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Synthesis of Aminoshikimic Acid

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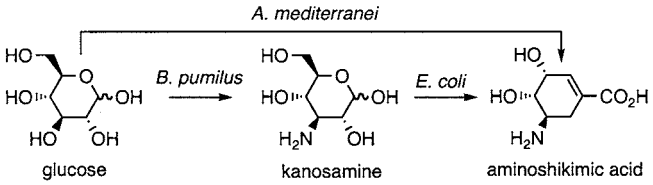
ABSTRACT



5-Amino-5-deoxyshikimic acid (aminoshikimic acid) was synthesized from glucose using recombinant *Amycolatopsis mediterranei* and also synthesized by a tandem, two-microbe route employing *Bacillus pumilus* and recombinant *Escherichia coli*.

5-Amino-5-deoxyshikimic acid (aminoshikimic acid, Scheme 1) is characterized by multiple stereogenic centers and functional groups arrayed around a six-membered carbocyclic ring. As with shikimic acid, aminoshikimic acid is an attractive candidate for use as the core scaffold for synthesis of combinatorial libraries.[1] Aminoshikimic acid is also an intriguing alternative to shikimic acid as a starting material for the synthesis of neuraminidase inhibitors such as the antiinfluenza agent Tamiflu.[2] The first microbe-catalyzed syntheses of aminoshikimic acid are described in this account. Amplified expression of *rifI*-encoded aminoshikimate dehydrogenase in *Amycolatopsis mediterranei* leads to synthesis of aminoshikimic acid. Alternatively, 3-amino-3-deoxy-D-glucose (kanosamine, Scheme 1) synthesized by *Bacillus pumilus* is converted into aminoshikimic acid using recombinant *E. coli* constructs.

Aminoshikimic acid is the namesake of the aminoshikimate pathway, which generates the 3-amino-5-hydroxybenzoic acid (AHBA, Scheme 1) starter unit required for the biosynthesis of the ansamycins and mitomycins.[3] Reduction of the aminoshikimate pathway intermediate, 5-amino-5-deoxy-3-dehydroshikimic acid (aminoDHS, Scheme 1), by *rifI*-encoded aminoshikimate dehydrogenase was anticipated to afford aminoshikimic acid in *A. mediterranei*. The *rifI* locus is situated in the *rif* biosynthetic gene cluster[4] and has been annotated as an aminoshikimate dehydrogenase, although this activity had not been previously established in vitro.[4]

Biosynthesis of aminoDHS in *A. mediterranei* results from intersection of kanosamine biosynthesis,[5b] 1-deoxy-1-imino-D-erythrose 4-phosphate (iminoE4P) biosynthesis,[5a] and the aminoshikimate pathway (Scheme 1).[6] To increase the quantities of synthesized aminoshikimic acid, an *A. mediterranei rifK* mutant lacking 3-amino-5-hydroxybenzoate synthase would be advantageous since competition between the RifI dehydrogenase and RifK dehydratase for in vivo supplies of 5-amino-5-deoxy-3-dehydroshikimic acid would be eliminated. However, RifK apparently functions as both a UDP-3-keto-D-glucose transaminase (Scheme 1) and as an AHBA synthase.[7] Inactivation of *rifK* would therefore block the biosynthesis of kanosamine from which iminoE4P is derived.

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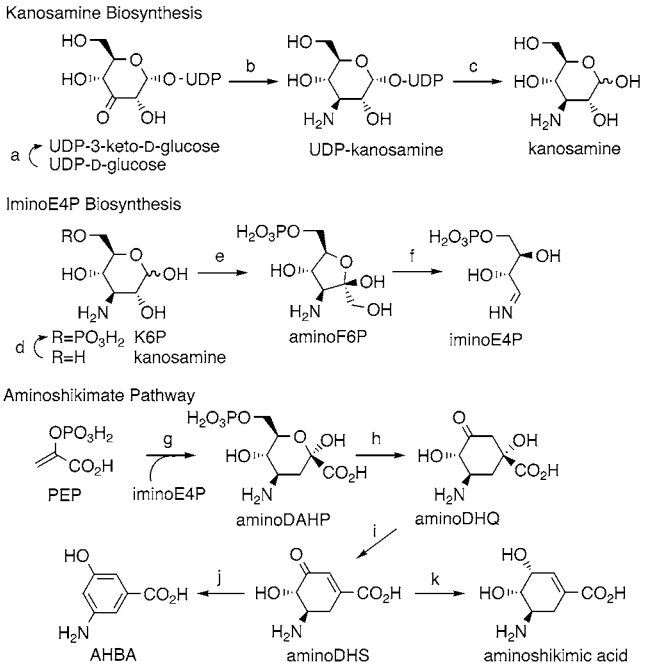
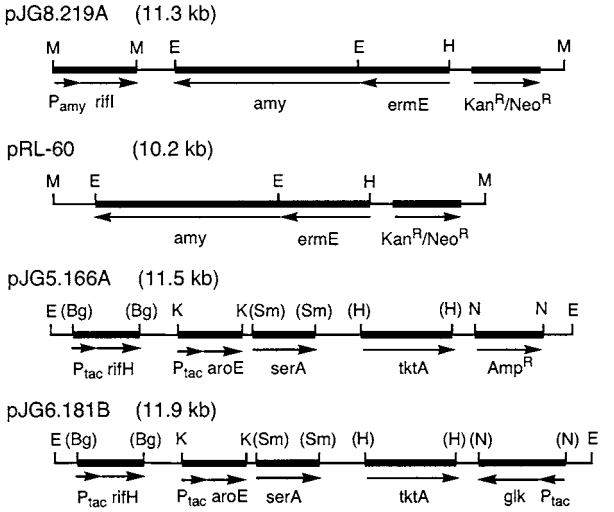
Scheme 1. Biosynthesis of Kanosamine, IminoE4P, AHBA, and Aminoshikimic Acid[a,b]



