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Biosynthesis of 1-Deoxy-1-imino-D-erythrose 4-Phosphate: A Defining Metabolite in the Aminoshikimate Pathway

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Biosynthesis of a spectrum of biologically active natural products shares the intermediacy of 3-amino-5-hydroxybenzoic acid (AHBA, Scheme 1), a metabolite generated by the aminoshikimate pathway. Delineation of this pathway has largely been due to the efforts of Floss and collaborators who have shown that 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate[1b] (aminoDAHP, Scheme 1) is converted[1a,c] into AHBA in cell-free lysates of *Amycolatopsis mediterranei* and characterized aminoshikimate pathway enzymes encoded by the *rif* biosynthetic gene cluster.[2] However, formation of 1-deoxy-1-imino-D-erythrose 4-phosphate (iminoE4P, Scheme 1) and its subsequent condensation with phosphoenolpyruvate (PEP, Scheme 1) leading to aminoDAHP have not yet been demonstrated.[1a,c] Transamination of D-erythrose 4-phosphate (E4P, Scheme 1) has been suggested[1b,c] to be the source of 1-deoxy-1-imino-D-erythrose 4-phosphate (Scheme 1). In this account, a different route for biosynthesis of 1-deoxy-1-imino-D-erythrose 4-phosphate is examined involving transketolase-catalyzed fragmentation of 3-amino-3-deoxy-D-fructose 6-phosphate (aminoF6P, Scheme 1).

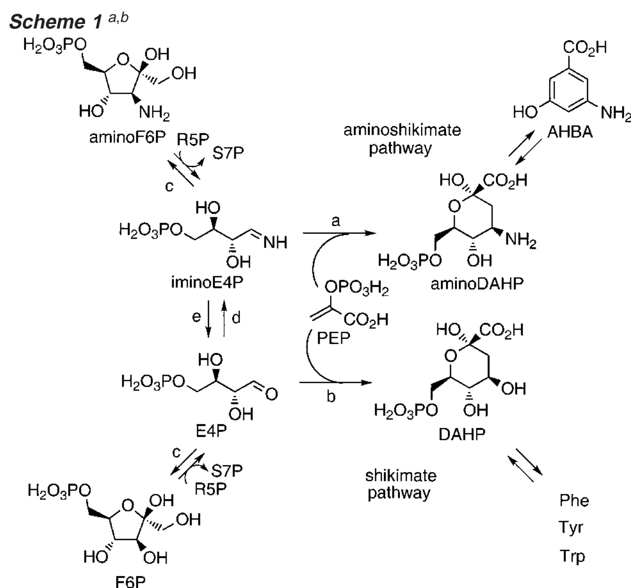
Direct chemical synthesis of 1-deoxy-1-imino-D-erythrose 4-phosphate was not attempted. Structurally related D-erythrose 4-phosphate exists as a monomer in dilute solution only under a very limited range of conditions.[3] It seemed likely that 1-deoxy-1-imino-D-erythrose 4-phosphate would display this same problematic solution chemistry in addition to being prone to hydrolytic loss of its nitrogen atom. An in situ generation/trapping strategy was therefore pursued. Generation of 1-deoxy-1-imino-D-erythrose 4-phosphate relied upon transketolase-catalyzed ketol transfer from 3-amino-3-deoxy-D-fructose 6-phosphate to D-ribose 5-phosphate (R5P, Scheme 1). The trapping reaction involved condensation of the resulting 1-deoxy-1-imino-D-erythrose 4-phosphate with phosphoenolpyruvate catalyzed by aminoDAHP synthase. Isolation of aminoDAHP would implicate formation of 1-deoxy-1-imino-D-erythrose 4-phosphate and in the process verify the enzyme activity proposed for aminoDAHP synthase.

3-Amino-3-deoxy-D-fructose 6-phosphate was obtained by hexokinase-catalyzed phosphorylation (f, Scheme 2) of 3-amino-3-deoxy-D-fructose (aminoF) derived by chemical synthesis (a−e, Scheme 2) from D-fructose. Use of citric acid as an activator[4] during phosphorylation increased the yield of 3-amino-3-deoxy-D-fructose 6-phosphate from 40% to 87% and decreased the amount of hexokinase required from 10 000 to 500 units.

