Biosynthesis of 4-Formyl-4-imidazoline-2-on, the Heterocyclic Base of Nikkomycin X

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

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The nucleoside-peptide antibiotic nikkomycin X contains the unusual heterocyclic base 4-formyl-4-imidazoline-2-on. Investigations on the biosynthesis of this base were accomplished in a resting cell system which was optimized in respect to nikkomycin production.

Incorporation experiments with [2-^{14}C]adenine, [8-^{14}C]adenine, [2-^{14}C]glycine, [2-^{14}C]uracil, [U-^{14}C]histidine, and [2-^{14}C]histidine revealed that only [2-^{14}C]adenine, [U-^{14}C]- and [2-^{14}C]histidine were specifically incorporated into nikkomycin X. An incorporation experiment with [2-^{13}C]adenine resulted in a 31-fold enrichment of the carbon atom 2 of 4-formyl-4-imidazoline-2-on. These results show that the heterocyclic base of nikkomycin X is a product of the histidine biosynthetic pathway.

Introduction

The nikkomycins, a complex of structurally closely related nucleoside-peptide antibiotics from *Streptomyces tendae* Tü 901, are potent inhibitors of chitin synthases from fungi and insects [1–6]. All biologically active nikkomycins posses a modified nucleoside moiety linked by a peptide bond to the uncommon amino acid hydroxypyridyl-homothreonine (HPHT) (Fig. 1).

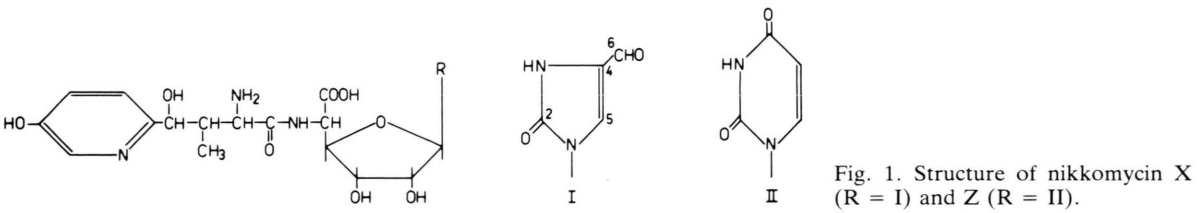

Fig. 1. Structure of nikkomycin X (R = I) and Z (R = II).

The investigations presented in this paper are related to the biosynthesis of 4-formyl-4-imidazoline-2-on, the heterocyclic base of the nikkomycins X, I and M [3]. On principle there exist three possibilities which could explain the biogenetic origin of the base (Fig. 2):

1. 4-Formyl-4-imidazoline-2-on is derived from the biosynthetic pyrimidine nucleotide metabolism by a rearrangement of uracil, similar to a reaction proposed by Uramoto *et al.* [10] for the biosynthesis of neopolyoxin B. If this is right, all C-atoms of the base should be derived from uracil.

2. The base is derived from the biosynthetic histidine metabolism. If this assumption is correct then the ring carbons should originate from C-atoms 1 and 2 of ribose and C-atom 2 (but not 8) of adenine or from the imidazole ring of histidine, respectively.

3. An intermediate of the purine nucleotide biosynthetic pathway represents the precursor of 4-formyl-4-imidazoline-2-on. This implies that carbons 4 and 5 of the base should be derived from glycine, and C-2 should originate from C-8 of adenine.

In order to test whether one of these hypotheses is correct, incorporation experiments with different ^{14}C- and ^{13}C-labeled precursors were performed.

