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ENZYMATIC BROMINATION OF NIKKOMYCIN Z

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Two brominated nikkomycins were produced by enzymatic halogenation of nikkomycin Z in the presence of a nonheme bromoperoxidase isolated from *Streptomyces aureofaciens* Tü 24. The monobrominated and dibrominated nikkomycin Z derivatives were substituted at the hydroxypyridyl moiety of the *N*-terminal amino acid of nikkomycin Z at position C-6‴ (ZBr) or C-4‴ and C-6‴ (ZBr_2).

The brominated nikkomycin Z derivatives had a decreased affinity to chitin synthase of *Coprinus cinereus* as compared to nikkomycin Z and exhibited a low inhibitory activity towards various fungi and yeasts.

Nikkomycins are nucleoside peptide antibiotics produced by *Streptomyces tendae* Tü 901, which act as competitive inhibitors of chitin synthase of fungi, yeasts and insects[2~6]. In previous studies, new nikkomycins with altered biological properties were obtained by isolation of minor components from the fermentation broth of *S. tendae* wildtype and mutants, and by directed fermentations and chemical synthesis[7~12]. Another possibility for increasing the number of available nikkomycins with new properties is enzymatic transformation, a method which has not yet been applied to this group of antibiotics. Enzymatic or microbial transformations may be used if chemical synthesis is difficult or impossible[13]. The advantages of enzyme catalyzed reactions are that they take place under mild reaction conditions and provide steroselectivity and specifity of the reaction.

In this study we used a heat-stable bromoperoxidase isolated from the chlorotetracycline producer *Streptomyces aureofaciens* Tü 24[14] whose gene has already been cloned in *Streptomyces lividans* TK64[15]. Haloperoxidases are enzymes isolated from various organisms which catalyse the halogenation of various compounds such as tyrosine, β-ketoacids, cyclic β-diketones and alkenes by substitution or addition mechanisms[16,17].

In the present paper, we demonstrate the enzymatic bromination of nikkomycin Z by a nonheme bromoperoxidase, and the subsequent isolation, structure elucidation and evaluation of biological prop-

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erties of the new compounds.

In addition, we transformed *S. tendae* Tü 901/8c with the cloned bromoperoxidase gene from *S. aureofaciens* to investigate the possibility of an *in vivo* modification of nikkomycins by bromoperoxidase action during cultivation in nikkomycin-production medium.

