Novel Nikkomycins L$_x$ and L$_z$ Produced by Genetically Engineered *Streptomyces tendae* Tü901

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Streptomyces tendae Tü901 produces the peptidyl nucleoside antibiotics nikkomycins I, J, X, and Z, which act as competitive inhibitors of chitin synthetases from fungi and insects (reviewed by FIEDLER, 1993[1]). The peptidyl side chain of these nikkomycins, 2-amino-4-hydroxy-3-methyl-4-(3'-hydroxy-6'-pyridyl)butanoic acid (hydroxypyridyl-homothreonine) (Fig. 1), is linked *via* a peptide bond to the nucleoside moiety. The nucleoside moiety consists of a 5-aminohexuronic acid with either *N*-glycosidically bound 4-formyl-1,3-dihydro-2*H*-imidazole-2-one (nikkomycin C$_x$; forming nikkomycin X) or *N*-glycosidically bound uracil (nikkomycin C$_z$; forming nikkomycin Z). Nikkomycins I and J have glutamic acid peptidically bound to the 6'-carboxyl group of the nikkomycin C$_x$ or C$_z$ component, respectively. More than 20 biologically active nikkomycin structures have been generated by mutasynthesis, directed fermentation, and enzymatic modification, and have been isolated from mutants and as minor components from the culture filtrate of the wild type strain.

Here we report the isolation, structure elucidation, and antimicrobial activity of two novel nikkomycin derivatives, nikkomycins L$_x$ and L$_z$, synthesized as main components by a genetically engineered mutant of *S. tendae* Tü901. The mutant was obtained by inserting a kanamycin resistance gene in the nikkomycin biosynthesis gene *nikF* encoding a cytochrome P450 monooxygenase (characterization and inactivation of the *nikF* gene will be separately published). To characterize the effect of the mutation on nikkomycin biosynthesis the *nikF* gene insertion mutant was cultivated in SP production medium[2] for seven days on a rotatory shaker at 27°C and the culture filtrate was analyzed for nikkomycins by HPLC[3] and for antifungal activity against the test organism *Paecilomyces variotii* by agar diffusion assay. The mutant did not not synthesize nikkomycins Z, X, J, and I, which exhibit retention times of 6.95, 7.20, 8.80, and 8.90 minutes, respectively, in HPLC chromatograms. In contrast, the mutant accumulated two compounds which eluted at 6.90 and 7.00 minutes. According to the high antifungal activity of culture filtrates and the following data they were expected to be novel nikkomycin derivatives that contain uracil or 4-formyl-imidazolone as the base, respectively, and were therefore named nikkomycins L$_z$ and L$_x$. The UV/Vis diode-array spectral analyses indicated an absorption maximum at 260 nm for nikkomycin L$_z$, and at 267 nm and 286 nm for nikkomycin L$_x$. The UV/Vis spectrum of nikkomycin L$_z$ was almost identical to that of nikkomycins containing the uracil base[4], and the spectrum of nikkomycin L$_x$ was very similar to that of nikkomycin K$_x$[5]. Nikkomycin K$_x$ contains 4-formyl-1,3-dihydro-2*H*-imidazole-2-one as the base and 2-amino-4-hydroxy-4-(2'-pyridyl)butanoic acid as the peptidyl moiety (Fig. 1). Nikkomycin L$_x$ reacted with the aldehyde reagent barbituric acid to form red-colored reaction compounds. This reaction is characteristic for nikkomycins containing the 4-formyl-imidazolone base, whereas nikkomycins with uracil as the base do not react with barbituric acid[6]. Furthermore, nikkomycins L$_z$ and L$_x$ did not co-elute with known nikkomycins in HPLC chromatograms.

