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Kanosamine Biosynthesis: A Likely Source of the Aminoshikimate Pathway's Nitrogen Atom

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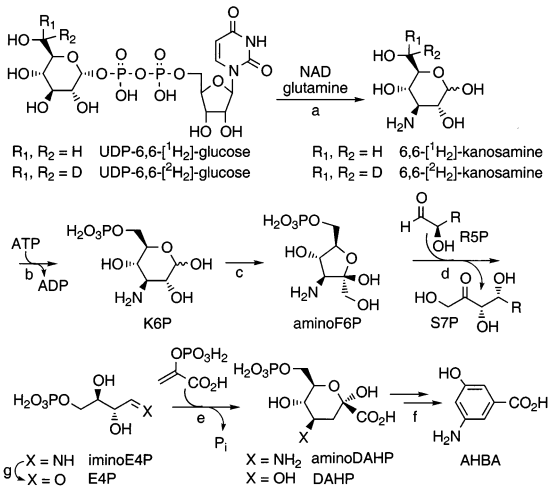
Many biologically active natural products including rifamycin, mitomycin, and ansamitocin are derived from 3-amino-5-hydroxybenzoic acid (AHBA, Scheme 1) by way of the aminoshikimate pathway.[1] Floss and co-workers have demonstrated the role of 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate[2] (aminoDAHP, Scheme 1) in the aminoshikimate pathway and delineated the loci in the *rif* biosynthetic gene cluster required for biosynthesis of 3-amino-5-hydroxybenzoic acid.[3] Recently, aminoDAHP was demonstrated to form in *Amycolatopsis mediterranei* cell-free lysate from 3-amino-3-deoxy-D-fructose 6-phosphate (aminoF6P, Scheme 1) via the inferred intermediacy of 1-deoxy-1-imino-D-erythrose 4-phosphate (iminoE4P, Scheme 1).[4] Biosynthesis of 3-amino-3-deoxy-D-glucose (kanosamine, Scheme 1) is now examined as a possible source of the aminoshikimate pathway's nitrogen atom.

With identification of 3-amino-3-deoxy-D-fructose 6-phosphate as a precursor to 3-amino-5-hydroxybenzoic acid, attention turned to delineation of the source of this alkaloid. One natural product that, by combination of an isomerization and phosphorylation, could be a precursor to 3-amino-3-deoxy-D-fructose 6-phosphate was kanosamine (Scheme 1). Various microbes generate kanosamine as a biosynthetic end-product.[5] As exemplified by the biosynthesis of kanamycin,[7] kanosamine is also an intermediate en route to other natural products. The possibility therefore needed to be explored that *A. mediterranei* might similarly utilize kanosamine as a biosynthetic intermediate as well as a vehicle for incorporation of the nitrogen atom into the aminoshikimate pathway. Working backward from the intermediacy of 3-amino-3-deoxy-D-fructose 6-phosphate, kanosamine 6-phosphate was first synthesized and tested as a precursor to aminoDAHP.

D-Glucose was selectively protected and the resulting C-3 hydroxyl group oxidized (Scheme 2). Subsequent diastereoselective reduction provided for an overall net inversion of configuration at C-3. Activation of the C-3 hydroxyl group as a triflate ester followed by nucleophilic displacement with NaN_3 introduced the requisite nitrogen atom at C-3. Reduction and subsequent deprotection gave kanosamine. Hexokinase-catalyzed phosphorylation of kansoamine employed citric acid as an activator[4] and afforded kanosamine 6-phosphate in an overall yield of 43% from starting D-glucose.

