attained by systematically varying the solvent, base and catalyst (HOBt). Optimal conditions found for preparation of 2 were DMSO as solvent, DIEA as base, and no catalyst. Indeed, the addition of HOBt did not result in any improvement and complicated purification of the product. The overall yield of coupling and deprotection was 72%, based on UPOC.

Since the solubility properties of the dipeptides were better, DMF/H₂O was used as the coupling solvent for preparing all tripeptides. We noticed that the yield of the coupling reaction varied significantly from batch to batch for (oct)Lys-(oct)Lys-UPOC (2). The decreases in yield were consistently accompanied by the appearance of an impurity during the coupling reaction. The impurity was detected during the monitoring of the reaction by TLC, had an R_f slightly higher than the starting unprotected amino acid or dipeptide, and was visible under UV light but did not give a positive reaction with ninhydrin, indicating that a free amine was not present. Small amounts of this impurity were isolated during the purification of 2, and NMR analysis showed it to be the N-formylated dipeptide. This formylation was correlated to the presence of variable amounts of formic acid in the starting dipeptide. Formate

salts can be formed during precipitation of the products of catalytic transfer hydrogenation where formic acid is the hydrogen donor. NMR analysis showed that 9 exists at least partially as a formate salt. This kind of chain terminating side reaction has been described for couplings with active esters of amino components present as acetate salts, and would involve the formation of mixed anhydride intermediates^{17, 18}. The side reaction was not detected during the synthesis of the other tripeptides (1, 3, 4, 5) where the starting dipeptides were previously purified by HPLC and were present as trifluoroacetates. Since many laboratories now routinely use catalytic transfer hydrogenation with formic acid as the donor for deprotection of peptides, it is advisable to exchange the formate salt prior to chain extension.

Polyoxin B was purified according to ¹⁹, and its structure confirmed by ¹H and ¹³C nmr spectroscopy. The proton NMR of Leucyl-polyoxin B (5) in DMSO showed the expected resonances, but the H-1' doublet as well as one of the CONH doublets were split (see experimental section). This was attributed to the coexistence of two stable conformers of roughly the same populations in DMSO. This phenomenon was not observed in any of the other tripeptides (1 to 4), although the spectrum of one of the dipeptides (m-F-Phe-UPOC, peak II) in DMSO-d₆ showed a split CONH

doublet, a split H-6 doublet, a broad NH-3 resonance and a complex H-1', H-5' region. For this latter compound the spectrum was not simplified even when run at 55°C. However when the nmr sample was frozen, thawed, and the spectrum run immediately at 25°C the split peaks collapsed into the expected pattern (analogous to that observed for peak I, see experimental section).

L-4-oxalysine¹² and (D,L)-*meta*-fluorophenylalanine were protected with benzoyloxycarbonyl groups (Z) by standard procedures²⁰. Boc-FMDP was prepared according to¹⁴. All three protected amino acids were activated as *p*-nitrophenyl esters, coupled to UPOC and deprotected, to yield dipeptides 10, 11 and 12. The two epimers of 11 were separated by HPLC, and the peak with faster mobility (peak I) identified as the L,L diastereomer on the basis of its hydrolysis by *C. albicans* peptidases and inhibition of chitin synthetase. In contrast the slow moving epimer (peak II) was resistant to hydrolysis under the same conditions and did not inhibit chitin synthetase (see Biological Results).

BIOLOGICAL RESULTS AND DISCUSSION

Inhibition of Chitin Synthetase

The synthetic polyoxins and nikkomycins fall into two distinct categories. As expected, none of the tripeptidyl derivatives (1-5) were efficient inhibitors of chitin synthetase (Table 7). The best inhibitor (5) was approximately 25 times less active than the parent compound (8, see Table 8). In comparison the nikkomycin derivatives (3, 4) were a minimum of 270 fold less active as compared to the parent compounds 6 and 7, respectively (Table 7 and Table 8). Of the dipeptidyl polyoxins (9, 10, 11, 12) only 9 and 11 (1,1 diastereomer) were reasonably good inhibitors (Table 8). In the case of the oxalysine containing polyoxin (10) the poor inhibition may be attributed to the high hydrophilicity of the Oly residue. In previous investigations, semisynthetic polyoxins with charged or polar side chains in the amine terminal residue were poor inhibitors of chitin synthetase from *C. albicans*^{11, 16} and phytopathogenic fungi²¹. Based on their poor inhibition of chitin synthetase, the tripeptidyl antibiotics are expected to be active against *C. albicans* only if they are efficiently transported into this yeast and hydrolyzed to an active drug. Of the four synthetic dipeptidyl derivatives, only 9 and 11 would be expected to kill the yeast by inhibition of cell wall biosynthesis.

Competition for peptide transport

The tripeptidyl nucleoside antibiotics synthesized in this study (Table 7) were designed to be transported by permeases able to handle tripeptides⁷. Once inside the cell, they were expected to be hydrolyzed by cytoplasmic peptidases, releasing dipeptide chitin synthetase inhibitors. As shown in Table 7, with the exception of 2 none of the tripeptides competed with the uptake of (Leu)₂. It was unexpected that tripeptide 2 would compete well with dipeptide uptake. Furthermore, compound 2 competed with tripeptide transport as well. However, all other tripeptidyl compounds (1, 3, 4, and 5) competed poorly with the tripeptide substrate (Met)₃. These results were surprising, because based on the structural specificity of the tripeptide transport system in *C. albicans*⁷ the additional leucyl residue at the amine terminus was predicted to result in increased affinity.

The nikkomycins, 6 and 7, have high affinity for the dipeptide transport system (Table 8), while polyoxin B, (8), does not compete with the uptake of either (Leu)₂ nor (Met)₃. Among the synthetic dipeptides, only 9 and 10 were reasonably well recognized by the system transporting (Leu)₂ (Table 8). In the case of 10 this is somewhat unexpected given its high hydrophilicity and the low affinity of the oligopeptide transport system of *C. albicans* for lysine containing peptides⁷. Perhaps more

surprising is the lack of transport inhibition shown by 11 which is structurally very close to the nikkomycins, although much more hydrophobic. Previous investigations had indicated that hydrophobic peptides are better inhibitors of peptide transport in *C. albicans* than are hydrophilic peptides⁷.

Hydrolysis by cell extracts

The tripeptidyl polyoxins are ineffective in vitro inhibitors but may be activated by intracellular enzymes to generate polyoxins and nikkomycins which are effective chitin synthetase inhibitors (Tables 7 and 8). We found that cell extracts from *C. albicans* H-317 degraded 3, 4 and 5 to leucine and the corresponding polyoxin and nikkomycins. Degradation of 3 and 4 was faster than that seen for norleucyl-uracil polyoxin C (Nle-UPOC)⁹ whereas degradation of 5 was somewhat slower. Under the conditions used for this assay, Nle-UPOC was completely hydrolyzed after a 1 hr incubation with cell extract, 3 and 4 after 0.5 hr, and 5 after approximately 3 hr. 1 and 2 were not degraded at all. These results, as well as the resistance to hydrolysis by 9 and other analogs^{16,11} suggest that the very hydrophobic and bulky residue in the second amino acid of 1 and 2 stabilizes both peptide bonds toward peptidase degradation by *Candida* cell extract. The only synthetic

dipeptide which was readily hydrolyzed by the cell extract was the L,L-diastereomer of 11 (peak I) (see Table 8). Both amino acid components were formed as products, as seen by TLC. Peak II was resistant to hydrolysis, indicating that it is the D,L diastereomer. The hydrolysis of the dipeptide 12 proceeded very slowly, half of the peptide being consumed in about 3 hr as monitored by HPLC.

Inhibition of growth of *C. albicans*

We studied the effect of peptides (1-12) and the toxic amino acids on the growth of three strains of *C. albicans* (Tables 7, 8 and 9). Two of the strains were wild type (H-317 and 124) whereas strain 124N5 is resistant to nikkomycin. It was necessary to determine the toxicity of the component amino acids prior to evaluation of 10-12. *m*-F-Phe has been found to be inactive against *C. albicans* (strains B311 and 759) unless it is transported inside the cell by peptide carriers^{13, 22}. Similar results were observed against the strains examined in this investigation (Table 9). Oxalysine shows some toxicity against all the strains tested (Table 9). In contrast to the reported lack of toxicity of FMDP when tested against *C. albicans* AMB 25 (MIC $\geq$ 200 ug/mL)²³, this amino acid showed high toxicity toward the three strains tested here causing growth inhibition at 7.8 ug/mL (Table 9).

The dipeptide 12 showed low toxicity (Table 8), a fact that can be explained in terms of its failure to inhibit chitin synthetase and its low transportability into the cell. On the other hand, the low toxicity of dipeptide 10, a poor chitin synthetase inhibitor which has a much higher affinity for the (Leu)₂ transport system, can be explained in terms of its resistance to hydrolysis to toxic Oly if it reaches the cytoplasm. The non-toxicity of 11 despite its being a good chitin synthetase inhibitor and a good substrate for peptidases, can be explained by its low transportability into the cytoplasm.

None of the tripeptidyl polyoxins were effective anticandidal agents (Table 7). Leu-nikkomycin X which was the most potent synthetic analog had an MIC=83 ug/mL against *C. albicans* 124. This MIC is lower than that found for the dipeptide 9 despite the fact that the dipeptide is a 60-fold more efficient inhibitor of chitin synthetase. Thus it appears that 3 behaves as a prodrug. It is transported into the pathogen and hydrolyzed to the toxic nikkomycin X inside the cell. Its low effectiveness is explained by its low affinity for both the di- and oligopeptide transport systems. The low toxicity of 1 and 2 is not surprising because neither of these compounds is hydrolyzed by cell extract peptidases and the intact

tripeptides are poor inhibitors of chitin synthetase. In comparison to 3, compound 5 is hydrolyzed at a slower rate inside the cell (see above) and releases polyoxin B, which is not as potent a chitin synthetase inhibitor as nikkomycin X (Table 8). Those differences explain the limited effectiveness of 5 against the yeast.

The difference in toxicities of 3 and 4 is difficult to explain, since their rate of transport, hydrolysis, and inhibiting power of the released moiety is very similar. One possible explanation is that although their extent of affinity for the oligopeptide transport system is almost the same, Leu-nikkomycin Z is not translocated to a significant extent to an intracellular site. Supporting this hypothesis are the data of Yadan *et al.*¹⁰ who showed that in *C. albicans* nikkomycin and dimethionine had similar K_m values for the dipeptide transport system (4 μ M and 6 μ M, respectively) whereas their respective V_m values were 0.15 pmoles min⁻¹ and 3.5 pmoles min⁻¹.

Recent findings²⁴⁻²⁶ indicate that two chitin synthetase activities are present in *Saccharomyces cerevisiae*. Chitin synthetase 1 is the major *in vitro* activity measurable but is not essential for cell growth and appears to be a repair or auxiliary enzyme. Chitin synthetase 2 of *S. cerevisiae* is essential for chitin

synthesis and septum formation. If multiple chitin synthetases exist in *C. albicans* as well, then it is possible that the *in vitro* activity we measure does not accurately reflect *in vivo* chitin synthesis of the cell. In this case, the correlation between growth inhibition and enzyme activity measured may not be valid. Further studies must be carried out to determine the number of isozymes of chitin synthetase present in *C. albicans*.

CONCLUSION

This study reports the successful synthesis of novel dipeptides and tripeptides which are analogs of polyoxins and nikkomycins. Dipeptides containing Oly or FMDP were resistant to Candidal peptidases and were consequently ineffective drugs. A polyoxin analog containing *m*-F-Phe was not recognized by the peptide transport system.

One tripeptidyl nikkomycin (3) was hydrolyzed to an active component after entering the yeast and therefore appears to behave as a prodrug. The results show that the structural specificity for peptide transport in *C. albicans* may be much more complex than previously believed. It is clear that additional knowledge of those molecular interactions which are involved in the actual translocation of a peptide into the yeast must precede the design of

chitin synthetase inhibitors which will efficiently kill this pathogen.

EXPERIMENTAL SECTION

High-performance liquid chromatography (HPLC, analytical, Semipreparative) was carried out on a Waters chromatograph (Waters Associates, Milford, MA) equipped with two Model 510 solvent delivery units, a U6K injector and a Model 481 variable wavelength UV detector, a Model 680 automated gradient controller, and a Model 730 data module. Analytical separations were carried out on a Waters micro-Bondapak C18 reversed-phase column (30 cm x 3.9 mm, particle size 10 μ m) or an Aquapore spheri-5 RP18 column (22 cm x 4.6 mm). The mobile phase was MeOH-H₂O-TFA at a flow rate of 1.4 mL/min. The absorbance of the column eluants was recorded at 254 nm for all polyoxins. For purification of final products two Waters C18 semipreparative columns (30 cm x 7.8 mm and 15 cm x 19 mm) or an Aquapore RP-18 (25 cm x 10 mm) were used. TLC analysis was carried out on silica gel 60 F₂₅₄ plates, 0.25 mm (Merck, Darmstadt).

The NMR spectra were recorded on a Bruker WP-200SY instrument, and the chemical shift values are reported in ppm relative to internal tetramethylsilane. Optical

rotations were determined on a Perkin-Elmer model 141 polarimeter. For elemental analyses, freeze-dried samples were further dried in vacuum over P_2O_5 at $65^\circ C$ for 48 hours. Elemental analyses were performed by Galbraith laboratories, Knoxville, Tennessee. Nikkomycin X was purified by chemochromatography⁸ from a crude obtained from Bayer Pflanzenschutz Leverkusen. Nikkomycin Z (Calbiochem, San Diego, CA) was purified before use by HPLC (solvent, TFA 0.10%; flow rate, 4.50 mL/min; 19 cm x 150 mm column; 2 mg shots). Polyoxin B was obtained from an agricultural fungicide preparation supplied by Kaken Chemical (Honkomagome, Tokyo, Japan) as described in ¹⁹, where it is referred to as peak I. UPOC (uracil polyoxin C) was prepared according to Damodaran *et al.*¹⁵, and obtained mainly as a zwitterion. N- α -benzyloxy-carbonyl-N- ϵ -octanoyl-lysine-*p*-nitrophenyl ester was prepared as described in ¹⁶. L-4-Oxalysine was obtained as a gift from Dr. Hong-Long Zhang, Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai.

N²-(*tert*-Butoxycarbonyl)-N³-(4-methoxyfumaroyle)-L-2,3-diaminopropanoic acid was prepared according to reference ¹⁴. (D,L)-*meta*-Fluoro-phenylalanine was purchased from Chemical Dynamics Corporation (South Plainfield, New Jersey).

Polyoxin B (8)

Polyoxin B was purified according to ¹⁹ and was HPLC homogeneous. Its structure was confirmed by ¹H and ¹³C nmr. The assigned proton resonances at 200 MHz in DMSO-d₆ were: δ_H 4.7 (1H, dd, H-5'), 5.3-5.7 (m, OH), 5.81 (1H, d, J=6 Hz, H-1'), 6.53 (2H, broad s, CONH₂), 7.39 (1H, s, H-6), 8.0 (3H, broad, NH₃⁺), 9.0 (1H, d, J=8.2 Hz, CONH), 11.48 (1H, broad s, NH-3). The presence of a H on the 5 position is ruled out because H-6 appears as a singlet at 7.39 ppm. A CH₃ group on this position is also ruled out because there is no resonance near 2 ppm corresponding to 5-CH₃. A carboxyl group on position 5 has also been ruled out ¹⁹ on the basis of the UV spectrum. The group on the 5 position is CH₂OH, as shown by the 50 MHz ¹³C proton decoupled spectrum. This resonance appears at 57.0 ppm (in water, C₆D₆ external reference δ =128.0) and is assigned by comparison to 5-hydroxymethyl uridine as a model compound (59 ppm in H₂O, DSS internal reference) and by the splitting to a triplet in the off-resonance proton decoupled spectrum. Polyoxin G, which also has an hydroxymethyl group in position 5 but differs from polyoxin B in that the OH group in position 3'' is replaced by an H atom, was ruled out on the basis of the ¹³C-¹H splitting patterns under off-resonance proton decoupling conditions. The other resonances were: δ 55.2, 56.8 (α -CH), 69.1, 70.2, 70.4 (CH, ribose and polyoxamic acid side chain), 73.0 (CH,

ribose), 83.3 (CH, ribose), 91.0 (CH, ribose), 65.8 (CH₂-5''), 114.4 (C-5), 140.4 (C-6), 152.0 (C-2), and 159.5, 165.3, 168.3, and 172.2 (C-4, CONH, OCONH, and CO₂H, unassigned).

N-ε-(Octanoyl)-lysyl-(uracil polyoxin C) ((oct)Lys-UPOC)
(9)

Z-ε-(C₇H₁₅CO)-Lys-ONp⁴ (116 mg, 0.22 mmol) and UPOC (60 mg, 0.2 mmol) were suspended in DMSO (2 mL). Diisopropylethyl amine was added (35 uL, 0.2 mmol) (pH ≈ 7) and the mixture stirred at room temperature for 20 hr. (The solution cleared up after a few hours). After acidification with acetic acid (0.1 mL), the solvents were evaporated in vacuo to dryness, the residue suspended in 1% acetic acid, triturated with a spatula, filtered, washed with water and dried under vacuum. The solid was resuspended in ether, triturated and filtered. Yield 0.108 g (80%). The product, Z-ε-(C₇H₁₅CO)-Lys-UPOC was homogeneous on silica thin layers using n-BuOH/AcOH/H₂O (4:1:2, R_f = 0.59) as eluant, and was subjected to transfer hydrogenation using Pd black (≈30 mg) and 90% formic acid (0.3 mL) in methanol (3 mL). The initial suspension clears with time and after 3 hr the catalyst was removed by filtration through celite, the filtrate evaporated to dryness, and the residue suspended in ether, triturated and filtered. Yield 78 mg (72%).

