METABOLIC PRODUCTS OF MICROORGANISMS. 252†

ISOLATION OF NEW NIKKOMYCINS FROM *STREPTOMYCES TENDAE*

HEINRICH DECKER, CHRISTIANE BORMANN, HANS-PETER FIEDLER and HANS ZÄHNER

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

HOLGER HEITSCH and WILFRIED A. KÖNIG

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

(Received for publication August 31, 1988)

Two new nikkomycins were isolated from the culture broth of *Streptomyces tendae* Tü 901/PF 53$^+$-3. The new compounds are the dipeptide nikkomycin pseudo-Z (ΨZ) and tripeptide nikkomycin pseudo-J (ΨJ), which are analogues to nikkomycins Z and J. Nikkomycins pseudo-Z and pseudo-J have a *C*-glycosidic linkage between uracil and 5-amino-5-deoxy-D-*allo*-furanuronic acid, which is comparable to the *C*-glycosidic bond in pseudouridine. The new *CC*-nucleoside nikkomycins exhibit a lower biological activity than the *CN*-nucleoside nikkomycins.

The nikkomycins are peptide nucleoside antibiotics which competitively inhibit the chitin synthetase of fungi and insects because of their structural similarity to UDP-*N*-acetylglucosamine[2,3]. The major biologically active components in the culture broth of the wild type strain of *Streptomyces tendae* Tü 901 are nikkomycins Z and X. These are composed of 5-amino-5-deoxy-D-*allo*-furanuronic acid *N*-glycosidically bound to uracil in the case of nikkomycin Z or to 4-formyl-4-imidazolin-2-one in the case of nikkomycin X[4]. 2-Amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)-3-methylbutyric acid, an unusual amino acid, is peptidically linked to the 5-aminohexuronic acid and completes the dipeptide structures of nikkomycins Z and X[5]. The tripeptide nikkomycins have an additional amino acid bound at the C-6 carboxyl residue of the nucleoside moiety[6]. New nikkomycins could be found by isolation of minor components from the fermentation broth of *S. tendae*[7], from mutants of *S. tendae* with an altered production spectrum[8], by mutasynthesis, and by directed fermentation[9]. Although the nikkomycins exhibit a high acaricidal activity and a low toxicity to rats (LD_{50}<5 g/kg)[10], the application of nikkomycin for agricultural purposes has been discontinued[11,12]. Nevertheless, the nikkomycins are still of interest, because they inhibit the growth of the pathogenic yeast *Candida albicans*[13]. We have tried to isolate nikkomycins with altered properties and increased activity against various fungi and yeasts. We present here the isolation of new nikkomycins produced by a mutant of *S. tendae*.

Materials and Methods

Microorganism

Conditions of protoplast fusion and media are described by HOPWOOD *et al.*[14]. *S. tendae* Tü 901/PF 53$^+$-3 is a prototrophic revertant of the adenine auxotrophic strain PF 53 which came out of a

† Part 251[1].

protoplast fusion of the mutant *S. tendae* Tü 901/390 (ade$^-$) and 901/391-11 which have an altered nikkomycin spectrum compared to the wild type strain. *S. tendae* Tü 901/395-11 produces nikkomycins K_z, K_x, O_z and O_x instead of nikkomycins Z and X.

Culture Conditions

The strain *S. tendae* Tü 901/PF 53$^+$-3 was maintained on the following medium: Yeast extract 0.4%, glucose 0.4%, malt extract 1% and agar 2%. The cultures were grown at 37°C for 3 days and stored at 4°C.

Fermentation

For the production of the new nikkomycins we used a medium consisting of: Soybean meal 3%, mannitol 4%, yeast extract 1%, starch 1%, uracil 0.4%, pH 6.8. The fermentation of *S. tendae* Tü 901/PF 53$^+$-3 was performed in a 10-liter stirred tank reactor (Biostat E, Braun Melsungen, FRG). The 9.5-liter production medium were inoculated with 0.5 liter starter culture grown in the same medium for 30 hours on a rotary shaker in Erlenmeyer flasks and fermented at 500 rpm, 27°C and aeration 0.5 liter/vvm for 7 days.

