METABOLIC PRODUCTS OF MICROORGANISMS. 263†

NIKKOMYCINS S_Z, S_X, So_Z and So_X, NEW INTERMEDIATES ASSOCIATED TO THE NIKKOMYCIN BIOSYNTHESIS OF *Streptomyces tendae*

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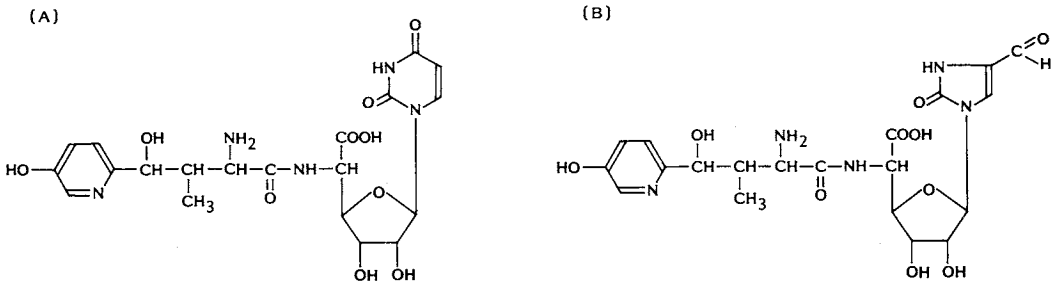
(Received for publication June 21, 1991)

New intermediates associated with nikkomycin biosynthesis, called nikkomycins S_Z, S_X, So_Z and So_X, were isolated and characterized from the culture broth of *Streptomyces tendae* Tü 901/S 2566. They are analogues to octosyl acids, shunt metabolites of polyoxin biosynthesis. The decreasing amounts of nikkomycins S_Z and S_X, produced in the culture medium, shows a significant correlation to the increasing amounts of the biologically active nikkomycins Z and X, dependent on the increasing concentration of iron.

Nikkomycins belong to the group of nucleoside peptide antibiotics[2] which act as potent competitive inhibitors of fungal and insect chitin synthase[3~8] (Fig. 1).

More than 20 biologically active nikkomycin structures have been isolated from the culture filtrates of *Streptomyces tendae* strains (reviewed by FIEDLER, 1989[9]). The biosynthesis of nikkomycins has been investigated by feeding labeled precursors to the mycelia. It is known that the pyridyl residue and the attached carbon atom of the amino acid moiety resulted from L-lysine[10]. The imidazolone base of nikkomycins originates from histidine[11]. For the aminohexuronic acid structure a similar mechanism was discussed, as described for the structurally related polyoxins[12,13]. This paper describes the fermentation and isolation of intermediates correlated to the biosynthesis of the nucleoside moiety, especially the aminohexuronic acid of the nikkomycins.

Fig. 1. Structures of nikkomycins Z (A) and X (B), the main compounds produced from *Streptomyces tendae* Tü 901/S 2566.



† Part 262: See ref 1.

Results

Detection by HPLC

The variation of the commonly used HPLC conditions[14] resulted in an improved resolution of hydrophilic compounds including the nikkomycin nucleosides C_Z and C_X and the new intermediates. The HPLC analysis of the culture filtrate of *S. tendae* Tü 901/S 2566 is shown in Fig. 2. The presence of the intermediates nikkomycins S_Z and S_X was postulated by UV/Vis diode-array spectral analysis.

Fermentation and Isolation

S. tendae Tü 901/S 2566 produced more than 1.2 g/liter of the intermediates nikkomycin S_Z and S_X without addition of $FeSO_4 \cdot 7H_2O$ to the production medium. Increasing concentrations of $FeSO_4 \cdot 7H_2O$ resulted in reduction of the titers of these intermediates and an increase in the levels of nikkomycins Z and X, as shown in Fig. 3.

Large amounts of nikkomycins S_Z and S_X were produced by 10-liter fermentation of *S. tendae* Tü 901/S 2566 for 160 hours of incubation. The isolation and purification was carried out according to an isolation procedure for nikkomycin nucleosides, which was investigated in our group. The intermediates were isolated from the culture filtrate by ion exchange column chromatography using Dowex 50 WX 2

Fig. 2. HPLC chromatogram of culture filtrate of *Streptomyces tendae* Tü 901/S 2566 monitored at 280 nm (A), and UV spectra of nikkomycins S_Z and Z (B), and of nikkomycins S_X and X (C), recorded during the HPLC analysis.