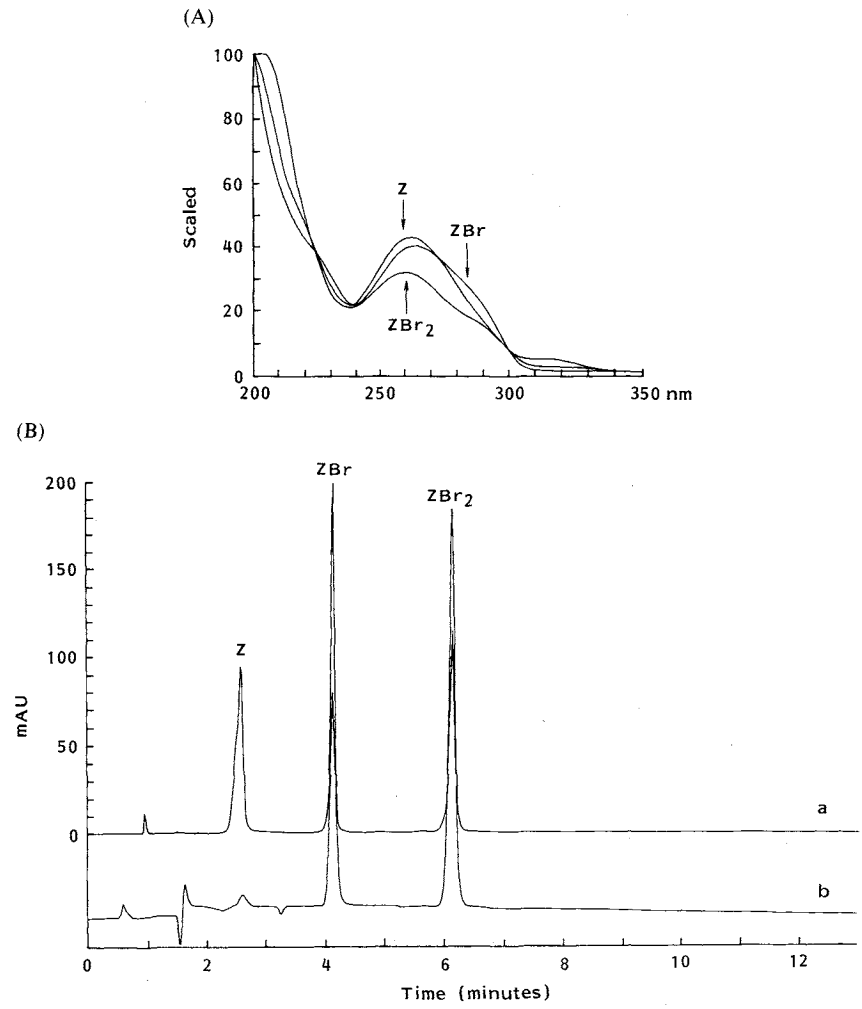
Results

Enzymatic Transformation and Isolation of Brominated Nikkomycins

By enzymatic modification of pure nikkomycin Z (95%) using the nonheme bromoperoxidase isolated from *S. aureofaciens*, we obtained mono- and dibrominated nikkomycin Z molecules which were substituted at the hydroxypyridyl moiety of the *N*-terminal amino acid of nikkomycin Z at position C-6‴ (**1**=ZBr) or C-4‴ and C-6‴ (**2**=ZBr_2).

Fig. 1. HPLC chromatogram of nikkomycin Z, mono- and dibrominated nikkomycin Z.

The UV spectra (A) were recorded during the HPLC (B) run. (a) UV signal at 260 nm, (b) conductivity signal.



The halogenated nikkomycins could be specifically detected by HPLC analysis using a photoconductivity detector, whereas pure nikkomycin Z could not be monitored by this detection method. The UV spectra of the brominated nikkomycins obtained with a photodiode array detector during the HPLC run[18] were almost identical to the UV spectra of nikkomycin Z (Fig. 1).

The purification of the brominated nikkomycins was performed by size exclusion chromatography on Fractogel TSK and preparative reversed-phase HPLC using Nucleosil-100 C-18 and gradient elution with water-methanol.

Physico-chemical Properties

Positive ion FAB mass spectra of **1** yielded protonated molecular ions at 574 and 576 (~1:1) corresponding to Br isotopes 79 and 81. This shift in molecular mass of 78 and 80 mass units, as compared to the FAB spectra of nikkomycin Z indicated mono-bromo-substitution.

The position of the bromo-substitution was determined from the ^{1}H NMR spectrum of **1** (Fig. 2). The proton signal of 6-H of the pyridine system at 8.1 ppm of nikkomycin Z[2,11] was not present in **1**, while all the other ring protons had about the same chemical shift values as in nikkomycin Z. The coupling constant $J_{3''',4'''}=8.2$ Hz of the ring protons at C-3 and C-4 also clearly indicated the *ortho*-relationship of these protons.

From the positive ion FAB mass spectrum of **2**, the presence of two Br atoms became apparent. Protonated molecular ion signals occurred at *m/z* 652, 654 and 656 with a relative intensity of 1:2:1 as expected for two Br atoms.

Although the ^{1}H NMR spectrum (Fig. 3) clearly indicated that both Br atoms are located on the pyridine ring, the assignment of their position was difficult. Again the downfield proton signal of 6-H of

Fig. 2. ^{1}H NMR spectrum (250 MHz) of **1** D_2O (nikkomycin ZBr).