Isolation

The quantification and characterization of nikkomycins by HPLC and a photodiode array detection were done as described by FIEDLER[15,16]. The isolation of the new nikkomycins was almost identical to the procedure described by BORMANN *et al.*[8]. Nikkomycins Z and pseudo-Z (ΨZ) were eluted from a SP-Sephadex C-25 column with 0.04 M pyridine acetate buffer (pH 4.7) and nikkomycins J and pseudo-J (ΨJ) with 0.05 M pyridine acetate buffer (pH 4.7). The fractions containing the new nikkomycins were pooled, concentrated in a rotatory evaporator and lyophilized. The separation of nikkomycin Z, pseudo-Z, nikkomycin J and pseudo-J was carried out by preparative HPLC (HPP-200/100, Kronwald, Sinsheim, FRG) and eluates were detected at 260 nm using a spectralphotometer, equipped with a preparative cell (Knauer, Berlin, FRG). The preparative column (250×16 mm) and pre-column (30×16 mm) were filled with Nucleosil-100 C-18 reversed phase material (particle size 10 μm; Grom, Ammerbuch, FRG). The nikkomycins could be successively eluted using pure water and a linear water-methanol gradient. The linear 0~10% methanol gradient started after 6 minutes and lasted for 10 minutes (flow rate: 24 ml/minute).

Biolocigal Activity

The MIC values for nikkomycin Z were determined by agar disc diffusion assay with a nikkomycin concentration ranging from 10 to 1,000 μg/ml. The activity of nikkomycin pseudo-Z, nikkomycins J and pseudo-J were compared in relation to nikkomycin Z with a concentration ranging from 100 to 2,000 μg/ml. Paper disks (diameter: 6 mm) with 10 μl nikkomycin solution were applied to the following agar plates (diameter 8.5 cm) seeded with test organisms:

Medium 1: Glucose 0.4%, yeast extract 0.4%, malt extract 1%, agar 1.5%, pH 5.5.

Medium 2: Malt extract 2%, agar 1.5%.

Medium 3: Na-Glutamate 8 mM, KH_2PO_4 3.6 mM, $MgSO_4$ 0.4 mM, NaCl 1.7 mM, glucose 0.2%, agar 1.5%, pH 5.5.

Medium 4: Corn meal agar (Difco).

Test plates were prepared by the following methods:

- Yeasts: 10 ml agar plates were inoculated with an overnight culture in the same medium (0.1 ml culture, $OD_{578\,nm}$=1.3).

- Fungi: 10 ml agar plates were inoculated with 10^5 spores per ml. In the case of *Botrytis* and *Alternaria* 200 ml agar medium were inoculated with the mycelium harvested from an agar plate. In this case the test plates were prepared with 17.5 ml medium. The incubation temperatures for the test organisms are listed in Table 1.

Chitin Synthetase Assay

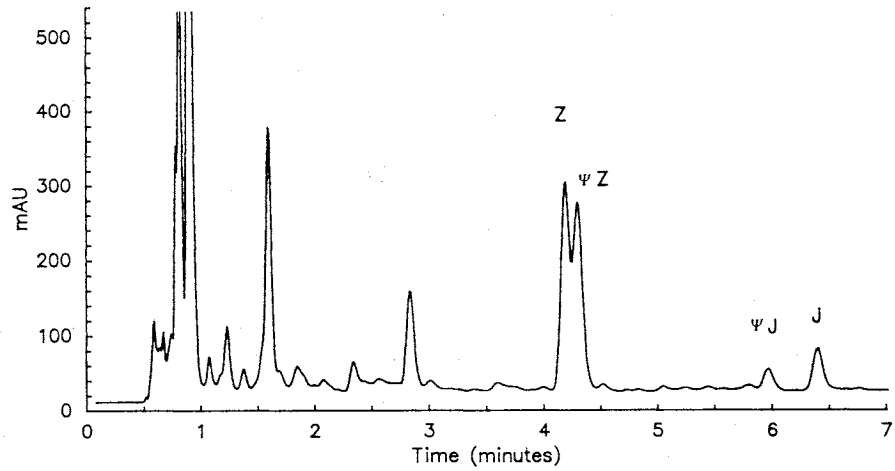
The preparation of the digitonin solubilized chitin synthetase from *Coprinus cinereus* was performed according to GOODAY and DE ROUSSET-HALL[17]. The *Ki*-values for nikkomycins Z and pseudo-Z were determined as described by DIXON[18].

