METABOLIC PRODUCTS OF MICROORGANISMS. 228†

NEW NIKKOMYCINS PRODUCED BY MUTANTS OF *STREPTOMYCES TENDAE*

CHRISTIANE BORMANN, WOLFGANG HUHN and HANS ZÄHNER

Institute of Biology II, University of Tübingen,
D-7400 Tübingen, FRG

RALPH RATHMANN, HOLGAR HAHN and WILFRIED A. KÖNIG

Institute of Organic Chemistry, University of Hamburg,
D-2000 Hamburg, FRG

(Received for publication May 7, 1984)

Four new dipeptidyl nikkomycins of the Z- and the X-series with a variation in the amino acid moiety of the molecule were isolated from the mutant *Streptomyces tendae* 901/395 and characterized. Nikkomycins K_z and K_x contain 2-amino-4-hydroxy-4-(2-pyridyl)butyric acid, and nikkomycins O_z and O_x 2-amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)butyric acid. In contrast to nikkomycins Z and X, nikkomycins K_x and O_x are quite stable at alkaline pH and exhibit a lower biological activity against various test organisms. From the mutant *S. tendae* 901/C37, which is auxotrophic for methionine and threonine, enhanced amounts of two tripeptidyl nikkomycins, Q_z and Q_x, were produced which are analogues of nikkomycins J and I and contain a homoserine residue instead of glutamic acid. These nikkomycins exhibit a high pH instability.

Nikkomycins are nucleoside peptide antibiotics which act as competitive inhibitors of fungal and insecticidal chitin synthetases[1,2,3]. From the culture filtrate of *Streptomyces tendae* a spectrum of active nikkomycins has been isolated[4~7]. The major components are the two dipeptidyl nikkomycins Z and X composed of 2-amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)-3-methylbutyric acid, an unusual amino acid, and a 5-aminohexuronic acid *N*-glycosidically bound to uracil in nikkomycin Z or to 5-formyl-4-imidazolin-2-one in nikkomycin X. The tripeptidyl nikkomycins J and I, minor components of the culture broth, are analogues of nikkomycins Z and X with a peptidically bound glutamyl residue linked to the carboxy group of the nucleoside moiety. By mutasynthesis and directed fermentation nikkomycin Z and J analogues containing thymine, 5-hydroxymethyluracil or isoorotic acid as base could be obtained[8]. In the present paper we describe new nikkomycins produced by mutants of *Streptomyces tendae*.

Materials and Methods

Microorganisms

Spores of the wild type strain *Streptomyces tendae* Tü 901 were mutagenized by UV light at 365 nm in the presence of 8-methoxypsoralen (MOP) according to TOWNSEND *et al.*[9]. 10^{10} spores per 10 ml saline plus 1 mg MOP were incubated for 10 minutes at 27°C and subsequently irradiated by UV light. Mutants were selected among 0.1% survivors.

Mutagenesis with ethyl methane sulfonate (EMS) was performed by incubating 10^9 spores of *S. tendae* Tü 901 per 10 ml sodium phosphate buffer, pH 7.0 with 5% (v/v) EMS at 27°C. Mutants were

† Part 227. WUTHIER, D.; W. KELLER-SCHIERLEIN & B. WAHL: Isolierung und Strukturauflärung von Rutamycin B. Helv. Chim. Acta 67: 1208~1216, 1984

selected among 10% survivors.

Culture Conditions

The strains were grown in batch cultures of 20 ml in 100-ml Erlenmeyer flasks and of 100 ml in 500-ml Erlenmeyer flasks, on a rotatory shaker for 5~7 days at 27°C. For nikkomycin production the following media were used: I. 3% mannitol, 1% starch, 2(3)% soybean meal, 1% yeast extract (Oxoid, FRG), pH 5.5; II. 2% glycerol, 2% soybean meal, pH 6.0. To investigate the influence of amino acids on nikkomycin production cells were grown for 3 days in medium I, washed twice with saline and transferred into resting-cell medium according to FIEDLER *et al.*[3] with different supplements.

