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Production of Nikkomycins B_x and B_z by Mutasynthesis with Genetically Engineered *Streptomyces tendae* TÜ901

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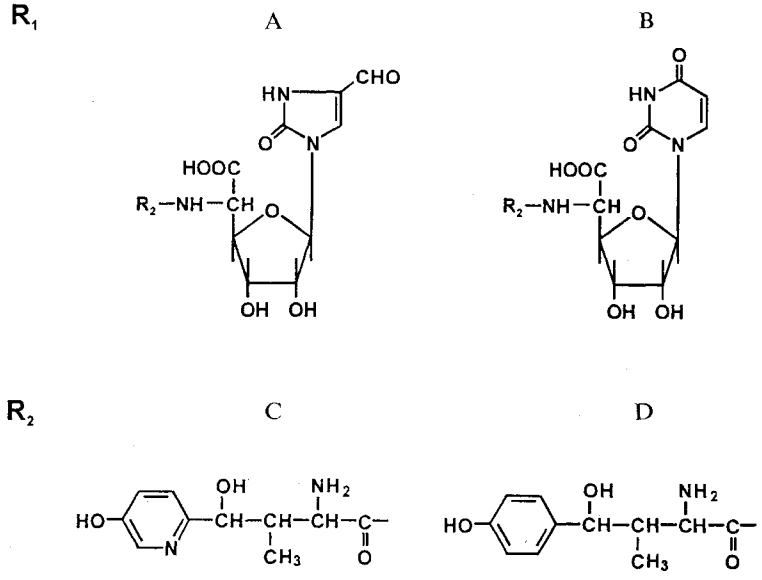
The previously described *Streptomyces tendae nikC::aph* mutant was used to mutasynthesize nikkomycins B_x and B_z. The mutant is deficient in L-lysine 2-aminotransferase, which transaminates lysine to form piperideine 2-carboxylate, the precursor of the peptidyl side chain of the biologically active nikkomycins I, J, X, and Z, and is therefore unable to produce these nikkomycins. The mutant accumulates the biologically inactive biosynthetic nucleoside precursors nikkomycins C_x and C_z. Resting cell cultures of the mutant fed with benzoic acid produced the biologically active nikkomycins B_x and B_z, which contain 2-amino-4-hydroxy-3-methyl-4-(4′-hydroxyphenyl)butanoic acid as the peptidyl side chain. The structures of nikkomycins B_x and B_z were confirmed by mass spectrometry and NMR. Nikkomycins B_x and B_z exhibit significantly higher pH stability than their analogues nikkomycins X and Z.

Nikkomycins are nucleoside-peptide antibiotics that are potent inhibitors of chitin synthetases and have antifungal, insecticidal, and acaricidal activity[1~4]. *Streptomyces tendae* Tü901 produces various nikkomycin structures, and the major components of the culture filtrate are nikkomycins I, J, X, and Z. The nucleoside moiety of these nikkomycins consists of a 5-aminohexuronic acid with *N*-glycosidically bound 4-formyl-4-imidazoline-2-one (nikkomycin C_x) or uracil (nikkomycin C_z). Nikkomycins I and J have glutamic acid peptidically bound to the 6′-carboxyl group of nikkomycins C_x and C_z, respectively. The peptidyl side chain, 2-amino-4-hydroxy-3-methyl-4-(3′-hydroxy-6′-pyridyl)butanoic acid (hydroxypyridylhomothreonine), is linked *via* a peptide bond to the nucleoside moiety (Fig. 1)[5,6]. Approximately 20 biologically active nikkomycin structures have been isolated from the culture filtrate of mutants and are minor components of the culture filtrate of the wild-type. In addition, these structures have been generated by mutasynthesis, directed fermentation, and enzymatic bromination (reviewed by FIEDLER, 1993[7]). The structure-activity relationship of various nikkomycins has been investigated with respect to their inhibition constants for chitin synthetase, uptake by peptide transport systems, and susceptibility to degradation by peptidases[8]. Nikkomycins X and Z and their analogues, nikkomycins B_x and B_z, which contain 2-amino-4-hydroxy-3-methyl-4-(4′-hydroxyphenyl)butanoic acid as the peptide side chain, are the most potent, and nikkomycin B_x displays the highest activity against *Candida albicans*. Nikkomycins B_x and B_z have been isolated as minor components ($<2\sim3$ mg/liter) from *S. tendae* Tü901 culture filtrate[9]. Since partial chemical synthesis of nikkomycins B_x and B_z is difficult[10], large amounts cannot be produced.