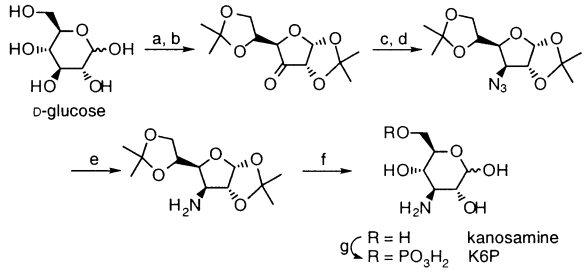
Initial attempts at the in vitro bioconversion of kanosamine 6-phosphate into aminoDAHP focused on an assembled system. Kanosamine 6-phosphate, D-ribose 5-phosphate, and phosphoenolpyruvate were incubated with yeast phosphoglucose isomerase, *Escherichia coli tktA*-encoded transketolase,[6a,b] and *E. coli aroF*^{FBR}-encoded DAHP synthase.[6b,c] Although no aminoDAHP was detected (entry 1, Table 1), formation of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) indicated that kanosamine 6-phosphate was a substrate for isomerase. The action of transketolase on isomerase-generated 3-amino-3-deoxy-D-fructose 6-phosphate and subsequent hydrolysis of 1-deoxy-1-imino-D-erythrose 4-phosphate account for the formation of DAHP.[4] The bioconversion of kanosamine 6-phosphate was then repeated upon substitution of *E. coli aroF*^{FBR}-encoded DAHP synthase (entry 1, Table 1) with *A. mediterranei rifH*-encoded aminoDAHP synthase (entry 2, Table 1). Along with DAHP, formation of aminoDAHP was observed (entry 2, Table 1).

Scheme 1 [a,b]



[a] Conversions (genes): (a) see ref 5c; (b) aminoglucokinase; (c) glucoisomerase; (d) transketolase (*tktA*); (e) aminoDAHP synthase (*rifH*), DAHP synthase (*aroF*^{FBR}); (f) aminoshikimate pathway; (g) hydrolysis. [b]Abbreviations: AT(D)P, adenosine 5′-tri(di)phosphate; K6P, kanosamine 6-phosphate; aminoF6P, 3-amino-3-deoxy-D-fructose 6-phosphate, R5P, D-ribose 5-phosphate; S7P, D-sedoheptulose 7-phosphate; iminoE4P, 1-deoxy-1-imino-D-erythrose 4-phosphate; E4P, D-erythrose 4-phosphate; P_i, inorganic phosphate; aminoDAHP; 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; AHBA, 3-amino-5-hydroxybenzoic acid.

Scheme 2 [a]



[a] Reactions: (a) acetone, $ZnCl_2$, H_3PO_4, 68%; (b) PDC, $(CH_3CO)_2O$, CH_2Cl_2, reflux, 91%; (c) $NaBH_4$, EtOH/H_2O (9:1), 0 °C, 90%; (d) (i) $(CF_3SO_2)_2O$, pyridine, CH_2Cl_2, −20 °C, quantitative, (ii) NaN_3, DMF, 50 °C, 92%; (e) $LiAlH_4$, Et_2O, 94%; (f) 2 N HCl, 25 °C, quantitative; (g) ATP, $MgCl_2$, hexokinase, citric acid, pH 8, 90%.

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Table 1. Biosynthesis of AminoDAHP from Kanosamine 6-Phosphate and Kanosamine from UDP-Glucose

entry	reaction conditions	products[c] (yield,[d] %)
1	kanosamine 6-phosphate, R5P, PEP; yeast phosphoglucose isomerase (60 units), *E. coli* TktA transketolase (9 units[a]), *E. coli* AroF^{FBR} DAHP synthase (660 units[b]), pH 7.3	DAHP (39)
2	kanosamine 6-phosphate, R5P, PEP; yeast phosphoglucose isomerase (60 units), *E. coli* TktA transketolase (9 units[a]), *A. mediterranei* RifH aminoDAHP synthase (64 units[b]), pH 7.3	aminoDAHP (2); DAHP (30)
3	kanosamine 6-phosphate, R5P, PEP; *A. mediterranei* cell-free extract (DAHP synthase activity of 0.2 unit[b]), pH 7.3	aminoDAHP (6); DAHP (20); AHBA (2); Tyr (3); Phe (2)
4	glucose 6-phosphate, R5P, PEP, glutamine, $(NH_4)_2SO_4$; *A. mediterranei* cell-free extract (DAHP synthase activity of 0.2 unit[b]), pH 7.3	DAHP (21)
5	UDP-6,6-[$^2H_2$]-glucose, NAD, glutamine; *A. mediterranei* cell-free extract, pH 6.8	6,6-[$^2H_2$]-kanosamine (5)

[a] Transketolase was assayed according to ref 6a. [b] AminoDAHP synthase was assayed as DAHP synthase activity according to ref 6a. [c] See the legend to Scheme 1 for abbreviations. [d] Yields are ^{1}H NMR yields of aminoDAHP, DAHP, and AHBA purified to homogeneity and of L-tyrosine and L-phenylalanine purified to a binary mixture. Response factors and quantification of product concentrations were based on integration relative to 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$.