Production of new nikkomycins by fermentation of mutant strain 901/395 was carried out in a 20-liter intensor system (b 20, Giovanola, Switzerland) with 18.5 liters medium II. The fermentor was inoculated with 1.5 liters of a culture grown for 3 days in the same medium. The fermentation was run under the following conditions: temperature 27°C, agitation 1,200 rpm, aeration 0.5 liter/vvm, pH adjusted to $\leq$6.0 with 1 N H_2SO_4; 500 ml feeding-solution contained 50 g L-isoleucine and 50 g L-tyrosine and was added after 72 and 96 hours.

Fermentation of mutant strain 901/C37 was performed in a 10-liter fermentor with impeller system (type F 0020, Chemap A.G., Switzerland). 9.5 liters medium I with 3% soybean meal was inoculated with 0.5 liter culture grown for 24 hours in the same medium and fermented at 300 rpm, 27°C and aeration 0.5 liter/vvm for 6 days.

Isolation

The procedure for isolation of nikkomycins K_z, K_x, O_z, O_x and Q_z, Q_x was almost identical to that described by DELZER[10]. Nikkomycins were analyzed by HPLC[11]. The fermentation broth was adjusted to pH 4.0 with acetic acid and filtered with 2% Celite. The filtrate was applied to a 0.8-liter column of Dowex 50WX4 (Na^+ form, 50~100 mesh) and eluted with 0.05 N ammonia. As nikkomycins are quite unstable at alkaline pH[12], fractions of the eluate were immediately deduced to a small volume in a rotatory evaporator and adjusted to pH 6.0 with acetic acid. Fractions containing nikkomycins were collected, adjusted to pH 7.5 with ammonia and chromatographed on Amberlite IRA 401 S (OH^- form, 0.5 liter). Nikkomycins were eluted with 1% formate and the pooled fractions, pH 2.0, were applied to a SP Sephadex C25 column (pyridinium form, 0.2 liter) with a flow rate of 100 ml/hour. Nikkomycins K_z, K_x, O_z and O_x were successively eluted by 0.01 M pyridine acetate (PAP), pH 4.7, while nikkomycins Z and X were eluted by 0.04 M; nikkomycins Q_z, Q_x, J and I were eluted by 0.05 M buffer. Fractions containing new nikkomycins were concentrated in a rotatory evaporator and lyophilized. Samples containing K_z, K_x, O_z and O_x (300 mg/ml) were chromatographed on Biogel P2 (0.5 liter, 100~200 mesh) with 0.1% acetic acid at a flow rate of 30 ml/hour. Samples with nikkomycins Q_z and Q_x were chromatographed on Biogel P2 (1.4 liters, 100~200 mesh) at 120 ml/hour and rechromatographed on Biogel P2 (1.0 liter, 200~300 mesh) at 15 ml/hour.

