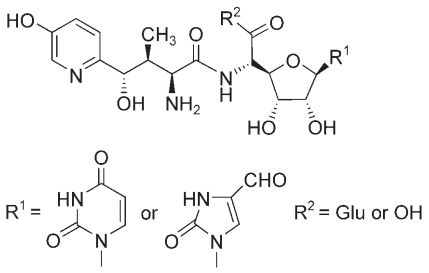
DOI: 10.1002/cbic.200500208

3′-*Enol*pyruvyl-UMP, a Novel and Unexpected Metabolite in Nikkomycin Biosynthesis

Cristian Ginj,[a] Heinz Rüegger,[c] Nikolaus Amrhein,[a] and Peter Macheroux*[a, b]

Nikkomycins are produced by several species of *Streptomyces* and exhibit fungicidal, insecticidal, and acaricidal properties due to their strong inhibition of chitin synthase.[1–4] Structurally, they can be classified as peptidyl nucleosides containing two unusual amino acids, that is, hydroxypyridylhomothreonine and aminohexuronic acid with an *N*-glycosidically linked base (Scheme 1).[5] Although the chemical structure of nikkomycins



Scheme 1. Basic structure of nikkomycin antibiotics.

has been known since the 1970s, information on their biosynthesis is scarce. Following the cloning of the entire set of structural genes involved in nikkomycin biosynthesis,[6] the enzymatic steps leading to the 4-formyl-4-imidazolin-2-one base were investigated in some detail.[7–9] The peptidyl moiety is synthesized by eleven enzymatic reactions, of which only two have been investigated in depth.[10,11]

The aminohexuronic acid is introduced into the nikkomycin skeleton by the transfer of 5-phosphoribosyl-1-pyrophosphate to the nucleobase (uracil or 4-formyl-4-imidazolin-2-one) followed by addition of an *enol*pyruvyl moiety from phospho*enol*-pyruvate (PEP), supposedly to the 5′-hydroxyl group of the ribose.[12,13] This putative intermediate is then further modified by rather speculative reactions to yield the aminohexuronic acid precursor.[13]

Based on amino acid sequence similarity, the *nikO* gene in the nikkomycin operon appears to encode an *enol*pyruvyl transferase.[13] This family of enzymes comprises two well-characterized enzymes, that is, 5-*enol*pyruvylshikimate 3-phosphate synthase (EPSPS, EC 2.5.1.19) and UDP-*N*-acetylglucosamine *enol*pyruvyltransferase (MurA, EC 2.5.1.7), which catalyze the transfer of the intact *enol*pyruvyl moiety from PEP to the 5-hydroxyl group of shikimate 3-phosphate and the 3′-hydroxyl group of UDP-*N*-acetylglucosamine, respectively. Therefore, NikO can be expected to catalyze an *enol*pyruvyl transfer reaction in nikkomycin biosynthesis.

In order to substantiate the role of NikO, we have cloned the gene from *Streptomyces tendae* Tü901 and heterologously expressed the protein in *Escherichia coli*. The recombinant protein was purified to homogeneity and analyzed for its activity.[14] Contrary to expectation, no *enol*pyruvyl transferase activity was found with uridine as the substrate.[13] Instead, UMP was found to serve as a substrate for the enzyme. The enzymatic assay was performed by measuring released phosphate by a colorimetric end-point method and a continuous spectrophotometric enzyme-coupled method that exploits the purine nucleoside phosphorylase reaction.[14–16] The phosphate release observed in these assays is accompanied by the generation of a new nucleotide compound from UMP and PEP, as demonstrated by HPLC analysis.[14]