Table 1. Biological activity of the new nikkomycins in relation to nikkomycin Z.

Microorganism	Temp (°C)	Medium	Biological activity in % nikkomycin[a]			
			Z	Pseudo-Z	J	Pseudo-J
Yeasts:						
Candida albicans Tü 565	27	1	100	0	0	0
Saccharomyces cerevisiae Tü 125	27	1	0	0	0	0
Yarrowia lipolytica ATCC 8662	27	1	100	7.5	0.15	0
Y. lipolytica ATCC 8662	27	3	100	3.2	0.05	0.02
Fungi:						
Alternaria kikuchiana CBS 107.53	27	4	100	49.5	14.9	14.9
Botrytis cinerea Tü 157	24	2	100	47.0	44.1	30.3
Mucor hiemalis Tü 180	24	1	100	46.0	4.6	1.4
M. miehei Tü 284	37	1	100	40.3	0	0
Paecilomyces variotii Tü 137	37	1	100	9.7	0	0

[a] MIC nikkomycin Z (μg/ml): 100%.
Tü: Culture collection of the Institute of Microbiology I, University of Tübingen.

Fig. 1. HPLC analysis of the culture broth of *Streptomyces tendae* Tü 901/PF 53$^+$-3 after 8 days of cultivation.



The classification of the peaks was carried out in the chromatogram.
mAU: Milli absorbance units.

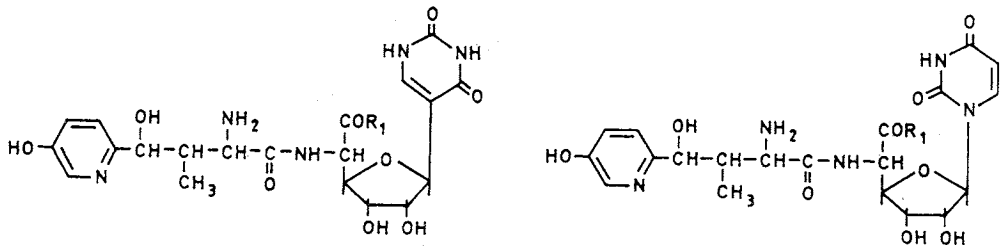
Results and Discussion

In an attempt to isolate new nikkomycins from mutants of *S. tendae*, two new substances were detected by HPLC analysis of the culture filtrate of prototrophic revertants of *S. tendae* Tü 901/PF 53 (spontaneous reversion rate: 5.8×10^{-5}). Among 60 tested revertants three strains *S. tendae* Tü 901/PF 53$^+$-3, -5 and -29 produced two new compounds with an UV-spectrum similar to nikkomycin Z. The UV-spectra were recorded during the HPLC run by a photodiode array detector. As *S. tendae* Tü 901/PF 53$^+$-3 produced the highest amount of the new substances, this strain was used for further experiments. An HPLC chromatogram and the classification of the peaks is shown in Fig. 1.

Isolation and Fermentation

The separation of nikkomycins Z and pseudo-Z from nikkomycins J and pseudo-J could be carried

Fig. 2. Structures of the dipeptide nikkomycins Z and pseudo-Z and tripeptide nikkomycins J and pseudo-J.