Nikkomycins L$_z$ and L$_x$ were isolated from fermentation broth (4 liter) containing approximately 50 and 450 mgl^{-1}, respectively. The culture filtrate was chromatographed on a column containing Dowex 50 WX 2 (H$^+$, 100~200 mesh)[2]; nikkomycins L$_x$ and L$_z$ were eluted with 0.05 N ammonia. The eluate was immediately concentrated *in vacuo* and chromatographed on Lewatit MP 64 Z (acetate$^-$) at pH 5.5; nikkomycin L$_z$ and 30% of the applied nikkomycin L$_x$ did not bind to the resin and were eluted with water. The eluate was concentrated *in vacuo* and lyophilized. To isolate nikkomycin L$_z$, 50 mg of the sample was dissolved in 850 μl of water, and barbituric acid (pH 7) was added to a final concentration of 1 mg per ml. After incubation for 2 hours at room temperature, nikkomycin L$_x$ reacted with barbituric acid. The reaction mixture was chromatographed on a column with Biogel P2 using water as the eluent. Fractions containing nikkomycin L$_z$ were

Fig. 1.   Chemical structures of nikkomycins L_x and L_z (A) and peptidyl moieties
of indicated nikkomycins[1] (B).

Nikkomycin L_x

Nikkomycin L_z

Nikkomycin I, J, X, Z

Nikkomycin K_x, K_z

Nikkomycin P_x, R_x
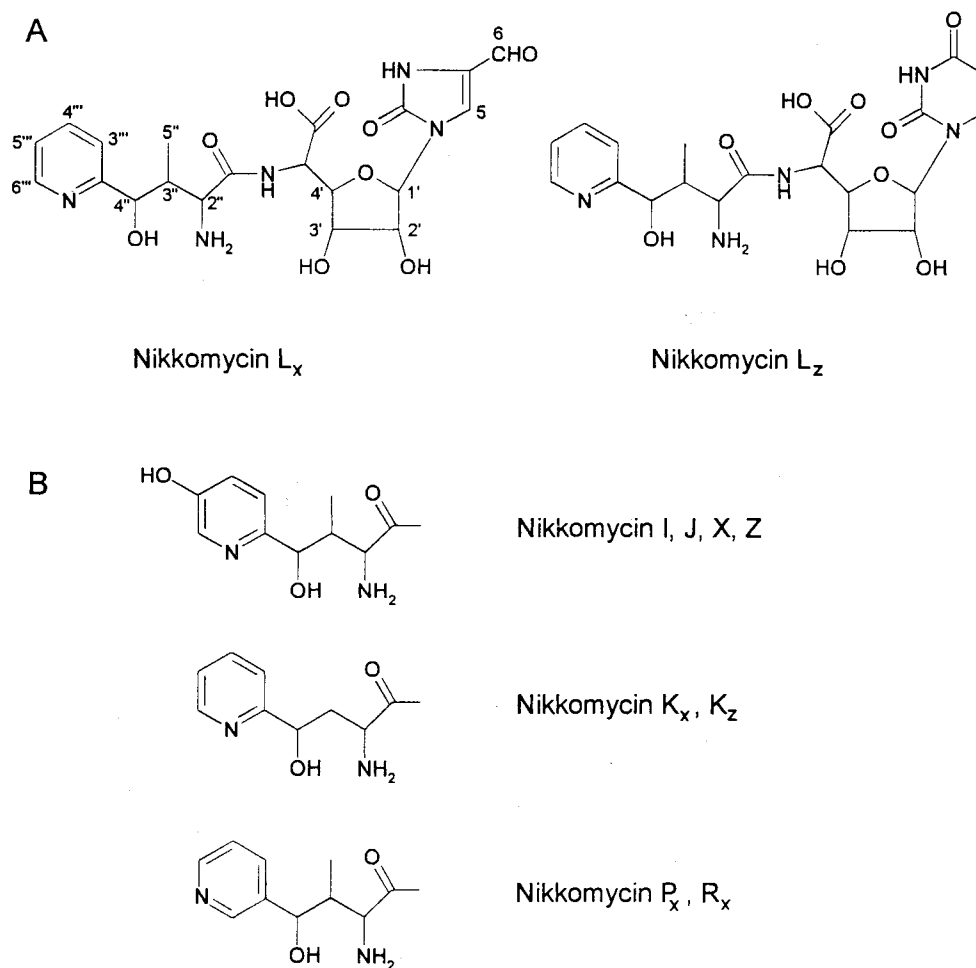
lyophilized and resulted in the isolation of nikkomycin L_z at 60 to 70% purity. The nikkomycin L_x that bound to Lewatit MP 64 Z (mentioned above) was eluted with 75 mM formic acid. The fractions were concentrated *in vacuo*, lyophilized, and chromatographed on a column containing Biogel P2 using water as the eluent. Fractions containing nikkomycin L_x were lyophilized and subsequently chromatographed on a column containing MCI gel CHP 20 P (75~150 μ; Mitsubishi Chemical Corporation; Tokyo) with water, and nikkomycin L_x was eluted with 10 % methanol, lyophilized and obtained at more than 95% purity.