Incubation of 3-amino-3-deoxy-D-fructose 6-phosphate with cell-free lysate of *A. mediterranei* (ATCC 21789) has previously been reported to give higher yields of aminoDAHP than incubations employing the assembled bioconversion system.[4] Accordingly, reaction of kanosamine 6-phosphate with D-ribose 5-phosphate and phosphoenolpyruvate in *A. mediterranei* cell-free lysate led to higher yields of aminoDAHP and formation of 3-amino-5-hydroxybenzoic acid (entry 3, Table 1). As a control experiment, D-glucose 6-phosphate, D-ribose 5-phosphate, and phosphoenolpyruvate were incubated in *A. mediterranei* cell-free lysate with glutamine and $(NH_4)_2SO_4$ as possible sources of nitrogen (entry 4, Table 1). No aminoDAHP formation was observed.

Reaction of kanosamine with ATP, D-ribose 5-phosphate, and phosphoenolpyruvate in *A. mediterranei* cell-free lysate did not produce quantifiable levels of either aminoDAHP or DAHP. As a consequence, attention turned to biosynthesis of kanosamine in *A. mediterranei*. Kanosamine biosynthesis was first observed and studied in *Bacillus pumilus* (formerly *Bacillus aminoglucosidicus*) in the 1960s.[5a−c] Incubation of UDP-[U-^{14}C]-glucose in *B. pumilus* cell lysate with NAD and glutamine led to the formation of [U-^{14}C]-kanosamine.[5c] Likewise, incubation of UDP-6,6-[$^2H_2$]-glucose with NAD and glutamine in *A. mediterranei* cell-free lysate led to the formation of 6,6-[$^2H_2$]-kanosamine (entry 5, Table 1). Based on analysis by electrospray mass spectrometry, UDP-6,6-[$^2H_2$]-glucose having an M + 2 ion with relative intensity 98.08% yielded kanosamine having an M + 2 ion with 97.83% relative intensity. Incubation of UDP-glucose with NAD, glutamine, ATP, D-ribose 5-phosphate, and phosphosphoenolpyruvate in *A. mediterranei* cell-free lysate did not produce quantifiable concentrations of amino-DAHP. Low levels of aminoglucokinase (b, Scheme 1) in lysed *A. mediterranei* cells may explain the formation of kanosamine from UDP-glucose as well as the lack of quantifiable aminoDAHP formation from UDP-glucose and kanosamine.

Kanosamine biosynthesis has been directly implicated as the source of the aminoshikimate pathway's nitrogen atom. This follows from the observed bioconversions of kanosamine 6-phosphate into aminoDAHP and 3-amino-5-hydroxybenzoic acid (entries 2 and 3, Table 1), along with the observed bioconversion of UDP-glucose into kanosamine (entry 5, Table 1). As a consequence, elaboration of the source of the aminoshikimate pathway's nitrogen atom must now include elaboration of the biosynthesis of kanosamine. In turn, biosynthesis of kanosmine emerges as a pathway possibly shared by microbes (biosyntheses) as diverse as *Bacillus* spp. (kanosamine),[5] *Streptomyces kanamyceticus* (kanamycin),[7] *A. mediterranei* (rifamycin),[3c] *Streptomyces lavendulae* (mitomycin),[8] and *Actinosynnema pretiosum* (ansamitocin).[9]

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Supporting Information Available: Synthesis of kanosamine 6-phosphate and its conversion to aminoDAHP; synthesis of UDP-6,6-[$^2H_2$]-glucose and its conversion to 6,6-[$^2H_2$]-kanosamine (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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