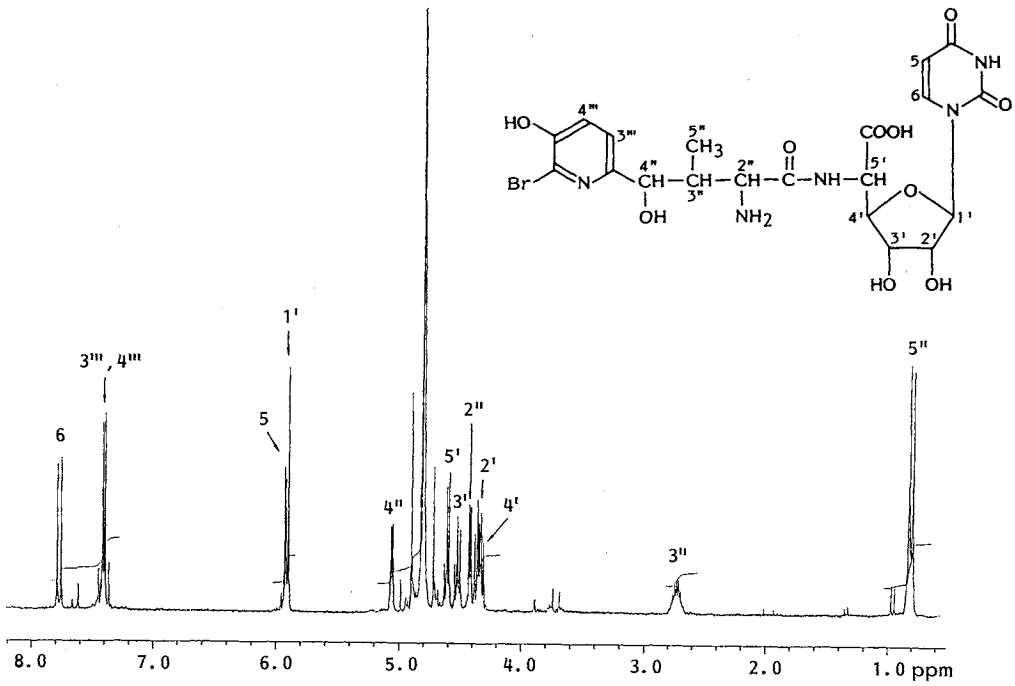


Fig. 3. ^{1}H NMR spectrum (250 MHz) of **2** D_2O (nikkomycin ZBr_2).