[a] Enzyme (encoding gene): (a) UDP-3-keto-D-glucose dehydrogenase (*rifL*); (b) UDP-3-keto-D-glucose transaminase (*rifK*); (c) UDP-kanosamine phosphatase (*rifM*); (d) kanosamine kinase (*rifN*); (e) phosphokanosamine isomerase; (f) transketolase (*orf15*); (g) aminoDAHP synthase (*rifH*); (h) aminoDHQ synthase (*rifG*); (i) aminoDHQ dehydratase (*rifJ*); (j) AHBA synthase (*rifK*); (k) aminoshikimate dehydrogenase (*rifI*). [b]Abbreviations: K6P, kanosamine 6-phosphate; aminoF6P, 3-amino-3-deoxy-D-fructose 6-phosphate; PEP, phosphoenolpyruvate; iminoE4P, 1-imino-1-deoxy-D-erythrose 4-phosphate; aminoDAHP, 4-amino-3,4-dideoxy-D-*arabino*-heptulosonic acid 7-phosphate; aminoDHQ, 5-amino-5-deoxy-3-dehydroquinic acid; aminoDHS, 5-amino-5-deoxy-3-dehydroshikimic acid; AHBA, 3-amino-5-hydroxybenzoic acid.

To compete for in vivo supplies of aminoDHS, *A. mediterranei* ATCC 21789/pJG8.219A was constructed to increase expression of *rifI*-encoded aminoshikimate dehydrogenase. The *rifI* insert in plasmid pJG8.219A is under the control of an amylase promoter (P_{amy})[8] and led to an aminoshikimate dehydrogenase specific activity of 0.2 units/mg in *A. mediterranei* ATCC 21789/pJG8.219A. For comparison, the specific activity of aminoshikimate dehydrogenase was 0.08 units/mg for *A. mediterranei* ATCC 21789/pRL-60 (Scheme 2) lacking plasmid-localized *rifI*. This

Scheme 2. Restriction Enzyme Maps of Plasmids[a]



[a] Restriction enzyme sites are abbreviated as follows: Bg = *Bgl*II, E = *Eco*RI, H = *Hin*dIII, K = *Kpn*I, M = *Mlu*I, N = *Nco*I, Sm = *Sma*I, X = *Xba*I. Parentheses indicate that the designated enzyme site has been eliminated. (—) vector DNA, (**—**) insert DNA.

increase in aminoshikimate dehydrogenase expression resulted in the synthesis of 0.2 g/L of aminoshikimic acid in 0.4% yield from glucose along with 1.4 g/L of rifamycin B when *A. mediterranei* ATCC 21789/pJG8.219A was cultured under fermentor-controlled conditions for 12 days at 28 °C (entry 1, Table 1). A cation-exchange resin was used for the purification of aminoshikimic acid from the culture medium of *A. mediterranei* ATCC 21789/pJG8.219A.

Table 1. Concentrations and Yields of Aminoshikimic Acid

		aminoSA[c]			
entry	construct	g/L	yield[b] (%)	Rf[c] (g/L)	SA[c] (g/L)
1	*A. mediterranei* ATCC 21789/pJG8.219A	0.2	0.4	1.4	0
2	*E. coli* SP1.1/pJG5.166A	0	0	0	2.1
3[a]	*E. coli* SP1.1/pJG5.166A	0.81	16/4	0	3.7
4[a]	*E. coli* SP1.1/pJG6.181B	1.1	19/5	0	3.4

[a] Kanosamine was added to the culture medium. [b] Percent yield (mol/mol) from kanosamine/overall percent yield (mol/mol) from glucose. [c] Abbreviations: aminoSA, aminoshikimic acid; Rf, rifamycin B; SA, shikimic acid.

