METABOLIC PRODUCTS OF MICROORGANISMS. 255†

NIKKOMYCINS W_z AND W_x, NEW CHITIN SYNTHETASE INHIBITORS FROM *STREPTOMYCES TENDAE*

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Two new dipeptidyl nikkomycins of the Z and X type were isolated from the culture broth of *Streptomyces tendae* TÜ 901/395-11/32 and characterized. They show a variation in the amino acid moiety of the molecule. Nikkomycin W_z is composed of L-tyrosine and 5-amino-5-deoxy-D-*allo*-furanuronic acid *N*-glycosidally bound to uracil, whereas nikkomycin W_x is composed of L-tyrosine and 5-amino-5-deoxy-D-*allo*-furanuronic acid *N*-glycosidally bound to 4-formyl-4-imidazolin-2-one. The new nikkomycins are good inhibitors of chitin synthetase from *Coprinus cinereus* but they did not inhibit growth of fungi and yeasts.

The wildtype strain *Streptomyces tendae* TÜ 901/8c produces the nucleoside peptide antibiotics nikkomycins Z, X, J and I[2] which act as potent competitive inhibitors of fungal and insectile chitin synthetase[3~6].

UV/MOP mutagenesis of the wildtype strain led to the mutant *S. tendae* TÜ 901/395 which produced in addition to nikkomycins Z, X, J and I, the compounds K_z, K_x, O_z and O_x[7]. By analyzing colonies derived from protoplasts of TÜ 901/395, strain TÜ 901/395-11 was obtained, which produced only nikkomycins K_z, K_x, O_z and O_x[8]. Mutation of *S. tendae* TÜ 901/395-11 by NTG and selection of fluorotyrosine-resistant clones led to the isolation of *S. tendae* TÜ 901/395-11/32 which produced in addition to nikkomycins K and O two new compounds, named nikkomycins W_z and W_x.

This paper discusses the fermentative production, isolation, structure elucidation and biological properties of the new nikkomycins W_z and W_x.

Results

Producing Organism

The fluorotyrosine-resistant strain *S. tendae* TÜ 901/395-11/32 producing nikkomycins W_z and W_x was obtained from *S. tendae* TÜ 901/395-11 by NTG mutagenesis as described in the Experimental section. The mutant produced in addition to nikkomycins K_z, K_x, O_z and O_x two compounds which were identified by HPLC and diode array detection as new nikkomycins. The UV spectra of the new nikkomycins W_z