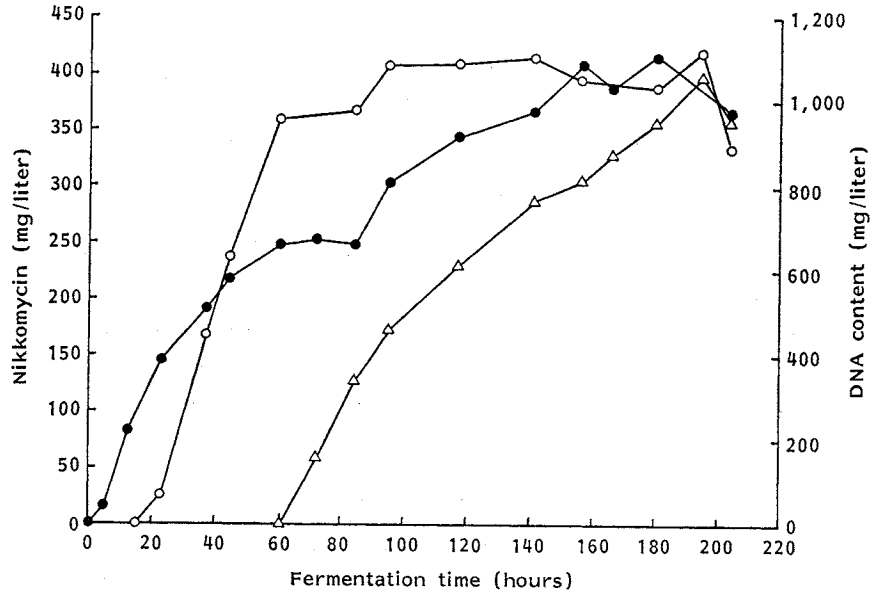


Nikkomycin pseudo-Z R_1=OH
Nikkomycin pseudo-J R_1=Glu

Nikkomycin Z R_1=OH
Nikkomycin J R_1=Glu

Fig. 3. Production of nikkomycins Z and pseudo-Z during fermentation of *Streptomyces tendae* Tü 901/PF 53$^+$-3.

○ Nikkomycin Z, ● DNA content, △ nikkomycin pseudo-Z.



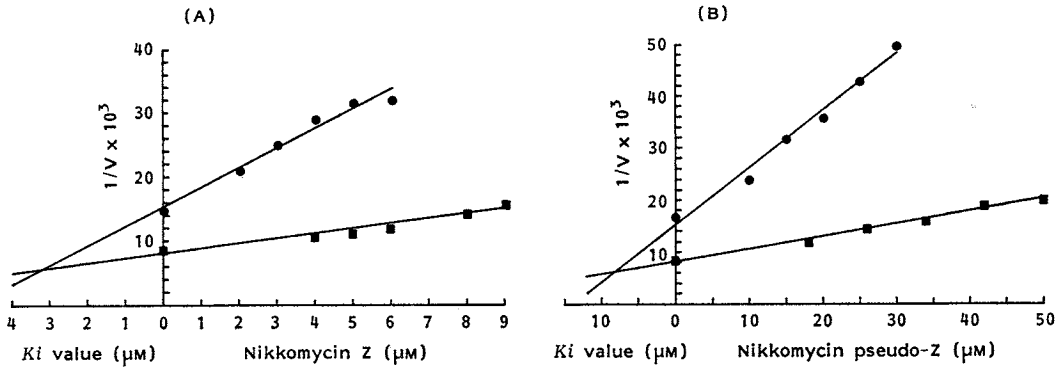
out on a SP-Sephadex C-25 column, as described in Materials and Methods. The dipeptidyl nikkomycins Z and pseudo-Z and the tripeptidyl pair nikkomycins J and pseudo-J could be completely separated by preparative HPLC. The structure elucidation† provided the result that *S. tendae* Tü 901/PF 53$^+$-3 synthesizes two new components, which are nearly identical to nikkomycins Z and J. In contrast to the *N*-glycosyl linkage between uracil and 5-aminohexuronic acid in nikkomycins Z and J, nikkomycins pseudo-Z and pseudo-J have a *C*-glycosidic bond at the same position. According to pseudouridine[19], which is a *CC*-nucleoside found in transfer-ribonucleic acid, we named the new components nikkomycins pseudo-Z and pseudo-J. Fig. 2 illustrates the structures of the new nikkomycins.

