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Characterization of the Early Stage Aminoshikimate Pathway in the Formation of 3-Amino-5-hydroxybenzoic Acid: The RifN Protein Specifically Converts Kanosamine into Kanosamine 6-Phosphate

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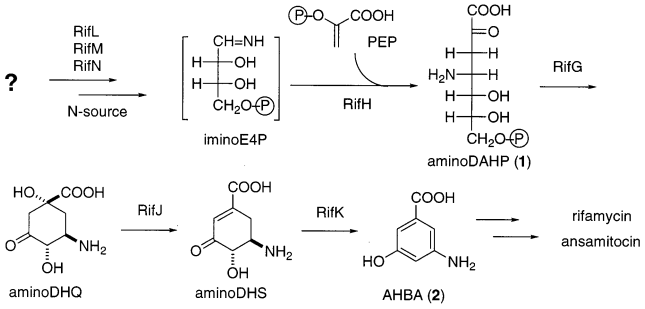
3-Amino-5-hydroxybenzoic acid (AHBA, **2**) is the precursor of the mC_7N units[1] found in mitomycin[2] and ansamycin[3] antibiotics, such as rifamycin B[4] and ansamitocin P-3.[5] The biosynthesis of AHBA proceeds via a novel variant of the shikimate pathway (Scheme 1) which appears to branch off from the normal pathway at the stage of 3,4-dideoxy-4-amino-D-*arabino*-heptulosonic acid 7-phosphate (aminoDAHP, **1**).[6] Purification of the last enzyme in this sequence, AHBA synthase, which aromatizes 5-amino-5-deoxy-3-dehydroshikimic acid (aminoDHS) to AHBA, and cloning of the encoding gene, *rifK*, by reverse genetics[7] set the stage for the cloning, sequencing, and analysis of the entire 95 kbp rifamycin (*rif*) biosynthetic gene cluster from *Amycolatopsis mediterranei* S699[8] and subsequently of the mitomycin[9] and ansamitocin (*asm)*[10] biosynthetic genes from *Streptomyces lavendulae* and *Actinosynnema pretiosum*, respectively.

Scheme 1



Further studies on the rifamycin biosynthetic gene cluster identified seven genes, *rifG*, *-H*, *-J*, *-K*, *-L*, *-M*, and *-N*, which are involved in the biosynthesis of AHBA.[8,11] Three of these, *rifG*, *-H*, and *-J*, encode homologues of shikimate pathway enzymes, and their products were identified as 5-amino-5-deoxy-3-dehydroquinic acid (aminoDHQ) synthase, aminoDAHP synthase, and aminoDHQ dehydratase, respectively, confirming the validity of the pathway from aminoDAHP to AHBA.[11] However, the mode of formation of aminoDAHP has remained enigmatic, although it is clearly not derived from DAHP.[6,11] Three additional gene products, RifL, RifM, and RifN, are absolutely essential for AHBA biosynthesis and function in the pre-aminoDAHP part of the pathway.[11] RifL closely resembles Pur10, an oxidoreductase involved in puromycin biosynthesis.[12] RifM is homologous to phosphatases belonging to the CBBY family,[13] and RifN is related to a glucose kinase from *S. coelicolor* A3 implicated in glucose repression.[14] Their role in aminoDAHP formation has so far remained unclear, as has the origin and mode of introduction of the nitrogen atom. No other plausible candidate gene for the nitrogen introduction step has been found in the *rif* cluster, and circumstantial evidence[11,15] suggests that RifK may have a second function in the pathway, that of introducing the nitrogen into a precursor of aminoDAHP, in addition to its well-characterized role as the AHBA synthase.[7,17]

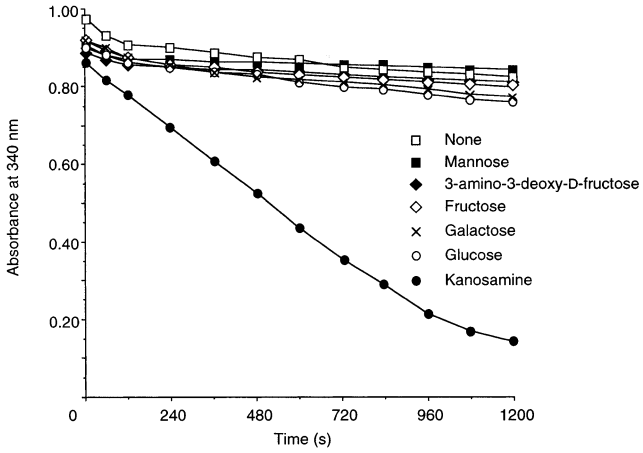


Figure 1. Substrate specificity of RifN.