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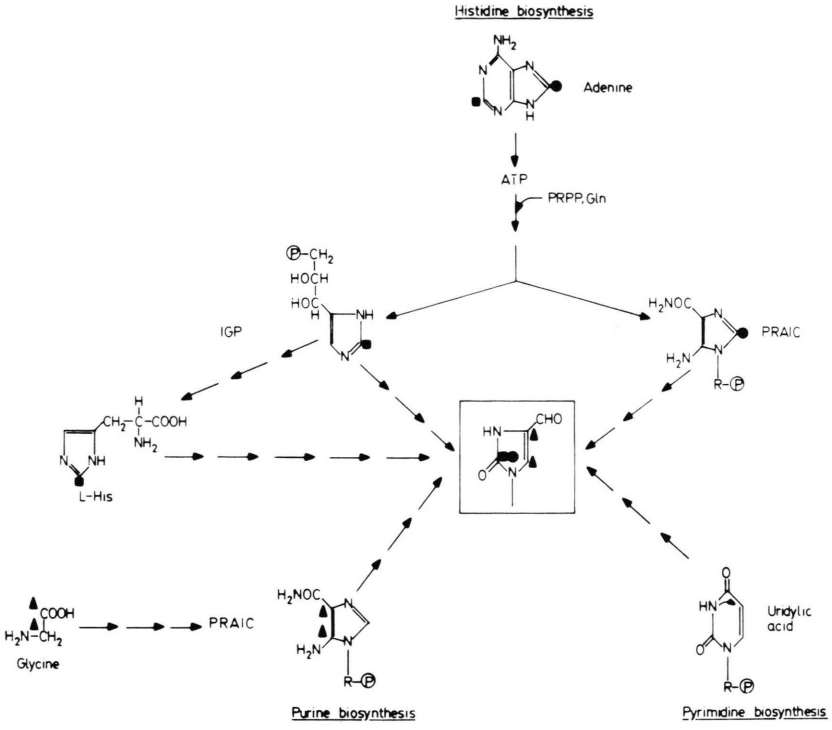


Fig. 2. Possible pathways for the biosynthesis of 4-formyl-4-imidazoline-2-on.
PRPP = 5-phosphoribosyl-1-pyrophosphate, IGP = imidazole glycerol phosphate, PRAIC = 5′-phosphoribosyl-5-amino-imidazole-4-carboxamide.

Materials and Methods

Chemicals

Soy peptone was purchased from Oxoid (Wesel, W.-Germany), yeast extract from Difco (Detroit, USA), and 3,3′-dimethylglutaric acid from Sigma (St. Louis, USA). [2-^{14}C]L-Histidine, [2-^{14}C]- and [2-^{13}C]adenine were from CEA (Gif-sur-Yvette, France), [2-^{14}C]glycine, [U-^{14}C]L-histidine, [2-^{14}C]uracil, and [8-^{14}C]adenine from NEN (Dreieich, W.-Germany). All other chemicals, analytical grade, were obtained from Merck (Darmstadt, Bundesrepublik Deutschland).

Organisms and culture conditions

Streptomyces tendae Tü 901 (a gift from H. Zähner, Tübingen) was grown in the following medium (75 ml in a 500 ml baffled flask): 3% mannitol, 1% starch, 1% yeast extract, 0,5% soy peptone, tap water. After incubation (28 °C) on a gyrotory shaker for 68 hours, the mycelium was harvested and washed with tap water by centrifugation. The cells were resuspended in tap water; the volume of this suspension was 50% of the volume claimed by the original culture. 10 ml of this cell suspension were transferred to 20 ml resting cell medium (21 mM 3,3′-dimethylglutaric acid, 0.67% mannitol, 4.3 mM NH_4NO_3, 2.7 mM $MgSO_4$ in tap water, pH 6.0 adjusted with NaOH) located in a 100 ml baffled flask. This culture was incubated at 28 °C on a gyrotory shaker (160 rpm).

Incorporation experiments

[2-^{14}C]Adenine (specific radioactivity 51 mCi/mmol or 0.14 mCi/mmol, respectively; see Table I), [2-^{14}C]-glycine (47 mCi/mmol), [U-^{14}C]histidine (10 mCi/mmol), [2-^{14}C]histidine (52 mCi/mmol), [8-^{14}C]-adenine (55 mCi/mmol), and [2-^{14}C]uracil (55 mCi/mmol) were added to resting cell cultures immediately after inoculation. 2 to 5 μCi of ^{14}C-labeled compound were used for 60 ml culture.

[2-^{13}C]adenine (90% enriched) was added to a final concentration of 0.5 mM in the resting cell culture.

After incubation for 24 to 48 hours, the mycelium was removed by centrifugation.

Concentration of biologically active nikkomycins was determined by plate diffusion assay with *Mucor hiemalis* Tü 179 according to [1].