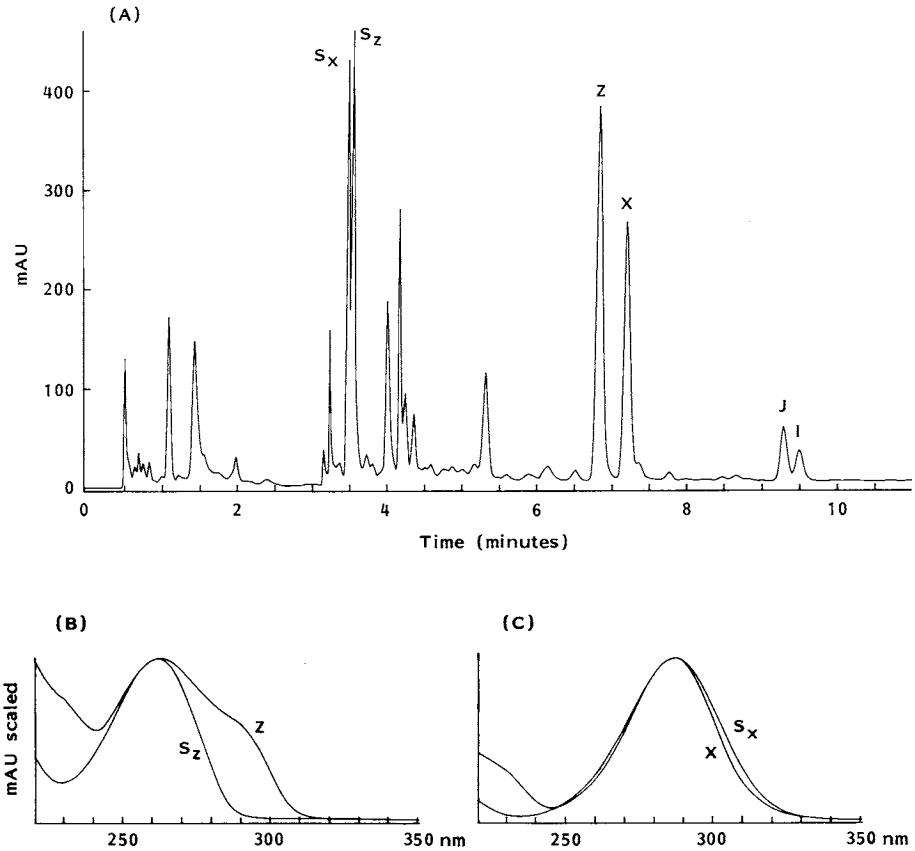


Fig. 3. Yields of nikkomycins Z+X (open bar) and S_Z+S_X (closed bar) produced by *Streptomyces tendae* Tü 901/S 2566 dependent on the concentration of $FeSO_4 \cdot 7H_2O$ in the production medium.

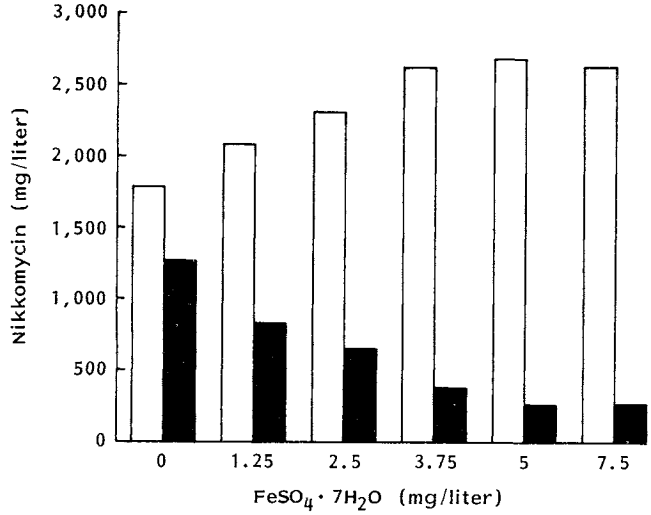
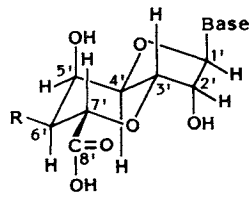
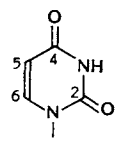
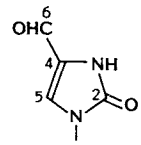
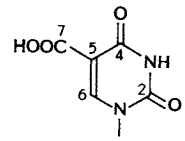