Recent work by Guo and Frost[18] has shed new light on the issue by demonstrating that the aminosugar, 3-amino-3-deoxy-D-fructose 6-phosphate (aminoF6P, **5**), can be converted into aminoDAHP (together with DAHP) or further into AHBA by the action of transketolase from *Escherichia coli*, with ribose 5-phosphate as acceptor, and the recombinant RifH protein or a cell-free extract of *A. mediterranei* plus phosphoenolpyruvate (PEP). Presumably, the transketolase converted **5** into the imino analogue of erythrose 4-phosphate (E4P), which then partly served directly as a substrate for the RifH-catalyzed condensation with PEP to give aminoDAHP and partly underwent hydrolysis to E4P to produce DAHP. As a biosynthetic source of the aminoF6P, Guo and Frost[18] proposed kanosamine (3-amino-3-deoxy-D-glucose, **3**), a known secondary metabolite of *Streptomyces* and other bacteria.[19] Since either kanosamine or its isomerization product would have to be phosphorylated to give aminoF6P, this suggests a possible role for the kinase encoded by *rifN*.

To examine its functional activity, RifN was overexpressed in *E. coli* as a His_6 fusion protein[20] and purified to near homogeneity on a Ni-NTA column (Qiagen). The standard coupled assay for kinase activity was performed as described by Seno and Chater,[21] measuring NADH consumption at 340 nm. Only kanosamine reacted specifically with RifN + ATP, while all other sugar derivatives examined (glucose, mannose, galactose, fructose, glucosamine, and 3-amino-3-deoxy-D-fructose) gave no change in absorbance at 340 nm (Figure 1). The product generated from

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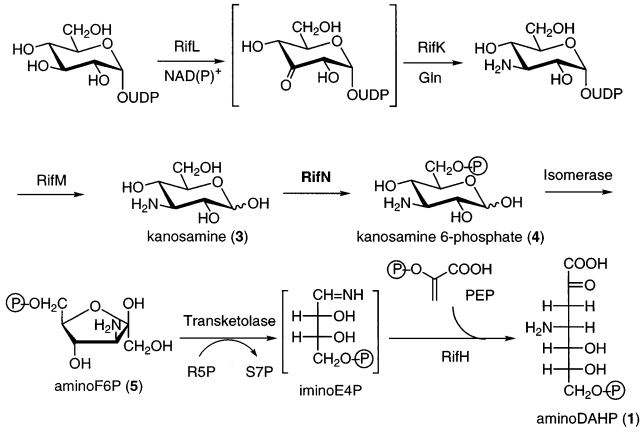
kanosamine was prepared on a preparative scale[22] and identified by ^{1}H NMR, ^{13}C NMR, and ESI-MS analyses[23] as kanosamine 6-phosphate (**4**). The ^{1}H NMR in D_2O showed two anomeric doublets at 4.57 and 5.12 ppm (J = 7.8 and 3.6 Hz, respectively) in a 1:1 ratio. ^{31}P-coupled signals in the ^{13}C NMR for C-6 (J = 3.6 Hz) and C-5 (J = 6.1 Hz) established the position of the phosphate group. K_m values of 1.9 and 0.39 mM, respectively, were determined for ATP and kanosamine, and V_{max} is 0.6 mmol min^{-1} mg^{-1} at 37 °C and pH 7.2. The enzyme is dependent on Mg^{2+}, with Mn^{2+}, Co^{2+}, and Ni^{2+} able to substitute at 21, 30, and 18% relative efficiency, whereas Zn^{2+}, Cu^{2+}, and Fe^{2+} are inhibitory.

The data identify RifN as a specific kanosamine 6-kinase, which together with the essential nature of the *rifN* gene[11] establishes kanosamine and its 6-phosphate as intermediates in AHBA formation. RifL and -M must function before RifN in the pathway, since a *rifN* mutant of *A. mediterranei* was able to complement both a *rifL* and a *rifM* mutant to restore rifamycin B production (data not shown). Keeping in mind the likely biosynthesis of kanosamine,[24] this allows us to propose a new pathway for aminoDAHP formation starting from UDP-glucose (Scheme 2). RifL and RifK jointly

Scheme 2



convert UDP-glucose into UDP-kanosamine,[25] which is cleaved by RifM to kanosamine. Following the action of RifN, a "housekeeping" isomerase (no candidate gene for a dedicated enzyme has been found in the *rif* cluster[8]) must convert kanosamine 6-phosphate into aminoF6P. The conversion of the latter into the imine of-E4P may be catalyzed by Rif Orf15, which is homologous to transketolases[8] and which may act in concert with the aminoDAHP synthase, RifH, to suppress hydrolysis of the imine. Work is underway to further test this hypothetical pathway.