Biological Activity

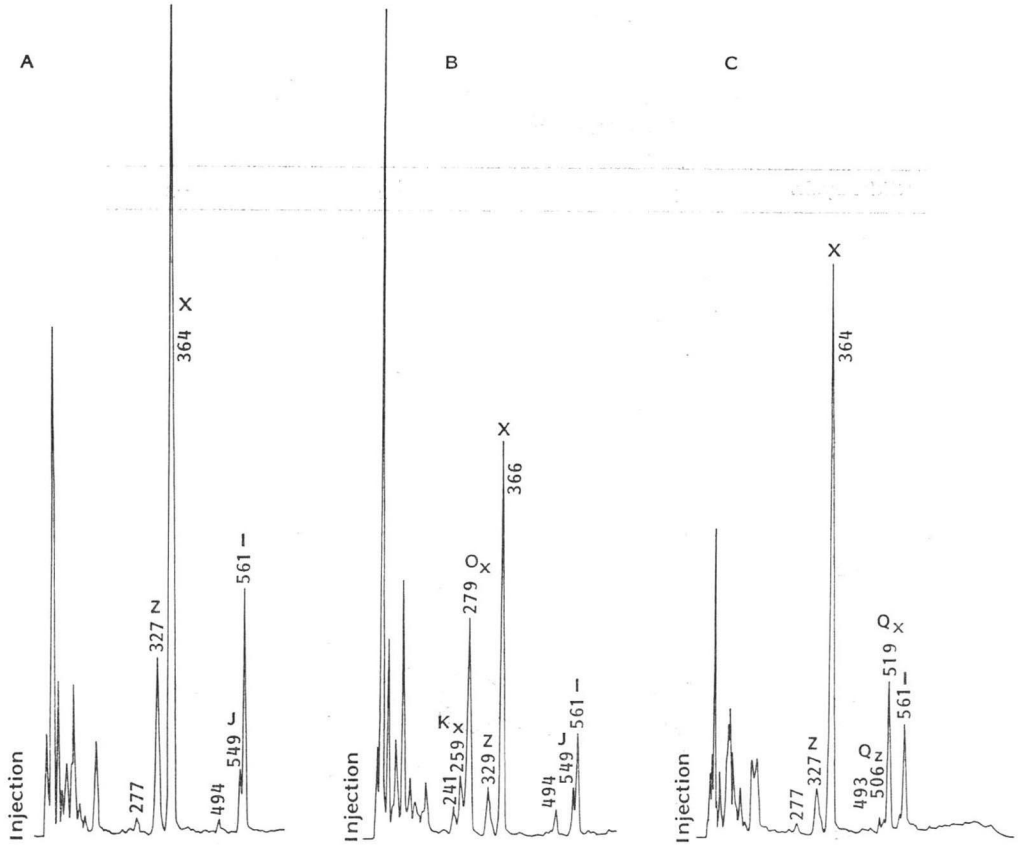
MIC's were determined by the disc diffusion assay with nikkomycin in a concentration range of 20~2,000 μg/ml. Paper disks (diameter: 6 mm) with 10 μl nikkomycin solution were applied to complete medium (CM)-agar plates (diameter: 8.5 cm) composed of 0.4% glucose, 0.4% yeast extract, 1% malt extract, 2% agar, pH 7.3, seeded with test organisms. Test plates for yeasts were prepared with 10 ml CM-agar inoculated with a 0.1-ml culture (OD_{578nm}=1.3) grown overnight in the same medium. Test plates for fungi were prepared with 17.5 ml CM-agar inoculated with 10^5 spores per ml. For *Alternaria, Botrytis, Chaetomium, Corticium, Piricularia* and *Scopulariopsis* 200 ml of CM-agar was inoculated with mycelium harvested from a CM-agar plate. Unless otherwise stated test plates were examined after 24-hour incubation. *Botrytis* and *Mucor hiemalis* were incubated at 24°C, *Mucor miehei* and *Paecilomyces* at 37°C and the other organisms at 27°C.

Results and Discussion

Mutants

We screened mutants of *S. tendae* which were auxotrophic or exhibited retarded growth on minimal

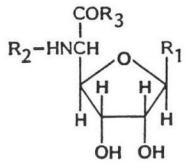
Fig. 1. HPLC analysis of the culture broth of *S. tendae* Tü 901 (A), 901/395 (B) and 901/C37 (C). The strains were cultivated in medium I for 6 days, 901/C37 for 8 days.

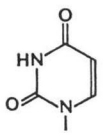
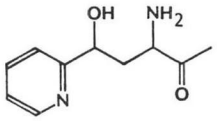
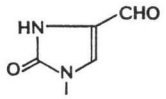
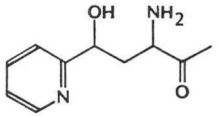
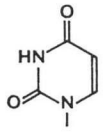
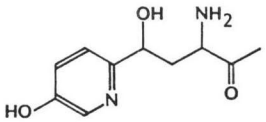
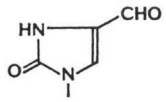
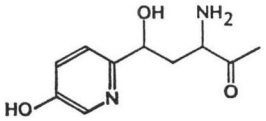
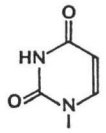
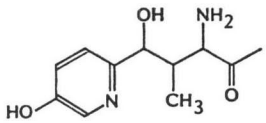
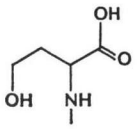
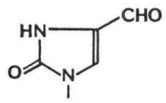
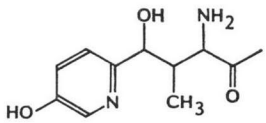
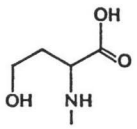