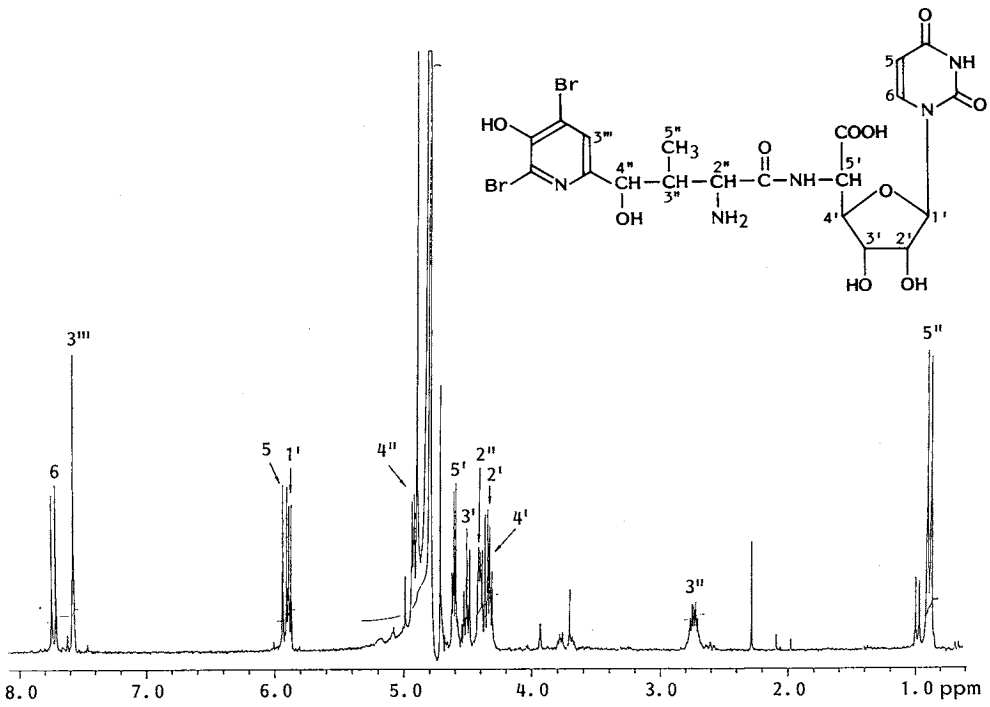


Table 1. ^{1}H chemical shifts (ppm) of nikkomycins ZBr (**1**) and ZBr_2 (**2**).

Nikkomycin ZBr:
δ 0.84 (3H, d, J=7.0 Hz, CHCH_3), 2.74 (1H, m, CHCH$_3$), 4.33 (1H, dd, $J_{3',4'}$=4.9 Hz, $J_{4',5'}$=4.0 Hz, 4'-H), 4.36 (1H, dd, $J_{1',2'}$=5.4 Hz, $J_{2',3'}$=5.8 Hz, 2'-H), 4.42 (1H, d, J=3.9 Hz, CHNH$_2$), 4.52 (1H, dd, $J_{2',3'}$=5.9 Hz, $J_{3',4'}$=4.9 Hz, 3'-H), 4.60 (1H, d, J=3.9 Hz, 5'-H), 5.05 (1H, d, J=3.4 Hz, 4''-H), 5.90 (1H, d, J=5.4 Hz, 1'-H), 5.91 (1H, d, J=7.9 Hz, 5-H), 7.34 and 7.40 (2 × 1H, d, J=8.2 Hz, 3'''-H and 4'''-H), 7.73 (1H, d, J=7.9 Hz, 6-H)
Nikkomycin ZBr_2:
δ 0.86 (3H, d, J=7.0 Hz, CHCH_3), 2.72 (1H, m, CHCH$_3$), 4.33 (1H, dd, $J_{3',4'}$=4.8 Hz, $J_{4',5'}$=4.0 Hz, 4'-H), 4.37 (1H, dd, $J_{1',2'}$=5.5 Hz, $J_{2',3'}$=5.4 Hz, 2'-H), 4.41 (1H, d, J=3.7 Hz, CHNH$_2$), 4.51 (1H, dd, $J_{2',3'}$=5.4 Hz, $J_{3',4'}$=4.8 Hz, 3'-H), 4.60 (1H, d, J=3.9 Hz, 5'-H), 4.92 (1H, d, J=4.2 Hz, 4''-H), 5.89 (1H, d, J=5.5 Hz, 1'-H), 5.93 (1H, d, J=8.0 Hz, 5-H), 7.58 (1H, s, 3'''-H), 7.74 (1H, d, J=8.0 Hz, 6-H).

nikkomycin Z was missing, indicating that one of the bromo substituents was located at C-6. The signal at 7.58 ppm could either have been a proton at C-3 or C-4 of the pyridine system. In the ^{1}H-^{1}H COSY spectrum of nikkomycin Z[11], a cross-peak of low intensity was present indicating a long-range-coupling of the 3'''-H proton with the proton in γ-position of the amino acid part of the molecule (4''-H). No such cross-peak was detectable in the ^{1}H-^{1}H COSY spectrum of **2**, however it was visible in the COSY-long range spectrum. This technique is particularly suited to detect weak long-range couplings. Thus, the Br substituents in **2** are located in positions 4 and 6 of the pyridine system.