(Oct)Lys-UPOC was 97% pure as judged by HPLC, and its mobility identical to a standard¹⁶. $R_f = 0.45$ (1-butanol:acetic acid:water, 4:1:2).

N- ϵ -(Octanoyl)-lysyl-N- ϵ -(octanoyl)-lysyl-UPOC (2)

9, (40 mg, 74 μ mol) from the previous step, and Z- ϵ -(C₇H₁₅CO)-Lys-ONp (46.8 mg, 89 μ mol) were dissolved in DMF (0.7 mL). N-methylmorpholine (8.15 μ L, 74 μ mol) was added and the mixture stirred at room temperature for 15 hr. After acidification with acetic acid (0.1 mL), the solvents were evaporated in vacuo to dryness. The residue was treated with ether, filtered, washed with 4% citric acid, water, and dried under vacuum. The product was subjected to transfer hydrogenation as described for 9 for 1.5 hr. The catalyst was removed by filtration through celite, the filtrate evaporated to dryness, and the residue suspended in ether and filtered. 36.2 mg of crude (oct)Lys-(oct)Lys-UPOC was obtained, 24 mg of which were purified by reversed-phase HPLC using a 250 x 10 mm semiprep Aquapore RP-18 column with 0.1% TFA/MeOH 47:53 as eluant. 13.9 mg of pure 2 was obtained after concentration, suspension in ether and filtration. Yield = 31%, based on 9. $R_f = 0.57$ (1-butanol:acetic acid:water, 4:1:2). $[\alpha]_D^{25} = +8.8$ (c=0.2, DMF). NMR. $\delta_H^{DMSO-d_6}$ 0.85 (6H, t, J=6.2 Hz, -CH₃), 1.1-1.8 (32H, m, -CH₂-), 2.03 (4H, m, α -CH₂ of

octanoyl), 3.00 (4H, m, ϵ -CH₂ of Lys), 3.76, 4.0-4.2 (4H, m, H-2', 3', 4', α -CH, unassigned), 4.35 (1H, m, α -CH), 4.50 (1H, m, α -CH), 5.26 (1H, broad, OH), 5.44 (1H, broad, OH) 5.68 (1H, d, J=8.1 Hz, H-5), 5.79 (1H, d, J=6.4 Hz, H-1'), 7.54 (1H, d, J=8.1 Hz, H-6), 7.79 (2H, t, 5.8 Hz, ϵ -NH of Lys), 7.8-8.5 (4H, unresolved, two broad peaks at 8.05 and 8.44 ppm when run at 400 MHz, NH₃⁺ and CONH respectively), 8.54 (1H, d, J=8.3 Hz, CONH), 11.38 (1H, broad s, NH-3). Elemental Analysis. Found: C, 51.47; H, 7.33; N, 10.70%. Calculated for C₃₈H₆₅O₁₁N₇ · CF₃CO₂H · H₂O : C, 51.77; H, 7.39; N, 10.56%.

N- α -Formyl-N- ϵ -octanoyl-lysyl-UPOC

Small amounts (1 mg) of this formylated dipeptide were isolated as an impurity during the HPLC purification of tripeptide 2. R_f=0.47 (1-butanol:acetic acid:water, 4:1:2). NMR. $\delta_{\text{H}}^{\text{DMSO-d}_6}$ 0.86 (3H, t, J=6.1 Hz, CH₃), 1.15-1.74 (16H, m, -CH₂-), 2.02 (2H, t, J=7.3 Hz, α -CH₂ of octanoyl), 2.99 (2H, m, ϵ -CH₂ of Lys), 3.95-4.15 (3H, m, H-2', 3', 4'), 4.43 (1H, m, α -CH), 4.56 (1H, m, α -CH), 5.28 (1H, broad, OH), 5.44 (1H, broad, OH), 5.64 (1H, d, J=8.3 Hz, H-5), 5.79 (1H, d, J=6.4 Hz, H-1'), 7.45 (1H, d, J=8.3 Hz, H-6), 7.75 (2H, broad t, ϵ -NH of Lys), 8.03 (1H, s, CH of formyl), 8.30 (1H, d, J=8.8 Hz, CONH), 8.41 (1H, broad, CONH), 11.37 (1H, s, NH-3). FAB mass spectrometry indicated

a molecular ion $[M + H^+]$ at 570.2 daltons. Calculated MW: 569.6 daltons.

Leucyl-N-(octanoyl)-lysyl-UPOC (1)

The procedure was similar to the preparation of 2, and was carried out using Z-Leu-ONp (51 mg, 132 μ mol) and 9 (72.1 mg, 110 μ mol) in 1 mL of DMF. 9 had been previously purified, according to ¹⁶, and was a TFA salt. The coupling time was 42 hr and 50 mg of Z-Leu-(oct)Lys-UPOC (Yield = 58%) was obtained. It was homogeneous on silica thin layers, with n-BuOH/AcOH/H₂O (4:1:2, R_f=0.67) as eluant. The deprotection took 1.5 hr, and 32.5 mg of the tripeptide 1 was obtained (overall yield = 42%) which was homogeneous on silica thin layers, with the same eluant (R_f=0.54) and did not need further purification. Reversed-phase HPLC analysis (spheri-5 RP-18 column, eluant 0.1% TFA/MeOH 45:55) showed higher than 99% purity. $[\alpha]_D^{25} = +8.9$ (c=0.09, DMF). NMR. $\delta_H^{DMSO-d_6}$ 0.86 (9H, m, -CH₃), 1.15-1.75 (19H, m, -CH₂- and CH), 2.02 (2H, t, J=7.2 Hz, α -CH₂ of octanoyl), 2.98 (2H, m, ϵ -CH of Lys), 3.67, 4.0-4.4 (6H, m, H-2', 3', 4', 3 α -CH), 5.26 (1H, d, J=4.4 Hz, OH), 5.60 (1H, d, J=8.1 Hz, H-5), 5.75 (1H, d, J=6.8 Hz, H-1'), 7.67 (1H, d, J=8.1 Hz, H-6), 7.82 (1H, t, J=5.1 Hz, ϵ -NH of Lys), 7.95 (1H, broad d, J=5.9 Hz, CONH), 8.49 (1H, d, J=7.3 Hz, CONH). Elemental Analysis. Found: C, 51.63; H,

7.50; N, 11.62%. Calculated for $C_{30}H_{50}O_{10}N_6 \cdot 2.5 H_2O$: C, 51.49; H, 7.92; N, 12.01% .

Leucyl-nikkomycin Z (4)

Nikkomycin Z (double TFA salt) (38 mg, 50 μ mol) and Z-Leu-ONp (23.2 mg, 60 μ mol) were dissolved in a mixture of DMF (0.5 mL) and H_2O (0.15 mL). NMM (17 mL, 150 μ mol) was added and the mixture allowed to react, with stirring, at room temperature, for 24 hr. After acidification with acetic acid (50 μ L), the solvents were evaporated in vacuo, the residue treated with ether, filtered and subjected to transfer hydrogenation for 2 hr as described above. After filtration through celite, washing the filter with water, evaporation and precipitation with ether, 35.4 mg of crude material was obtained, which was purified by HPLC on a semiprep Aquapore RP-18 column with 0.1% TFA/MeOH 95:5 as eluant. Homogeneous 5 (26.1 mg) was obtained. (Yield = 45%). $[\alpha]_D^{25} = +17.0$ ($c=0.1$, AcOH). $R_f = 0.27$ (1-butanol: acetic acid:water, 4:1:2). NMR. $\delta_H^{DMSO-d_6}$ 0.66 (3H, d, $J=6.4$ Hz, CH_3-3''), 0.89 (6H, m, CH_3 of Leu), 1.45-1.70 (3H, m, $\beta-CH_2$ and $\gamma-CH$ of Leu), 3.8-4.2, 4.5-4.7 (H-2', 3', 4', 5', 2'', $\alpha-CH$ of Leu, unassigned), 4.75 (1H, broad, H-4''), 5.70 (1H, d, $J=7.8$ Hz, H-5) 5.81 (1H, d, $J=5.4$ Hz, H-1'), 7.2-7.4 (2H, m, H-3''', 4'''), 7.49 (1H, d, $J=7.8$ Hz, H-6), 8.0-8.2 (4H, broad, NH_3^+ and H-6'''), 8.43 (1H,

d, $J=8.3$ Hz, CONH), 8.63 (1H, d, $J=7.8$ Hz, CONH), 9.95 (1H, broad, ArOH), 11.42 (1H, s, NH-3). Elemental Analysis.

Found: C, 34.70; H, 4.23; N, 7.45%. Calculated for

$C_{26}H_{36}O_{11}N_6 \cdot 3.5 CF_3CO_2H \cdot 8 H_2O$: C, 34.41; H, 4.86; N, 7.30%.

Leucyl-nikkomycin X (3)

Nikkomycin X (double TFA salt) (40 mg, 53 μ mol) was processed as described above for nikkomycin Z, with the exception that 1.2 equivalents of Boc-Leu-ONp were used as the coupling reagent. Coupling time was 30 hr. Deprotection was accomplished with TFA/ CH_2Cl_2 (50:50) in 0.5 hr. The solvents were evaporated under vacuo, and the residue precipitated with ether and filtered. Crude product (36.2 mg) was purified by HPLC using a 15 cm x 19 mm C-18 column, with TFA 0.05% / MeOH 96:4 as the eluant. Homogeneous 3 (20.5 mg) was obtained after concentration and freeze-drying (Overall yield = 39%). $[\alpha]_D^{25} = +18.0$ ($c=0.1$, AcOH). $R_f = 0.29$ (1-butanol:acetic acid:water, 4:1:2). NMR. $\delta_H^{DMSO-d_6}$ 0.66 (3H, d, $J=6.6$ Hz, CH -3''), 0.89 (6H, m, CH_3 of Leu), 1.45-1.70 (3H, m, β - CH_2 and γ -CH of Leu), 3.75 - 4.65 (H-2', 3', 4', 5', 2'', α -CH of Leu, unassigned), 4.76 (1H, broad, H-4''), 5.47 (1H, d, $J=4.3$ Hz, H-1'), 7.2-7.4 (2H, m, H-3''-4''), 7.72 (1H, s, H-5), 8.0-8.2 (4H, broad m, NH_3^+ and H-6''), 8.46 (1H, d, $J=9.0$ Hz, CONH),

8.64 (1H, d, $J=9.5$ Hz, CONH), 9.24 (1H, s, CHO), 11.2 (1H, s, NH-3). Elemental Analysis. Found: C, 38.96; H, 4.57; N, 8.84%. Calculated for $C_{26}H_{36}O_{11}N_6 \cdot 3 CF_3CO_2H \cdot 2.5 H_2O$: C, 38.60; H, 4.45; N, 8.44%.

Leucyl-polyoxin B (5)

Polyoxin B (TFA salt) (62 mg, 0.1 mmol) and Z-Leu-ONp (46 mg, 0.12 mmol) were dissolved in a mixture of DMF (1 mL) and H_2O (0.3 mL). NMM (22.4 μ L, 0.2 mmol) was added and the mixture stirred at room temperature for 44 hr. After acidification with acetic acid (0.1 mL), the solvents were evaporated in vacuo, and the residue treated with ether and filtered. 40 mg of Z-Leu-polyoxin B (yield = 53%) was obtained. $R_f = 0.72$ on silica thin layers with n -BuOH/AcOH/ H_2O (1:1:1) as the eluant.

This product was subjected to transfer hydrogenation in methanol (2 mL) with palladium black (≈ 30 mg) and 90% formic acid (0.2 mL) for 24 hr. The crude product was purified on a C_{18} reversed-phase column using 0.05% TFA/MeOH (100:1) as eluant. The fractions containing pure 4 were pooled, evaporated under vacuum and freeze-dried. 7.2 mg of material, showing more than 92% purity by HPLC analysis, was obtained. Other fractions containing 4 were concentrated under vacuum and reinjected onto the 19 cm x

150 mm C-18 column with 0.05% TFA/MeOH (100:2) as the eluant. 4.5 mg of 100% HPLC homogenous material was obtained after concentration and freeze-drying. (Overall yield: 11%). $[\alpha]_D^{26} = +14.0$ (c=0.1, AcOH). Rf = 0.22 (1-butanol:acetic acid:water, 4:1:2). NMR. $\delta_H^{DMSO-d_6}$ 0.89 (6H, d, J=5.4 Hz, CH₃ of Leu), 1.45-1.80 (3H, m, β -CH₂ and γ -CH of Leu), 3.6-4.3, 4.4-4.7 (m, unresolved, H-2', 3', 4', 3'', 4'', CH₂-5'', CH₂OH, 3 α -CH), 5.81 and 5.82 (1H, split d, J=5.9 Hz, H-1'), 6.4-6.8 (2H, broad, OCONH₂), 7.42 (1H, s, H-6), 8.0-8.2 (3H, m, NH₃⁺), 8.65 and 8.90 (1H, split d, J=8.1 Hz, CONH), 8.74 (1H, d, J=7.8 Hz, CONH), 11.50 (1H, s, NH-3), 13.15 (1H, broad s, CO₂H). Elemental Analysis. Found: C, 34.60; H, 3.90; N, 8.46%. Calculated for C₂₃H₃₆O₁₄N₆ · 3.5 CF₃CO₂H · H₂O : C, 34.73; H, 4.03; N, 8.10%.

L-4-Oxalysyl-UPOC. (Oly-UPOC), (10)

N²,N⁶-di-Benzoyloxycarbonyl-L-4-oxalysine was prepared by standard procedures²⁰ from L-4-oxalysine, and crystallized from ether. (Rf = 0.52, CH₂Cl₂/MeOH 10:3.2). It was converted into the *p*-nitrophenyl ester by coupling to *p*-nitrophenol with dicyclohexylcarbodiimide in THF and crystallizing from isopropanol. Yield: 50%. This product was homogeneous on silica thin layers (Rf=0.76, CH₂Cl₂/MeOH 95:5). NMR. $\delta_H^{CDCl_3}$ 3.32-3.47 (2H, m, ϵ -CH₂), 3.53-3.66

(2H, m, δ -CH₂), 3.84 (1H, dd, J=9.3 Hz, 3.0 Hz, β -CH), 4.08 (1H, dd, J=9.3, 3.0 Hz, β -CH), 4.77 (1H, m, α -CH), 4.99 (1H, broad, ϵ -NH), 5.08 (2H, s, benzylic CH₂), 5.15 (2H, s, benzylic CH₂), 5.79 (1H, d, J=7.2 Hz, α -NH), 7.2-7.4 (12H, m, phenyl), 8.17 (2H, d, J=8.5 Hz, meta of *p*-NO₂-phenyl). (α,ϵ -dibenzylloxycarbonyl)-Oly-ONp (85.7 mg, 160 μ mol) was coupled to UPOC·HCl (46.9 mg, 145 μ mol) in DMSO (1.45 mL) in the presence of DIEA (50.5 μ L, 290 μ mol) at room temperature for 25 hr. The reaction mixture was acidified with acetic acid (300 μ L) and the solvents evaporated in vacuo to dryness. The residue was suspended and triturated in AcOH 1%, filtered, suspended and triturated in ether, and filtered. This diprotected dipeptide was homogeneous on silica gel thin layers (Rf=0.29, 1-butanol:acetic acid:water, 4:1:2). It was suspended in MeOH (4 mL) and subjected to transfer hydrogenation with palladium black ($\approx$ 40 mg) and 90% formic acid (0.4 mL) with stirring at room temperature overnight. The catalyst was removed by filtration through Celite, the filtrate evaporated under vacuo to dryness, and the residue suspended in Et₂O, triturated and filtered. Yield 33.4 mg (45%). Rf=0.09 (1-butanol:acetic acid:water, 4:1:2). Due to the extremely high hydrophilicity of this material, no satisfactory reversed-phase HPLC analysis could be performed, but its homogeneity was supported by TLC, NMR and elemental analysis. $[\alpha]_D^{25} = +25.8$ (c=0.12, H₂O). NMR. $\delta_H^{DMSO-d_6}$

2.95 (2H, m, ϵ -CH₂), 3.57 (m, β -CH₂, γ -CH₂), 4.02-4.35 (m, H-5', α -CH, H-2'-3'-4'), 5.62 (1H, d, J=8.0 Hz, H-5), 5.78 (1H, d, J=6.6 Hz, H-1'), 7.76 (1H, d, J=8.0 Hz, H-6), 8.01 (1H, d, J=7.7 Hz, CONH), 8.24 (2H, s, formate counterion). Elemental analysis. Found: C, 38.64; H, 5.92; N, 13.25%. Calculated for C₁₅H₂₃O₉N₅ · 2 CH₂O₂ · H₂O : C, 38.71; H, 5.54; N, 13.28%.

meta-Fluorophenylalanyl-UPOC (11)

Benzoyloxycarbonyl-(D,L)-*m*-fluorophenylalanine was prepared by standard procedures²⁰ from (D,L)-*m*-fluorophenylalanine and crystallized from ether. Yield, 81%, (Rf=0.56, n-butanol:acetic acid:water, 4:1:2). It was converted into the *p*-nitrophenyl ester by coupling to *p*-nitrophenol with DCC in THF and crystallizing from isopropanol. Yield 52%. NMR. $\delta_{\text{H}}^{\text{DMSO-d}_6}$ 3.0-3.3 (2H, m, β -CH₂), 4.61 (1H, m, α -CH), 5.05 (2H, s, benzylic CH₂), 7.03-7.43 (11H, m, phenyl), 8.14 (1H, d, J=7.7 Hz, OCONH), 8.32 (2H, d, J=9.0 Hz, *meta* of *p*-NO₂-phenyl). (D,L)-Z-*m*-fluorophenylalanine-*p*-nitrophenylester was coupled to UPOC·HCl as described for compound 10. The protected dipeptide was homogeneous on silica gel thin layers (Rf=0.46, 1-butanol:acetic acid:water, 4:1:2). It was subjected to transfer hydrogenation as described above. Yield 59 mg (55%). The two diastereomers were observed on TLC (Rf=0.17 and 0.21, 1-butanol: acetic acid: water,

4:1:2) and were separated by semipreparative HPLC. 37 mg of the product were injected onto the Waters C₁₈ column and eluted with 0.025% TFA in H₂O:acetonitrile, linear gradient of 5% to 10% acetonitrile in 1 hr. In this way, 18.5 mg of one diastereomer (peak I) and 11.5 mg of the other (peak II) were obtained.