medium plates for new nikkomycins. Among 200 tested strains two mutants 901/395 and 901/C37 produced substances during growth in nikkomycin-production medium I which appeared as unique peaks in the HPLC analysis of their culture filtrates (Fig. 1) and exhibited attributes of nikkomycins. In the case of strain 901/395 two compounds with retention times (RTs) of 259 seconds and 279 seconds, respectively, and with mutant 901/C37 one substance (RT 519) reacted with barbituric acid, an aldehyde reagent used to identify the 4-formyl-4-imidazoline-2-one base of nikkomycins[8]. Furthermore, using resting-cell conditions with 20 mM uracil as supplement these peaks were repressed analogous to the findings with nikkomycins X and I[3], whereas synthesis of another compound, RT 241, by strain 901/395 and RT 506 produced by strain 901/C37, was stimulated as noted in the case of nikkomycins Z and J[3]. From these results we considered that the strains 901/395 and 901/C37 produced new nikkomycins with 4-formyl-4-imidazoline-2-one and uracil as base. Strain 901/395 was obtained from UV-MOP mutagenesis and exhibited retarded growth on minimal medium, which could be improved by supplementation with L-tryptophan. The novel substances observed on HPLC were isolated and characterized* as four new dipeptidyl nikkomycins of the Z- and X-series which varied in the amino acid moiety of the molecule (Fig. 2). From culture filtrates nikkomycins K_z and O_z were usually not separated by HPLC. During cultivation in medium I strain 901/395 produced nearly identical amounts of the

* Results concerning the determination of the chemical structure will be published elsewhere.

Fig. 2. Structure of new nikkomycins.



Nikkomycin	R_1	R_2	R_3
K_z			—OH
K_x			—OH
O_z			—OH
O_x			—OH
Q_z			
Q_x			

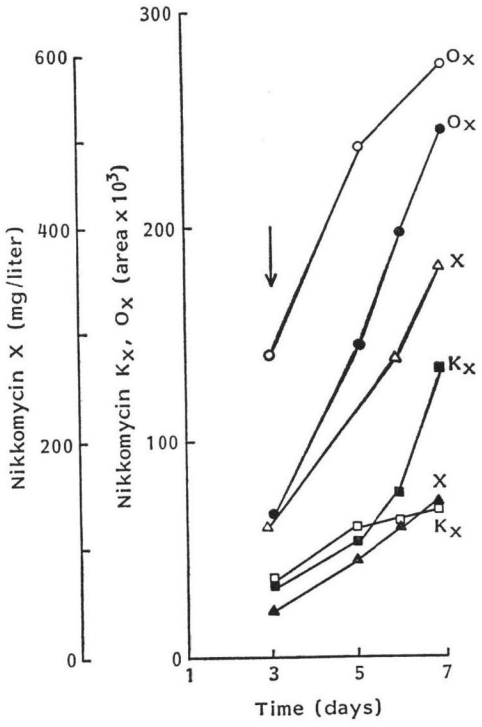
new nikkomycins K_z, K_x, O_z, O_x and nikkomycins Z and X. After 6 days cultivation the sum of nikkomycins Z, X was about 450 mg/liter compared to 1,100 mg/liter produced by the wild type strain. Nikkomycins K_z, K_x, O_z and O_x were produced by the wild type strain, too, but in small quantities (Fig. 1A).

The strain 901/C37 derived from *S. tendae* by EMS mutagenesis is auxotrophic for methionine and threonine. The mutagenic block of methionine biosynthesis is due to a defect in the cystathionine lyase-locus, as the mutant grew on minimal medium supplemented with threonine and homocysteine (each at 0.1 mg/ml), whereas no growth occurred with threonine and cystathionine. Threonine biosynthesis is blocked at the homoserine kinase and/or threonine synthetase locus as the mutant did not grow on minimal medium supplemented with methionine and homoserine. The compounds which appeared as extra peaks in the HPLC analysis of the culture filtrate (Fig. 1C) were isolated and analyzed* as new tripeptidyl nikkomycins, Q_z and Q_x. Nikkomycins Q_z and Q_x are analogues of J and I with homoserine

* See footnote page 11.

instead of glutamic acid peptidically bound to the C_6 of the aminohexuronic acid (Fig. 2). With resting-cells, synthesis of nikkomycins Q_z and Q_x was stimulated up to 300% with 30 mM D,L-homoserine.