Fig. 4. Structures of the isolated components and of octosyl acid A.



	S_Z	So_Z	S_X	So_X	Octosyl acid A
Base					
R	H	OH	H	OH	H

and Lewatit MP 64 Z II. By size exclusion chromatography using Bio-Gel P 2 the intermediates nikkomycins S_Z and S_X were separated from a small fraction containing the compounds nikkomycins So_Z and So_X. These substances were separated from each other by preparative reversed-phase HPLC using Nucleosil-100 C-18[15] with water as eluent. After concentration and freeze-drying, all compounds were obtained as white powders.

Physico-chemical Properties

All four isolated compounds are nucleosides having a 3,7-anhydrooctofuranose structure in the sugar part of the molecule (Fig. 4). This structural element with *trans*-connected ring systems was already found in octosyl acid A, a shunt metabolite in biosynthesis of the polyoxins[12].

The chemical shifts and coupling constants of the ^1H NMR spectrum are listed in Table 1. From the

Table 1. ^1H chemical shift data of nikkomycins S_Z, S_X, So_Z and So_X and octosyl acid A.

a) Chemical shifts (δ in ppm) (solvent: D_2O for $S_{Z/X}$ and $So_{Z/X}$ and DMSO-d_6 for octosyl acid A):

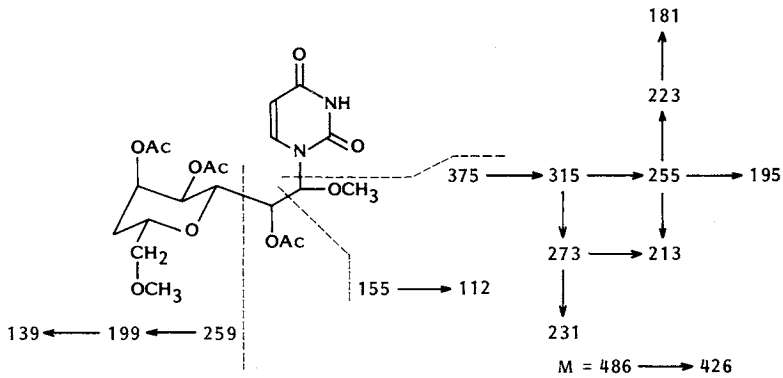
	1′-H	2′-H	3′-H	4′-H	5′-H	6′-H_a	6′-H_e	7′-H	5-H	6-H
S_Z	5.82	4.41	3.97	4.04	4.68	1.84	2.20	4.37	5.86	7.87
S_X	5.74	4.48	4.13	4.01	4.60	1.84	2.27	4.40	7.78	9.29
So_X	5.76	4.48	4.19	4.03	4.61	3.78	—	4.11	7.76	9.28
So_Z	5.86	4.41	4.02	4.08	4.64	3.78	—	4.07	5.88	7.92
Octosyl acid A[a]	5.68	4.28	3.80	4.00	4.94	1.75	2.05	4.41	—	8.94

b) Approximate J values (Hz):

	1′,2′	2′,3′	3′,4′	4′,5′	5′,6′a	5′,6′e	6′a,6′e	6′a,7′	6′e,7′	5-6
S_Z	0.0	4.2	10.6	2.2	~2.4	~3.6	15.0	12.6	2.8	8.1
S_X	0.0	4.6	10.4	2.4	~2.4	~3.6	15.0	12.6	2.8	—
So_X	0.0	4.6	10.4	2.2	2.8	—	—	10.1	—	—
So_Z	0.0	4.3	10.5	1.9	2.8	—	—	10.2	—	8.1
Octosyl acid A[a]	0.0	4.0	10.5	2.5	~2	~2	14.0	11.5	3.2	—

[a] Data taken from ref 16.