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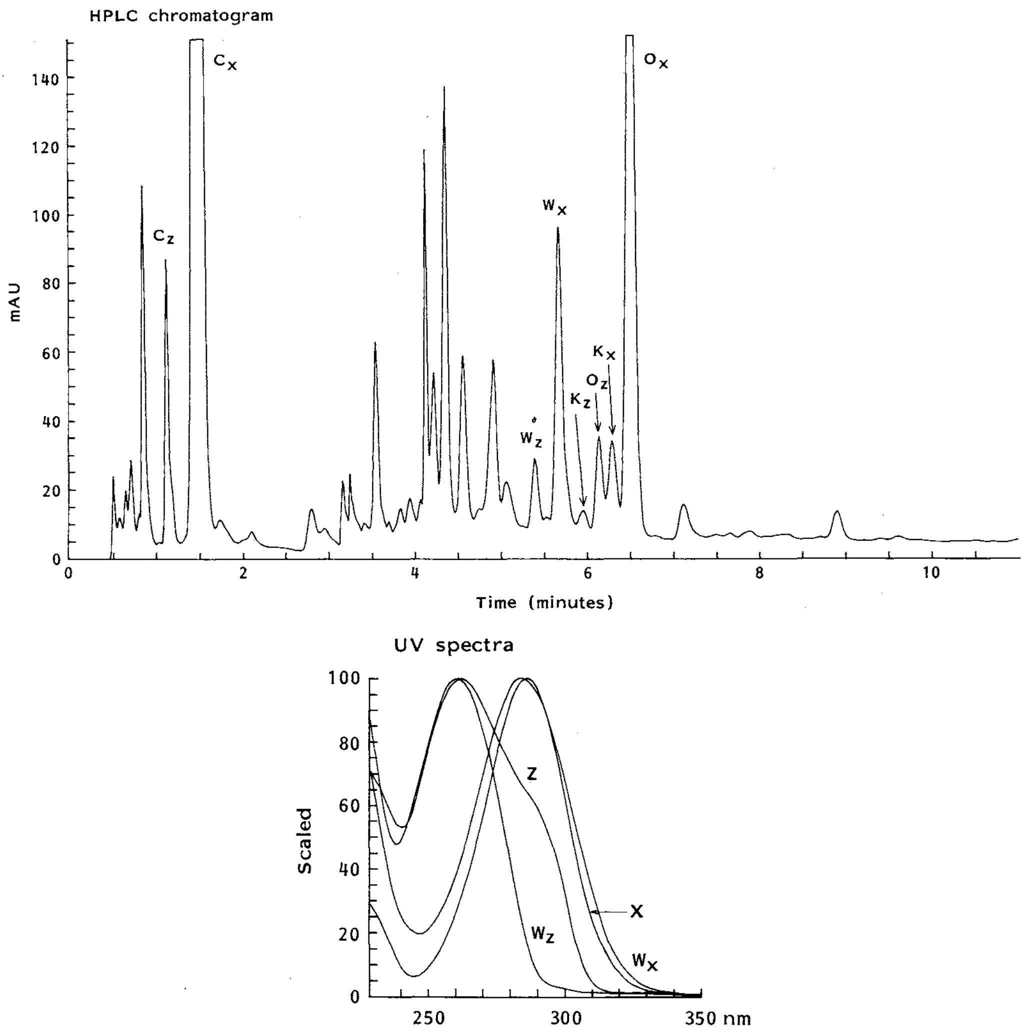
Fig. 1. HPLC chromatogram at 270 nm of culture filtrate of *Streptomyces tendae* TÜ 901/395-11/32.

The UV spectra of nikkomycins W_z and W_x were recorded during the HPLC run and overlayed with the spectra of nikkomycins Z and X which were stored on a computer library.



and W_x are similar to those of nikkomycins Z and X, as shown in Fig. 1.

Fermentation and Isolation

Large amounts of nikkomycins W_z and W_x were produced by 10-liter fermentations of *S. tendae* TÜ 901/395-11/32. The maximal production of total 300 mg/liter nikkomycins W_z and W_x was reached after 148 hours of incubation.

The isolation and purification was carried out according to the separation scheme for nikkomycins as described by FIEDLER[9]. Nikkomycins were isolated from the culture filtrate and purified by ion exchange column chromatography using Dowex 50W-X4, Amberlite IRA 401-S and SP-Sephadex C-25. Nikkomycins W_z and W_x were separated from each other to homogeneity by preparative reversed-phase HPLC using Nucleosil-100 C-18 and gradient elution with water - methanol. After concentration and freeze-drying, both compounds were obtained as white powders.

Physico-chemical Properties

Acid hydrolysis of nikkomycins W_x and W_z, with subsequent methylation (HCl-methanol) and trifluoroacetylation, yielded a volatile derivative which was identified by GC-MS as *N,O*-bistrifluoroacetyl-tyrosine-*O*-methyl ester (*m/z* 387, M^+; *m/z* 274, $M-TFANH_2$; *m/z* 203, TFAO-C_6H_4-CH_2). The L-configuration was assigned by chiral capillary GC using XE-60-L-valine-(*S*)-α-phenylethylamide as chiral stationary phase[10].