Fig. 3. Production of nikkomycins K_x (□), O_x (○) and X (△) by *S. tendae* 901/395 cultivated in medium I (open symbols) and in medium II supplemented (arrow) with 40 mM tyrosine and 40 mM isoleucine (closed symbols).



Fermentation and Isolation

In resting-cell experiments with strain 901/395 we observed a 200 to 300% stimulation of the synthesis of nikkomycins K_x and O_x by L-isoleucine (40 mM) similar to that observed previously with nikkomycin X synthesis[13]. Also addition of L-tyrosine (40 mM) resulted in a stimulation of the synthesis of nikkomycins K_x and O_x up to 200%. As the production of nikkomycins Z and X was significantly repressed using medium II (Fig. 3), a batch fermentation was employed for the isolation of the new nikkomycins in medium II supplemented with L-isoleucine and L-tyrosine. For the isolation of nikkomycins Q_z and Q_x strain 901/C37 was cultivated in medium I with 3% soybean meal.

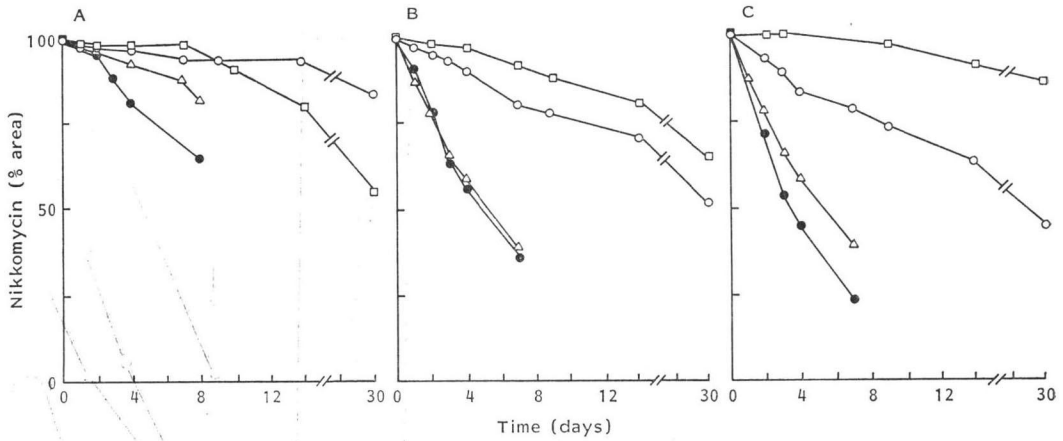
The purification of nikkomycins K_x and O_x and nikkomycins Q_z and Q_x is summarized in Table 1. Chromatography on SP-Sephadex C25 resulted in a separation of nikkomycins K_z, K_x, O_z and O_x and nikkomycins X, Z, J and I, respectively. Homogeneous nikkomycins K_x and O_x, as judged by HPLC analysis, were obtained on Biogel P2. In contrast, nikkomycins Q_z, Q_x, J and I were eluted as a mixture on SP-Sephadex C25 by 0.05 N PAP. Chromatography on Biogel P2 and rechromatography resulted in a 5:1 enrichment of nikkomycins Q_z and Q_x relative to J and I.

Table 1. Purification of nikkomycins K_x, O_x, and, Q_z and Q_x from mutants of *S. tendae*.
Total amount of nikkomycins K_x, O_x, and, Q_z and Q_x expressed as integration units of HPLC analyses.

Purification step	Nikkomycin K_x		Nikkomycin O_x		Nikkomycins Q_z, Q_x	
	Vol (liter)	Total amount (area $\times 10^3$)	Vol (liter)	Total amount (area $\times 10^3$)	Vol (liter)	Total amount (area $\times 10^3$)
Culture filtrate	16.6	2,208	16.6	5,876	8	694
Dowex 50 WX4 0.05 N NH_4OH	0.8	1,184	0.8	4,152	1.9	503
Amberlite IRA 401S 1% HCOOH	4.4	1,144	4.4	2,864	2.7	248
SP-Sephadex C25						
0.01 N PAP, pH 4.7	0.54	658	0.54	2,367		
0.05 N PAP, pH 4.4					0.03	98

Fig. 4. pH stability of nikkomycins K_x (□), O_x (○), Q_x (●) and X (△).