In a production medium, without uracil, *S. tendae* Tü 901/PF 53$^+$-3 produced a mixture of nikkomycins of the Z- and X-series. High levels of uracil suppressed the production of the nikkomycins with

† Results concerning the determination of the chemical structure will be published in the next time in the same journal.

Fig. 4. Dixon plot for the determination of the *Ki* value of chitin synthetase of *Coprinus cinereus* for nikkomycins Z (A) and pseudo-Z (B).

V: Incorporation rate of substrate into chitin (nmol/minute/mg protein).
● 0.5 mM UDP-GlcNAc, ■ 1.0 mM UDP-GlcNAc.



4-formyl-4-imidazoline-2-one as base[10]. Nikkomycins pseudo-Z and pseudo-J could only be detected under uracil supplemented conditions, together with the analogous compounds nikkomycins Z and J. In fermentations the nikkomycin Z production started 40 hours earlier than nikkomycin pseudo-Z synthesis (see Fig. 3). After 8 days of incubation concentrations of nikkomycins Z and pseudo-Z were 420 mg/liter and 400 mg/liter, respectively. The tripeptidyl nikkomycins were only minor components of the culture broth. After 8 days 63 mg/liter nikkomycin pseudo-J and 75 mg/liter nikkomycin J could be detected. The regulation and the enzymes, which are involved in the biosynthesis of the nucleoside of nikkomycin pseudo-Z are still unknown. Enzymes which catalyze pseudouridine biosynthesis are pseudouridine synthase I[19], for the modification of uridine residues in the tRNA and pseudouridylate synthetase[20], which catalyzes the direct synthesis of pseudouridine 5′-phosphate from ribose 5-phosphate and uracil.

Biological Activity

Table 1 summarizes the results of the biological activities of the new nikkomycins. In all investigated cases nikkomycin pseudo-Z was less active than nikkomycin Z and nikkomycin pseudo-J less active than nikkomycin J. The lower biological activity of the tripeptidyl nikkomycins compared to the dipeptidyl nikkomycins has been previously described[9]. The *Ki* value of chitin synthetase of *C. cinereus* was for nikkomycin pseudo-Z approximately three times lower than for nikkomycin Z. Fig. 4 is showing the Dixon plots for the determination of the *Ki* values (*Ki* values: Nikkomycin Z 3.2 μM; nikkomycin pseudo-Z 8.5 μM). Studies on the mode of action of the polyoxins[21], which are related nucleoside peptide antibiotics, have shown that the uridine moiety has a specific site for binding to the enzyme and C-5 substituents (*e.g.*, COOH, CH_2OH or CH_3) also fit to this binding site. From our results we can conclude that the lower biological activity of the *C*-nucleoside nikkomycins is connected with a lower affinity of nikkomycin pseudo-Z to the uridine binding site of the chitin synthetase.

Acknowledgments

This work was supported by Deutsche Forschungsgemeinschaft (SFB 323). H.D. was supported by a Dechema grant. We thank A. WÖRN and I. RINDFLEISCH for the HPLC analysis.