Peak I: $[\alpha]_D^{25} = +44.1$ ($c=0.17$, H₂O). NMR. $\delta_H^{DMSO-d_6}$ 2.8-3.2 (m, β -CH), 3.99 (1H, t, $J=3.8$ Hz, H-4'), 4.0-4.3 (3H, m, α -CH, H-2', H-3'), 4.62 (1H, m, H-5'), 5.35 (broad, OH), 5.51 (1H, d, $J=5.4$ Hz, OH), 5.67 (1H, d, $J=8.1$ Hz, H-5), 5.80 (1H, d, $J=5.8$ Hz, H-1'), 7.02-7.45 (4H, m, phenyl), 7.53 (1H, d, $J=8.1$ Hz, H-6), 8.2 (very broad s, NH₃⁺), 8.96 (1H, d, $J=8.1$ Hz, CONH), 11.4 (1H, broad s, NH-3). Elemental analysis. Found: C, 43.00; H, 4.08; N, 9.53%. Calculated for C₁₉H₂₁O₈N₄F · C₂F₃O₂H · H₂O : C, 43.16; H, 4.14; N, 9.59%.

Peak II: $[\alpha]_D^{25} = -28.6$ ($c=0.07$, H₂O). NMR. $\delta_H^{DMSO-d_6}$ 2.8-3.2 (m, β -CH₂), 3.9-4.0 (1H, m, H-4'), 4.0-4.3 (3H, m, α -CH, H-2', H-3'), 4.53 (1H, m, H-5'), 5.63 (1H, d, $J=8.3$ Hz, H-5), 5.76 (1H, d, $J=5.8$ Hz, H-1'), 6.97-7.44 (4H, m, phenyl), 7.61 (1H, d, $J=8.3$ Hz, H-6), 8.83 (1H, broad d, $J=7.0$ Hz, CONH), 11.38 (1H, broad s, NH-3). Elemental analysis. Found: C, 40.44; H, 3.94; N, 9.10%. Calculated for C₁₉H₂₁O₈N₄F · 1.3 C₂F₃O₂H · 2.2 H₂O : C, 40.52; H, 4.20; N, 8.75%.

N-(4-Methoxyfumarooyl)-L-2,3-diaminopropanoyl-UPOC.
(FMDP-UPOC), (12)

N²-(*tert*-Butoxycarbonyl)-N³-(4-methoxyfumarooyl)-L-2,3-diaminopropanoic acid was converted into the *p*-nitrophenyl ester by using dicyclohexylcarbodiimide in THF and crystallizing from ethyl acetate:ether. Yield: 75%. This product was homogeneous on silica thin layers using CH₂Cl₂/MeOH (8:2, R_f=0.80) as eluant. NMR $\delta_{\text{H}}^{\text{DMSO-d}_6}$ 1.41 (9H, s, *tert*-butyl), 3.68 (2H, m, β -CH₂), 3.73 (3H, s, OCH₃), 4.40 (1H, m, α -CH), 6.63 (1H, d, J=15.4 Hz, C=CH), 7.02 (1H, d, J=15.4 Hz, C=CH), 7.41 (2H, d, J=8.9 Hz, *ortho*-phenyl), 7.58 (1H, d, J=7.5 Hz, OCONH), 8.34 (2H, d, J=8.9 Hz, *meta*-phenyl), 8.82 (1H, t, J=5.7 Hz, β -NH). Boc-FMDP-ONp (104 mg, 238 μ mol) was coupled to UPOC·HCl (70 mg, 216 μ mol) in DMSO (2.16 mL) with added DIEA (75.3 μ L, 432 μ mol) by stirring at room temperature for 17 hr. The solvent was evaporated in vacuo to dryness, and the residue suspended and triturated in ether, and filtered. (R_f=0.32, 1-butanol:acetic acid:water 4:1:2). The protecting group was removed by treatment with 2 mL of 2N HCl/dioxane, and 93.1 mg of product were obtained (R_f=0.21, 1-butanol:acetic acid:water, 4:1:2, yellow color with ninhydrin spray). 60 mg of this crude product were purified by injection onto the Aquapore RP-18 column in 10 mg shots (solvent: TFA 0.025%; flow rate, 4.50 mL/min). 12 mg of a $\geq$ 98% pure

fraction was obtained, as well as 26.5 mg of a $\approx$ 95% pure fraction. Overall yield (coupling-deprotection-

purification): 41%. $[\alpha]_D^{25} = +20.0$ ($c=0.11$, H_2O). NMR.

$\delta_H^{DMSO-d_6}$ 3.55 (2H, m, $\beta-CH_2$), 3.73 (3H, s, OCH_3), 3.94-4.26 (4H, m, H-2'-3'-4' and $\alpha-CH$), 4.68 (1H, dd, H-5'), 5.35 (1H, d, $J=5.0$ Hz, OH), 5.54 (1H, d, $J=5.7$ Hz, OH), 5.67 (1H, d, $J=8.3$ Hz, H-5), 5.78 (1H, d, $J=6.0$ Hz, H-1'), 6.61 (1H, d, $J=15.6$ Hz, $C=CH$), 6.98 (1H, d, $J=15.6$ Hz, $C=CH$), 7.45 (1H, d, $J=8.3$ Hz, H-6), 8.24 (broad, NH_3^+), 8.69 (1H, m, $\beta-NH$), 9.09 (1H, d, $J=8.2$ Hz, CONH), 11.44 (1H, s, NH-3) Elemental analysis. Found: C, 35.76; H, 4.00; N, 10.16%. Calculated for $C_{18}H_{23}O_{11}N_5 \cdot 1.5 C_2F_3O_2H \cdot 2.6 H_2O$: C, 35.86; H, 4.26; N, 9.96%.

Biological Methods

Organisms: The following yeast strains were studied: *C. albicans* H-317, a clinical isolate from the Center for Disease Control, Atlanta, GA; *C. albicans* 124 and *C. albicans* 124 Nik^R, nikkomycin sensitive and resistant strains, respectively, from Dr. William Kingsbury, Smith, Kline and French Laboratories, Philadelphia, PA.

Chitin Synthetase Assay

Total chitin synthetase activity obtained in a mixed

membrane fraction from *C. albicans* H-317 was assayed in the presence and absence of polyoxin compounds by measuring the incorporation of N-acetylglucosamine into chitin. The detailed procedures have been previously reported^{27, 28}.

Peptidase Assay

The procedures used to prepare a cell extract of *C. albicans* H-317 in assays for hydrolysis of the antibiotics were previously reported). To assay for hydrolysis, TLC as well as HPLC were used. 0.5 mg of the antibiotic was dissolved in 50 uL of H₂O:DMSO (50:50) and mixed at 36°C with 200 uL of cell extract (3.75 mg protein/mL). The mixture was incubated at 36°C, and at 20 min intervals small portions were withdrawn and directly chromatographed. Nle-UPOC⁹ was used as a control, and was completely hydrolyzed after 1 hr under these conditions.

Transport Studies

The procedures and transport conditions to assay for the uptake of labeled trimethionine and dileucine in the presence of polyoxin compounds have been previously described^{27, 16}. Cells of strain H-317 were harvested at 8×10^6 cells/mL by centrifugation after growth in yeast nitrogen base with isoleucine (0.5%) as nitrogen source.

After two washes with sterile distilled water, the cells were resuspended in 2% glucose and warmed at 37°C for 10 min. An equal volume of cells was added to citric acid (10 mM)-KH₂PO₄ (10mM) buffer at pH 3.5 with radioactive trimethionine (2.9×10^{-5} M, 1 μ Ci/ μ mol)²⁹ or pH 5.5 buffer with radioactive dileucine (5.6×10^{-5} M, 44 μ Ci/ μ mol)³⁰ to a total volume of 1 mL. Analogs were added at 10-fold the molar concentration of the transport substrates for competition. At intervals of 0, 1, 2, 3, and 4 min, 180 μ L of the reaction mixtures were withdrawn and applied to prewet filters (pore size 0.45 μ m) and washed twice with 2mL of cold distilled water. The filters were placed in 5 mL of scintillation cocktail and counted. The rate of uptake was calculated as nanomoles of peptide taken up/milligram of dry weight cells per minute.

Determination of MIC

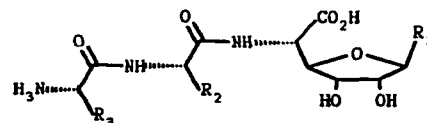
The methods and procedures employed for the determination of the MIC values in the presence of the polyoxin analogs have been described previously except that ammonium sulfate (0.5%) was used for most experiments as the nitrogen source^{27, 16}. The MIC was recorded as the lowest concentration of drug that resulted in 5% morphologically aberrant cells as visualized by microscopy after a 24-48 h incubation at 37°C in yeast nitrogen base

(Difco Labs, Detroit, MI).

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Abbreviations used: DMF, dimethylformamide; DMSO, dimethylsulfoxide; DSS, sodium 3-(trimethylsilyl)-1-propanesulfonate; FAB, fast atom bombardment; FMDP, N³-(4-methoxyfumaryl)-L-2,3-diaminopropanoic acid; HOBt, hydroxybenzotriazole; ID₅₀, concentration at which chitin synthetase is inhibited by 50%; MIC, minimum inhibitory concentration; lowest concentration that clearly inhibits growth; Nle, norleucine; NMM, N-methylmorpholine; (oct)Lys-, N-ε-(octanoyl)-lysyl; Oly, L-4-oxalysine; TFA, trifluoroacetic acid.

Table 7 Synthetic tripeptide chitin synthetase inhibitors

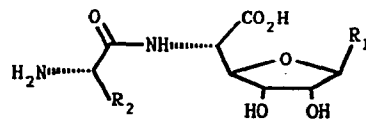


COMPOUNDS	R ₁	R ₂	R ₃	Chitin Synthetase Inhibition (ID ₅₀ ^a , ug/mL)	NIC ^b (ug/mL)			Hydrolysis ^c	% Transport inhibition ^d	
					H-317	124	124N5		(Leu) ₂	(Met) ₃
<u>1</u> Leu-(oct)Lys-UPOC		CH ₃ (CH ₂) ₆ CONH-(CH ₂) ₄ -	(CH ₃) ₂ CHCH ₂ -	50	> 666	> 666	> 666	-	0	32
<u>2</u> (oct)Lys-(oct)Lys-UPOC		CH ₃ (CH ₂) ₆ CONH-(CH ₂) ₄ -	CH ₃ (CH ₂) ₆ CONH-(CH ₂) ₄ -	80	> 666	> 666	> 666	-	60	56
<u>3</u> Leu-Nikkomycin X			(CH ₃) ₂ CHCH ₂ -	30	166	83	333	+	0	22
<u>4</u> Leu-Nikkomycin Z			(CH ₃) ₂ CHCH ₂ -	100	> 666	666	> 666	+	0	28
<u>5</u> Leu-Polyoxin B			(CH ₃) ₂ CHCH ₂ -	20	333	333	666	+	0	27

^aConcentration at which chitin synthetase is inhibited by 50%. ^bMinimum inhibitory concentration; lowest concentration of drug that clearly inhibited growth of strains H-317, 124 and 124N5 of *C. albicans*. ^c(+) compound was hydrolyzed by *C. albicans* H-317 extract, (-) no hydrolysis by cell extract.

^dPercent inhibition of transport of (Leu)₂ or (Met)₃ into *C. albicans* H-317. A 10-fold molar excess of analogue was used in comparison to the transport substrates (Leu)₂ or (Met)₃.

Table 8 Dipeptide chitin synthetase inhibitors



COMPOUNDS	R ₁	R ₂	Chitin Synthetase Inhibition (ID ₅₀ ,ug/mL)	MIC (ug/mL)			Hydro-lysis	% Transport inhibition	
				H-317	124	124N5		(Leu) ₂	(Met) ₃
<u>Naturally occurring:</u>									
<u>6</u> Nikkomycin X			0.1	5	2.5	333	-	90	30
<u>7</u> Nikkomycin Z			0.3	20	10	666	-	80	20
<u>8</u> Polyoxin B			0.8	40	20	166	n.d. ^a	0	0
<u>Synthetic:</u>									
<u>9</u> (oct)Lys-UPOC		CH ₃ (CH ₂) ₆ CONH-(CH ₂) ₄ -	0.5	>666	666	>666	-	50	10
<u>10</u> Oly-UPOC		H ₂ N(CH ₂) ₂ OCH ₂ -	100	>1000	>1000	>1000	-	58	0
<u>11</u> m-F-Phe-UPOC			2.0 (>100) ^b	>1000 ^c	>1000 ^c	>1000 ^c	+ (-) ^b	6 (3) ^b	0
<u>12</u> FMDP-UPOC			100	250	500	1000	-	7	0

^an.d. is not done. ^bPeak I (peak II). ^cMixture of diastereomers.

Table 9 Effect of various toxic amino acids on growth of *C. albicans* strains^a

Amino acid	MIC (ug/mL)		
	H-317	124	124N5
Oxalysine	31	31	31
(D,L)-m-F-phenylalanine	>1000	>1000	>1000
FMDF ^b	7.8	7.8	15.6

^a*C. albicans* strains H-317, 124, and 124N5 were grown in the presence of various amounts of toxic amino acid and their minimum inhibitory concentration (MIC) determined. ^bFMDF is N³-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid.

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Appendix A: Hydrolysis of nikkomycin by cell extracts from
C. albicans.

Polyoxin D has been shown to resist hydrolysis by intracellular peptidases from *C. albicans* (61). This resistance to peptidases is necessary for the fungicidal action of the polyoxins, since the constituent amino acids are not chitin synthetase inhibitors. Nikkomycins are more toxic than the polyoxins and it is reasonable to expect that they would also resist enzymatic hydrolysis. There is no previous report in the literature about the stability of nikkomycins toward hydrolysis by fungal peptidases and we had to investigate this point. It has been mentioned at the end of Chapter I that nikkomycins X and Z are slowly degraded in a neutral aqueous solution at room temperature.

It is seen in Figures 5 and 6 that the negative slopes for the HPLC areas of nikkomycin X versus time are very similar when cell extracts are or are not present. The same is seen for the positive slopes of the degradation products. This indicates that nikkomycin X is not hydrolyzed by cell extracts from *C. albicans*.

During the non-enzymatic hydrolysis, half of the original nikkomycin is consumed in 44 hr., under the conditions of the assay (see Figure 5).