Isolation of nikkomycin X

1. Incorporation experiments with ^{14}C-labelled precursors: After acidification to pH 4.0 cell-free culture fluid (1–2 cultures) was applied to SP-Sephadex C-25 (pyridinium form, 2 × 20 cm). Elution of nikkomycins was carried out with a discontinuous gradient of pyridine acetate buffer pH 4.7 (10, 20, 30, 40, and 50 mM). Fractions containing biologically active nikkomycins (eluted with 50 mM buffer) were pooled and lyophilized after determination of radioactivity. The lyophilisate was redissolved in 0.1% acetic acid, and the different nikkomycins were separated by preparative HPLC (RP-18 column; 4.6 × 125 mm; Shandon Hypersil ODS 5 μ) according to the method of Fiedler [9].

2. Incorporation experiments with [2-^{13}C]adenine: Cell-free culture supernatant from 10 cultures containing approx. 70 mg biologically active nikkomycins was applied to a column of SP-Sephadex C-25 (pyridinium form, 3 × 35 cm). Nikkomycin was eluted with a discontinuous gradient consisting of 10, 30, 40 and 50 mM pyridine acetate buffer pH 4.7. Fractions containing nikkomycin were pooled and lyophilized. The lyophilisate was redissolved in 0.1% acetic acid and chromatographed several times on Biogel P-2 (200–400 mesh; 2.6 cm × 76 cm) equilibrated with 0.1% CH_3COOH. Flow rate was held at 14 ml/hr. Purified nikkomycin X was obtained with a yield of approx. 30%.

Isotope determination: Radioactivity was determined by liquid scintillation counting (Philipps PW 4700 scintillation spectrometer). ^{13}C-NMR spectra of purified nikkomycin X (dissolved in D_2O/DCl pH 2.5) were recorded at 75 MHz with a Bruker WM 300 NMR spectrometer. Mass spectrometric investigations were done by the static SIMS-method [7, 8]; nikkomycin was dissolved in 0.1% acetic acid and applied on silver foil.

Results and Discussion

Fermentative production and isolation of nikkomycin: The resting cell medium used was developed by Sommer [20] who reported a maximal nikkomycin yield of approx. 200 mg/l after 72 hours of incubation. By selecting for a high-producing wild type clone and increasing the oxygen transfer into the culture medium it was possible to increase the nikkomycin production of *Streptomyces tendae* up to

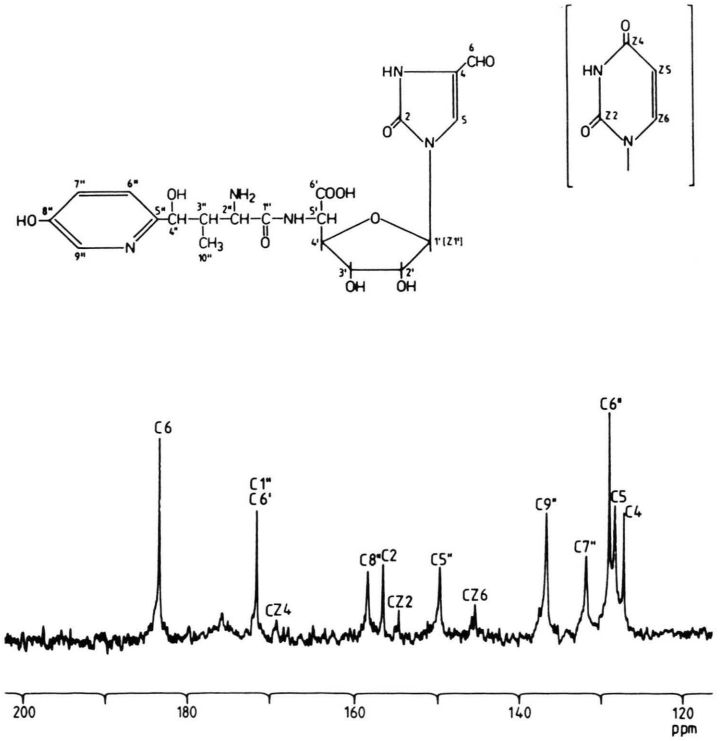


Fig. 3. ^{13}C-NMR spectrum of nikkomycin X (section, chemical shift range 120–200 ppm). Signals contributed by an impurity of nikkomycin Z are indicated.

250–260 mg nikkomycin/l after 48 hours of incubation.

The purification procedure used for the isolation of nikkomycin X did not yield a 100% homogenous substance; the product still contained a certain amount of nikkomycin Z (6–10% as determined by HPLC). However, this degree of purity proved to give satisfactory results in ^{13}C-NMR measurements (Fig. 3).