Fig. 5. Treatment of nikkomycin S_Z with HCl saturated methanol followed by acetylation yields a product with cleaved furanose ring.



coupling constants $J_{3',4'}$ and $J_{6'a,7'}$ it can be concluded that the corresponding protons have axial positions in a 6-membered ring. An equatorial position can be derived from the coupling constant of the proton 5′-H. This leaves only one possibility to assign the stereochemistry of all the other substituents at C-5′, C-6′ and C-7′ and the type of binding of the 5-membered ring at C-3′ and C-4′. The coupling constant $J_{1',2'}$ has a value of 0 Hz, thus yielding a singlet for 1′-H. This phenomenon is already known from the octosyl acid A[16] and related to the *trans*-arrangement of the two ring systems.

Ring strain is probably responsible for the unusual cleavage of the furanose ring when the compounds are treated with hydrogen chloride saturated methanol. S_Z is thus, after acetylation with acetic anhydride and pyridine, converted into the product shown in Fig. 5.

The same compound was obtained by ISONO *et al.* starting from decarboxylated octosyl acid A[16]. The spectroscopic data of both compounds are identical.

In Table 2 the ^{13}C NMR data of nikkomycins S_X and S_Z are compared with those of octosyl acid A[17]. Again close similarities are observed. The signal assignment was obtained from ^1H-^{13}C COSY spectra.

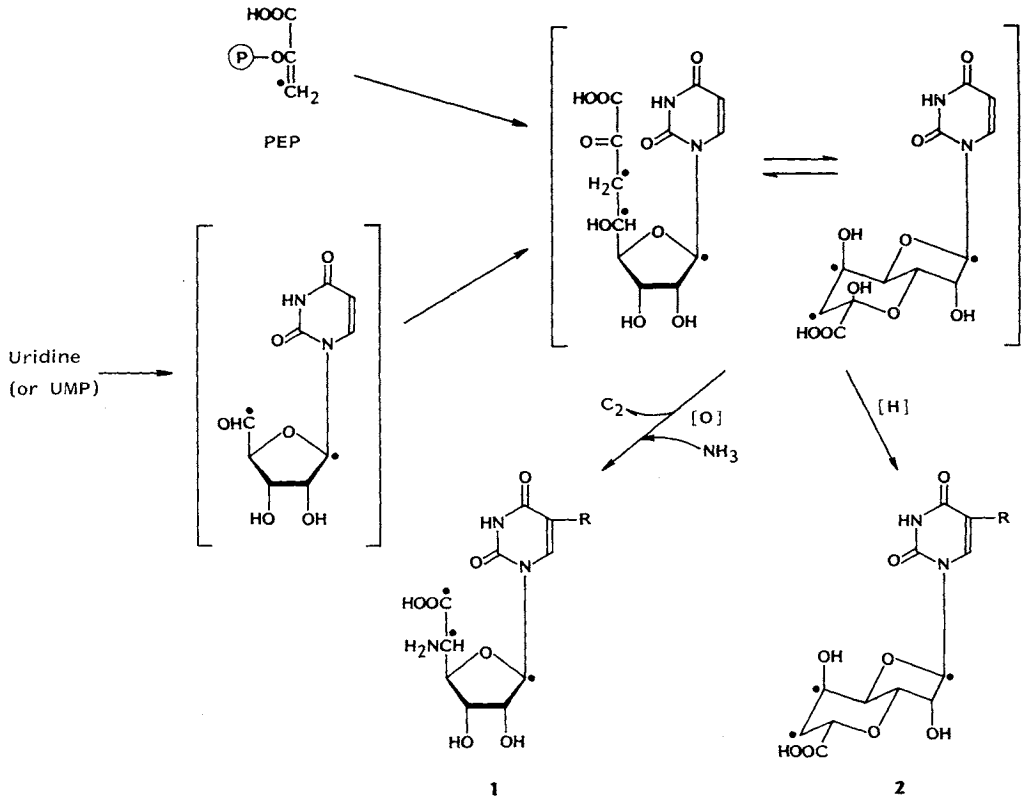
In the positive ion FAB spectra $(M+H)^+$ and $(M+Na)^+$ ion signals at m/z 315 and m/z 337 for

Table 2. ^{13}C NMR chemical shift (δ in ppm) (solvent: D_2O for $S_{Z/X}$ (methanol as internal reference) and DMSO-d_6 for octosyl acid A).