The structures of nikkomycin L_x and L_z were elucidated by electrospray mass spectrometry and ¹H- and ¹³C-NMR spectroscopy[2]. ES-MS gave identical [M + H]⁺ signals at m/z 480.0 for nikkomycins L_x and L_z. The observed mass difference of 16 amu corresponded

to the expected mass of nikkomycin Z and X analogues which lack the oxygen atom of the pyridyl residue. The results were proved by high accuracy measurements (ES-FTICR-MS) comparing the mass of the measured mass spectra ([M + H]⁺ = 480.1718) with the theoretically expected exact molecular mass (480.1725 amu) of the elemental composition $C_{20}H_{26}N_5O_9$. The deviations were below 1.5 ppm. However, fragments of the MS/MS experiments could not be unambigously assigned to the expected structure of nikkomycins L_x and L_z.

The complete structure elucidation of nikkomycin L_x was based on the following interpretation of NMR spectra (TOCSY, ROESY, HSQC, and HMBC) (Table 1). In the TOCSY spectrum, three spin systems were found; two spin systems were assigned to the aliphatic and aromatic part of the arylhomothreonine amino acid, and the third spin system was assigned to the sugar unit

of the nucleoside. Because of the lack of a further spin system, which should have been observed if uracil were present as the base, and because of the presence of a single signal in the HSQC spectrum, which exhibited a characteristic chemical shift for aldehydes [$\delta(^1H) = 9.10\,ppm/\delta(^{13}C) = 183.9\,ppm$], we postulated a 4-formyl-1,3-dihydro-2H-imidazole-one structure as part of nikkomycin L_x. This was verified by the interpretation of the HMBC spectrum. Except for the structure of the aromatic ring attached to the homothreonine moiety, the structure of nikkomycin L_x is identical to that of nikkomycin X. A closer investigation of the aromatic ring structure revealed four coupling protons in the TOCSY spectrum. Some of these aromatic signals showed an extreme low-field shift in the ^1H- and the ^{13}C-domain, which is characteristic of a pyridyl nitrogen

atom in the aromatic ring. The relative positions of the hydrogen atoms were finally assigned by three ROE contacts of neighboring protons, which points to an α-bound nitrogen, as also expected by comparison with the original structure of nikkomycin X. For a β-bound nitrogen, only two ROE contacts would have been observed. Thus, the structure of nikkomycin L_x is identical to that of nikkomycin X, except that the hydroxy group in the p-position of the aryl-homothreonine is lacking.

The one-dimensional NMR spectrum of nikkomycin L_z revealed a pattern very similar to that of nikkomycin L_x. However, the corresponding signals from the formyl-imidazolone base were not detected, and two novel signals [$\delta(^1H) = 5.85\,ppm$; $\delta(^1H) = 7.67\,ppm$] that assign a uracil base were observed (data not shown). Therefore, we concluded that nikkomycin L_z is an analogue of nikkomycin L_x that contains uracil instead of 4-formyl-1,3-dihydro-2H-imidazole-2-one as the base (Fig. 1).

The stability of nikkomycins is influenced by the peptidyl moiety of the compounds[2]. Therefore, the stability of nikkomycins L_x and L_z was studied at various pH values using nikkomycins X and Z as references (Fig. 2). Nikkomycins L_x and L_z were significantly more stable under alkaline condition than nikkomycins X and Z. Approximately 50% of both nikkomycins L_x and L_z was present after 28 days of incubation at pH 8.0, while nikkomycins X and Z were almost completely inactivated by hydrolysis of the peptide bond, yielding the peptidyl moiety, hydroxypyridylhomothreonine, and the nucleoside moieties, nikkomycins C_x and C_z. Under neutral

Table 1. ^1H and ^{13}C chemical shifts (ppm) of nikkomycin $L_x{}^a$.