Analysis of the reaction products revealed that phosphate was released stoichiometrically with the formation of the new compound during the enzyme reaction; this indicated that one phosphate group still resided in the nucleotide. This result was substantiated by using ^{32}P-labelled UMP in the enzymatic reaction: 90% of the radiolabel is retained (6.5% in residual substrate); this indicated that the phosphate was released from PEP rather than UMP. Corroborating evidence was obtained from ^{31}P and ^{1}H{^{31}P} NMR spectroscopy, which revealed a single phosphorus attached to the 5′-position in the nucleotide product. Consequently, it can be concluded that the 5′-position is not the site of *enol*pyruvyl attachment. In order to determine the actual acceptor site of the *enol*pyruvyl moiety, the reaction product was isolated, purified by RP-HPLC, and subjected to ^{1}H, ^{13}C, and 2D ^{13}C,^{1}H-heteronuclear NMR spectroscopy. This analysis showed unambiguously that the *enol*pyruvyl moiety is attached at the 3′-position of the ribose sugar moiety (Figure 1). Therefore, we propose that NikO catalyzes the generation of 3′-*enol*pyruvyl-UMP (3′-EPUMP), as shown in Scheme 2, and not 5′-*enol*pyruvyluridine, as postulated earlier.[13] In fact, no reaction product was observed when NikO was incubated in the presence of uridine and PEP. These results raise the question of how 3′-EPUMP is further utilized in the generation of the aminohexuronic acid precursor. In the original proposal by Isono and co-workers, attachment of the *enol*pyruvyl moiety at the 5′-position (by reaction with a putative aldehyde group) would directly yield an octofuranose uronic acid nucleoside. This reaction is then thought to be followed by oxidative elimination of the two distal carbon atoms and introduction of an amino group at the 5′-position.[12,17]

[a] *Dipl.-Chem. C. Ginj, Prof. Dr. N. Amrhein, Prof. Dr. P. Macheroux*
Institut für Pflanzenwissenschaften, ETH-Zentrum
Universitätsstraße 2, 8092 Zürich (Switzerland)

[b] *Prof. Dr. P. Macheroux*
Current address:
Institut für Biochemie, Technische Universität Graz
Petersgasse 12, 8010 Graz (Austria)
Fax: (+43) 316-873-6952
E-mail: peter.macheroux@tugraz.at

[c] *Dr. H. Rüegger*
Laboratorium für Anorganische Chemie, ETH-Hönggerberg
Wolfgang-Pauli-Straße 10, 8093 Zürich (Switzerland)

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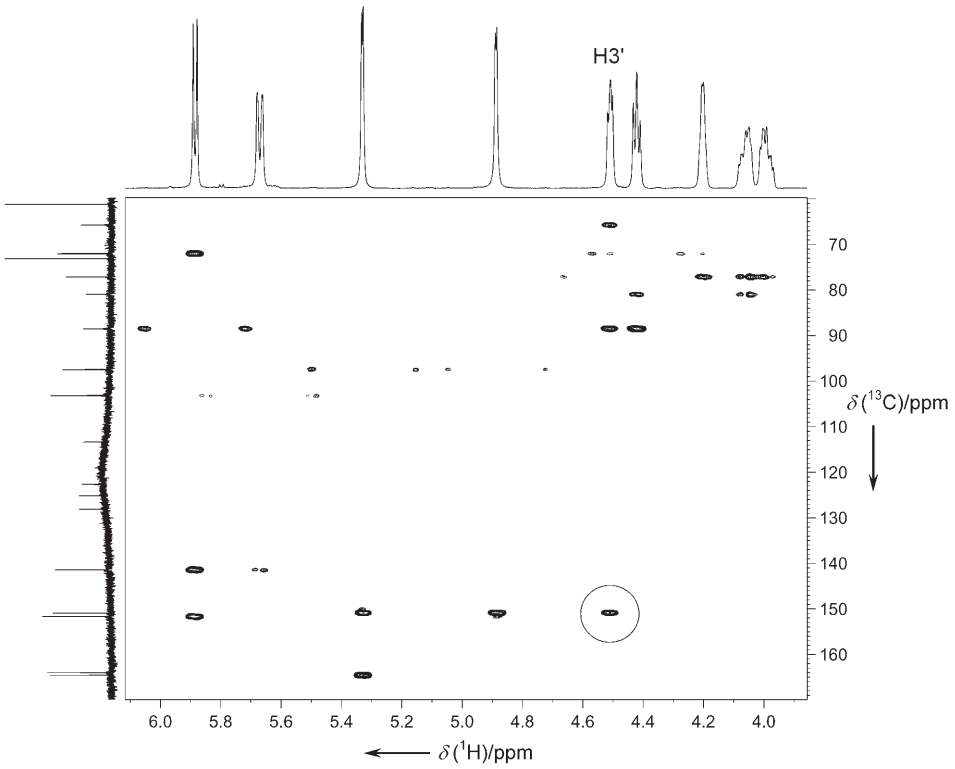
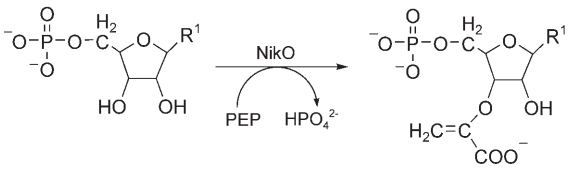