Negative ion FAB-MS gave identical $(M-H)^-$ signals at *m/z* 449 for nikkomycins W_x and W_z and fragment ions at *m/z* 111 for the base (4-formyl-4-imidazolin-2-one and uracil, respectively).

The ^1H NMR spectrum of nikkomycin W_x is shown in Fig. 2. The chemical shift data and coupling constants of both metabolites are given in Table 1. The singlets (Fig. 2) at δ 7.58 (5-H) and δ 9.13 (aldehyde proton) are typical for the 4-formyl-4-imidazolin-2-one residue, while the uracil moiety of nikkomycin W_z shows the dublets of 5-H and 6-H at δ 5.77 and δ 7.54, respectively. The signals of the AA′BB′ coupling system of the tyrosine moiety appear at δ 6.70 and δ 7.00 for nikkomycin W_x and at δ 6.74 and δ 7.03 for nikkomycin W_z with similar coupling constants. The signals of the sugar moiety and of the α- and β-protons of the tyrosine residue were unambiguously assigned on the basis of ^1H-^1H COSY spectra.

The configuration of the 5-amino-5-deoxy-β-D-*allo*-furanuronic acid moiety was found to be identical to nikkomycins X and Z by comparing their ^1H NMR data[11].

From these spectroscopic investigations the structures of both metabolites could be determined.

Fig. 2. ^1H NMR spectrum (400 MHz, D_2O) of nikkomycin W_x.

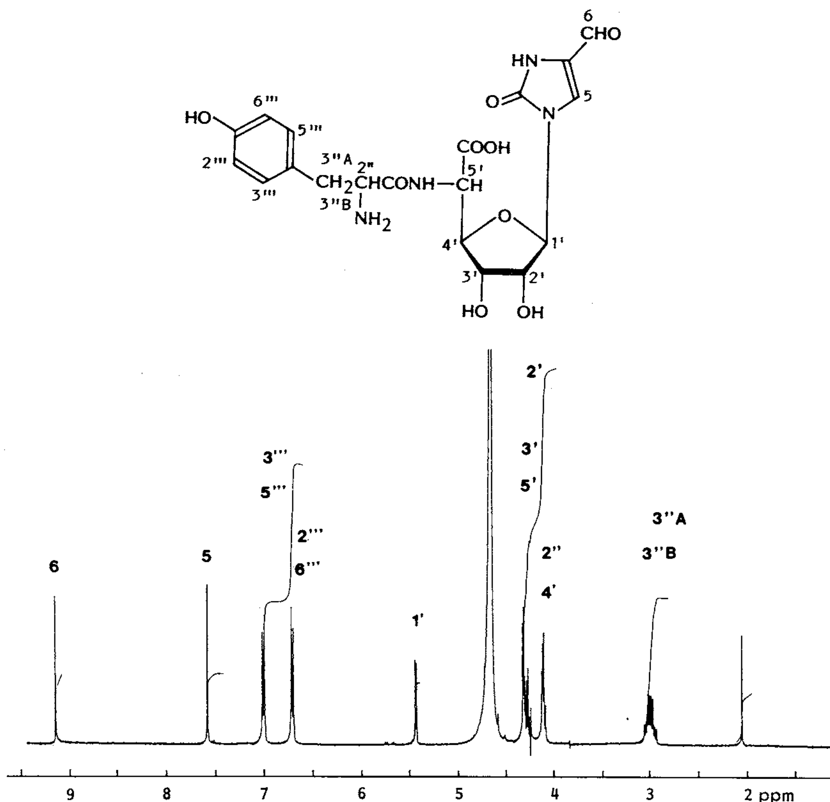


Table 1. ^{1}H chemical shifts (ppm) of nikkomycins W_x and W_z.