^1H	δ 7.68 (5-H), 9.10 (6-H), 5.46 (1'-H), 4.28 (2'-H), 4.38 (3'-H), 4.17 (4'-H), 4.40 (5'-H), 4.24 (2''-H), 2.60 (3''-H), 4.98 (4''-H), 0.60 (5''-H), 7.40 (3'''-H), 7.75 (4'''-H), 7.23 (5'''-H), 8.33 (6'''-H).
^{13}C	δ 127.8 (1-C), 157.1 (3-C), 129.2 (5-C), 183.9 (6-C), 90.2 (1'-C), 76.5 (2'-C), 73.8 (3'-C), 87.8 (4'-C), 60.1 (5'-C), 177.1 (6'-C), 172.4 (1''-C), 60.1 (2''-C), 47.7 (3''-C), 78.3 (4''-C), 10.0 (5''-C), 163.2 (2'''-C), 125.1 (3'''-C), 142.2 (4'''-C), 126.9 (5'''-C), 151.5 (6'''-C).

^{a} Numbering of atoms see Figure 1.

Fig. 2.   pH stability of nikkomycins L_x (■), L_z (♦), X (▲), and Z (●).
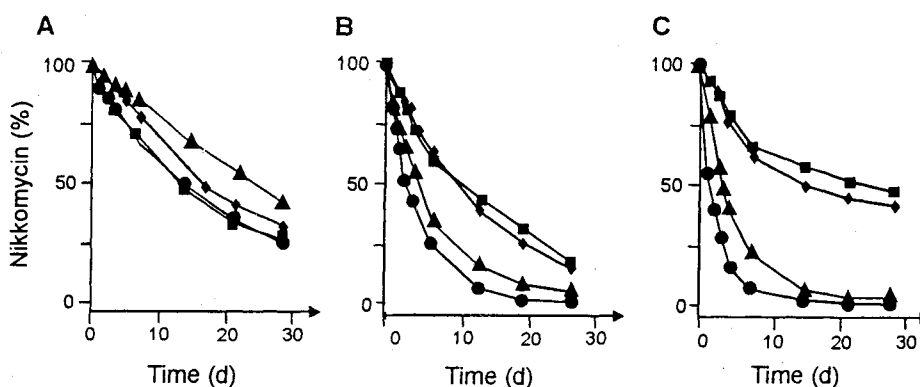
Purified nikkomycins (L_x, L_z, and Z: 1 mg/ml; X: 0.5 mg/ml) were incubated in sterile 50 mM buffer at room temperature. (A) MES, pH 5.5; (B) TES, pH 7.0; (C) Tricine, pH 8.0. Samples were analyzed by HPLC.

condition (pH 7.0), nikkomycins L_x and L_z were slightly more stable than nikkomycin X and Z, and under acid condition (pH 5.5), the investigated nikkomyins revealed similar stabilities.

The antifungal activity of nikkomycin L_x and X were compared in agar diffusion assays[5]. The activity of nikkomycin L_x against various test organisms was slightly lower or similar to that of nikkomycin X[5]. MIC's have been not determined for nikkomycin L_z, as it was not purified to homogeneity. Biological activities similar to those described for nikkomycin Z would be expected[7].

Synthesis of nikkomycins L_x and L_z has been generated by molecular genetic manipulation of S. tendae Tü901 wild type. The peptidyl moiety of these nikkomycin derivatives, 2-amino-4-hydroxy-3-methyl-4-(2'-pyridyl)-butanoic acid (nikkomycin E) was previously isolated as a minor compound from the S. tendae Tü901 culture filtrate[8]. Nikkomycin E is probably a biosynthetic intermediate that is hydroxylated by the nikF encoded P450 monooxygenase yielding hydroxypyridylhomo-threonine, the peptidyl moiety of nikkomycins I, J, X, and Z. Nikkomycins L_x and L_z complete the series of nikkomycin derivatives that lack the hydroxy group at the pyridyl ring of the peptidyl moiety, i.e. nikkomycins K_x and K_z synthesized by a mutant obtained by chemical mutagenesis of the wild type[5], and nikkomycins P_x and R_x purified as minor components from the S. tendae Tü901 culture filtrate (Fig. 1)[8].

Acknowledgments

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