Purified nikkomycin (K_x, O_x: 0.8 mg/ml; Q_x: 3 mg/ml; X: 0.55 mg/ml) was incubated in 0.05 M buffer at 20°C and under sterile conditions. A: MES, pH 5.5; B: TES, pH 7.0; C: Tricine, pH 8.0. Probes were analyzed by HPLC.



pH Stability

Nikkomycins Z and X are relatively stable at low pH values, but unstable under alkaline conditions[12]. In Fig. 4 the pH stability of nikkomycins K_x, O_x, X and Q_x is shown. Nikkomycins K_x and O_x exhibited good stability at pH 7.0 and 8.0 compared to nikkomycins X and Q_x. In particular nikkomycin K_x was extremely stable at pH 8.0. A comparison of the stability of nikkomycins K_x and O_x suggests that interaction of the hydroxyl group of the pyridyl residue and the N atom of the peptide bond seem to promote hydrolytic cleavage at alkaline pH.

Biological Activity

As described in a previous paper[8] analogues of the tripeptidyl nikkomycin J are 7 times less effective against chitin synthetase than the corresponding Z-analogues. Recently NAIDER *et al.*[14] reported that the tripeptidyl polyoxins, which are related nucleoside peptide antibiotics, also are poor inhibitors of chitin synthetase. Nikkomycins Q_z and Q_x were not purified to homogenity, since a similar low biological activity could be expected. In Table 2 the MICs of nikkomycins K_x, O_x, Z and X are shown. Nikkomycin K_x exhibited slight biological activity, which was 3 ~ 10 times lower than that of nikkomycin O_x except for almost identical activity against *Scopulariopsis brevicaulis*. Nikkomycin O_x in comparison to nikkomycins Z and X was significantly less active against most test organisms; for *Botrytis, Mucor miehei, Piricularia* and *Candida albicans* however, the MICs were within the same range. These results indicate that the hydroxyl group of the pyridyl residue markedly enhances the biological efficiency of nikkomycins. In addition, the methyl group of the amino acid is important for activity as noted for the polyoxins[15]. These authors reported that the inhibitory activity of the polyoxins with a hydroxyl group at the C_3 atom of the carbamoylpolyoxamic acid were higher than those of polyoxins without a substituent at this position. For practical use of nikkomycins K_x and O_x as insecticidal and antifungal agents, field tests are necessary to elucidate whether the high pH stability compensates for lower biological activity.

Table 2. MICs of nikkomycins against fungi and yeasts.

Test organism	MIC (μg/ml)			
	Nikkomycin K_x	Nikkomycin O_x	Nikkomycin Z	Nikkomycin X
Alternaria kikuchiana CBS 107.53[a]	>100	>100	0.5	1
Aspergillus viride-nutans CBS 127.56[a]	>100	>100	>100	>100
Botrytis cinerea Tü 157[b]	25	1.5	1.5	1
Chaetomium globosum Tü 138[a]	>100	>100	>100	60
Colletotrichum acutatum CBS 979.69[a]	11	1	0.15	0.4
Corticium sasakii CBS 255.33[a]	>100	>100	>100	>100
Mucor hiemalis Tü 179/180	7	2	0.09	0.02
M. miehei Tü 284	14	2.5	1.3	5
M. racemosus DSM 62760	9	3	0.4	0.2
Paecilomyces varioti Tü 137[a]	6	1.5	0.14	0.04
Piricularia oryzae Tü 692[b]	20	6	4	2
Scopulariopsis brevicaulis Tü 8058[b]	7	6	7	1
Candida albicans Tü 164	10	3	2	1
C. guilliermondii CBS 6021	>100	>100	>100	>100
C. utilis DSM 70167	>100	>100	>100	>100
C. vulgaris Tü 565	20	8	3	2
Dipodascus magnusii Tü 167	1	0.13	0.07	0.05
Metschnikowia pulcherrima Tü 652	>100	>100	>100	20
Saccharomyces cerevisiae Tü 125	>100	>100	>100	>100
Yarrowia lipolytica ATCC 8662	10	4	0.23	0.2
Wingea robertsii DSM 70870	>100	>100	20	7

[a] 48-hour, [b] 72-hour incubation. Tü: Culture collection of the Institute of Microbiology I, University of Tübingen.