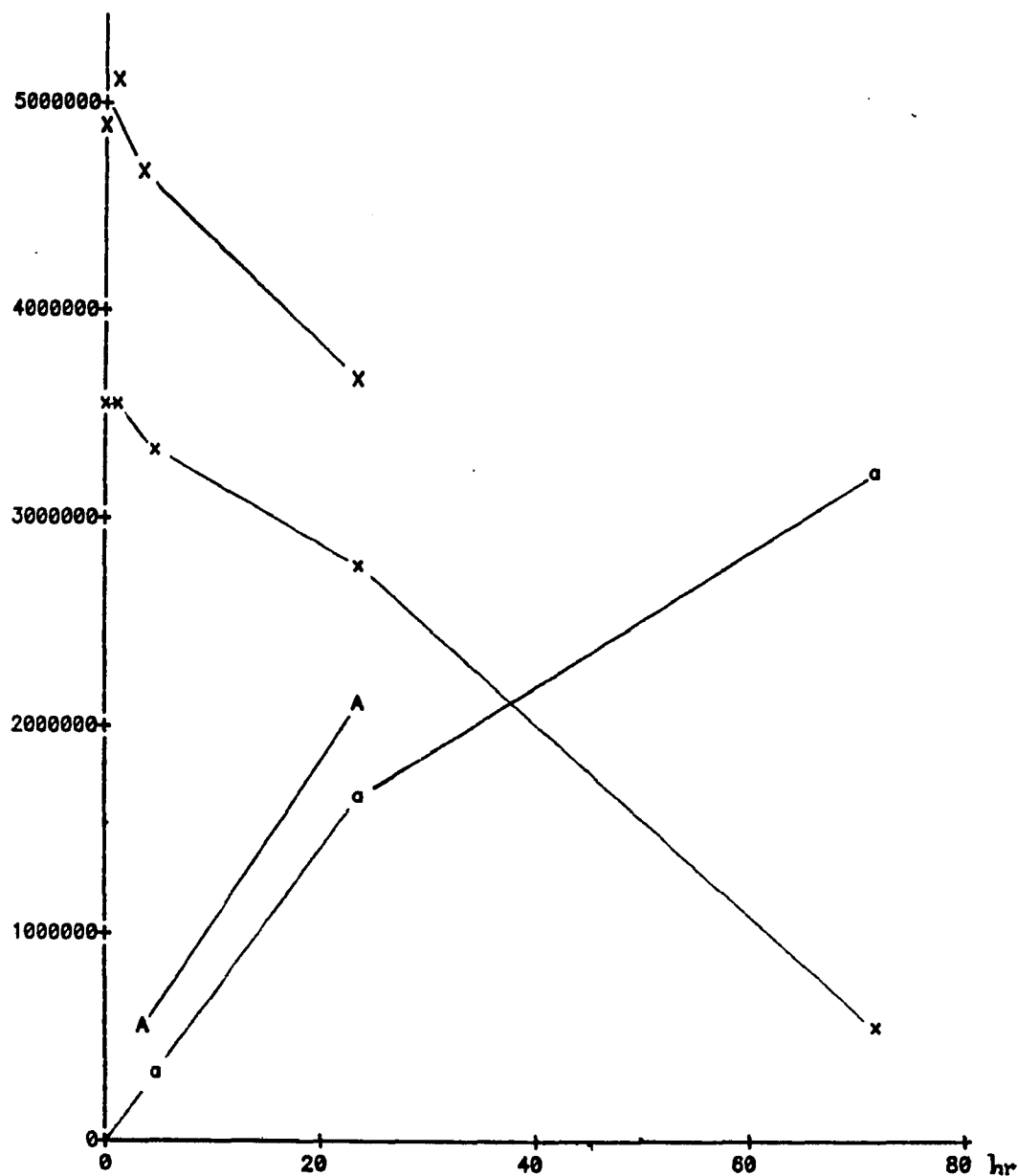


Figure 5 Plot of concentration of nikkomycin X vs time. Numbers on vertical axis are areas of HPLC peaks. X, x: nik X with and without *C. albicans* cell extract. A, a: degradation product(s) with and without cell extracts. 0.5 mg of antibiotic was dissolved in 250 μ L buffer tris.HCl 0.1 M, NaCl 0.1 M, pH 7.23 @ 20°C with or without cell extract and incubated at 37°C. 10 μ L samples were injected onto an Aquapore spheri-5 RP18 column (22 cm x 4.6 mm). The eluant was H₂O with 0.1% TFA and with a linear gradient of MeOH from 0 to 10% in 30 min. The flow rate was 1.4 mL/min and the UV detector was set to 254 nm. The cell extracts were described in the previous section.

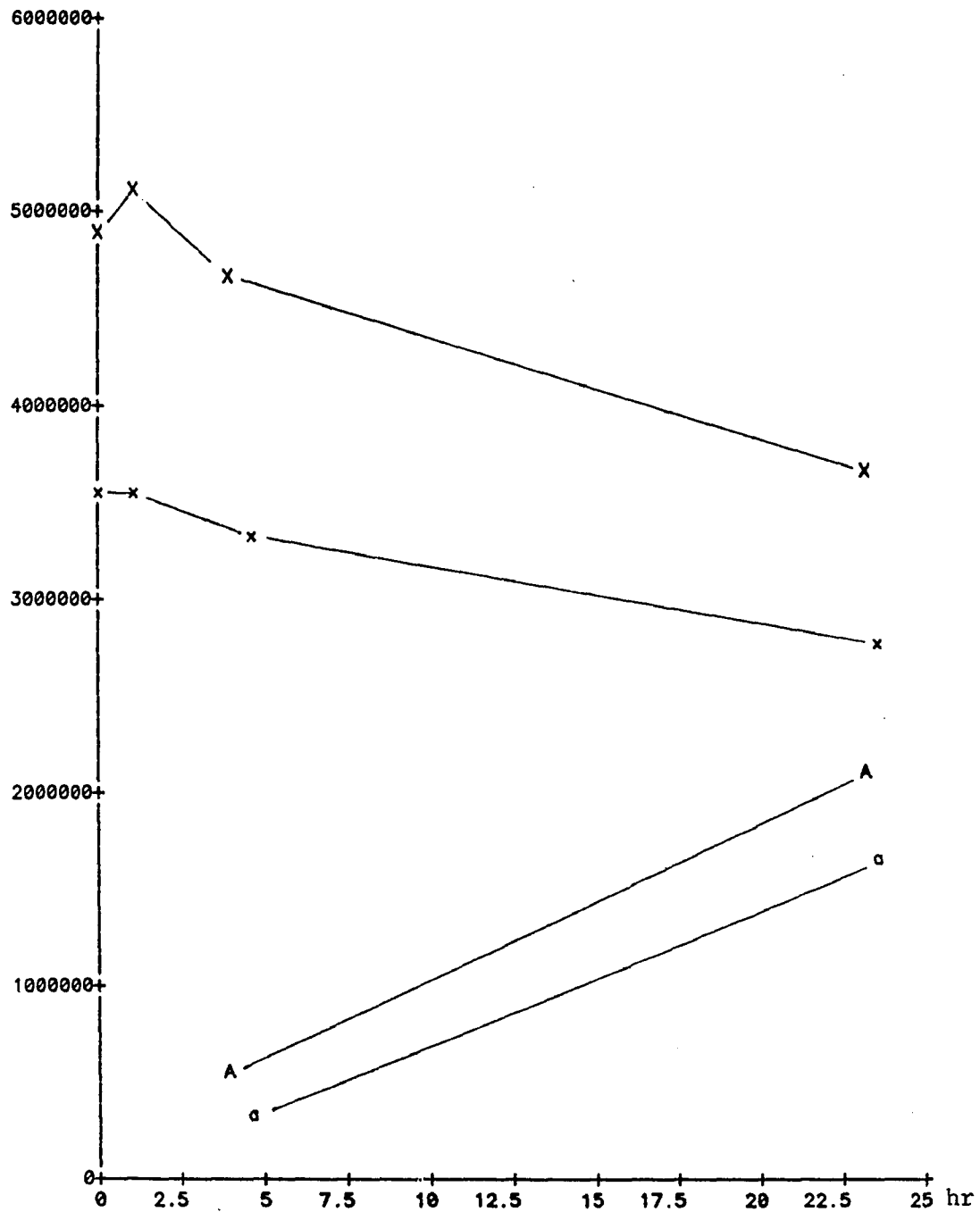


Figure 6 Plot of concentration of nikkomycin X vs time.
Blow up of Figure 5.

Chapter IV. Conformational studies on nikkomycin X.

Solution studies.

The conformational study of nikkomycin X in aqueous solution is a first step toward understanding the molecular interactions of this antibiotic with the proteins which recognize it as a substrate (peptide permeases) or as an inhibitor (chitin synthetase). Once these interactions are understood, it should be possible to design a drug that would be translocated more efficiently into the cytoplasm, and/or that would be a better chitin synthetase inhibitor.

In 1984, Rathman *et. al.* (50) presented conformational parameters in DCl/D₂O for the aminoacids nikkomycin C, [1-(5'-amino-5'-deoxy- β -D-allofuranosyluronic acid)uracil], and nikkomycin C_x, [1-(5'-amino-5'-deoxy- β -D-allofuranosyluronic acid)uracil]. These two antibiotics are the carboxy terminus aminoacids of nikkomycin X and nikkomycin Z respectively (see Table 2, Chapter I). Using coupling constants $^3J(C-2,H-1')$, they deduced an anti conformation for both nucleosides, with an angle of the uracil ring plane $\chi = 32^\circ$ or 88° for nikkomycin C, and an angle of the imidazolinone ring plane $\chi = 22^\circ$ or 98° for nikkomycin C_x. They also calculated relative populations of rotamers of the exocyclic C-4' C-5' bond using proton-proton and

carbon-proton coupling constants. For both antibiotics the trans (τ) rotamers were the most populated ones (70%). Finally, using the coupling constants of the ribose ring protons they calculated pseudorotational parameters and relative populations of types N and S conformers. They assumed a geometry for the S-type conformers identical to the one found in the crystal (50).

As a preliminary experiment for the study of the conformation of nikkomycin X in aqueous solution, ^1H -nmr spectra of dilute samples of nikkomycin X (≈ 2.5 mg/mL) were run in water and different techniques for suppressing the immense solvent peak were tried. A closed capillary with D_2O was placed inside the nmr tube for signal lock. The top spectrum in Figure 7 was obtained without suppression. The presence of the water resonance introduced serious distortions associated with the high dynamic range of the spectrum, including inadequate analog-to-digital converter resolution, and computer memory overflow. The solvent resonance, with its first and higher order spinning sidebands, obscures most of the spectrum and severely distorts the baseline. The bottom spectrum (Figure 7) was obtained by irradiating the sample with the $\overline{1331}$ sequence proposed by Hore (82), which is designed to selectively excite all the protons except those of the solvent. In this

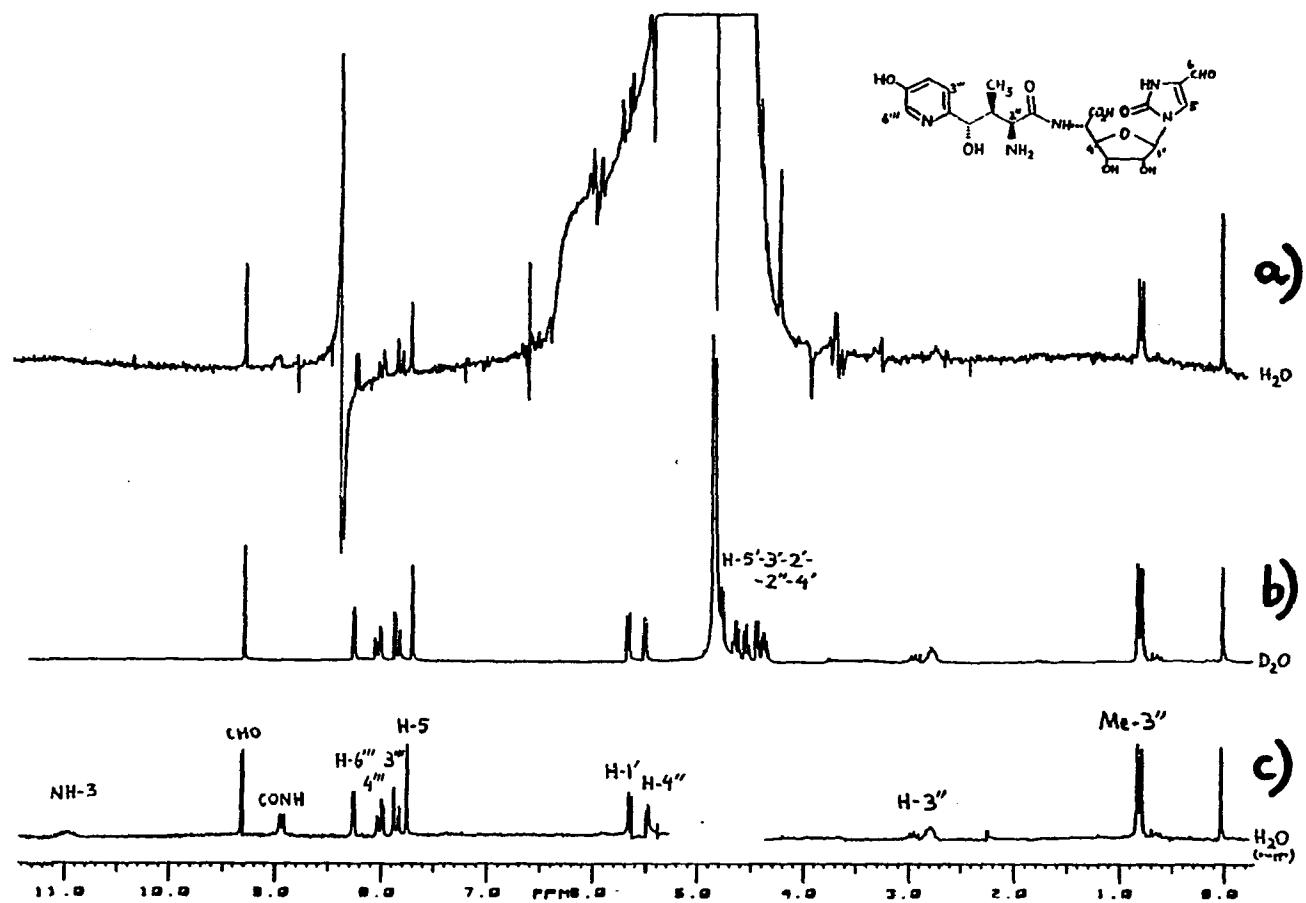


Figure 7 200 MHz ^1H -nmr spectrum of nikkomycin X, obtained
 a) in H_2O ; b) in D_2O ; c) in H_2O with the 1331
 pulse sequence. Reference: internal DSS.

way it was possible to obtain a higher than 250-fold suppression of the solvent signal and observe exchangeable protons, such as the amide NH (≈ 8.95 ppm) and the imidazolinone NH-3 (≈ 11 ppm). These latter resonances cannot be seen in D₂O solution (Figure 7, middle spectrum).

Attempts were also made to suppress the water peak by irradiating the sample with a selective low power saturating pulse prior to pulsing and observation. This procedure, although straightforward to implement, runs the risk of saturation transfer by chemical exchange from the solvent to the solute spins. Indeed, even though the information that could be obtained in a run at pH 5.65 was roughly the same as when using the $\overline{1331}$ sequence, the amide NH and the imidazolinone NH-3 resonances did not show up (data not shown). Therefore the saturating pulse technique proved inferior to the $\overline{1331}$ sequence in this particular case.

The problems associated with the $\overline{1331}$ pulse technique were the following. First of all, the resonances within ≈ 0.6 ppm from the water peak could not be detected. This region includes both α -CHs and all the ribose ring protons except H-1'. Secondly, due to the limited spectral region that is excited, three independent runs were necessary in

order to obtain a complete spectrum. Specifically, the bottom spectrum in Figure 7 is a composite of three separated spectra where the regions of interest were selectively excited by using three different sets of delays between pulses (0.7, 1.2 and 2.5 msec).

In the next section it will be shown how pH titration studies yielded information on the conformation of nikkomycin X in aqueous solution. Since the $1\bar{3}3\bar{1}$ pulse sequence allowed the monitoring of both exchangeable and non-exchangeable protons, it was the method of choice for carrying out those studies.

Conformational studies of nikkomycin X in aqueous solution.

Conformational Studies of Nikkomycin X in Aqueous Solution

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SYNOPSIS

Nikkomycin X is a peptidyl-nucleoside antibiotic that inhibits chitin synthesis in fungi. Information on its conformation in aqueous solution was obtained from pH titration studies in which the nmr shifts of exchangeable and nonexchangeable protons were monitored. These studies and nuclear Overhauser effects support an unfolded or conformationally flexible structure for the antibiotic, and the *syn* and *anti* conformations of the nucleoside moiety were found to coexist. The conformation of the ribose ring was determined using a two-state model; a slight shift from type N to type S conformers occurred as the pH was raised from 1 to 6.

INTRODUCTION

Nikkomycin X (also called neopolyoxin A; Figure 1), a peptidyl nucleoside antibiotic produced by *Streptomyces tendae*, is a strong inhibitor of chitin synthetase from pathogenic fungi.¹⁻³ This antibiotic is toxic to the zoopathogenic fungus *Candida albicans* only at relatively high concentrations, a fact that is very likely due to its poor binding and translocation into the cell by permeases.^{4,5}

Determination of the potential conformations and flexibility of nikkomycin X in aqueous solution is a first step toward understanding the molecular interactions of this and other related antibiotics with large membrane associated proteins such as peptide permeases and chitin synthetase. Previous analysis⁶ of long-range coupling constants ³*J*(C-2, H-1') for the isolated carboxy terminus amino acid of nikkomycin X [1-(5'-amino-5'-deoxy β-D-allofuranosyluronic acid)-4-formyl-4-imidazoline-2-one] in DCl/D₂O indicated that the nucleoside moiety has an *anti* conformation, with an angle of the imidazolinone ring plane of $\chi = 22^\circ$ or 98° . The relative

populations of rotamers of the exocyclic C4'-C5' bond, as well as N- and S-type ribose conformers in DCl/D₂O, were calculated using proton-proton and carbon-proton coupling constants.⁶

Since *C. albicans* grows well at a pH of approximately 4-4.5 while the cytoplasmic pH in yeast is very close to neutrality,⁷ we decided to study the conformation of nikkomycin X at different pH values. We therefore measured ¹H-nmr chemical shifts and coupling constants in the pH range from 1 to 6. These titrations were carried out on dilute aqueous nikkomycin, using the ¹³³I pulse sequence proposed by Hore⁸ to generate the nmr spectra. Under these conditions the strong water resonance was barely excited and we were able to gain access to information on exchangeable protons, such as the amide and the imidazoline NH resonances, without the risk of saturation transfer. Information on these exchangeable protons would not be available from samples run in D₂O. We also explored the possible existence of a folded conformation of nikkomycin X by measuring nuclear Overhauser effects (NOEs) upon irradiation of the resolved proton signals.