Incorporation of ^{14}C-labelled compounds

To test the different hypotheses for the biogenetic origin of the nikkomycin X base 4-formyl-4-imidazoline-2-on (as summarized in Fig. 2), adenine, glycine, histidine, and uracil labelled with ^{14}C were administered to cultures of *S. tendae* during nikkomycin production. After isolation of the nikkomycin complex by ion exchange chromatography, nikkomycins X and Z were separated by HPLC. The labelling of the two different compounds is shown in Table I.

Radioactivity from [2-^{14}C]adenine, [U-^{14}C]L-histidine, and [2-^{14}C]L-histidine is very effectively incorporated into the nikkomycins (incorporation rates 15%, 8% and 20%, resp.). These three compounds lead to a significantly higher specific radioactivity in nikkomycin X compared to nikkomycin Z (ratios 17:1 to 25:1). Glycine, the precursor of carbons 5 and 6 in adenine, is incorporated to a much lower extent (incorporation rate 1.7%) into the nikkomycin complex; both, X and Z are nearly equally labelled by this amino acid. A similar results was obtained with [8-^{14}C]adenine: a rather low incorporation of label (0.9%) and no significant discrimination between nikkomycins X and Z. Uracil obviously is a very specific precursor of nikkomycin Z and not of the X isomer (specific radioactivity ratio X:Z = 1:46), excluding an earlier proposal for the biosynthesis of the neopolyoxins [10].

These results imply that the unusual heterocyclic base of nikkomycin X is formed *via* adenine (*i.e.* probably ATP) and histidine. Partial oxidation of the histidine heterocycle and cleavage of the side chain between the α- und β-carbons could lead to the 4-formyl-4-imidazoline-2-on. The somewhat better incorporation of [2-^{14}C]L-histidine compared to [U-^{14}C]L-histidine (see column 6 of Table I) could support this assumption. If this holds true, C-2 of the histidine imidazole ring and C-2 of the "pyrimidine" ring of adenine should be the immediate precursors of C-2 in nikkomycin X.

Table I. Incorporation of ^{14}C-labeled compounds.

Compound	Total incorporation [%][a]	Nikkomycin X + Z produced [μmol]	Specific radioactivity nikkomycin X [dpm/μmol]	Specific radioactivity nikkomycin Z [dpm/μmol]	Ratio of specific radioactivities (nikkomycin X : nikkomycin Z)
[2-^{14}C] adenine[b]	12.9	7.4	1.31×10^5	9.23×10^3	14.2
[2-^{14}-C] adenine[c]	15.7	11.0	4.98×10^4	2.90×10^3	17.2
[U-^{14}C] L-histidine	8.4	10.6	6.42×10^4	2.49×10^3	25.8
[2-^{14}C] L-histidine	20.8	5.8	1.91×10^5	1.00×10^4	19.1
[8-^{14}C] adenine	0.9	8.0	4.84×10^3	2.69×10^3	1.8
[2-^{14}C] glycine	1.7	7.9	5.76×10^3	2.73×10^3	2.1
[2-^{14}C] uracil	2.7	11.9	1.12×10^3	5.24×10^4	0.021

[a] Percentage of radioactivity recovered in the nikkomycin complex after chromatography on SP-Sephadex.
[b] Specific radioactivity 51 mCi/mmol.
[c] Specific radioactivity 0.14 mCi/mmol.

C-atom	ppm	A Integral	R[a]	B Integral	R'[a]	r[b]
2	156.4	4.60	0.22	57.48	6.91	31.41
4	127.1	10.37	0.50	3.81	0.46	0.92
5	128.2	28.61	1.39	11.69	1.41	1.01
6	183.3	16.93	0.82	7.06	0.85	1.04
1'	90.3	20.57	1.00	8.32	1.00	1.00
2'	75.5	32.31	1.57	9.45	1.14	0.73
3'	73.2	28.73	1.40	10.01	1.20	0.86
4'	85.9	21.05	1.02	9.68	1.16	1.14
5'	59.6	–	–	–	–	–
6'	171.6	–	–	–	–	–
1''	171.6	–	–	–	–	–
2''	59.6	–	–	–	–	–
3''	42.5	32.69	–	12.09	–	–
4''	73.8	46.17	–	11.33	–	–
5''	149.5	10.68	–	4.02	–	–
6''	128.8	39.69	–	10.66	–	–
7''	131.5	24.11	–	8.00	–	–
8''	158.3	7.34	–	2.89	–	–
9''	136.7	35.37	–	10.16	–	–
10''	8.9	22.50	–	12.69	–	–

Table II. ^{13}C-NMR spectral data of nikkomycin X isolated from resting cell cultures.
A: natural abundance ^{13}C-content; B: after incorporation of [2-^{13}C]adenine.