	2	4	5	6	7	1′	2′	3′	4′	5′	6′	7′	8′
S_Z	152.3	167.4	102.8	142.6	—	93.5	73.3	71.5	78.6	65.2	36.0	76.0	179.3
S_X	154.0	125.0	126.5	181.3	—	90.7	73.3	71.7	78.0	65.0	35.6	75.7	178.6
Octosyl acid A[a]	148.6	164.0	101.4	147.7	162.9	91.8	71.2	70.4	77.2	63.0	34.8	72.1	171.8

[a] Data taken from ref 17.

Fig. 6. Proposed biosynthetic pathway for the nucleoside skeleton of the polyoxins.



(Reprinted from ref 12 with kindly permission of Prof. K. ISONO, Riken Institute, Saitama, Japan.)

nikkomycins S_Z and S_X, respectively, and 331 and 353 for nikkomycins So_Z and So_X, respectively, were observed. From accurate mass measurements by high resolution FAB-MS the elemental compositions for nikkomycins S_Z and S_X ($C_{12}H_{14}O_8N_2$) and for nikkomycins So_Z and So_X ($C_{12}H_{14}O_9N_2$) could be derived. Reaction with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) gave a derivative with 3 TMS groups in the case of S_X and with partly 4 TMS groups in the case of S_Z with an additional TMS group at the uracil.

Discussion

Based on the analogy of structure and action of nikkomycins to polyoxins there was assumed that the biosynthetic pathway of the aminohexuronic acid part of the nikkomycin molecule followed the scheme shown in Fig. 6, as described for the polyoxins[12].

The formation of the intermediate octofuranuloseuronic acid was manifested by the precursors uridine and phosphoenolpyruvate. Subsequently oxidative elimination of the two terminal carbons forms the carbon skeleton of the polyoxins (**1**). Conversely, reduction at the ketal carbon (C-7') results in formation of the anhydro ring structure of octosyl acid (**2**). The same biosynthetic pathway seems to be realized in case of nikkomycins. Nikkomycins S_Z and S_X are analogues to octosyl acid (**2**) consisting of the corresponding bases, uracil and 4-formyl-4-imidazolin-2-one of nikkomycins Z and X, respectively. The isolation of these compounds and the conversion to nikkomycins Z and X in dependence of the iron concentration of the production medium gave strong evidence for the same biosynthetic procedure in *S. tendae* as described for *Streptomyces cacaoi* var. *asoensis*. The function of nikkomycins So_Z and So_X as reductive products, arrived from further intermediates on the way of oxidative elimination of the two terminal carbons by a similar mechanism as described above, or as preparative artefacts of a nonbiological oxidation of nikkomycins S_Z and S_X, will be subject of further studies.

Experimental

Bacterial Strains

The used nikkomycin over-producing mutant *Streptomyces tendae* Tü 901/S 2566[18] was originally obtained from *Streptomyces tendae* mutant Tü 901/172 ura$^-$[19].

Shaking Cultures

S. tendae Tü 901/S 2566 was cultivated in 500-ml Erlenmeyer flasks containing 100 ml of a production medium consisting of mannitol 4.3%, starch 1.2%, soya peptone 2%, yeast extract 1% in tap water (pH 6.3) and variable amounts of $FeSO_4 \cdot 7H_2O$ from 0 to 7.5 mg/liter.