MATERIALS AND METHODS

Nikkomycin X was purified as described previously⁹ and obtained as a double trifluoroacetate salt. All nmr samples were 0.1 M in KCl in order to avoid a

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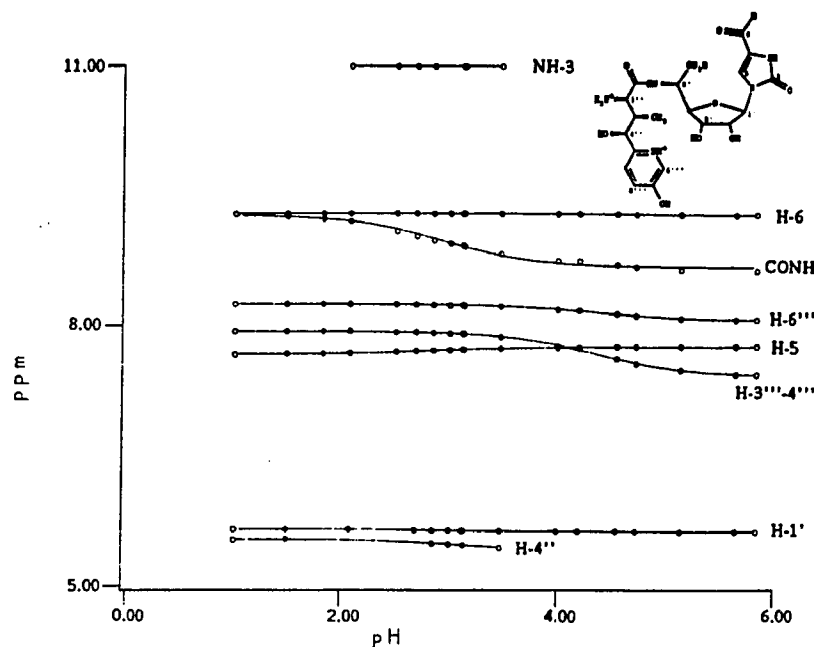


Figure 1 The pH titration shifts of some nikkomycin X resonances at 25°C. The assignments are indicated on the right.

change in ionic strength upon addition of the titrants (0.1 N HCl or 0.1 N NaOH). The concentration of nikkomycin X was ≈ 2.5 mg/mL. Sodium 3-(trimethylsilyl)-1-propanesulfonate (DSS) was used as an internal reference, and a closed capillary containing D₂O was placed inside the nmr tube for signal lock. The reference DSS has been shown to be essentially independent of pH when compared to acetone in D₂O (pH 1–11).¹⁰ Therefore no attempt was made to correct for possible pH-dependent shifts. ¹H-nmr spectra at 200 MHz were obtained on a Bruker spectrometer equipped with an ASPECT 2000A computer, at $25 \pm 1^\circ\text{C}$. The temperature was controlled with a Bruker variable temperature unit (VTU) by passing heated air through the probe. The VTU was calibrated before each run with an ethylene glycol thermometer. This consisted of a closed capillary containing ethylene glycol, inside a 5-mm nmr tube with DMSO-*d*₆. The separation in hertz between the methylene and hydroxyl proton resonances was previously correlated with the temperature by obtaining direct temperature measurements with a thermocouple at different VTU settings. Water suppression was achieved with a ¹³³I pulse sequence.⁸ The transmitter pulse was attenuated (9 db) and a net tip angle of $\approx 45^\circ$ was obtained with

a total ¹³³I pulse width of 24 μs . The delay between pulses was set at 0.7, 1.2, and 2.5 ms, in order to examine different regions of the spectrum. In each case, 200 transients were recorded and the relaxation delay was 8.0 s.

The pH readings were obtained with a Radiometer pH meter, model PHM82, using a combination glass pH electrode (Wilma, Buena, NJ) specially designed to fit into a 5-mm nmr tube. Since the solutions were not buffered, the pH was measured prior to and after the nmr runs, and the readings were averaged. In virtually all cases the variation in the pH was less than 0.05 pH units. The titration curves reflect points obtained by first adding base until the pH rose to pH ~ 6 and additional points obtained by back titrating with acid. Curve fitting of the titration data was carried out with the regression analysis routine of the Lab One NMR 1 software from New Methods Research, Inc. (Syracuse, NY). The iterative method used is based on the Levenberg-Marquardt algorithm.¹¹

Equilibrium NOEs were measured at 200 MHz on the Bruker spectrometer and at 400 MHz on a JEOL spectrometer. Nikkomycin concentrations were 4 mg/mL at 200 MHz and 8.5 mg/mL at 400 MHz in D₂O containing 0.10 M KCl. Measured pD's

CONFORMATIONAL STUDIES OF NIKKOMYCIN X

(meter readings uncorrected for the isotope effect on the glass electrode) were 4.0 (at 400 MHz) and 4.5 (at 200 MHz). At this latter pD* the H-5 resonance was resolved from the hydroxypyridine resonances. Irradiation times of 3.5 s (400 MHz) and 2.5 s (200 MHz) were used, and all measurements were made at room temperature. In the run at 200 MHz the following precautions were taken in preparing the sample: D₂O low in paramagnetic impurities (Aldrich, Milwaukee, WI) (minimum isotopic purity 99.96 atom%D) was used; the glassware was washed with a solution of EDTA in D₂O prior to use, and the final solution was subjected to eight freeze-pump-thaw cycles at 0.5 mm. Hg under dry helium. The magnitudes and number of NOEs observed were basically equivalent for both sets of experiments. Spectral analysis by computer simulation was carried out in some cases (see the results and discussion section) with PANIC (a LAOCOON type program¹²) on the ASPECT computer.

RESULTS AND DISCUSSION

The different protons in nikkomycin X produce titration curves reflecting the deprotonation of acidic groups. In the simplest case the curves are described by the Henderson-Hasselbach relationship:

$$\text{pH} = \text{pK}_a + \log \frac{A}{AH} \quad (1)$$

wherein AH and A are concentrations of protonated and deprotonated species, respectively.

Within the fast exchange condition, the chemical shift δ is linearly related to the fractional populations of species A and AH:

$$\delta = \frac{A}{A + AH} \delta_A + \frac{AH}{A + AH} \delta_{AH} \quad (2)$$

Table I ¹H-NMR Titration Parameters of Nikkomycin X in H₂O

Proton	δ_{AH}^a	$\Delta\delta^b$	pK _a ^c	J (Hz)
NH-3	11.00	$\pm 0.02^d$	—	—
H-6	9.28	$\pm 0.01^e$	—	—
CONH	9.28	-0.61	2.95 ± 0.05	7.0
H-6'''	8.247	-0.189	4.341 ± 0.025	nd ^f
H-3'''-4'''	7.933	-0.520	4.353 ± 0.020	nd
H-5	7.675	+0.080	2.82 ± 0.06 (2.90 ± 0.04) ^h	—
H-1'	5.654	-0.030	2.85 ± 0.06	$J_{1,2'} = 5.09 \pm 0.05^j$
H-4''	5.49	$\pm 0.05^g$	—	2.3 ^k
H-5' ^l	4.75 ^l	nd	nd	$J_{4',5'} = 3.62 \pm 0.05^j$
H-3' ^l	4.62 ^l	nd	nd	$J_{3',4'} = 5.45 \pm 0.05^j$
H-2' ^l	4.52 ^l	nd	nd	$J_{2',3'} = 5.33 \pm 0.05^j$
H-2'' ^l	4.42 ^l	nd	nd	4.6
H-4' ^l	4.35 ^l	nd	nd	—
H-3''	2.72	$\pm 0.02^g$	—	—
Me-3''	0.778	$\pm 0.007^g$	—	7.0

^a Calculated chemical shift in parts per million from internal DSS in the fully protonated nikkomycin, or average δ when the titration pattern was not simple or curve fitting was not attempted (see footnotes d and e). For samples run in D₂O, observed chemical shift (see footnote i).

^b Calculated $\Delta\delta = (\delta_A - \delta_{AH})$. ^lH-nmr titration shift in parts per million. Negative numbers indicate upfield shifts upon deprotonation.

^c Calculated pK_a corresponds to microconstant pk_i, within the assumptions referred to in the Appendix.

^d The variation in δ for NH-3 did not follow any discernible curve. It is likely that the true $\Delta\delta$ is less than 0.04 ppm.

^e No simple titration, or curve fitting not calculated. The chemical shift varied within the limits indicated about average δ .

^f Not determined.

^g Doublet broadened by "benzylic" long-range coupling to H-3''.

^h Values in parentheses obtained when point at lowest pH is omitted from the correlation (see Figure 2 and text).

ⁱ Sample run in D₂O at pD* 2.2.

^j Coupling constant calculated through computer simulation for sample run in D₂O at pD* 2.2.

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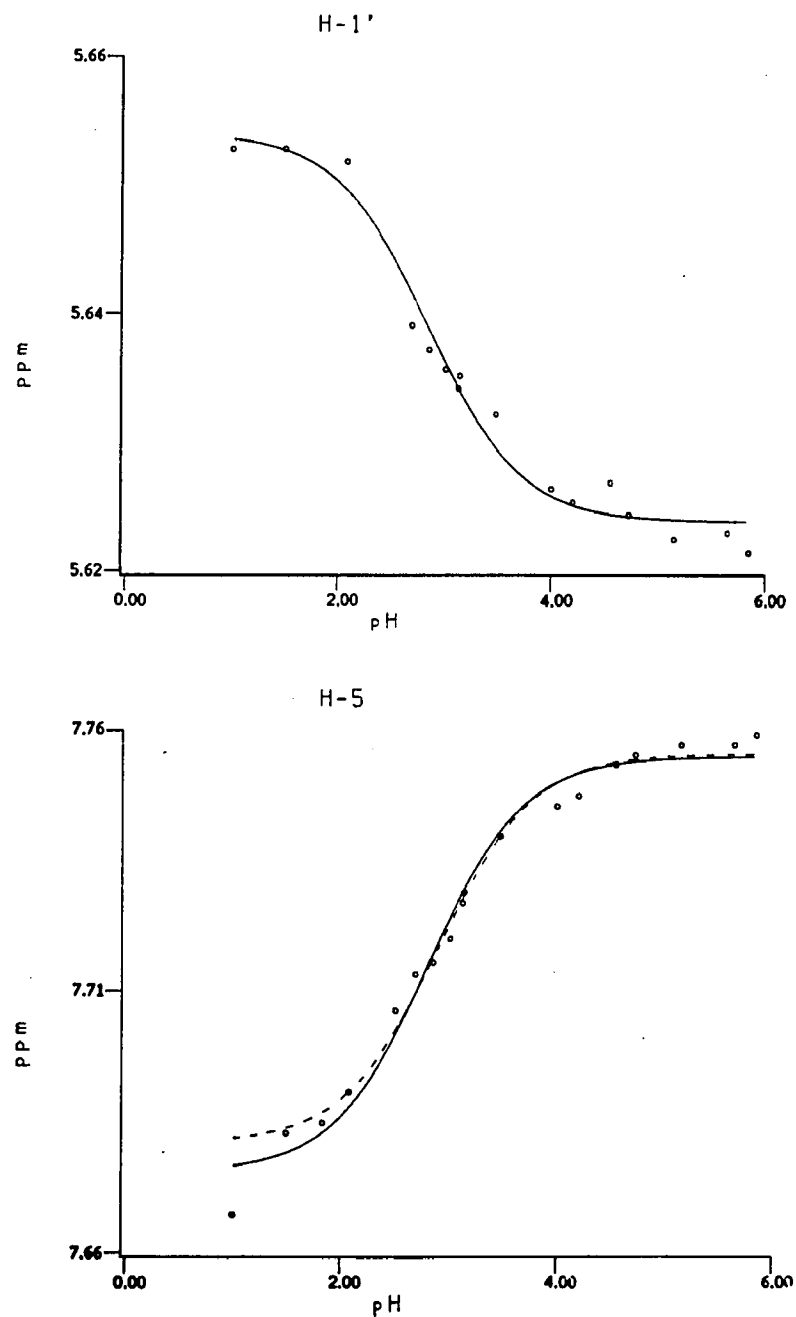


Figure 2 The pH titration shifts of H-1', H-5, and H-6". The dashed line on the plot for H-5 is obtained when the point at lowest pH is omitted from the correlation.

CONFORMATIONAL STUDIES OF NIKKOMYCIN X

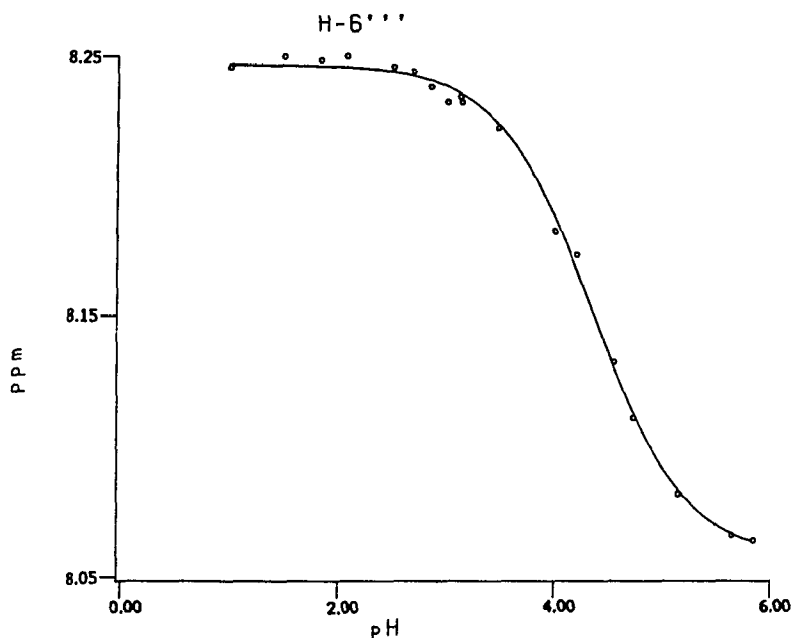


Figure 2. (Continued)

Thus, Eq. (1) can be written as

$$\text{pH} = \text{pKa} + \log \frac{\delta - \delta_{\text{AH}}}{\delta_{\text{A}} - \delta} \quad (3)$$

or rearranged to

$$\delta = \frac{\delta_{\text{AH}} + \delta_{\text{A}} 10^{(\text{pH}-\text{pKa})}}{1 + 10^{(\text{pH}-\text{pKa})}} \quad (4)$$

Equation (4) was used for nonlinear least-squares curve fitting of the titration data, treating the pKa and the chemical shifts of the fully protonated (δ_{AH}) and fully deprotonated species ($\delta_{\text{A}} = \delta_{\text{AH}} + \Delta\delta$) as adjustable parameters. In this way we were able to determine pKa values to better than ± 0.1 pKa unit and to test the fit of the data to the Henderson-Hasselbach model. The titration curves are shown in Figure 1, and best-fit values for the parameters are given in Table 1.

The amide proton resonance shows an upfield titration shift of -0.61 ppm. Intrinsic titration shifts (shifts that arise from interaction with an ionizable group mediated only via covalent bonds) are always to higher field upon deprotonation and have been shown to be on the order of -0.3 to -0.6 ppm for the amide proton of the C-terminal amino acid res-

idue in some short peptides.¹³ In those studies the pKa for the titration curve was identical to the pKa of the α -carboxyl group determined by ^{13}C -nmr.¹⁴ In the case of nikkomycin X, we found that the amide proton titration data agrees well with a curve generated by Eq. (4) (Figure 1). The calculated pKa for the ionization of the carboxyl group of nikkomycin X is 2.95 ± 0.05 . As the pH is raised the amide proton resonance of nikkomycin X starts to broaden due to an increase of the rate of exchange with the solvent, catalyzed by OH^- ions.^{15,16} The coupling constant for the doublet is no longer measurable at pH 5.15, and no attempts were made to run spectra above pH 6.

The protons of the pyridine ring show intrinsic titration shifts due to deprotonation of the pyridinium ion. These hydrogen atoms form an ABX system and the chemical shift of the center of the AB portion—H-3''' and H-4'''—in addition to the shift for the X part—H-6'''—are plotted in Figure 1. From both curves the same pKa value, 4.35, is obtained for the pyridine nucleus (Table I).