The synthesis of ten proteins (P1-P10), identified by comparing patterns on 2-D gels of *S. tendae* Tü901 proteins in crude extracts, correlates with nikkomycin production[11]. The *N*-terminal sequences of two of these proteins led to the cloning of the nikkomycin biosynthesis gene cluster. Analysis of the nucleotide sequence of the gene cluster revealed the *nikC* gene, which encodes protein P8. This protein is L-lysine 2-aminotransferase, which catalyzes the initial reaction in

Fig. 1. Structures of nikkomycins.



Nikkomycin	R_1	R_2
X	A	C
Z	B	C
B_x	A	D
B_z	B	D
C_x	A	H
C_z	B	H

the biosynthesis of the biosynthetic precursor hydroxypyridylhomothreonine[12,13]. L-Lysine is transaminated to form piperideine-2-carboxylate, which is oxidized to picolinic acid and then incorporated to form hydroxypyridylhomothreonine[14]. The mutant *S. tendae nikC::aphII*, in which the *nikC* gene is inactivated, is unable to produce the biologically active nikkomycins I, J, X, and Z and accumulates the nucleoside moieties, nikkomycins C_x and C_z. This *nikC* mutant has been complemented to nikkomycin I, J, X, and Z production by adding picolinic acid to the production medium[14].

Here we describe the mutational synthesis of nikkomycins B_x and B_z by resting-cell cultures of *S. tendae nikC::aphII* supplemented with benzoic acid.

Materials and Methods

Microorganism and Growth Conditions

The mutant *S. tendae nikC::aphII*, in which *nikC* gene is inactivated, used in the present study has been described previously[11]. The mutant was grown in 15- and 400-ml batch cultures in 100-ml and 2-liter Erlenmeyer flasks, respectively, on a rotatory shaker at 27°C. The production medium SP (pH 6.0) consisted of 3% mannitol, 1% starch, 0.8% yeast extract (Oxoid), 0.5% neutralized soya peptone (Oxoid), and 15 μg kanamycin/ml. Resting cell medium RC[15] consisted of 7% glucose, 0.16% L-asparagine, and 50 mM MES (2-[*N*-morpholino]ethanesulfonic acid), pH 5.5; separately autoclaved trace solution was added to autoclaved RC medium (final concentration, mg/liter): $MgSO_4 \cdot 7H_2O$ (10), KCl (10), $CaCl_2$ (10), $FeSO_4 \cdot 7H_2O$ (2), $ZnSO_4 \cdot 7H_2O$ (1), $CuSO_4 \cdot 5H_2O$ (0.5), $Na_2MoO_4 \cdot 2H_2O$ (0.1). Supplements were dissolved in dimethylsulfoxide and added to final concentrations of 0.5 mM and 1 mM. Fifteen-milliliter medium was inoculated with a spore suspension, and 400-ml medium with 4 ml of a culture grown for 3 days in the same medium. For resting-cell experiments, cultures of the *nikC* mutant were grown for 3 days in medium SP, washed three times with deionized water and once with medium RC, and suspended in half the volume of the original culture in medium RC, and cultivated for 3 days.

Nikkomycin Analysis

Nikkomycin structures in the culture supernatant were determined by HPLC analysis with photodiode array detection according to SCHÜZ *et al.*[16]. The Thermo Separation Products Spectra System consisted of pump P2000, a vacuum degasser, detector UV3000HR, autosampler AS3000, controller SN4000, and PC1000 software v3.0.

Antifungal activity of culture filtrates was determined in agar diffusion assays using *Paecilomyces variotii* Tü137 as a test organism. Paper disks (diameter: 6 mm) with 10 μl culture filtrate were applied to test plates composed of 0.4% glucose, 0.4% yeast extract, 1% malt extract, 2% agar, pH 7, and seeded with 10^5 spores per ml. Test plates were incubated for 24 hours at 37°C.

Isolation of Nikkomycins

The resting-cell culture filtrates were adjusted to pH 3.5 with acetic acid and chromatographed on a column containing Dowex 50 WX 2 (H^+, 100~200 mesh); nikkomycins B_x and B_z were eluted with 0.05 N ammonia. The eluate was immediately concentrated *in vacuo* and lyophilized. The samples were dissolved in distilled water and chromatographed on Biogel P2 with distilled water. Fractions containing nikkomycins B_x and B_z were lyophilized.