Given the ability of *E. coli* shikimate pathway enzymes to bind aminoshikimate pathway substrates,[1c] 3-amino-3-deoxy-D-fructose 6-phosphate, D-ribose 5-phosphate, and phosphoenolpyruvate were incubated with *E. coli tktA*-encoded transketolase[5a,b] and *E. coli aroF*[FBR]-encoded DAHP synthase[5b,c] (entry 1, Table 1). Although only 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP, Scheme 1) was formed, this product indicated that 3-amino-3-deoxy-D-fructose 6-phosphate was a substrate for transketolase and that 1-deoxy-1-imino-D-erythrose 4-phosphate was formed and subsequently hydrolyzed to D-erythrose 4-phosphate.

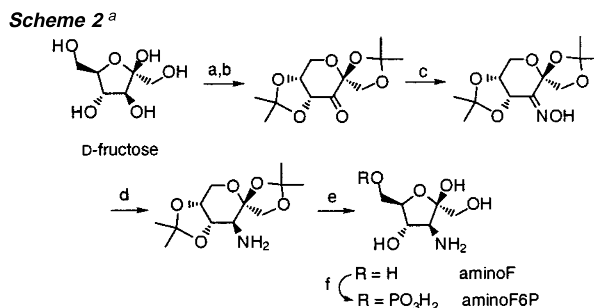
TktA-catalyzed reaction of 3-amino-3-deoxy-D-fructose 6-phosphate was then examined in the presence of *A. mediterranei* RifH (entry 2, Table 1), which has been suggested to be an aminoDAHP synthase due to its sequence similarity to plant-like DAHP synthases

Scheme 1 [a,b]



[a] Enzymes (genes): (a) aminoDAHP synthase (*rifH*); (b) DAHP synthase (*aroF*[FBR]); (c) transketolase (*tktA*); (d) transaminase; (e) hydrolysis. [b] Abbreviations: iminoE4P, 1-deoxy-1-imino-D-erythrose 4-phosphate; aminoDAHP, 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate; aminoF6P, 3-amino-3-deoxy-D-fructose 6-phosphate; E4P, D-erythrose 4-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; F6P, D-fructose 6-phosphate; PEP, phosphoenolpyruvate; R5P, D-ribose 5-phosphate; S7P, D-sedoheptulose 7-phosphate; AHBA, 3-amino-5-hydroxybenzoic acid; Tyr, L-tyrosine; Phe, L-phenylalanine; Trp, L-tryptophan.

Scheme 2 [a]



[a] Reactions: (a) H_2SO_4, acetone, 52%; (b) $RuCl_3$, $NaIO_4$, K_2CO_3, $Et_3(PhCH_2)NCl$, $CHCl_3/H_2O$ (1:1), reflux, 99%; (c) $H_2NOH{\cdot}HCl$, NaOAc, CH_3CN/H_2O (1:1), 91%; (d) $LiAlH_4$, THF, reflux, 11%, (e) 2 N HCl, 25 °C, quant. (f) ATP, $MgCl_2$, hexokinase, citric acid, pH 8, 87%.

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Table 1. Reaction of AminoF6P in the Presence of Transketolase, DAHP Synthase, and AminoDAHP Synthase

entry	reaction condition	products[c] (% yield)[d]
1	aminoF6P, R5P, PEP; *E. coli* TktA transketolase (9 units[a]), *E. coli* AroF[FBR] DAHP synthase (660 units[b]), pH 7.3	DAHP (53)
2	aminoF6P, R5P, PEP; *E. coli* TktA transketolase (9 units[a]), *A. mediterranei* RifH aminoDAHP synthase (64 units[b]), pH 7.3	aminoDAHP (2 ± 0.3); DAHP (35)
3	aminoF6P, R5P, PEP; *A. mediterranei* cell-free extract (DAHP synthase activity of 0.2 units[b]), pH 7.3	aminoDAHP (7 ± 0.2); DAHP (19); AHBA (3); Tyr (5); Phe (5)
4	F6P, R5P, PEP, glutamine, $(NH_4)_2SO_4$; *A. mediterranei* cell-free extract (DAHP synthase activity of 0.2 units[b]), pH 7.3	DAHP (29)