Figure 1. Section of the 500.23 MHz 2D $^{13}C,^{1}H$-HMBC NMR spectrum of NikO product, showing the correlation (circled) between the quaternary alkene carbon of the *enol*pyruvyl and the H3′ proton of UMP, thus establishing the structure of the product.



Scheme 2. Proposed enzymatic *enol*pyruvyl transferase reaction catalyzed by NikO.

Clearly, our findings that NikO introduces the *enol*pyruvyl moiety at the 3′- rather than the 5′-position contradicts the current proposal for nikkomycin biosynthesis. Isono and coworkers have demonstrated the incorporation of ^{13}C from D-[1-^{13}C]glucose at the 5′- and 6′-positions of the nucleoside.[12] In order to rationalize this labeling, rearrangement of the carbon skeleton must be postulated, catalyzed by enzymes operating downstream of NikO. The characterization of these enzymes will shed more light on the chemical reactions in nikkomycin biosynthesis and might provide us with useful tools to explore the synthesis of novel nikkomycin antibiotics.

Experimental Section

General procedures: ^{1}H, $^{13}C\{^{1}H\}$, and $^{31}P\{^{1}H\}$ NMR spectra were recorded in [D_6]DMSO on a Bruker AVANCE-500 NMR spectrometer. Chemical shifts (δ) are given in ppm, relative to TMS and H_3PO_4.[18] ^{1}H and ^{13}C spectral assignments were made based on $^{1}H,^{13}C$ heteronuclear correlation NMR spectroscopy.

Analytical HPLC was carried out by using a Beckman Gold System equipped with detector module 168 (UV/Vis diode array detector, Beckman, Switzerland) and a LiChroCART® 250–4 column (from Merck KGaA, Darmstadt, Germany) packed with Lichrosphere 100, C-18 and 5 μm diameter. The elution was carried out by applying a linear gradient of 0.05% trifluoroacetic acid to acetonitrile over 15 min with a flow rate of 1 mL min^{-1}. The preparative HPLC purification was performed isocratically with 5% acetonitrile. The purified product was lyophilized.

Enzyme assay (spectrophotometric continuous assay): The continuous enzyme-coupled assay was carried out by using the EnzChek® Phosphate Assay Kit (Molecular Probes Europe BV, The Netherlands), as described in the manual provided by the manufacturer. The orthophosphate (P_i) released in the NikO-catalyzed reaction was used in the coupled reaction in which 2-amino-6-mercapto-7-methylpurine riboside (MESG) was converted to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP). Enzymatic conversion of MESG to the phosphorylated product resulted in a shift of the absorbance maximum from 330 to 360 nm. All reagents provided with the kit were prepared according to the manufacturer's instructions, except that our test buffer (50 mM Tris pH 7.5, 2 mM DTT, pH 7.5 at 25 °C) was used as reaction buffer instead of the provided reaction buffer. An Uvikon 933 spectrophotometer (Kontron, Switzerland) was used for spectrophotometry.