Fermentation

S. tendae Tü 901/S 2566 was cultivated in a 10-liter fermenter (Biostat S, Braun Diessel Biotech, Melsungen, Germany). 10 liters of medium consisting of mannitol 4.3%, starch 1.2%, soya peptone 1%, yeast extract 1% in tap water (pH 6.3) were inoculated with 0.5 liter of shaking cultures grown for 30 hours in an preculture medium consisting of starch 2% and soybean meal 2% in tap water (pH 6.3). The fermenter was kept at 27°C and agitated at 300 rpm with an aeration of 0.5 v/v/m.

HPLC Analysis

The chromatographic system consisted of an HP 1090 M liquid chromatograph equipped with a diode-array detection system and workstation (Hewlett-Packard, Waldbronn, Germany). A detection wavelength of 280 nm and a reference wavelength of 550 nm were used.

The HPLC column filled with Shandon Hypersil ODS (5 μm, 125 mm × 4.6 mm i.d.), fitted with a precolumn (20 mm × 4.6 mm i.d.) obtained from Grom (Herrenberg, Germany).

Samples of the fermentation broth were centrifuged and 10 μl of the supernatant were injected onto the HPLC column and analysed by gradient elution. Solvent A was water, containing 10 mM hexanesulfonic acid and 2 ml of acetic acid per liter; solvent B was water-acetonitrile (6:4), also containing 10 mM hexanesulfonic acid and 2 ml of acetic acid per liter. The separation started with an isocratic elution of 100% solvent A for 2 minutes, followed by a shift within 0.1 minute to 15% solvent B, a linear gradient elution to 45% solvent B in 9 minutes and a post-time of 5 minutes under the initial conditions. The flow rate was 2 ml/minute.

Acetonitrile (HPLC grade) was obtained from Merck (Darmstadt, Germany). Hexanesulfonic acid sodium salt monohydrate was purchased from Fluka (Neu-Ulm, Germany). Water was purified by means of a Milli-Q system (Millipore, Eschborn, Germany).

Isolation

The fermentation broth was adjusted to pH 2.5 with 3 N H_2SO_4 and filtered with addition of 2% Hyflo Super-cel. The culture filtrate was chromatographed on a column containing Dowex 50 WX 2 (H^+, 100~200 mesh). The intermediates, which the resin did not bind, were separated from nikkomycins D, Z, X, J and I. The eluate was chromatographed on a column containing Lewatit MP 64 Z II (OH^-). The

intermediates were eluted with a gradient of 0.1 to 1.5 N formic acid, concentrated *in vacuo* and lyophilized. The powder was dissolved in water and chromatographed on a Bio-Gel P 2 column with water. The fractions containing the intermediates nikkomycins S_Z and S_X and nikkomycins So_Z and So_X were concentrated *in vacuo* and lyophilized. The separation of the intermediates nikkomycin S_Z from S_X and So_Z from So_X was achieved by preparative HPLC using a Nucleosil-100 C-18 column (10 μm, 250 mm × 32 i.d., precolumn 30 mm × 16 i.d.; Grom, Herrenberg, Germany), and isocratic elution with water over a period of 10 minutes and a flow rate of 48 ml/minute (pump HPP-200/100, gradient controller GCU-311; Kronwald, Sinsheim, Germany). The eluate was detected at 260 nm using a spectrophotometer equipped with a preparative cell (Knauer, Berlin, Germany). Nikkomycins S_Z, S_X, So_Z and So_X containing fractions were concentrated and lyophilized.

Structure Elucidation

The NMR spectra were obtained by using a Bruker WM 400 (400 MHz) and a WM 250 (250 MHz) spectrometer. D_2O was used as a solvent and the DOH-resonance signal served as an internal reference. For the ^{13}C spectra methanol was used as a reference.

Positive ion FAB mass spectra were analyzed on a VG 70-250S instrument with *m*-nitrobenzylalcohol as a matrix substance and with xenon as a collision gas.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 323) and the Bundesministerium für Forschung und Technologie (Zentrales Schwerpunktprojekt Bioverfahrenstechnik BCT 0501 4). T.C.S. was supported by a doctoral scholar-ship of the Fraunhofer Institut für Grenzflächen und Bioverfahrenstechnik, Stuttgart. We thank G. GREWE, I. WERNER, J. WACHTER and R. UTZ for their support in fermentation and HPLC.

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