Nikkomycin W_x:	δ 2.96 (1H, dd, J=7.2, 14.4 Hz, PhCH_2CH), 3.30 (1H, dd, J=6.8 Hz, PhCH_2CH), 4.10 (1H, dd, $CH_2CH NH_2$), 4.11 (1H, t, $J_{3',4'}=J_{4',5'}$=4.0 Hz, 4'-H), 4.26 (1H, dd, $J_{2',3'}$=5.4 Hz, 2'-H), 4.30 (1H, dd, 3'-H), 4.31 (1H, d, 5'-H), 5.43 (1H, d, $J_{1',2'}$=5.8 Hz, 1'-H), 6.70 (2H, m, J=8.4 Hz, Ph-H), 7.00 (2H, d, Ph-H), 7.58 (1H, s, 5-H), 9.13 (1H, s, 6-H)
Nikkomycin W_z:	δ 3.01 (1H, dd, J=7.0, 14.4 Hz, PhCH_2CH), 3.07 (1H, dd, J=7.0 Hz, PhCH_2CH), 4.10 (1H, dd, $J_{3',4'}$=5.2 Hz, 4'-H), 4.14 (1H, t, $CH_2CH NH_2$), 4.16 (1H, dd, $J_{2',3'}$=5.6 Hz, 2'-H), 4.28 (1H, dd, 3'-H), 4.35 (1H, d, $J_{4',5'}$=4.0 Hz, 5'-H), 5.70 (1H, d, $J_{1',2'}$=5.4 Hz, 1'-H), 5.77 (1H, d, $J_{5,6}$=8.0 Hz, 5-H), 6.74 (2H, m, J=7.8 Hz, Ph-H), 7.03 (2H, m, Ph-H), 7.54 (1H, d, 6-H)

Biological Activity

The new nikkomycins are good inhibitors of the chitin synthetase from *Coprinus cinereus*. The inhibition constants (*Ki*) ranged from 4.5 μM for nikkomycin W_x and 12 μM for nikkomycin W_z. The *Ki* value for nikkomycin Z is 3.2 μM[12] and is in the same order of magnitude. In contrast, the new nikkomycins did not inhibit growth of fungi and yeasts which are sensitive to nikkomycin Z. Nikkomycin W_x was 1,000 ~ 2,000 times less active as nikkomycin Z using *Yarrowia lipolytica* as test organism (MIC of nikkomycin Z: 0.23 μg/ml[7]).

In order to examine the discrepancy between chitin synthetase inhibition and activity of nikkomycins W_z and W_x against whole cells, we investigated the stability of the new nikkomycins in a crude cell extract of *Y. lipolytica*. Nikkomycins W_z and W_x were rapidly hydrolyzed to inactive nikkomycin C_z or C_x and L-tyrosine. Under identical experimental conditions, nikkomycin Z was not degraded by a cell extract of *Y. lipolytica*.

Discussion

So far only nikkomycins which contain unusual *N*-terminal amino acids have been isolated. This is the first description of nikkomycins produced by mutants of *S. tendae* with a proteinogenic amino acid peptidically bound to the amino hexuronic acid residue. In contrast to *S. tendae* TÜ 901/395-11 the mutant strain TÜ 901/395-11/32 excreted up to 1 mM tyrosine during the fermentation. Presumably, the enzyme catalyzing the formation of the peptide bond between amino acid and nucleoside moiety is unspecific so that high intracellular concentrations of L-tyrosine lead to the incorporation of this amino acid in the nikkomycin molecule.

The new nikkomycins do not inhibit growth of fungi and yeasts because of their rapid degradation by intracellular peptidases. In accordance, a synthetic compound, which is identical to nikkomycin W_z was also rapidly metabolized and inactivated by cell extracts from *Candida albicans*[13].

Experimental

Bacterial Strains

The NTG mutagenesis was performed with *S. tendae* TÜ 901/395-11 as described by BORMANN *et al.*[8]. The fluorotyrosine resistant mutant *S. tendae* TÜ 901/395-11/32 was selected among 1% survivors on minimal medium[14] which was supplemented with 10 μg/ml fluorotyrosine.