Acknowledgments

This research was supported by the Bundesministerium für Forschung und Technologie. We thank Dr. H.-P. FIEDLER and Miss A. DÖHLE for the HPLC analyses, Miss S. WINTER for technical assistance and Mr. K. WUSCH for preparation of the graphs.

References

1) BRILLINGER, G. U.: Metabolic products of microorganisms. 181. Chitin synthetase from fungi, a test model for substances with insecticidal properties. Arch. Microbiol. 121: 71~74, 1979
2) MÜLLER, H.; R. FURTER, H. ZÄHNER & D. M. RAST: Effect of nikkomycin Z, nikkomycin X and polyoxin A on chitosomal chitin synthetase and growth of *Mucor rouxii*. Arch. Microbiol. 130: 195~197, 1981
3) FIEDLER, H.-P.; R. KURTH, J. LANGHÄRIG, J. DELZER & H. ZÄHNER: Nikkomycins: Microbial inhibitors of chitin synthetase. J. Chem. Tech. Biotechnol. 32: 271~280, 1982
4) DÄHN, U.; H. HAGENMAIER, H. HÖHNE, W. A. KÖNIG, G. WOLF & H. ZÄHNER: Nikkomycin, ein neuer Hemmstoff der Chitinsynthese bei Pilzen. Arch. Microbiol. 107: 143~160, 1976
5) HAGENMAIER, H.; A. KECKEISEN, H. ZÄHNER & W. A. KÖNIG: Stoffwechselprodukte von Mikroorganismen. 182. Aufklärung der Struktur des Nukleosidantibiotikums Nikkomycin X. Liebigs Ann. Chem. 1979: 1494~1502, 1979
6) KÖNIG, W. A.; W. HASS, W. DEHLER, H.-P. FIEDLER & H. ZÄHNER: Stoffwechselprodukte von Mikroorganismen. 189. Strukturaufklärung und Partialsynthese des Nukleosidantibiotikums Nikkomycin B. Liebigs Ann. Chem. 1980: 622~628, 1980
7) HAGENMAIER, H.; A. KECKEISEN, W. DEHLER, H.-P. FIEDLER, H. ZÄHNER & W. A. KÖNIG: Stoffwechselprodukte von Mikroorganismen. 199. Konstitutionsaufklärung der Nikkomycine I, J, M und N. Liebigs Ann. Chem. 1981: 1081~1024, 1981
8) DELZER, J.; H.-P. FIEDLER, H. MÜLLER, H. ZÄHNER, R. RATHMANN, K. ERNST & W. A. KÖNIG: New nikkomycins by mutasynthesis. J. Antibiotics 37: 80~82, 1984

9) TOWNSEND, M. E.; H. M. WRIGHT & D. A. HOPWOOD: Efficient mutagenesis by near ultraviolet light in the presence of 8-methoxypsoralen in *Streptomyces*. J. Appl. Bacteriol 34: 799~801, 1971
10) DELZER, J.: Mutasynthese und gerichtete Fermentation in der Nikkomycinreihe. Dissertation, Universität Tübingen, 1983
11) FIEDLER, H.-P.: Quantification of nikkomycins in biological fluids by ion-pair reversed-phase high performance liquid chromatography. J. Chromatogr. 204: 313~318, 1981
12) DEHLER, W.: Nikkomycine. Studien zur Fermentation und Isolierung. Dissertation, Universität Tübingen, 1979
13) KURTH, R.: Untersuchungen zur Regulation der Nikkomycinbildung und Optimierung einer pO_2-Feeding-Fermentation. Dissertation, Universität Tübingen, 1981
14) NAIDER, F.; P. SHENBAGAMURTHI, A. S. STEINFELD, H. A. SMITH, C. BONEY & J. M. BECKER: Synthesis and biological activity of tripeptidyl polyoxins as antifungal agents. Antimicrob. Agents Chemother. 24: 787~796, 1983
15) HORI, M.; K. KAKIKI, S. SUZUKI & T. MISATO: Studies on the mode of action of polyoxins. III. Relation of polyoxin structure to chitin synthetase inhibition. Agric. Biol. Chem. 35: 1280~1291, 1971