The chemical shift data and coupling constants of both brominated nikkomycins are given in Table 1.

A recent paper published by ANDO *et al.*[19] described the chemical synthesis of mono-, di- and tribromo derivatives of nikkomycin Z. The monobrominated nikkomycin Z is identical with nikkomycin ZBr (**1**), which was derived in our work by enzymatical modification of nikkomycin Z.

Table 2. Biological activity of the brominated nikkomycins in relation to nikkomycin Z.

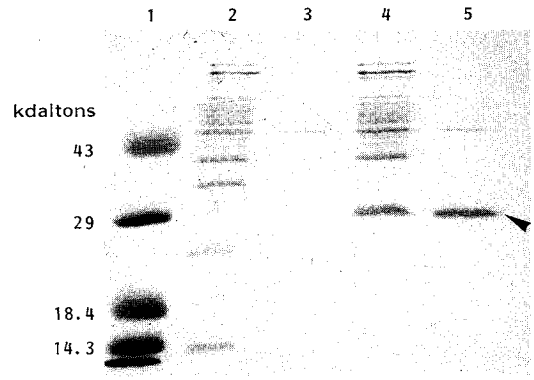
Microorganism	Temperature (°C)	Medium	Biological activity in % nikkomycin[a]		
			Z	ZBr	ZBr_2
Yeasts:					
Candida albicans CBS 2718	27	1	100	—	—
Saccharomyces cerevisiae CBS 1369	27	1	—	—	—
Yarrowia lipolytica ATCC 8662	27	1	100	2.5	0.19
Fungi:					
Alternaria kikuchiana Tü 169	27	3	100	5.6	—
Botrytis cinerea Tü 157	24	2	100	11.2	—
Mucor rouxii ATCC 24905	27	1	100	15.0	1.3
Paecilomyces variotii Tü 137	37	1	100	2.8	—
Bacteria:					
Bacillus subtilis ATCC 6051	37	1	—	—	—
Escherichia coli K-12	37	1	—	—	—

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

Biological Activity

The substition of hydrogen by bromine at the hydroxypyridyl ring at position C-6‴ or C-4‴ and C-6‴ of nikkomycin Z decreased the binding affinity to chitin synthase of *Coprinus cinereus*. The inhibition constants (*Ki*) of the new nikkomycins towards chitin synthase of *C. cinereus* were determined according to the method of DIXON[20] and ranged from 7 μM for the monobrominated nikkomycin to 8 μM for the dibrominated nikkomycin Z (nikkomycin Z: $Ki = 3.0\,\mu M$). As could be expected, the inhibitory activity of the new brominated nikkomycins towards various microorganisms was lower as compared to nikkomycin Z (Table 2). The dibrominated nikkomycin Z derivative was less active than the monobrominated nikkomycin Z. Gram-positive and Gram-negative bacteria, as well as *Saccharomyces cerevisiae*, which are insensitive to nikkomycin Z under the experimental conditions, were also not affected by the brominated nikkomycins.

Fig. 4. SDS-PAGE of crude extracts from *Streptomyces tendae* (lanes 2 and 3) and *S. tendae* (pHM626) (lanes 4 and 5).



Crude extracts applied on lanes 3 and 5 were heat-treated (80°C for 30 minutes). 10 μg of protein was applied to each lane. Bromoperoxidase subunits are indicated by an arrow. Lane 1 contains a molecular standard indicated on the left in kdaltons.

Transformation of *S. tendae* with a Plasmid Carrying the Bromoperoxidase Gene

We transformed *S. tendae* Tü 901/8c with the plasmid pHM626 containing the bromoperoxidase gene from *S. aureofaciens* Tü 24. The plasmid was constructed by subcloning the 1.1-kb *Hind* III-*Nco* I DNA fragment from pHM625[14] into the *Hind* III site of pIJ486[21] after filling the ends of the DNA fragments with Klenow enzyme. The presence of plasmid pHM626 led to the expression of heat-stable bromoperoxidase protein in *S. tendae* (Fig. 4). Heat-treated crude extracts exhibited a specific bromoperoxidase activity of 2.5 U/mg protein, which was also estimated for *S. lividans* TK64 (pHM626) crude extracts.