Both pKa values (carboxylic acid and pyridinium ion) are in excellent agreement with previously reported pKas.² The nearest additional ionizable group ($\alpha\text{-NH}_3^+$) has a pKa = 7.3² and therefore nikkomycin X behaves as a diprotic acid over the pH range

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of this study. As seen in Figure 1, deprotonation of the two acidic groups overlaps to a small extent (the pyridinium ion begins to deprotonate before carboxylate formation is complete). Equation (4) holds exactly for monoprotic acids or for stepwise deprotonation of polyprotic acids. Nevertheless, it can be used to a very good approximation for fitting our titration data for the following reasons: (a) The chemical shifts of the resonances studied show only one inflection point; they thus depend on the extent of protonation of only one of the acidic groups. (b) Since the two acidic groups are far apart in the molecule, the microscopic dissociation constant for one of them will not appreciably change if the other group is protonated or deprotonated (see Appendix).

The imidazolinone proton H-5 and the ribose proton H-1' show smaller titration shifts of +0.08 and -0.03 respectively. Expanded titration curves are shown in Figure 2. The calculated pKa values for these curves are in good agreement with the pKa deduced from the amide titration (Table I), and thus correspond to the ionization of the carboxyl group. For H-5 the correlation (and also the match of pKas) is improved if the point at lowest pH is omitted. This point (which shows the greatest deviation from the calculated curve) was obtained using a separate sample of nikkomycin dissolved directly in 0.1 N HCl without KCl (see the materials and methods section). All other points were obtained on the same nikkomycin sample.

We conclude, then, that the CONH, H-5 and H-1' shifts can be described by eq. (4), with an inflection at the pKa of the carboxylic acid. In the case of H-5 and H-1', this implies an intramolecular through-space interaction between these protons and the carboxyl group. For direct interaction of the carboxyl group and H-5 to occur, the nucleoside moiety has to adopt a conformation in which the imidazolinone ring is *anti* (Figure 3). Furthermore, the signs of the H-5 and H-1' titration shifts (Table I) can be rationalized on the basis of repulsion between the negative charge on the carboxylate anion and σ electrons in the C-H bonds.¹⁷ In the geometrical arrangement expected for nikkomycin in an *anti* conformation, these interactions will tend to shield H-1' and deshield H-5 from the magnetic field.

The possibility of an indirect effect of the carboxyl group titration on H-5 when the imidazolinone ring is in the *syn* conformation cannot be totally excluded. If the carboxyl group hydrogen bonds with the heterocyclic oxygen (oxygen on C-2), titration would affect this interaction and might result in a change in chemical shift of the H-5 resonance. Nevertheless, we view this possibility as unlikely since both H-6 and NH-3 exhibited significantly smaller changes than H-5 upon titration of the carboxyl group (Table I). We conclude that the titration behavior of H-5 supports the presence of an *anti* conformation for the heterocyclic ring. In any event the titration experiment provides evidence that the car-

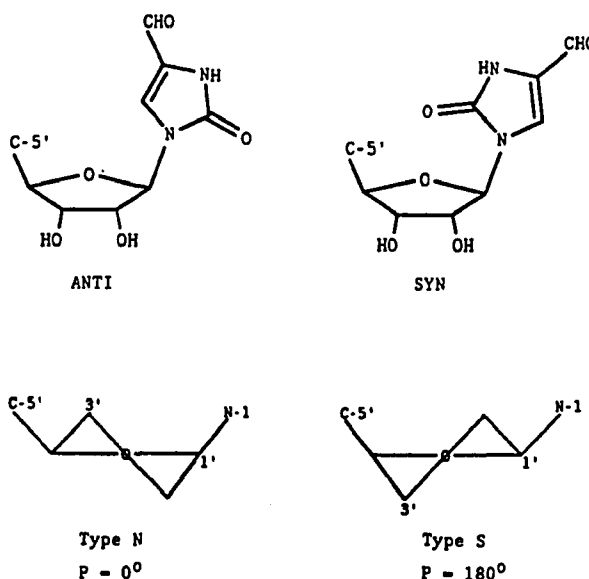


Figure 3 Conformations of imidazolinone nucleosides.

boxyl group is situated above the plane of the ribose ring and close to the imidazolinone heterocycle.

The imidazolinone NH-3 proton could only be observed between pH 2.0 and 3.5 as a broad resonance at 11.0 ppm. At other pH values, a fast rate of exchange with the solvent, catalyzed either by H^+ (low pH values) or by OH^- (high pH values), prevented its detection. For other resonances observed, the titration shifts were much smaller (Table I) and did not show a simple sigmoidal pattern.

The only ribose ring proton that could be observed during the titration experiments in H_2O was H-1'. Resonances falling within 1 ppm of the water peak (other ribose ring protons, H-5', H-2'', and at high pH values H-4'') could not be detected using Hore's sequence. When nikkomycin was run in D_2O at $pD^* = 2.2$, all the ribose protons were resolved at 200 MHz. Computer simulation of the ribose ring portion of the spectrum at $pD^* = 2.2$ gave some of the parameters shown in Table I.

The conformation of ribose rings can be described unequivocally by the puckering amplitude (τ_m) and the phase angle of pseudorotation (P).¹⁸ In nucleosides and nucleotides in solution, the furanose ring is engaged in a conformational equilibrium described by N- and S-type conformers (Figure 3).¹⁸ An exact treatment of a two-state model is not possible, since only three observables are available from the nmr experiment ($J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$) whereas the conformation in solution is fully described by five parameters (P_N , τ_N , P_S , τ_S , and the mole fraction of N-conformer X_N), resulting in an underdetermined system. Nevertheless, an approximate approach is possible,¹⁹ in which $J_{2'3'}$ is assumed to have the same value for the two "pure" conformers and the sum $J_{1'2'} + J_{3'4'}$ is assumed to be practically independent of the magnitude of the equilibrium constant. A classical Karplus-type two-parameter equation²⁰ is used for the calculations. The above assumptions, as well as the derivation of the Karplus parameters, rely on geometrical considerations in the solid state for an extensive set of nucleosides and nucleotides. In this way a fairly accurate value of X_N can be obtained, as well as estimates of the remaining parameters. (For possible alternative treatments, see Refs. 21 and 22).

According to the above treatment, we obtained at $pD^* = 2.2$, $\tau_N = 35^\circ$, $P_N = 16^\circ$, $\tau_S = 37.5^\circ$, $P_S = 165.5^\circ$, and $X_N = 0.50$. Therefore there is no particular preference for either the N- or S-type conformer at pH 2.2, and the values of the phase angles fall within the preferred pseudorotational regions for ribose nucleosides.¹⁸

Inspection of the H-1' resonance throughout the

titration experiment reveals that $J_{1'2'}$ gradually increases with the pH, from a value of 4.3 Hz at pH 1.0, to 5.9 Hz at pH 5.85 (Figure 4). However, the $J_{1'2'}$ values are based on a first-order analysis of the spectra, since the other pentose resonances are buried under the water peak. To analyze the putative effects of higher order spin-spin interactions on $J_{1'2'}$, we simulated nmr spectra run in D_2O at $pD^* = 2.2$ (200 MHz, see above and the materials methods section) and at $pD^* = 4.0$ (400 MHz), and used these to calculate coupling constants for the ribose protons. At $pD^* = 4.5$ and 200 MHz the spectrum of nikkomycin was complicated by overlap of H-3' and H-5' and second-order coupling interactions. The higher magnetic field (400 MHz) led to significant spectral simplification. The coupling constants at $pD^* = 2.2$ are listed in Table I. At $pD^* = 4.0$ they were $J_{1'2'} = 5.75 \pm 0.08$ Hz, $J_{2'3'} = 5.34 \pm 0.08$ Hz, $J_{3'4'} = 4.65 \pm 0.08$ Hz, and $J_{4'5'} = 4.04 \pm 0.08$ Hz. As seen in the plot in Figure 4, the calculated $J_{1'2'}$ s are slightly higher than the observed ones at both low (2.2) and high (4.0) pD^* values. This supports our conclusion that the true coupling constant does increase with the pH. Our interpretation, based on the approximation¹⁹ $X_N \approx 1 - 0.10 \times J_{1'2'}$, is that the variation shown corresponds to a shift of the conformational equilibrium from $X_N \approx 0.6$ to $X_N \approx 0.4$ as the pH increases from 1 to 6.

That $J_{4'5'}$ increases from 3.62 ± 0.05 Hz at $pD^* = 2.2$ to 4.04 ± 0.08 Hz at $pD^* = 4.0$ indicates that the shift of the equilibrium from N- to S-type conformers is accompanied by rotational changes along the C4'-C5' bond. Previous studies in both solution and the solid state have also found a correlation between the nature of the ribose ring puckering and the conformation of the CH_2OH group in pyrimidine nucleosides.^{19,23}

The coupling constant $J_{NH-\alpha CH}$ did not vary from pH 1 to 5 (Table I), indicating that there are no significant conformational changes along the N- αC bond. Its value of 7.0 Hz is close to the mean value for NH- αCH coupling constants in peptides²⁴ and suggests conformational freedom for this particular bond (see below).

NOEs are displayed in Figure 5. Due to the size of the molecule, the contribution to the relaxation processes from the double- and zero-quantum pathways are approximately equal in magnitude, and the NOEs detected are therefore small and positive. Because the small size of the NOEs makes two-dimensional NOE experiments (NOESY) impractical, one-dimensional experiments were run, using long irradiation times (2.5–3.5 s) in order to obtain maximum NOEs. Quantitative determinations of dis-

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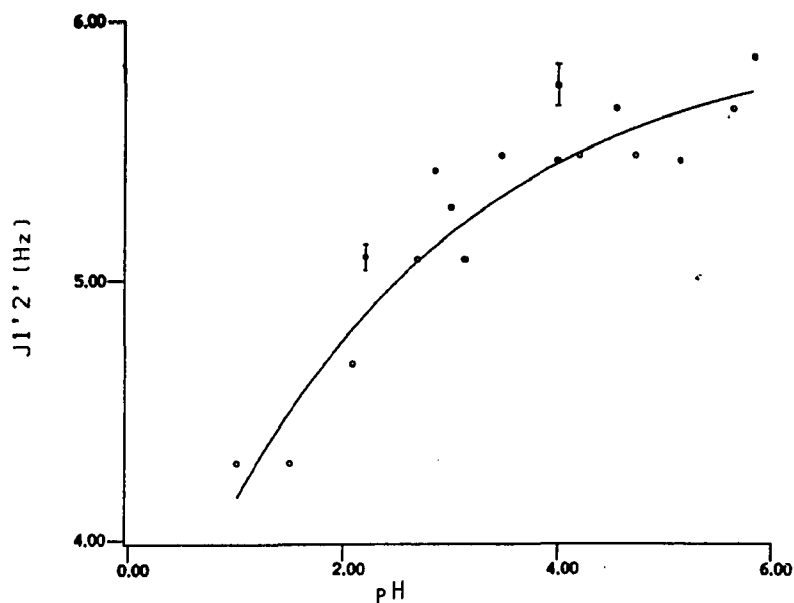


Figure 4 Plot of $J_{1,2'}$ vs pH, at 25°C. Open circles represent data obtained by first-order analysis from spectra run in H_2O . Solid circles represent points obtained by computer simulation of spectra run in D_2O .

tances by measuring initial slopes of the time development of NOEs were not attempted due to the signal-to-noise problem at short times. There were no through-space interactions between hydrogen

atoms of the two amino acids, although NOEs between hydrogen atoms within a given amino acid were observed. We conclude that nikkomycin X is conformationally flexible in water and that there is

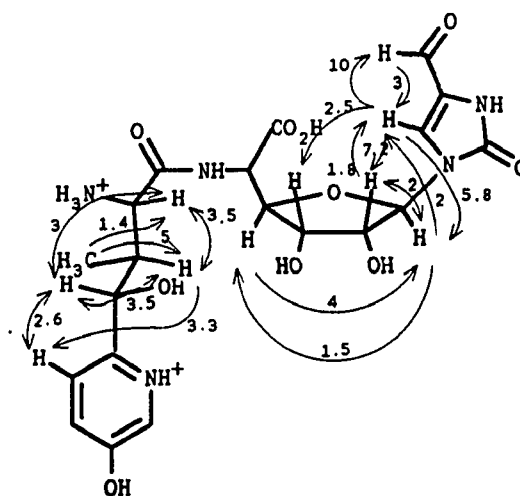


Figure 5 NOE measurements on nikkomycin X (8.5 mg/mL) in D_2O , at 400 MHz. The solution was 0.10 M in KCl and the pD^* was 4.0. Arrows go from irradiated to enhanced protons. Numbers are NOEs percent relative to the intensity of the saturated peak in the difference spectrum.

CONFORMATIONAL STUDIES OF NIKKOMYCIN X

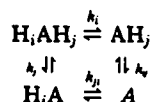
no detectable stabilized folded conformation. The NOEs observed between H-5 and H-2', and H-5 and H-3', support the existence of an *anti* conformation in the nucleoside moiety. Surprisingly, NOEs were measured between H-5 and H-1' (Figure 5). Calculations carried out on a closely related uracil nucleoside have demonstrated²⁵ that a measureable NOE between H-5 (H-6 in uracil) and H-1' is not expected for the *anti* conformer, and thus our result shows that the *syn* conformation is significantly populated as well. The existence of the *syn* conformation is in contrast to previous reports⁶ that have indicated the conformation of the carboxy terminus amino acid of nikkomycin X in aqueous solution to be *anti*. Nevertheless, these latter studies⁶ were based on coupling constants and the calculated conformation might be a weighted average of populations with very different angles of the imidazolinone ring plane (χ).

CONCLUSION

The following conformational changes were detected during the titration of nikkomycin X from pH 1 to 6: a shift from N-type ribose to S-type of approximately 20% upon deprotonation and rotational changes along the C4'-C5' bond. The titration data supports the existence of a conformer with the carboxylic group above the ribose plane and the imidazolinone ring in an *anti* position, whereas there is evidence from the NOE experiments that both the *anti* and *syn* conformations are populated. No NOEs could be detected between protons of the two amino acids, and this supports an unfolded or conformationally flexible structure for nikkomycin X in aqueous solution. These studies define the solution conformation of nikkomycin and provide a basis for the examination of this molecule bound to chitin synthetase or the peptide permease of *C. albicans*.

APPENDIX

A diprotic acid ionizes according to the following scheme:



where k_i , k_j , k_{ij} and k_{ji} are the microscopic acid dissociation constants, and the subscripts i and j indicate the protons on acidic groups i and j .²⁶ The microconstants are defined as

$$\begin{aligned} k_i &= \frac{\text{AH}_j \times \text{H}}{\text{H}_i\text{AH}_j}; & k_j &= \frac{\text{H}_i\text{A} \times \text{H}}{\text{H}_i\text{AH}_j}; \\ k_{ij} &= \frac{\text{A} \times \text{H}}{\text{AH}_j}; & k_{ji} &= \frac{\text{A} \times \text{H}}{\text{H}_i\text{A}} \end{aligned} \quad (\text{A1})$$

For a unique resonance (a resonance with a chemical shift dependent on the degree of protonation of only one of the acidic groups, say group i), the observed chemical shift [compare to Eq. (2)] is

$$\delta = f_{i,p}\delta_{i,p} + f_{i,d}\delta_{i,d} \quad (\text{A2})$$

where $f_{i,p}$ and $f_{i,d}$ are the fractional protonation and deprotonation of group i , and $\delta_{i,p}$ and $\delta_{i,d}$ the chemical shifts of species with group i fully protonated and fully deprotonated for that particular unique resonance.²⁶ Since $(f_{i,p} + f_{i,d}) = 1$, Eq. (A2) can be written as

$$\frac{\delta - \delta_{i,p}}{\delta_{i,d} - \delta} = \frac{f_{i,d}}{f_{i,p}} \quad (\text{A3})$$

Therefore,

$$\frac{\delta - \delta_{i,p}}{\delta_{i,d} - \delta} = \frac{\text{A} + \text{AH}_j}{\text{H}_i\text{AH}_j + \text{H}_i\text{A}} \quad (\text{A4})$$

Substituting Eqs. (A1) into Eq. (A4) and taking logarithms, gives

$$\text{pH} = \text{p}k_i + \log \frac{\delta - \delta_{i,p}}{\delta_{i,d} - \delta} + \log \frac{\left(\frac{k_j}{\text{H}} + 1\right)}{\left(\frac{k_{ji}}{\text{H}} + 1\right)} \quad (\text{A5})$$

When $k_{ij} \simeq k_j$, the last term vanishes and Eq. (A5) is formally analogous to Eq. (3). Since $k_i k_{ij} = k_j k_{ji}$, the condition $k_{ij} \simeq k_j$ is equivalent to $k_i \simeq k_{ji}$ and means that an acidic group i will dissociate with the same dissociation microconstant whether the acidic group j is protonated or not. That is a reasonable assumption if groups i and j are far away from each other in the molecule and cannot interact. Notice also that the pKa being measured with the model used in this study [Eq. (4)] corresponds to the microconstant $\text{p}k_i$.

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Appendix B: Conformation of the ribose ring of nikkomycinX.

