NEW NIKKOMYCINS BY MUTASYNTHESIS AND DIRECTED FERMENTATION

Sir:

The nikkomycins, nucleoside peptide antibiotics, produced by *Streptomyces tendae*[1,2,3] inhibit the chitin synthases of fungi and insects[4,5]. Uracil and 4-formyl-4-imidazoline-2-one are the variable bases of the natural nikkomycins. Since uracil originates from the pyrimidine metabolism, *pyr* mutants, which are not able to form uracil[6], produce only nikkomycins with the 4-formyl-4-imidazoline-2-one base.

To differentiate between the two natural heterocyclic bases barbituric acid can be used. It acts as an aldehyde reagent only with the nikkomycins containing the 4-formyl-4-imidazoline-2-one system, with a reaction rate about sixty times faster than dimedone[2] (pH 6 and 20°C). The products are red colored pigments, which can easily be separated from the nikkomycins. Another advantage of barbituric acid over dimedone is that it does not impair high performance liquid chromatography (HPLC). This was essential, as the new nikkomycins were directly detected by an especially developed HPLC-system[7].

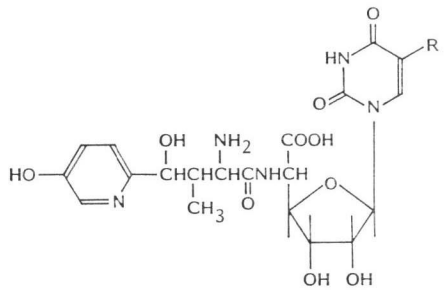
Twenty-three *N*-heterocyclic compounds, including pyrimidines, purines and imidazoles, were fed to the nikkomycin producer (directed fermentation) and to the *pyr* mutants descended from it (mutasynthesis). Upon administration of the three pyrimidine — thymine, 5-hydroxymethyluracil and 5-formyluracil — interesting additional peaks were detected in the HPLC. To qualify as new nikkomycins the peaks must have certain characteristics: pH stability and binding to cation and anion exchange resins. Furthermore, the sequence of the nikkomycins in the HPLC corresponds to that of their bases, and from one base both nikkomycin Z- and J-analogues are produced.

Isoorotic acid, another base, did not show any new peaks; instead, production of nikkomycins Z and J was increased. The combination of high voltage electrophoresis with various biological tests and HPLC analysis showed that a new weakly active nikkomycin was produced.

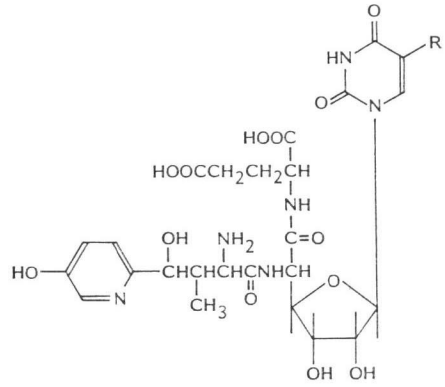
The new nikkomycins (Fig. 1) were isolated by chromatography on ion exchange resins, gel chromatography and preparative HPLC, and their structure was elucidated by ^{1}H NMR investigations in D_2O solution. The ^{1}H NMR spectra of the nucleoside moieties of the nikkomycins have been thoroughly studied and described in previous publications[2,9]. The signals were assigned by doubled resonance. In addition to the signals of nikkomycin Z in the new nikkomycins the singlets of a CH_3 group at 1.94 ppm (nikkomycin Z_T) and at 1.86 ppm (nikkomycin J_T) were assigned. Correspondingly, additional singlets of a CH_2 group at 4.38 ppm (nikkomycin Z_H) and at 4.36 (nikkomycin J_H) were found. The details of the structure elucidation will be published elsewhere.

The optimal precursor concentration was also determined in medium M (g/liter): starch 10, mannitol 30, yeast extract 10, soybean meal 20. For thymine, it was 1 g/liter; for 5-formyluracil and 5-hydroxymethyluracil, it was over 4 g/liter. The precursor was added to the culture 50 hours after inoculation, which corresponded to the beginning of the production stage. The maxi-

Fig. 1. Structures of the new nikkomycins.



Nikkomycin Z-series

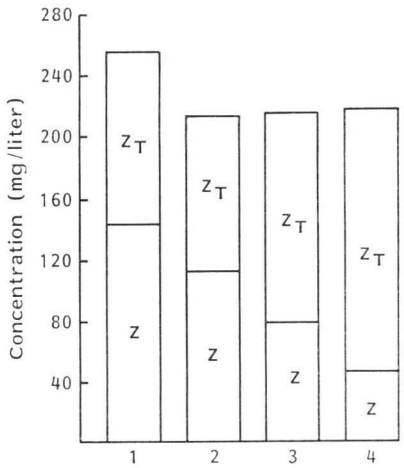


Nikkomycin J-series

Nikkomycin Z_T, J_T R = $-CH_3$
Nikkomycin Z_H, J_H R = $-CH_2OH$

Fig. 2. Optimization of the medium.
1. Medium M.
2. Medium M without yeast extract.
3. Medium M without yeast extract +isoleucine (2 g/liter).
4. Medium M without yeast extract +isoleucine (2 g/liter) +asparagine (4 g/liter).

Z=Nikkomycin Z, Z_T=nikkomycin Z_T.



mum production rate using *pyr* mutants in a fed batch fermentation, was at about 100 hours; then the rate decreases towards the end of the process (200 hours) to a quarter of the maximum rate. For this reason, and because 5-hydroxymethyluracil, 5-formyluracil and thymine inhibit pyrimidine synthesis, it was possible to use the wild strain instead of the *pyr* mutants. However, to obtain the desired product ratio (high Z_T/Z) in the directed fermentation it was necessary to omit from medium M the yeast extract, which is rich in purines and pyrimidines. It was also necessary to add L-isoleucine and L-asparagine to the medium. The results are shown in Fig. 2. The new medium so obtained (4 in Fig. 2) not only led to high Z_T/Z ratio, but also to higher yield of new nikkomycins. The explanation could be that the natural nikkomycins are more sensitive to nitrogen-repression than the new compounds. The advantage of directed fermentation in the wild type over mutasynthesis in the mutant was higher yield over a longer production period (over 280 hours).

The assumption that the products isolated after addition of 5-formyluracil and 5-hydroxymethyluracil are identical was verified by ^{1}H NMR spectroscopy.

The K_i values with the chitin synthase from *Coprinus cinereus* (Dixon plot)[3] were determined for the four new nikkomycins, Z_T, J_T, Z_H and J_H (Fig. 1):

nikkomycin Z_T K_i=2.1 μM
nikkomycin J_T K_i=14.6 μM
nikkomycin Z_H K_i=2.8 μM
nikkomycin J_H K_i=19.2 μM
nikkomycin Z K_i=6.6 μM
nikkomycin X K_i=0.7 μM

These values show that the new nikkomycins Z_T and Z_H are more active than nikkomycin Z and less active than nikkomycin X. The additional glutamate group (nikkomycins J_T and J_H) causes a seven-fold decrease in activity against chitin synthase. The biological activity of nikkomycin Z_T and Z_H against different fungi ranges from three times stronger to four times weaker than nikkomycin Z. The corresponding J-analogues, the tripeptides, are between four and forty times less active.

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