[a] R (R') = integral C_i/integral C_1.
[b] r = R'/R.

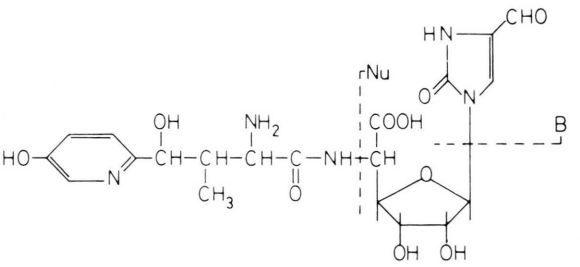
Incorporation of ^{13}C-enriched adenine

Final proof for the assumption presented above was obtained by an experiment with ^{13}C-enriched adenine. The analysis of ^{13}C-NMR spectra – accomplished according to Rathmann [11] and Sommer [20] – showed that unequivocal signal assignment is possible even if the nikkomycin X preparation does contain small amounts of the other nikkomycins including Z (Fig. 3, Table II). After incorporation of [2-^{13}C]adenine (90% enriched) only the signal at 156.4 ppm (*i.e.* C-2 of the nikkomycin X base) had increased in intensity compared to the natural abundance NMR spectrum (Fig. 3). Using C-1' (*i.e.* the glycosidic carbon atom of the hexosaminuronic acid portion) as the reference signal, a specific incorporation into C-2 of the heterocyclic base was obvious; a 31 fold enrichment was calculated (Table II, Fig. 3; calculation of incorporation rate according to Wright *et al.* [12]).

Mass spectrometric studies (SIMS) with nikkomycin X gave the following results: the fragment with $m/z = 111$ represents the formylimidazoline moiety. If the relative intensity of this signal is set as 100%, the relative intensity of the fragment $m/z = 112$ increases from 11.6% (nikkomycin with natural abundance ^{13}C content) to 27.1% in nikkomycin obtained after incorporation of [2-^{13}C]adenine. A specific incorporation of 15–16% is calculated from these data.

The difference between the results obtained by NMR measurement and mass spectrometry is at least partly due to the fact that the ^{13}C-enriched nikkomycin preparation contained a small amount (approx. 9%) of nikkomycin Z. Whereas the NMR method discriminates between the two isomers (C-2 of 4-formyl-4-imidazoline-2-on in nikkomycin X at 156.4 ppm, C-2 of uracil in nikkomycin Z at

Table III. Relevant fragments of nikkomycin X (natural ^{13}C-abundance) observed by SIMS (negative ions).



Mass number [m/z]	Interpretation	Relative intensity of negative ions [%]
94	$[M-(Nu-C_5N_2O_2H_{10})]^-$	5.0
111	$[B]^-$	100.0
125	$[M-(Nu-C_4N_2-OH_7)]^-$	5.0
134	$[M-(Nu-C_2N_2O_2H_6)]^-$	7.9
181	$[M-(Nu-CNOH)]^-$	96.2
494	$[M-(H)]^-$	0.3

154.5 ppm), both nikkomycins X and Z give fragments of $m/z = 111$ and $m/z = 112$.

Nevertheless the results show quite clearly that the 4-formyl-imidazoline base of nikkomycin X is biogenetically derived from adenine via the histidine biosynthetic pathway (see Fig. 2). Histidine itself appears to be an immediate precursor. Concerning the biochemical reactions leading from histidine to 4-formyl-4-imidazoline-2-on, only speculations are possible presently. Known reactions in histidine metabolism which could initiate a partial degradation of this amino acid include deamination to urocanic acid [13–15], transamination to imidazolyl-pyruvate [16, 17] and decarboxylation to histamine [18, 19]. This aspect is subject of further investigations.

Acknowledgements

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