References

1) KELLER-SCHIERLEIN, W.; A. GEIGER, H. ZÄHNER & M. BRANDL: Die Esmeraldine A und B, tief grüne Farbstoffe aus *Streptomyces antibioticus*, Tü 2706. Helv. Chim. Acta 71: 2058~2070, 1988
2) 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
3) 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
4) 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
5) KÖNIG, W. A.; K. P. PFAFF, H. H. BARTSCH, H. SCHMALLE & H. HAGENMAIER: Konfiguration von 2-Amino-4-hydroxy-4-(5-hydroxy-2-pyridyl)-3-methylbuttersäure, der *N*-terminalen Aminosäure der Nikkomycine. Liebigs Ann. Chem. 1980: 1728~1735, 1980
6) 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: 1018~1024, 1981
7) KÖNIG, W. A.; H. HAHN, R. RATHMANN, W. HASS, A. KECKEISEN, H. HAGENMAIER, C. BORMANN, W. DEHLER, R. KURTH & H. ZÄHNER: Drei neue Aminosäuren aus dem Nikkomycinkomplex – Strukturaufklärung und Synthese. Liebigs Ann. Chem. 1986: 407~421, 1986
8) BORMANN, C.; W. HUHN, H. ZÄHNER, R. RATHMANN, H. HAHN & W. A. KÖNIG: Metabolic products of microorganisms. 228. New nikkomycins produced by mutants of *Streptomyces tendae*. J. Antibiotics 38: 9~16, 1985
9) DELZER, J.; H.-P. FIEDLER, H. MÜLLER, H. ZÄHNER, R. RATHMANN, K. ERNST & W. A. KÖNIG: New nikkomycins by mutasynthesis and directed fermentation. J. Antibiotics 37: 80~82, 1984
10) FIEDLER, H.-P.; R. KURTH, J. LANGHÄRIG, J. DELZER & H. ZÄHNER: Nikkomycins: Microbial inhibitors of chitin synthase. J. Chem. Technol. Biotechnol. 32: 271~280, 1982
11) CRÜGER, W.; W. FROMMER, C. GOELKER, J. W. KAISER, H.-F. MOESCHLER, O. SALCHER, M. SCHEDEL & H. WEHLMANN: Neue Pflanzenschutzwirkstoffe aus Mikroorganismen. Ausarbeitung von Herstellungsverfahren für Nikkomycine. Pflanzenschutznachr. Bayer 38: 305~348, 1985
12) ZOEBELEIN, G. & U. KNIEHASE: Labor-, Gewächshaus- und Freilanduntersuchungen zur Wirkung von Nikkomycin gegen Insekten und Milben. Pflanzenschutznachr. Bayer 38: 203~304, 1985
13) MCCARTHY, P. J.; P. F. TROKE & K. GULL: Mechanism of action of nikkomycin and the peptide transport system of *Candida albicans*. J. Gen. Microbiol. 131: 775~780, 1985
14) HOPWOOD, D. A.; M. J. BIBB, K. F. CHATER, T. KIESER, C. J. BRUTON, H. M. KIESER, D. J. LYDIATE, C. P. SMITH, J. M. WARD & H. SCHREMPF: Genetic Manipulation of *Streptomyces*, a Laboratory Manual. The John Innes Foundation, Norwich, 1985
15) FIEDLER, H.-P.: Quantitation of nikkomycins in biological fluids by ion-pair reversed-phase high performance liquid chromatography. J. Chromatogr. 204: 313~318, 1981
16) FIEDLER, H.-P.: Screening for new microbial products by high performance liquid chromatography using a photodiode array detector. J. Chromatogr. 316: 487~494, 1984
17) GOODAY, G. W. & A. DE ROUSSET-HALL: Properties of chitin synthetase from *Coprinus cinereus*. J. Gen. Microbiol. 89: 137~145, 1975
18) DIXON, M.: The determination of enzyme inhibitor constants. Biochem. J. 55: 170~171, 1953
19) KAMMEN, H. O.; C. C. MARVEL, L. HARDY & E. E. PENHOET: Purification, structure and properties of *Escherichia coli* tRNA pseudouridine synthase I. J. Biol. Chem. 263: 2255~2263, 1988
20) BREITMANN, T. R.: Pseudouridylate synthetase of *Escherichia coli*: Correlation of its activity with utilisation of pseudouridine for growth. J. Bacteriol. 103: 264~265, 1970
21) HORI, M.; K. KAKIKI & T. MISATO: Further study on the relation of polyoxin structure to chitin synthetase inhibition. Agric. Biol. Chem. 38: 691~698, 1974