As Altona *et al.* have shown (83), the conformation of ribose rings can be described unequivocally by the puckering amplitude (τ_m) (or maximum angle of torsion) and the phase angle of pseudorotation (P). According to their treatment, the endocyclic torsion angles about the bonds C(1')-C(2'), C(2')-(3'), (3')-C(4'), C(4')-O(4'), and O(4')-C(1'), denoted τ_1 , τ_2 , τ_3 , τ_4 , and τ_0 respectively, are related to τ_m and P by the pseudorotation equations (84):

$$\tau_1 = \tau_m \cos(P - 144^\circ)$$

$$\tau_2 = \tau_m \cos P$$

$$\tau_3 = \tau_m \cos(P + 144^\circ)$$

$$\tau_4 = \tau_m \cos(P + 288^\circ)$$

$$\tau_0 = \tau_m \cos(P + 72^\circ)$$

As the molecule completes a full pseudorotational cycle, P goes from 0° to 360° and the dihedral angle τ_2 passes

through the values τ_m , 0° , $-\tau_m$, 0° , and τ_m again. The torsion angle τ_3 moves in the same way, but with a phase shift of $+144^\circ$, τ_4 with a phase shift of $+288^\circ$, and so on.

In cyclopentane the phase angle P rotates without significant change in potential energy. However, in ribonucleosides, the presence of endocyclic and exocyclic substituents gives rise to an energy barrier that opposes free pseudorotation. The authors (84) called type N conformers those with conformations occupying the northern half of the pseudorotational circle ($P = 0 \pm 90^\circ$), and type S conformers the ones that occupy the southern half of the pseudorotational circle ($P = 180 \pm 90^\circ$). Based on X-ray data for 60 nucleosides, they showed that β -purine and β -pyrimidine ribosides prefer two narrow pseudorotational ranges, each occupying less than 10% of all the possible values of P (0 to 360°). Thus there is a type N potential energy well for β -pyrimidine sugars with values of P ranging from 2 to 25° , and a type S energy well with P of 161 to 175° . Although in the solid state either the N or the S type conformers are found, in solution both conformers are assumed to be present in dynamic equilibrium. The observed ^1H -nmr parameters in solution can be fully explained by this two-state dynamic conformational equilibrium model (83).

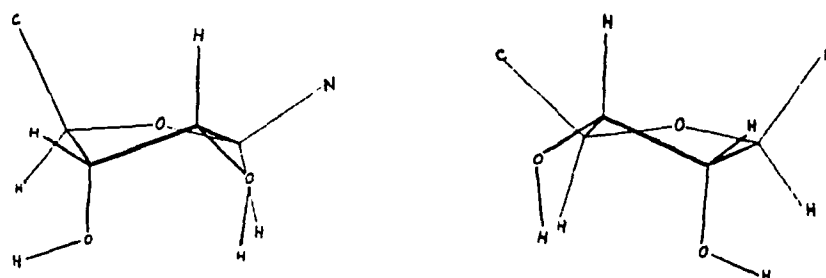


Figure 8 Two ribose ring conformers of nikkomycin X in aqueous solution. At pD* 2.2, the population of each conformer is 50%.

The following parameters were deduced for nikkomycin X from proton-proton coupling data in D₂O solution at pD* 2.2 (see previous section):

$$\tau_N = 35^\circ ; \quad P_N = 16^\circ ; \quad \tau_S = 37.5^\circ ; \quad P_S = 165.5^\circ ; \quad X_N = 0.50$$

The two conformers in equilibrium are shown in Figure 8

Based on the observed change of $J_{1,2'}$ with the pH, it was also shown in the previous section that the conformational equilibrium shifts from $X_N \approx 0.6$ to $X_N \approx 0.4$ as the pH increases from 1 to 6. This change is accompanied by rotational changes along the C4'-C5' bond. It was necessary to prove that the change in $J_{1,2'}$ was not due to high order spin-spin interactions, and spectra run at $pD^* = 2.2$ and $pD^* = 4.0$ were simulated using PANIC (a LAOCOON type program (85)) on an ASPECT 2000A computer (IBM). The simulated spectra are shown in Figures 9 and 10.

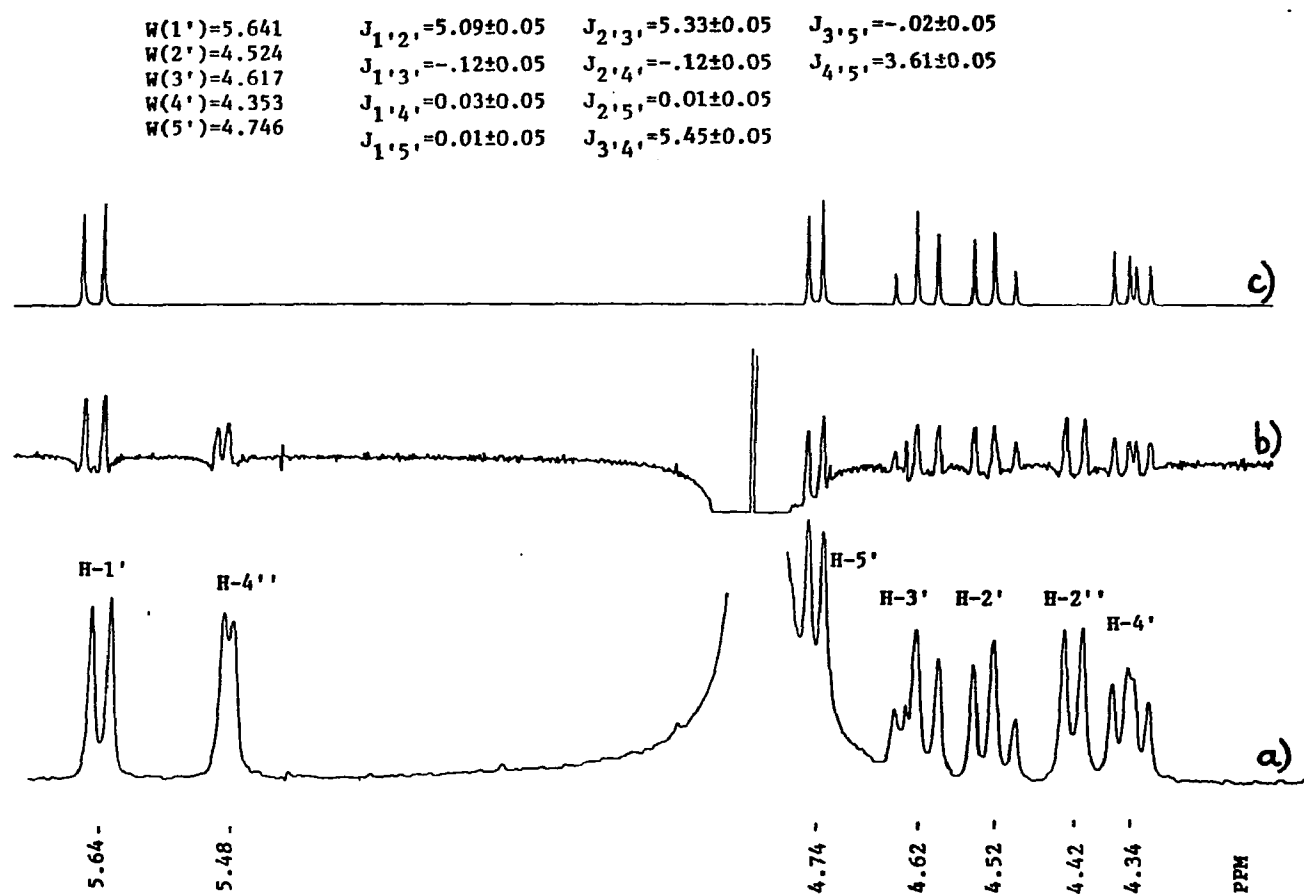


Figure 9 Ribose region of nikkomycin X. a) 200 MHz- ^1H -nmr spectrum in D_2O , $\text{pD}^* = 2.2$ (DSS internal reference). b) A sine bell multiplication and then zero filling was applied to FID of spectrum (a) before FT in order to enhance the resolution. c) Simulated spectrum. Calculated parameters are shown in upper panel, W: chemical shifts in ppm, J in Hz.

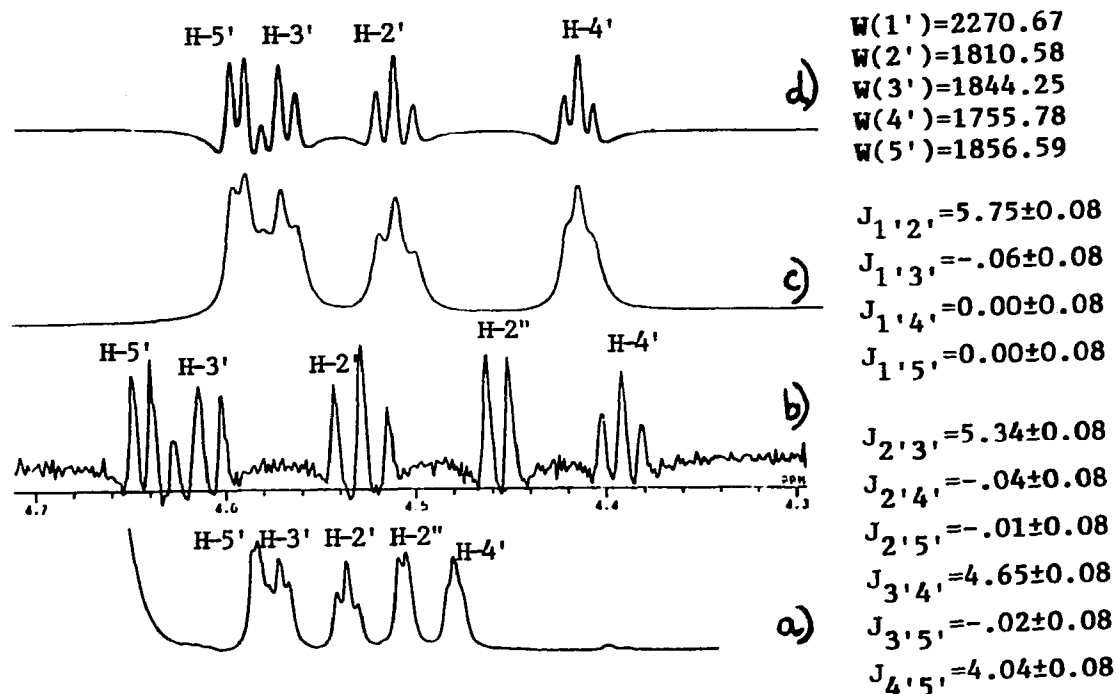


Figure 10 Ribose region of nikkomycin X. a) 400 MHz- ^1H -nmr spectrum in D_2O , $\text{pD}^* = 4.0$ (DSS internal reference). b) A sine bell multiplication and then zero filling was applied to FID of spectrum (a) before FT in order to enhance the resolution. c) Simulated spectrum. Linewidth: 4.7 Hz (lorentzian). Calculated parameters for this spectrum are shown at right. d) Sine bell multiplication and zero filling were applied to simulated FID and then FT in order to simulate spectrum (b). W (chemical shifts) and J in Hz. Horizontal axis corresponds to spectrum (b) only. The ribose protons are indicated above the resonances.

Transferred nuclear Overhauser effect.

The nuclear Overhauser effect (nOe) is a change in the intensity of an nmr signal when the transitions of another one are perturbed. The perturbation of interest is the saturation of the particular nmr resonance. $\eta_i(s)$ is the nOe at nucleus i when nucleus s is saturated, and occurs due to cross relaxation (simultaneous transitions of two spins) between nuclei. For a two-spin system relaxing via dipolar coupling,

$$\eta_i(s) = \frac{W_2 - W_0}{2W_1^i + W_2 + W_0}$$

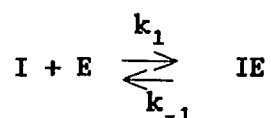
where W_2 and W_0 are the rate constants for the double and zero quantum transitions respectively, and W_1^i is the rate constant for the single quantum transition of nucleus i . The strength of the dipolar interaction depends on the internuclear distance. The numerator in the above equation is the net cross relaxation of nuclei i and s

$$\sigma_{is} \equiv W_2 - W_0$$

and the denominator is the total relaxation for nucleus i

$$\rho_i \equiv 2W_1^i + W_2 + W_0$$

If we have an inhibitor or small ligand, I , interacting with an enzyme E , generally a protein of much higher molecular weight,



it is possible that information relative to the cross relaxation between two protons in I can be transferred from the bound (IE) to the free state by chemical exchange. In such case, irradiation of free I protons will result in negative nOes in other free ligand protons, yielding information about the proximity in space of nuclei in the bound ligand (86). The use of the transferred nOe (trnOe) does not require that the proton resonances of the protein (or eventually the resonances of the bound ligand) be resolved, extending its usefulness to the study of the very largest systems (86).

A theoretical study of the dependency of the observed trnOe on the relevant parameters has been carried out by Clore *et al.* (86). The parameters are: the rate constants of chemical exchange, the expected nOes in the absence of chemical exchange, the relative concentration of free ligand to protein, the total spin-lattice relaxation rates of the free and bound ligand resonances, and the molecular weight of the protein. It is shown that in general, in the slow chemical exchange limit on the spin-lattice relaxation time scale of the free ligand ($k_1[E] + k_{-1} \ll \rho_{IF}$), attempts to obtain structural information on the bound ligand by measuring trnOes will be fruitless (except in special

cases). Conversely, in the fast chemical exchange limit ($k_1[E] + k_{-1} > 10\rho_{IF}$), trnOes can be used for obtaining information on the bound state, provided a negative trnOe is observed. Thus, for large proteins it is feasible to use large excess of ligand and still obtain substantial negative nOes and therefore useful conformational information. Structural information of a quantitative nature can be obtained from the trnOes by measuring their initial build-up rates (87). Attempts to observe trnOes on nikkomycin X in the presence of yeast membranes containing active enzymes are described in the next section.

Studies in the presence of active enzymes.

An understanding of how nikkomycins interact with chitin synthetase and peptide permeases may lead to the rational design of analogs with improved biological properties (for instance increased inhibitor activity, or increased permeability). The trnOe method has been used recently to determine the conformation of various enzyme-bound ligands (88,89) and agonists bound to receptors (90). It may be possible, in principle, to isolate chitin synthetase from *C. albicans* and carry out trnOe measurements on its inhibitors. Pure chitin synthetase in its active form has been isolated in an ingenious way (co-precipitation with its product) from another yeast, *Saccharomyces cerevisiae* (13).

In order to investigate the feasibility of trnOe experiments with pure *C. albicans* enzymes, nOe measurements on nikkomycin X in the presence of *C. albicans* membrane fractions containing activated chitin synthetase (the same membrane preparations used for the chitin synthetase inhibition assays described in Chapter III) were performed. Those preliminary experiments are described here.

EXPERIMENTAL PROCEDURES

Mixed membrane fractions from various strains of *Candida albicans* and from *Schizosaccharomyces pombe* were obtained in the same way as described for chitin synthetase assays (see Chapter III, third section), with slight modifications. Phosphate buffer in D₂O (99.996 % D, Aldrich, Milwaukee, WI) was used in order to avoid interferences in the nmr spectra. The following reagents (at the specified concentrations) were present in the enzyme preparation during the measurement of activity as well as during the nmr experiments: K₂HPO₄ , 30 to 60 mM; EDTA, 0.06 mM; DTE, 0.16 mM; MgCl₂ , 6.2 mM; GlcNAc, 3.2 mM. For the nmr runs, the above reagents had been previously freeze-dried twice from deuterium oxide in order to replace the exchangeable protons. The pD* of the buffer was adjusted to a value of 6.45 with DCl/D₂O. The buffer was microfiltered (0.4 μ m) before use. In contrast to enzyme preparations used previously for chitin synthetase assays (see Chapter III), the ones studied here lacked ATP. Chitin synthetase was activated with trypsin (0.04 mg/mL) at 37°C for 10 min, and then STI (0.06 mg/mL) was added. These two soluble proteins were eliminated from the sample by centrifuging at 20,000 rpm (4°C) 50 minutes and replacing the buffer three times.

Lipid vesicles and lamellae were prepared by suspending 1- α -dipalmitoylphosphatidylcholine, chain

deuterated (DPPC- d_{62} , obtained from Cambridge Isotope Laboratories, Woburn, Massachusetts) in phosphate buffer (pD* 6.45) in a glass vial. A few small glass beads were added and the suspension was vortexed for approximately one minute. In order to prepare the vesicles, the sample was transferred to a polypropylene vial and sonicated with a Heat Systems W-380 Sonicator, according to the procedure of Barrow et al. (91). The water bath temperature was maintained 10°C above the lipid phase transition temperature ($T_c \approx 41^\circ\text{C}$) and sonication was continued up to the point where the sample was almost clear. This required less than an hour. The sample was then centrifuged for 15 minutes at 10,000 rpm in a microcentrifuge in order to remove large and multilamellar vesicles, and was stored at room temperature. The vesicle diameters were around 1500 Å, ($\mu/\gamma^2 = 0.3$), as determined by quasielastic light scattering measurements using a Lexel Model 65 Argon-Ion Laser and Langley Ford Model 1096 correlator.

Nmr experiments were performed at 200 MHz on a Bruker spectrometer equipped with an Aspect 2000A computer. All experiments were run at 300°K. NOes were measured by using the following pulse sequence: [RD-preirradiate(t, ω) -observation pulse] $_n$ for a list of frequencies ω . RD, the relaxation delay, was set to 7.0 sec; t , the duration of the selective proton preirradiation, was varied from 0.50

to 3.0 sec for different samples. The duration of the observation pulse was adjusted for a tip angle of 90° . The nOes were observed by subtraction of a control FID (obtained by off-resonance preirradiation) from the experimental FID, multiplication of the difference FID by a decaying exponential, and then Fourier transformation. The magnitudes of the nOes were determined by peak height measurements.