Structure Elucidation

NMR spectra (TOCSY, HMBC, and HSQC) were recorded on a Bruker AMX 400 (400 MHz) spectrometer. Samples were dissolved in DMSO-d_6. Mass spectra were recorded on an API III triple quadrupole (Perkin-Elmer Sciex, Thornhill, Ontario, Canada) equipped with an electrospray ionization (ESI) source. Tandem experiments were performed using argon as a collision gas.

Results and Discussion

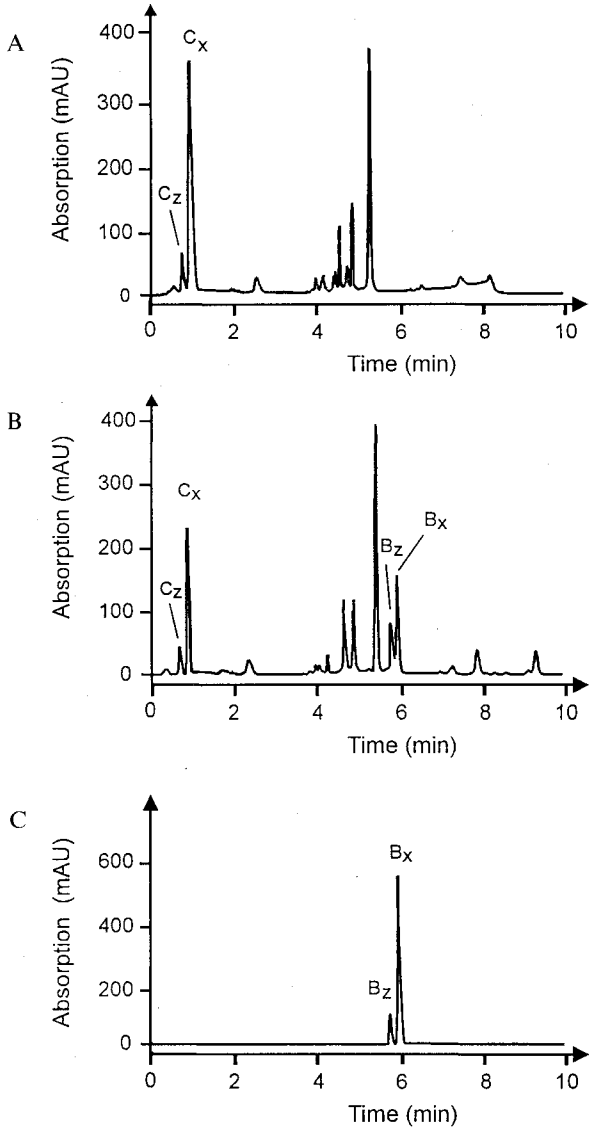
Mutasynthetic experiments with *S. tendae nikC::aphII*

Resting-cell cultures of *S. tendae nikC::aphII* were fed various precursor analogues. The antifungal activity of culture filtrates was tested by agar diffusion tests, and formed nikkomycin structures were determined by HPLC analysis. Active antifungal substances were not obtained when resting-cell cultures were fed with 6-hydroxypicolinic acid, 2-hydroxynicotinic acid, or 3-hydroxybenzoic acid. Cultures supplemented with 3-hydroxypicolinic acid, 3-hydroxymethylpyridine, 6-hydroxynicotinic acid, or 4-hydroxybenzoic acid produced antifungal activity; however, the biologically active compounds were synthesized in low amounts (<2~3 mg/liter) and could not be detected as peaks in HPLC chromatograms. In contrast, resting-cell cultures fed with benzoic acid were antifungally active and HPLC chromatograms of the supernatants revealed peaks at 5.8 and 6.0 minutes (Fig. 2).

Nikkomycins B_z and B_x, the expected mutasynthetic products, had retention times identical to those of the actual products (Fig. 2C), and cochromatography of nikkomycins B_z and B_x with culture filtrate containing the mutasynthetic products revealed single peaks at 5.8 (nikkomycin B_z) and 6.0 minutes (nikkomycin B_x) (data not shown). In addition, UV spectra of the substances that eluted at 5.8 and 6.0 minutes revealed an absorption maximum at 263 nm and 287 nm, respectively, and were