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(15) The circumstantial evidence includes the following: (a) No aminoDHS or earlier precursor is accumulated by a *rifK* mutant of *A. mediterranei* S699. (b) Two homologues of *rifK* are present in the *asm* gene cluster. (c) *rifK* and *rifL* homologues are juxtaposed identically in every AHBA biosynthesis gene cluster examined,[8−10, 16] suggesting a close functional interaction. (d) AHBA synthase can bind PMP as well as PLP (Yu, T.-W.; Floss, H. G., unpublished data).
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(20) The *rifN* gene was amplified by polymerase chain reaction (PCR), introducing *Bgl*II and *Eco*RI restriction sites at the two ends, and cloned into the expression vector pRSET-B (Invitrogen) linked to a His_6 tag. The resulting plasmid, pRM74, was transformed into *E. coli* BL21(DE3)-pLysS. The transformants were grown at 37 °C in LBBS medium (1% tryptone, 0.5% yeast extract; 1% NaCl; 2 M D-sorbitol; 2.5 mM betaine, pH 7.0) with 100 μg/mL carbenicillin and 25 μg/mL chloramphenicol. Protein synthesis was induced at OD_{600} = 0.4 by adding isopropyl β-D-thiogalactopyranoside to 0.2 mM.
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(22) A 15 mL aqueous solution of **3** (18 mg, 0.10 mmol), ATP (110 mg, 0.20 mmol), $MgCl_2 \cdot 6H_2O$ (0.25 mmol), purified RifN (0.5 mg), and citric acid (1 mmol) was incubated at 37 °C and pH 7.5−8.0. The enzymatic conversion was monitored by thin-layer chromatography, developing with either $CHCl_3$−methanol−ammonium hydroxide (10:7:3) or *n*-butanol−acetic acid−acetone−H_2O (140:100:33:80). The reaction mixture was passed through Centricon-10 centrifugal filter devices (Amicon) to remove the protein. The filtrate was lyophilized, and the resulting powder was dissolved in a minimum of water, applied to Dowex 1-X8 anion-exchange resin (65 mL, acetate form), and eluted with a linear gradient (0−2 M) of acetic acid. The fraction containing the product was lyophilized to give **4** as a white fluffy powder (yield 57%, 1:1 anomeric mixture).
(23) **4**: ESI-MS *m*/*z* 258 (M − H$^+$); HR ESI-MS calcd for $C_6H_{13}NO_8P$ (M − H$^+$) 258.0379, found 258.0387; ^{1}H NMR (300 MHz, D_2O) δ 3.08 (1H, d, J = 10.4 Hz, 4-Ha), 3.26 (1H, t, J = 10.4 Hz, 4-Hb), 3.34 (1H, dd, J = 7.8 and 10.4 Hz, 2-Ha), 3.44 (1H, br d, J = 8.8 Hz), 3.63−3.83 (6H, m), 3.90−4.50 (2H, br) 4.57 (1H, d, J = 7.8 Hz, 1-Ha) 5.12 (1H, d, J = 3.6 Hz, 1-Hb); ^{13}C NMR (75 MHz, D_2O with 5% CD_3OD) δ 55.5 and 58.3 (C-3), 63.2 (d, J = 3.6 Hz, C-6), 66.4 and 66.6 (C-4), 69.3 and 71.8 (C-2), 72.0 and 77.3 (d, J = 6.1 Hz, C-5), 92.5 and 97.4 (C-1).
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(25) Support for this notion comes from work by Guo and Frost reported in the accompanying communication[26] and from the following preliminary observations from our laboratory: (a) RifK and -L when coexpressed in *E. coli* form a complex which can be isolated on a Ni-NTA column when only RifL carries a His_6-tag. (b) The RifKL complex catalyzes NADH formation from NAD^+ dependent on UDP-glucose, but not dTDP-glucose as substrate. (c) The rate of NADH formation increases when glutamine is added as nitrogen source, asparagine and NH_4^+ were less effective, and glutamate and aspartate were inactive.
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