Fermentation

Strain TÜ 901/395-11/32 was cultivated in a 10-liter fermenter (Biostat E, Braun-Melsungen, FRG). 9.5 liters of medium containing starch 2%, mannitol 2%, soybean meal 2%, yeast extract 0.5% and tyrosine 10 mM in tap water (pH 6.8) were inoculated with 0.5 liter of shaking cultures grown for 30 hours

in the same medium. The fermenter was kept at 27°C and agitated at 400 rpm with an aeration of 0.5 v/v/m.

Isolation

The fermentation broth was adjusted to pH 4 with acetic acid and filtered with addition of 2% Hyflo Super-cel. The culture filtrate was chromatographed on a column containing Dowex 50W-X4 (Na^+, 50~100 mesh) and nikkomycins were eluted with 0.05 N ammonia. The eluate was immediately concentrated *in vacuo* to remove ammonia, adjusted to pH 8 and chromatographed on a column containing Amberlite IRA-401 S (OH^-). Nikkomycins were eluted with 1% formic acid, concentrated, adjusted to pH 3 and chromatographed on a SP-Sephadex C-25 (pyridine$^+$) column. Nikkomycins W_x and W_z were eluted with water and separated from nikkomycins K_x, K_z, O_x and O_z, which were completely bound to the ion exchange material. The fractions containing the new nikkomycins were concentrated and lyophilized. The separation of nikkomycins W_x and W_z was achieved by preparative HPLC using a Nucleosil-100 C-18 column (10 μm, 16 i.d. × 250 mm, precolumn 16 i.d. × 30 mm; Grom, Ammerbuch, FRG). Both compounds were quantitatively separated by elution with water over a period of 6 minutes, followed by a linear methanol gradient (0~10%) within 8 minutes, flow rate 24 ml/minute (pump HPP-200/100, gradient controller GCU-311; Kronwald, Sinsheim, FRG). The eluate was detected at 260 nm using a spectrophotometer equipped with a preparative cell (Knauer, Berlin, FRG). Nikkomycin-containing fractions were concentrated and lyophilized.

MS

Mass spectra were recorded on a Hewlett-Packard instrument HP 5985A (GC-MS) and on a Finnigan MAT 311A (negative ion FAB with glycerol as matrix and xenon as collision gas).

GC

For GC-MS investigations a 25-m fused silica capillary column with SE 30, for the configurational analysis a 25-m capillary with XE-60-L-valine-(*S*)-α-phenylethylamide[10] was used.

NMR Spectroscopy

The ^1H and ^1H-^1H COSY NMR spectra were recorded on a Bruker WM 400 (400 MHz) spectrometer using the DOH-resonance at δ 4.64 as an internal reference.

Derivatives

Samples of about 1 mg were hydrolyzed in screw cap vials in 0.5 ml of 3 N HCl for 2 hours at 100°C. After removal of HCl, 1 ml of a solution of hydrogen chloride in methanol was added to the sample and heated for 30 minutes at 100°C. The reagent was removed in a stream of nitrogen and 200 μl of dichloromethane and 50 μl of trifluoroacetic anhydride were added. The mixture was heated for 30 minutes at 100°C. Again the excess reagent was removed in a stream of nitrogen; the residue was dissolved in 100 μl of dichloromethane and used for the GC investigations.

Biological Assays

The disc diffusion assay was performed under the same experimental conditions as described by DECKER *et al.*[12] using identical test organisms.

The *Ki* values for nikkomycins W_z and W_x were determined for the digitonin solubilized chitin synthetase from *C. cinereus*[15] as described by DIXON[16].