Although the bromoperoxidase gene was expressed during cultivation of *S. tendae* (pHM626) in a nikkomycin production medium supplemented with 1 g/liter NaBr and 50 μg/ml thiostrepton, brominated nikkomycins were not detected by HPLC analysis, whereas nikkomycins Z and X were produced during an incubation time of 6 days in a reduced amount of 20% (Z: 50 mg/liter, X: 90 mg/liter) compared with the non-transformed strain.

Discussion

The nonheme bromoperoxidase isolated from the chlorotetracycline producer *S. aureofaciens* Tü 24 has proved to be a suitable tool for the bromination of the hydroxypyridyl moiety of nikkomycin Z. The reduced biological activity of the brominated nikkomycins might be attributed to their lower affinity towards chitin synthase, as the halogenated nikkomycins were not degraded by peptidases (data not shown). Whether their transport by the peptide transport system is affected by the bromine substituents will be investigated in further studies.

The reaction mechanism of the nonheme bromoperoxidase is not yet clear. Enzymatic activity could only be detected *in vitro* if acetate was present in the reaction mixture[14]. Although the bromoperoxidase gene was expressed in *S. tendae* (pHM626) and enzyme activity could be measured in crude extracts, the transformed strain did not produce any brominated nikkomycins. Explanations could be unsuitable intracellular conditions for the action of the bromoperoxidase or the existence of a yet unknown inhibitor produced by *S. tendae* as described for several other *Streptomyces* strains[14,22]. Another explanation may be a very low concentration of brominated nikkomycins, caused by the reduced nikkomycin production of the transformed strain which could not be detected by HPLC analysis.

Experimental

Microorganisms and Culture Conditions

For the production of nikkomycin Z, *S. tendae* Tü 901/8c was cultivated in the following medium: Soybean meal 2%, mannitol 3%, yeast extract 1%, starch 1% and uracil 0.4% (pH 6.8). The fermentation of *S. tendae* Tü 901/8c was performed under the same conditions as described for *S. tendae* Tü 901/PF 53^{+}-3[10].

For the production of bromoperoxidase, a medium consisting of soybean meal 2% and mannitol 2% or the nikkomycin production medium supplemented with thiostrepton (*S. lividans*: 10 μg/ml, *S. tendae*: 50 μg/ml) was used.

For preparation of protoplasts and isolation of DNA, the strains were grown in a medium consisting of sucrose 10.3%, tryptic soy broth 2%, $MgCl_2$ 1% and yeast extract 1%.

All strains were maintained on the following medium: Yeast extract 0.4%, glucose 0.4%, malt extract 1.0% and agar 1.5%.

Enzymatic Transformation of Nikkomycin Z and Isolation of the New Compounds

Nikkomycin Z was isolated according to the separation scheme for nikkomycins to a purity of 95% by ion exchange chromatography and preparative HPLC as described by FIEDLER[23] and FIEDLER *et al.*[24].

For the enzymatic modification of nikkomycin Z, an enzyme preparation partially by heat treatment of a crude extract of *S. lividans* TK64 (pHM626)[15] was used. The reaction was performed in a buffer containing 1 M sodium acetate, 91 mM sodium bromide, 7 mM H_2O_2, 5 mM nikkomycin Z and 300 μl of heat-treated enzyme preparation (protein 8.9 mg/ml, 8.8 U/mg of protein), pH 5.5. The reaction was stopped after 48 hours and 40 ml of the reaction mixture were applied on a Fractogel TSK HW-40 F column (Merck, Darmstadt, FRG; 40 × 8 cm, flow rate 120 ml/hour). Nikkomycin Z and the degradation products nikkomycin D and C_z were eluted with water. Then the brominated nikkomycins were eluted with water-methanol (90:10). The fractions containing the new nikkomycins were pooled, concentrated *in vacuo* and lyophilized.