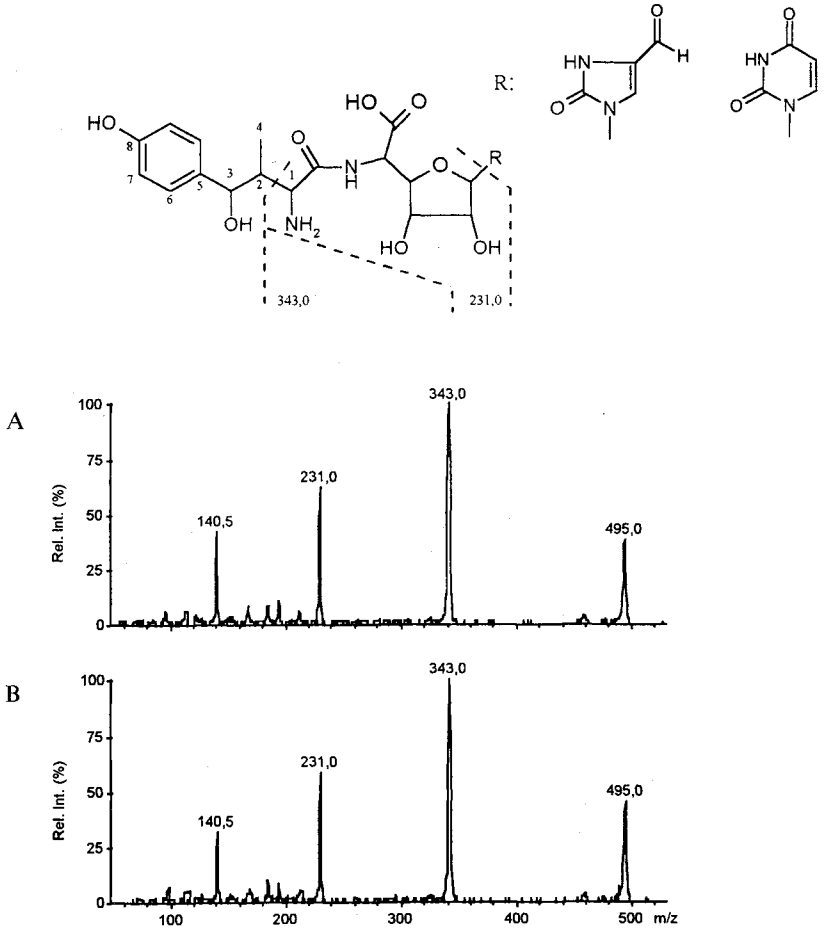
Fig. 2. HPLC chromatograms ($\lambda = 280$ nm) of culture filtrate and purified nikkomycins.

Streptomyces tendae nikC:: aphII was transferred to (A) resting-cell medium RC and (B) medium RC supplemented with 1 mM benzoic acid and cultivated for 3 days. (C) purified nikkomycins B_z (50 mg/liter) and B_x (450 mg/liter).



identical to those of nikkomycins B_z and B_x (data not shown). Structural analyses (see below) confirmed that the products obtained by mutasynthesis were nikkomycins B_z and B_x. Using concentrations of 0.5 mM and 1 mM benzoic acid in a 1-1 volume, approximately 0.3 mmol benzoic acid was utilized to form 0.07 mmol nikkomycin B_z and 0.26 mmol nikkomycin B_x after 3 days of resting cell cultivation. Concomitantly, 0.87 mmol of the nucleoside precursors nikkomycins C_z and C_x appeared when the culture was fed with 0.5 mmol benzoic acid in

Fig. 3. ESI-MS/MS spectra of the product isolated after feeding resting cell cultures of *Streptomyces tendae nikC::aphII* with benzoic acid (A) and of the reference substance nikkomycin B_x isolated from culture filtrate of the *S. tendae* wild-type[9] (B).



a 1-1 volume and 0.44 mmol appeared when the culture was fed with 1 mmol benzoic acid in a 1-1 volume, indicating that nikkomycin synthesis was inhibited at 1 mM benzoic acid. Benzoic acid also inhibited nikkomycin synthesis of the wild-type strain, which produced in the presence of 1 mM benzoic acid approximately 60% of the normal level of nikkomycins I, J, X, and Z; nikkomycins B_x and B_z were not synthesized in detectable amounts.