RESULTS AND DISCUSSION

When a ^1H -nmr spectrum of a membrane fraction from *Candida albicans* H-317 (16 mg protein/mL) containing active chitin synthetase was run at 200 MHz, only a few broad peaks were seen (Figure 11, trace b). Methyl groups and other resonances were observed at high fields, whereas no resonances were seen in the region from 5.5 to 11 ppm. When nikkomycin X was added (8 mg/mL) most of the antibiotic resonances, although broadened, could be observed (see trace c). When a nikkomycin resonance, such as H-5, was irradiated, negative nOes were observed (trace e). This is in sharp contrast to nOes observed in the absence of the enzyme-containing membranes, which were positive (see previous section, and trace d). The negative nOes strongly suggest that it is the enzyme-bound antibiotic which was being "observed". However it is possible that changes in

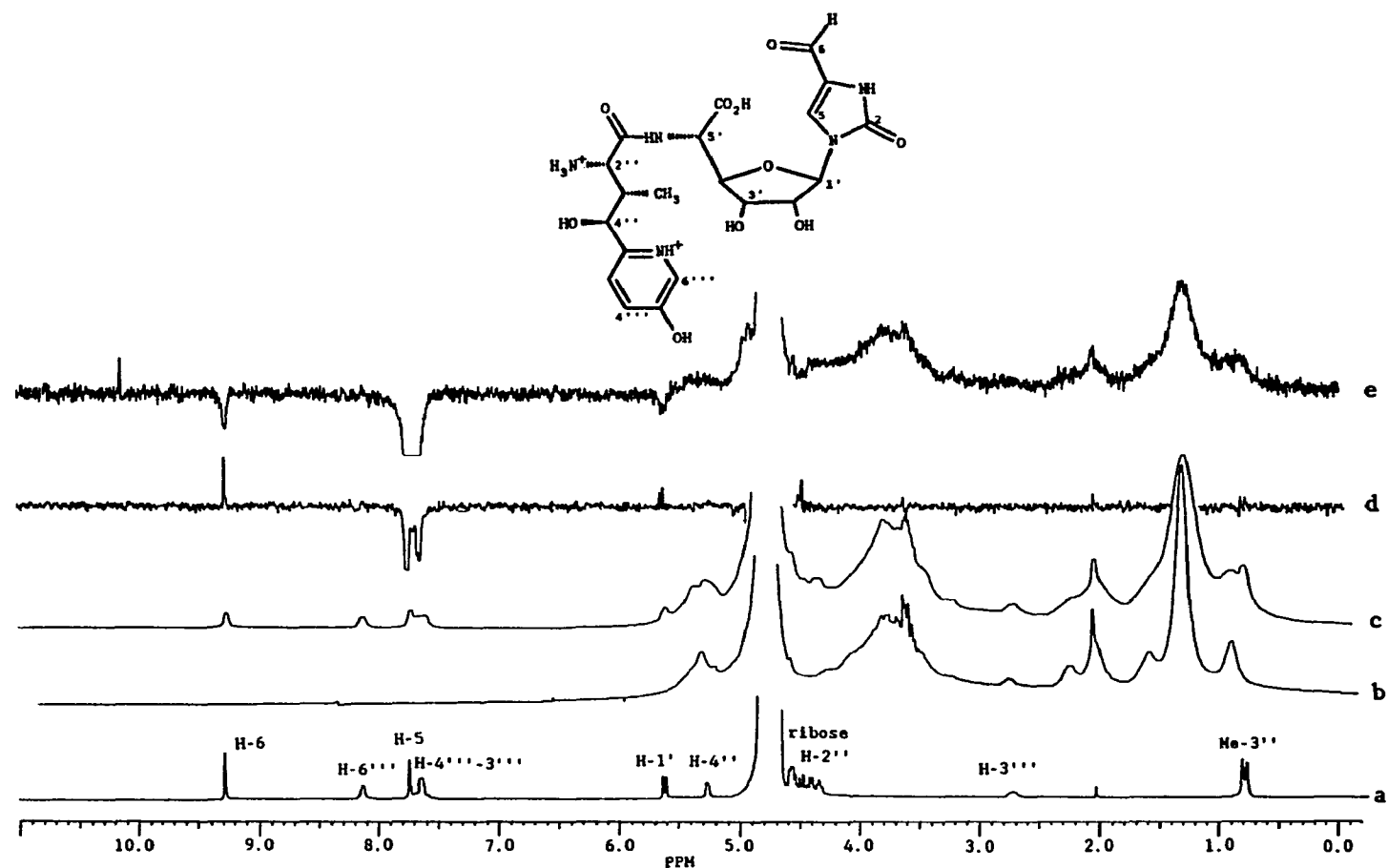


Figure 11 ^1H -nmr spectra in D_2O of (a) Nikkomycin X, and (b) membrane fractions from *Candida albicans* H-317 containing chitin synthetase; (c) Nikkomycin X plus the membrane fractions; (d) NOe difference spectrum, same conditions as in (a), H-5 irradiated (3 sec); (e) NOe difference spectrum, same conditions as in (c), H-5 irradiated (500 msec). This figure appears in reference [92]).

the solution viscosity were responsible for the change in the sign of the nOes. Furthermore, it is also conceivable that nikkomycin binds non-specifically to lipids or to membrane proteins unrelated to chitin synthetase.

An ideal control experiment would necessitate membranes from a strain of *C. albicans* unable to synthesize chitin. Since such a hypothetical strain is not viable, we ran control experiments which consisted of measuring nOes for nikkomycin in the presence of DPPC vesicles (16 mg/mL), DPPC lamellae (16 mg/mL), or DPPC vesicles plus BSA (16 mg/mL of each). These lipids and protein mimic the cellular components found in the membrane fractions of *C. albicans*, probably generating a sample of comparable viscosity. In all the control experiments the signs of the nOes were identical to those obtained for free nikkomycin in solution. These results are displayed in Table 10 (three last columns), and support the conclusion that the negative nOes observed in the presence of membrane fractions provide conformational information on nikkomycin bound to chitin synthetase.

It is possible that the above membrane fractions from *C. albicans* contain peptide permeases, and that these proteins are still operative. In that case, they would interact with nikkomycin and possibly yield negative nOes.

Membranes	none	H-317	124	124N5		<i>S. pombe</i>	DPFC 16 mg/mL vesicles	DPFC/BSA 16 mg/mL vesicles	DPFC 16 mg/mL lamellae		
Buffer (mM)	0	31	62	62		62	25	50	50		
Protein (mg/mL)	0	16	90	88		160	0	16	0		
Activity (nmol/mg.min)	0	0.95	0.25	0.25		0	0	0	0		
(nmol/mL.min)	0	15.2	22.5	22.0		0	0	0	0		
Nikkomycin (mg/mL)	3.9	8.0	8.0	8.0	26.2	7.8	8.0	8.0	8.2		
pD*	4.5	4.4	4.1	4.6	4.8	4.6	2.9	4.3	2.5		
irradiation time (sec)	2.50	0.500	0.600	0.600		0.600	3.0	3.0	3.0	0.500	
irrad. enhanc. CHO	H-5 H-1'	+5.8	-8.4	-3.2	-6.3	-5.1	-12.5 -2.4	positive	positive	+4.5	+1.9
H-6'''	Py-4-3 Me-3''	n.d.	n.d.	0	n.d.	n.d.	-3.7 -2.3	n.d.	n.d.	n.d.	
H-5	CHO H-1' H-6''' Me-3''	+8.8 +7.8	-7.0 -6.9	-5.23 -?	-8.7 -3.8	n.d.	-17.8 -6.3 -4.5 -3.5	positive positive	positive positive	+11.5 +6.8	+2.8 +2.6
Py-4-3	CHO H-6''' H-1' H-4'' H-3'' Me-3''	n.d.	n.d.	n.d.	n.d.	0 -1.6 0 -1.2? 0 -1.7	-2.2 -4.1 -1.5 -3.7 -2.7 -5.7	n.d.	n.d.	n.d.	
H-1'	H-5 CHO H-6''' Py-3-4 H-4''	+5.8	negative	0	-7.8	n.d.	-11.0 -6.8 -1.4 -2.1 -4.3(?)	positive	positive	+small	
H-3''	Me-3'' H-6''' H-5 Py-4-3 H-4'' H-2''	0	n.d.	0	n.d.	n.d.	-25 -10 -6.2(?) -20 -25 -25	n.d.	n.d.	n.d.	
Me-3''	H-3'' CHO H-6''' H-5 Py-4-3 H-4'' H-2'' H-1'	+small	negative	0	-3.4 0 0 0 0 -2.1 0	n.d.	-4.2 -2.6 -3.9 -2.1 -6.8 -6.2 -4.2(?) -2.1	0	positive	+3.6	
H-2'	H-1' H-5	n.d.	negative negative	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
H-4'	H-1'	+8.4	negative	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	

Table 10 Results of nOe experiments at 200 MHz on nikkomycin X in the presence of yeast membranes or lipid vesicles. Solvent: buffer phosphate in D₂O. Temperature: 300°K. The magnitudes of the nOes were determined by peak height measurements.

nOe experiments in the presence of membranes from *C. albicans* 124N5, a nikkomycin resistant mutant lacking a dipeptide transport system were run. Those membranes are not expected to have permeases able to interact with nikkomycin. Membranes from the corresponding wild strain, *C. albicans* 124, were used as control. Conversely, membrane fractions from *Schizosaccharomyces pombe*, a yeast believed to lack chitin synthetase, were used in parallel nOe experiments. The results of all these experiments are summarized in Table 10, and the nOe experiments with *S. pombe* membranes are shown in Figure 12.

The nOes in the presence of the nikkomycin resistant mutant were negative, suggesting an interaction with chitin synthetase. It was observed in this nmr run that the H-3'''-4''' signal did split ca. 0.3 ppm, whereas the H-6''' signal was split 0.18 ppm (data not shown). When the amount of nikkomycin was increased more than 3-fold, no significant change in the magnitude of nOes was detected (Table 10), but the split signals collapsed into the expected pattern. At this point we are unable to give a satisfactory explanation for the splitting of the pyridine signals.

In the experiment with *S. pombe* membranes, negative nOes were observed. Further biological tests showed that

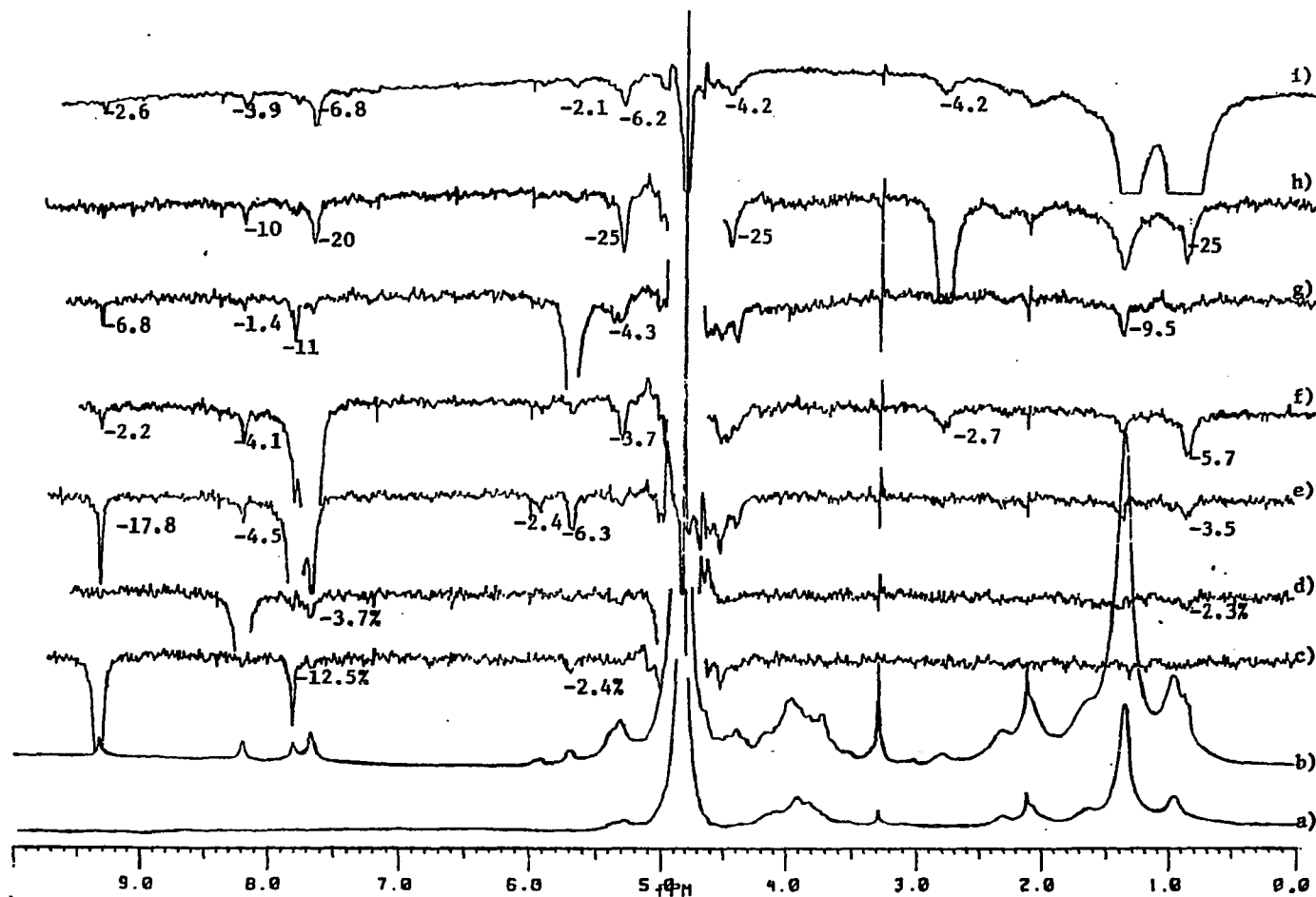


Figure 12 ^1H -nmr spectra in D_2O of (a) Membrane fractions from *Schizosaccharomyces pombe*; (b) plus nikkomycin X. (c) to (i) NOe difference spectra with various resonances irradiated. (For experimental conditions, see Table 7). Magnitudes of NOes are indicated next to peaks.

this yeast's growth is inhibited at very high concentrations of nikkomycin and it is therefore possible that chitin synthetases are present in the membranes. In addition to the nOes seen with other yeast membranes, an extra set of small negative nOes was observed (Table 10 and Figure 12). Many of those nOes, if they are primary nOes, could indicate folding of nikkomycin. However, some degree of spin diffusion seems to have occurred in this particular experiment. Thus, it is very likely that the nOe from CHO to H-1' was mediated by H-5, since the spatial relationship between H-1' and CHO would preclude direct dipolar interaction. The appearance of spin diffusion in this experiment could be due to the higher concentration of the membranes (Table 10), or possibly to the different sizes of the cellular components.

CONCLUSION

The above experiments are preliminary and they raise more questions than they answer. We cannot at this point distinguish clearly between a fast chemical equilibrium or a viscosity effect as the cause for the negative nOes observed in the presence of membranes. The positive nOes in the experiments with lipids suggest the chemical equilibrium hypothesis to be the correct one, but this cannot be conclusive. We think that this question can be

addressed by using a viscosity probe, a small molecule which should not interact with the membranes and on which nOes can be measured. If positive nOes are observed in the presence of membranes, the viscosity effect can be ruled out as a cause for the negative nOes in nikkomycin. Amino acids from the D-series would be suitable candidates for these experiments since they are not expected to interact with the membranes.

If transferred nOes are being measured, it will be necessary to get an estimate of the exchange rate, in order to know if quantitative structural data can be extracted from those measurements. On the other hand it is important to determine whether small nOes that could indicate folding, such as some of the ones observed with *S. pombe* membranes, are primary or due to spin diffusion. This can be done by measuring nOe build-ups (87), preferably at higher magnetic fields.

Summary and perspectives

This thesis has described a combination of synthetic and spectroscopic approaches to design improved inhibitors against *C. albicans*. A novel mode of chromatography was devised and used successfully for the separation and purification of nikkomycins X and Z. The conditions for the coupling of amino acids in the synthesis of nikkomycin and polyoxin analogs were optimized. Some of the synthetic analogs behaved as prodrugs but had low toxicity against *C. albicans*. Although the drug design strategies described deserve further exploration, it was found that the structural specificity for peptide transport in this yeast is more complex than previously thought and the permeability of new antibiotics cannot be predicted with a reasonable degree of certainty.

The spectroscopic studies showed nikkomycin X to be a very flexible molecule in aqueous solution and defined the configuration of the ribose ring and the relative spatial orientation of the imidazolinone heterocycle. Attempts to measure trnOes for this antibiotic in the presence of yeast membranes containing chitin synthetase resulted in data that was very difficult to interpret due to the complexity of the system and to the absence of a good control. It would be of great interest to run nOe experiments on

nikkomycins in the presence of purified chitin synthetase and of purified peptide permease. Such experiments should shed important structural information on the interaction of chitin synthetase inhibitors with critical components of the candidal cell membrane.

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