Crude cell extract was prepared from *Y. lipolytica*. The culture was incubated at 27°C on a rotary shaker in 500-ml Erlenmeyer flasks, harvested in the early stationary growth phase and washed twice with 50 mM KH_2PO_4 buffer, pH 5.5. The cells were homogenizied as described by LOGAN[17]. Glass beads and unbroken cells were removed by centrifugation (9,000 × *g*). Glycerol was added to a final concentration of 20% and the extract was stored at −20°C. The reaction mixture consisted of 50 μl cell extract (5 mg protein/ml), 75 μl 50 mM KH_2PO_4 (pH 6.5) and 25 μl nikkomycin solution (1 mg/ml). After 15 and 30 minutes, 50 μl were removed and 10 μl acetic acid were added to stop the reaction. The sample was centrifuged (13,000 × *g*) and analyzed by HPLC[18].

Acknowledgments

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References

1) Heitsch, H.; W. A. König, H. Decker, C. Bormann, H.-P. Fiedler & H. Zähner: Metabolic products of microorganisms. 254. Structure of the new nikkomycins pseudo-Z and pseudo-J. J. Antibiotics 42: 711～717, 1989
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) Brillinger, G. U.: Chitin synthase from fungi, a test model for substances with insecticidal properties. Arch. Microbiol. 121: 71～74, 1979
4) Müller, H.; R. Furter, H. Zähner & D. M. Rast: Inhibition of chitosomal chitin synthetase and growth of *Mucor rouxii* by nikkomycin Z, nikkomycin X, and polyoxin A: A comparison. Arch. Microbiol. 130: 195～197, 1981
5) 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
6) Cohen, E. & J. E. Casida: Properties and inhibition of insect integumental chitin synthetase. Pestic. Biochem. Physiol. 17: 301～306, 1982
7) 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
8) Bormann, C.; S. Mattern, H. Schrempf, H.-P. Fiedler & H. Zähner: Isolation of *Streptomyces tendae* mutants with an altered nikkomycin spectrum. J. Antibiotics 42: 913～918, 1989
9) Fiedler, H.-P.: Nikkomycins and polyoxins. *In* Natural Products Isolation. Separation Methods for Antimicrobials, Antivirals and Enzyme Inhibitors. *Eds*., G. H. Wagman & R. Cooper, pp. 153～189, Elsevier, 1989
10) König, W. A.; I. Benecke & S. Sievers: New results in the gas chromatographic separation of enantiomers of hydroxy acids and carbohydrates. J. Chromatogr. 217: 71～79, 1981
11) Rathmann, R.; W. A. König, H. Schmalle, G. Carlsson, R. Bosch, H. Hagenmaier & W. Winter: Untersuchungen zur Konfiguration und Konformation der Nucleosidbausteine der Nikkomycine. Liebigs Ann. Chem. 1984: 1216～1229, 1984
12) Decker, H.; C. Bormann, H.-P. Fiedler, H. Zähner, H. Heitsch & W. A. König: Metabolic products of microorganisms. 252. Isolation of new nikkomycins from *Streptomyces tendae*. J. Antibiotics 42: 230～235, 1989
13) Shenbagamurthi, P.; H. A. Smith, J. M. Becker, A. Steinfeld & F. Naider: Design of anticandidal agents: Synthesis and biological properties of analogues of polyoxin L. J. Med. Chem. 26: 1518～1522, 1983
14) Hopwood, D. A.; H. M. Wright, M. J. Bibb & S. N. Cohen: Genetic recombination through protoplast fusion in *Streptomyces*. Nature 268: 171～174, 1977
15) Gooday, G. W. & A. De Rousset-Hall: Properties of chitin synthetase from *Coprinus cinereus*. J. Gen. Microbiol. 89: 137～145, 1975
16) Dixon, M.: The determination of enzyme inhibitor constants. Biochem. J. 55: 170～171, 1953
17) Logan, D. A.: Partial purification and characterization of intracellular carboxypeptidase of *Candida albicans*. Exp. Mycol. 11: 115～121, 1987
18) Fiedler, H.-P.: Screening for new microbial products by high-performance liquid chromatography using a photodiode array detector. J. Chromatogr. 316: 487～494, 1984