These results indicate that the nikkomycin biosynthetic enzyme that converts picolinic acid to hydroxypyridylhomothreonine exhibits high substrate specificity, assuming that the substrates permeate the cytoplasmic membrane to similar extents. This enzyme is thought to activate the precursor to allow the formation of the C–C bond of the peptidyl chain of nikkomycins. Among the tested compounds, benzoic acid was the only substrate that was incorporated to form new biologically active nikkomycin structures at a reasonable level, about 40% compared to picolinic acid. In contrast, 4-hydroxybenzoic acid was poorly accepted by the biosynthetic enzyme, indicating that the hydroxylation of the phenyl group and of the pyridyl ring might occur at a later stage in the biosynthesis of the peptidyl chain of nikkomycins. The antifungally active structures obtained with 3-hydroxymethylpyridine are presumably nikkomycins P_x and R_z, which contain 2-amino-4-hydroxy-3-methyl-4-(3′-pyridyl) butanoic acid as the peptidyl moiety. These compounds have previously been isolated as a minor components of the culture filtrate of *S. tendae* wild-type[17].

Isolation and Physicochemical Properties of the Mutasynthetic Products

Since resting cell cultures were used for the production of nikkomycins B_z and B_x, isolation to more than 95% purity with an approximately 70% yield was obtained by ion-exchange chromatography on Dowex 50 WX 2

(H^+) and subsequent gel filtration on Biogel P2. The purified samples consisted of approximately 20% nikkomycin B_z and 77% nikkomycin B_x.

Electrospray ionzation mass spectra of the isolated nikkomycins B_z and B_x revealed a single $[M+H]^+$ peak at *m/z* 495.0. The identical result was obtained with the reference substance nikkomycin B_x. In addition, the daughter ion mass spectra of ESI-MS/MS measurements showed identical fragmentation patterns for both samples (Fig. 3). The signal at *m/z* 141.0 can be assigned to the loss of the formyl-imidazolinone base and of the uracil base ($C_4H_3N_2O_2CHO+H$).

The position of the hydroxy group of the phenyl ring was determined by NMR spectroscopy (Table 1). The occurrence of only two aromatic signals in the ^1H-^1H-TOCSY spectrum and the corresponding signals in the HSQC spectrum clearly confirm that the hydroxy group is in the *para*-position. The signals of the AA'BB' coupling system appeared at δ 6.72 and δ 7.06. The HMBC spectrum revealed that the chemical shifts of the carbon atoms 5-C and 8-C are chemical shift values typical for *para*-substituted moieties of similar structures[9].

Table 1. ^1H and ^{13}C chemical shifts (ppm) of nikkomycin B_x[a].

^1H	δ 4.0 (1-H), 2.17 (2-H), 4.85 (3-H), 0.63 (4-H), 7.06 (d, J=8.4 Hz, 6-H), 6.72 (7-H).
^{13}C	δ 56.7 (1-C), 40.5 (2-C), 73.0 (3-C), 5.9 (4-C), 134.1 (5-C), 126.5 (6-C), 114.3 (7-C), 156.0 (8-C).

[a] Numbering of atoms see Figure 3.

pH Stability of Nikkomycins B_x and B_z

The peptidyl moiety influences the stability of nikkomycins[18]; therefore, the stability of nikkomycin B_x was studied at various pH values using nikkomycin X as a reference (Fig. 4). Nikkomycin B_x was almost completely stable at pH 5.5 (95% was present after 28 days of incubation), and approximately 40% was degraded at pH 7.0 and at pH 8.0 after 28 days of incubation. Similar results were obtained with nikkomycin B_z (data not shown). In contrast, nikkomycin X was rapidly degraded at pH 7.0 and at pH 8.0 and at a lower rate at pH 5.5. Similar degradation kinetics were obtained for nikkomycin Z (data not shown)[19]. Figure 5 shows the mechanisms we propose for intramolecular rearrangements of nikkomycins at the various pH values that lead to hydrolysis of the peptide bond and cleavage into the biologically inactive nucleoside and peptidyl moieties. The instability of nikkomycins B_x and B_z under neutral and alkaline conditions might be caused by the nucleophilic attack of the oxygen atom on the carbon atom of the carbonyl group, leading to the cleavage of the amide bond (Fig. 5A). The nucleophilicity of the oxygen atom would decrease at lower pH values and result in increased stability of nikkomycins B_x and B_z. This hypothesis has actually been confirmed. In the case of nikkomycins X and Z, two further degradation mechanisms seem to be conceivable: Under acidic conditions, the pyridyl nitrogen would be partially protonated by the hydroxy proton, thereby causing an increased nucleophilicity of the oxygen atom, which could attack the carbonyl carbon and result in cleavage of the amide bond (Fig. 5B). Under neutral and alkaline conditions, the nucleophilic pyridyl nitrogen of nikkomy-

Fig. 4. pH stability of nikkomycins B_x and X.