The separation of mono- and dibrominated nikkomycin Z was carried out by preparative HPLC as described by FIEDLER *et al.*[24] and DECKER *et al.*[12].

Analytical Methods

The quantification and characterization of the nikkomycins by HPLC and photodiode array detection (HP 1090M, Hewlett-Packard, Waldbronn, FRG) during the HPLC run was performed as described by FIEDLER[18].

Bromination of nikkomycin Z was identified by coupling the HPLC and photodiode array detector with a photoconductivity detector (Tracor 965; Austin, TX, U.S.A.). The analytical column (125 × 4.6 mm) and pre-column (20 × 4.6 mm) were filled with Nucleosil-100 C-18 (7 μm; Grom, Herrenberg, FRG). Solvent A was 0.0005% aqueous TFA; solvent B was methanol. The elution started with 100% solvent A for 2 minutes, followed by a linear gradient to 15% solvent B in 1.5 minutes, increasing to 30% solvent B in 4.5 minutes. The flow rate was 2 ml/minute. The compounds were successively detected by UV absorbance at 260 nm and by conductivity after exposure of the eluate to UV light (mercury lamp, sensitivity 100 × 200).

Brominating activity was assayed by the monochlorodimedone assay as described previously[14]. One U of bromoperoxidase activity was defined as the formation of 1 μmol of monobromomonochlorodimedone per minute (specific activity = U/mg of protein).

Protein concentrations were measured by the method of BRADFORD[25] with bovine serum albumin as the standard.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to LAEMMLI[26] using 1 mm-slab gels at pH 8.8 containing 12% acrylamide. Proteins were stained with Coomassie Brilliant Blue G-250.

Structure Elucidation

The positive ion FAB mass spectra were analyzed on a VG 70-250S instrument (VG Instruments) using xenon as a collision gas and *m*-nitrobenzylalcohol as a matrix substance.

The ^1H and ^1H-^1H COSY NMR spectra were obtained by using a Bruker WM 400 (400 MHz) and a WM 250 (250 MHz) spectrometer. Samples were dissolved in D_2O. The DOH-resonance signal was used as an internal reference.

Biological Assays

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 activities of brominated nikkomycins were compared in relation to nikkomycin Z with a concentration ranging from 100 to 2,000 μg/ml. Paper discs (6 mm diameter) with 10 μl nikkomycin solution were applied to the following agar plates (8.5 cm i.d., 10 ml medium) seeded with test organisms:

Medium 1: Glucose 0.5%, yeast extract 0.4%, malt extract 1%, agar 1.5% (yeasts and other fungi: pH 5.5; bacteria: pH 7.3).

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

Medium 3: Corn meal agar (Difco) 1.7%.

The test plates were prepared as described by DECKER *et al.*[10]. The incubation temperatures for the test organisms are listed in Table 2.

The *Ki* values of the new nikkomycins were determined for digitonin-solubilized chitin synthase of *C. cinereus*[27] as described by DIXON[20].

DNA Preparation and Transformation of Protoplasts

Plasmid DNA from *S. tendae* Tü 901/8c and *S. lividans* TK64 was isolated by alkaline lysis[28]. Digestions with restriction endonucleases were carried out as recommended by the suppliers.

Protoplast formation, protoplast transformation and regeneration of the protoplasts of *S. tendae* were performed according to BORMANN *et al.*[29]. The recombinant plasmid pHM626 consisted of the 6.2 kb multicopy plasmid pIJ486 and a 1.1-kb insert carrying the bromoperoxidase gene, which was subcloned from an original 18.5 kb DNA fragment[15].

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