[a] Transketolase was assayed according to ref 5a. [b] AminoDAHP synthase was assayed as DAHP synthase activity according to ref 5a. [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*$_4$.

and location in the *rif* biosynthetic gene cluster.[2c,d] 3-Amino-3-deoxy-D-fructose 6-phosphate, D-ribose 5-phosphate, and phosphoenolpyruvate reacted in the presence of TktA and RifH to form DAHP along with a 2% yield of aminoDAHP (entry 2, Table 1). Addition of 3-amino-3-deoxy-D-fructose 6-phosphate, D-ribose 5-phosphate, and phosphoenolpyruvate to crude cell lysate prepared from *A. mediterranei* (ATCC 21789) afforded a 7% yield of aminoDAHP (entry 3, Table 1) along with formation of DAHP, AHBA, L-tyrosine, and L-phenylalanine. Far fewer units of DAHP synthase activity were present in *A. mediterranei* cell-free lysate (entry 3, Table 1) relative to the units of RifH activity (assayed as a DAHP synthase) employed with TktA transketolase (entry 2, Table 1). As a control experiment, D-fructose 6-phosphate, D-ribose 5-phosphate, phosphoenolpyruvate, and glutamine and $(NH_4)_2SO_4$ as possible sources of nitrogen were incubated in crude *A. mediterranei* cell-free extract (entry 4, Table 1). No aminoDAHP formation was detected.

The possibility remained that 3-amino-3-deoxy-D-fructose 6-phosphate might undergo a transamination reaction thereby being the source of the nitrogen atom but not of 1-deoxy-1-imino-D-erythrose 4-phosphate in its entirety. To address this possibility, 3-[^{15}N]-amino-3-deoxy-D-6,6-[$^2H_2$]-fructose 6-phosphate was synthesized from D-6,6-[$^2H_2$]-fructose and $^{15}NH_2OH{\cdot}HCl$ via the synthetic route specified in Scheme 1. Dilution with unlabeled 3-amino-3-deoxy-D-fructose 6-phosphate afforded material that gave M + 3, M + 2, and M + 1 ions with relative intensities of 10.97%, 0.24%, and −0.6%, respectively, when analyzed by negative ion electrospray mass spectrometry. Incubation with phosphoenolpyruvate and D-ribose 5-phosphate in *A. mediterranei* cell-free extract provided aminoDAHP after purification giving M + 3, M + 2, and M + 1 ions with relative intensities of 10.24%, 0.5%, and 0.42%. On the basis of this observed retention of both ^{15}N and ^{2}H labeling, 3-amino-3-deoxy-D-fructose 6-phosphate is apparently serving as a sequestered form of 1-deoxy-1-imino-D-erythrose 4-phosphate and is not merely a transaminase source of nitrogen.

Enzyme-catalyzed fragmentation of 3-amino-3-deoxy-D-fructose 6-phosphate and enzyme-catalyzed trapping of the resulting 1-deoxy-1-imino-D-erythrose 4-phosphate to form aminoDAHP has led to the identification of a defining metabolite in the aminoshikimate pathway. These observations add to our understanding of the aminoshikimate pathway as well as raise new questions. Does 1-deoxy-1-imino-D-erythrose 4-phosphate partition between formation of aminoDAHP and hydrolysis to D-erythrose 4-phosphate and formation of DAHP in intact *A. mediterranei*? Complexation of transketolase with aminoDAHP synthase, which might facilitate channeling[6] of 1-deoxy-1-imino-D-erythrose 4-phosphate from its formation of 3-amino-3-deoxy-D-fructose 6-phosphate to its condensation with phosphoenolpyruvate, remains to be explored. Also, how is 3-amino-3-deoxy-D-fructose 6-phosphate biosynthesized? Attention now turns to tracing the steps by which the nitrogen atom of the aminoshikimate pathway is derived from ammonium ion.

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Supporting Information Available: Synthesis of aminoF6P and 3-[^{15}N]-6,6-[$^2H_2$]-aminoF6P and enzymatic formation of aminoDAHP and 3-[^{15}N]-6,6-[$^2H_2$]-aminoDAHP (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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