Purified nikkomycins (B_z/B_x: 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. ■: Nikkomycin B_x, ▲: Nikkomycin X.

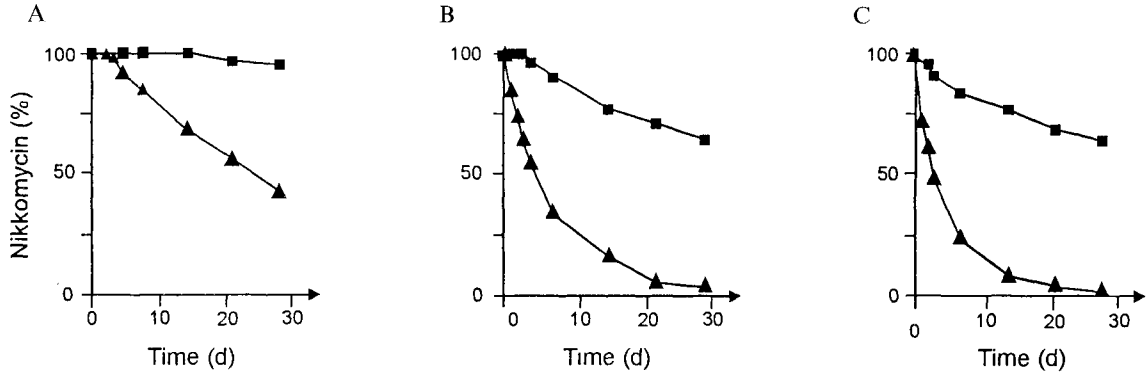
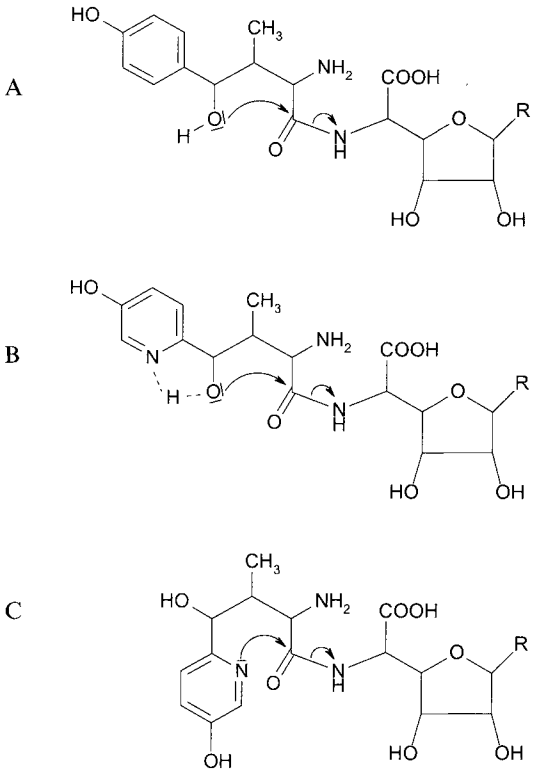


Fig. 5. Proposed models for intramolecular rearrangements of nikkomycins B_x and B_z at pH 7.0 and pH 8.0 (A), of nikkomycins X and Z at pH 5.5 (B) and of nikkomycins X and Z at pH 7.0 and pH 8.0 (C).



The residues indicated by R are identical to those shown in Fig. 3.

cins Z and X could directly attack the carbonyl carbon, thereby forming a six-membered transition state (Fig. 5C). In addition, the intramolecular rearrangement proposed for nikkomycin B_x and B_z (Fig. 5A) could occur in nikkomycins X and Z.

Nikkomycin K_x, which contains the peptidyl side chain 2-amino-4-hydroxy-4-(2′-pyridyl) butanoic acid, has the highest stability of all nikkomycins at pH 8.0, and stability at pH 7.0 is similar to that of nikkomycin B_x; however, nikkomycin K_x has significantly lower antifungal activity than nikkomycins B_x, X, and Z[10,18].

Nikkomycin Z is a potent oral antifungal agent against coccidioidomycoses and blastomycoses[20,21]. Because of their high stability, nikkomycins B_z and B_x might be alternative potent antifungal drugs, and could be easily produced by mutasynthesis.

Acknowledgements

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