Solutions of UMP and PEP were prepared by dissolving the solid compounds in the test buffer; MESG and PNP were dissolved directly in ultrapure Millipore water. The final concentrations in the assay mixture were 200 μM, 1 mM, and 10 μM for MESG, PEP and NikO, respectively. PNP solution (3 μL, containing 0.3 units of activity) was used, and the reaction was started by adding UMP.

Product analysis by RP-HPLC chromatography: The enzymatic reactions were set up by incubating PEP (1 mM), UMP (1 mM), and NikO (10 μM) at pH 7.5 and 30 °C for 1 h. Negative controls lacking PEP, UMP, or NikO enzyme were run in parallel. Aliquots were removed from each reaction mix and diluted with 0.05% trifluoroacetic acid, then this solution (20 μL) was injected into the RP-HPLC column. Compounds were analyzed by recording the absorbance at 260 nm (for UMP and 3′-EPUMP) and 230 nm (for PEP).

Analysis of the product of the reaction with [^{32}P]-UMP: The enzymatic reaction was carried out as described above. The cold mix was supplemented with a radiolabeled aliquot of [^{32}P]-UMP (Hartmann, Analytic GmbH, Braunschweig, Germany, 220 TBq mmol^{-1} specific activity). The radioactivity of samples was determined in a

Beckman scintillation counter by using the filters and settings according to the manufacturer's recommendations. Total recovery of the injected radioactivity was about 99.2% (unreacted substrate, product, and flow trough were not included into the collected peaks).

Identification of 3′-EPUMP: ^{1}H NMR ([D$_6$]DMSO, 500.23 MHz, 26 °C): $\delta = 11.41$ (br, 1H; H3), 7.74 (d, $^{3}J_{5\text{-}6} = 8.1$ Hz, 1H; H6), 5.88 (d, $^{3}J = 6.4$, 1H; H1′), 5.67 (dd, $^{3}J_{6\text{-}5} = 8.1$, $^{4}J_{3\text{-}5} = 1.4$ Hz, 1H; H5), 5.33 (d, $^{2}J = 2.4$ Hz, 1H; =CH$_2$), 4.88 (d, $^{2}J = 2.4$ Hz, 1H; =CH$_2$), 4.5 (dd, $^{3}J = 5.2$, 3.2 Hz, 1H; H3′), 4.42 (dd, $J = 6.4$, 5.2 Hz, 1H; H2′), 4.20 (m, $J = 3.5$, 3.2, 3.0 Hz, $J_{PH} = 0.9$ Hz, 1H; H4′), 4.06 (m, $J = 11.3$, 3.0 Hz, $J_{PH} = 6.5$ Hz, 1H; H5′), 3.99 (m, $J = 11.3$, 3.5 Hz, $J_{PH} = 6.9$ Hz, 1H; H5″); the protons of carboxyl and 2′-OH were very likely exchanged due to the small amount of water present in the sample, and therefore not assessed. ^{31}P{^{1}H} NMR ([D$_6$]DMSO, 202.5 MHz, 26 °C): $\delta = 0.1$ (s, 1P, H5′P); ^{13}C{^{1}H} NMR ([D$_6$]DMSO, 125.9 MHz): $\delta = 164.6$ (COOH), 164.0 (C4), 151.7 (C2), 150.9 (C=), 103.2 (C5), 97.5 (=CH$_2$), 88.5 (C1′), 80.9 (d, $^{3}J_{PC} = 7.9$ Hz, C4′), 77.1 (C3′), 72.0 (C2′), 65.7 (d, $^{2}J_{PC} = 4.3$ Hz, C5′).

Acknowledgements

This work was supported by an internal research grant from the ETH Zürich to P.M. and N.A. (00462). The authors are grateful to Philippe Roy for his invaluable help in HPLC analysis and the experiments involving radiochemicals.

Keywords: antibiotics · natural products · nikkomycin · phospho*enol*pyruvate · transferases

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Received: May 19, 2005
Published online on October 5, 2005