The aminoshikimic acid concentrations synthesized by *A. mediterranei* ATCC 21789/pJG8.219A are important for reasons beyond establishing a de novo synthesis of aminoshikimic acid from glucose using a single microbe. Because quinate dehydrogenases and shikimate dehydrogenases can have overlapping specificities,[11a,b] RifI could conceivably be an aminoquinate dehydrogenase catalyzing the reduction of aminoDHQ (Scheme 1) to 5-amino-5-deoxyquinic acid. The absence of this product during cultivation of *A. mediterranei* ATCC 21789/pJG8.219A

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establishes RifI as an in vivo aminoshikimate dehydrogenase. The synthesis of aminoshikimic acid when expression of RifI is increased also adds to questions relating to why *A. mediterranei* possesses the ability to synthesize this molecule.[7]

As an alternative route to aminoshikimic acid, we elaborated a synthesis that did not employ *A. mediterranei* constructs and that minimized the number of *A. mediterranei* genes requiring heterologous expression. This was accomplished using *Bacillus pumilus* ATCC 21143 to synthesize kanosamine from glucose.[9] Derivatives of *E. coli* SP1.1 were then employed to synthesize aminoshikimic acid from kanosamine.

Synthesis of kanosamine required careful attention to the purity of the *B. pumilus* strain employed as well as culture conditions. A multistep strain purification procedure was utilized[9,10] which included evaluation for catalase activity, starch hydrolysis, catabolism of citric acid and various carbohydrates, growth under anaerobic conditions, growth in the presence of elevated NaCl concentrations, and a Voges−Proskauer test for acetoin formation. The purified *B. pumilus* ATCC 21143 synthesized 1−2 g/L of kanosamine using culture conditions reported in the literature.[9] Culture conditions were subsequently modified to improve the concentrations of kanosamine synthesized by *B. pumilus* ATCC 21143. Employing glucose as the carbon source, soybean meal or peanut meal as the nitrogen source, and fermentor-controlled conditions, *B. pumilus* ATCC 21143 synthesized 25 g/L of kanosamine in 28% yield from glucose (Figure 1).

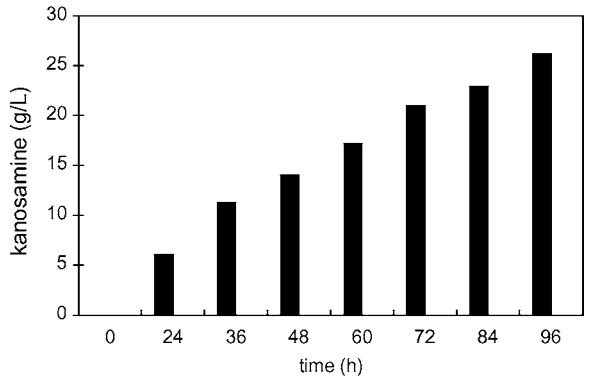

Figure 1. Synthesis of kanosamine from glucose by *B. pumilus* ATCC 21143 under fermentor-controlled conditions.

E. coli SP1.1, which had been previously prepared in our laboratory for synthesis of shikimic acid,[11] was chosen as the host strain for the synthesis of aminoshikimic acid from kanosamine. Both isozymes of shikimate kinase are inactive in *E. coli* SP1.1, which prevented phosphorylation of aminoshikimic acid. The corresponding absence of shikimic acid phosphorylation and de novo biosynthesis of aromatic amino acids required the addition of L-phenylalanine, L-tyrosine, and L-tryptophan to all cultures of *E. coli* SP1.1. These aromatic amino acid supplements also feedback inhibited the native isozymes of DAHP synthase.[11b] This inhibition minimized unwanted biosynthesis of shikimic acid from glucose.

Plasmid pJG5.166A carried *aroE* and *tktA* loci encoding shikimate dehydrogenase and transketolase, respectively, from *E. coli* along with the *rifH* locus from *A. mediterranei*. The resulting amplified expression of *aroE*-encoded shikimate dehydrogenase was intended to prevent unwanted synthesis of aminoDHS (Scheme 1) as a byproduct. Amplified expression of *tktA* was designed to increase the rate of transketolase-catalyzed ketol transfer from 3-amino-3-deoxy-D-fructose 6-phosphate (aminoF6P, Scheme 1) to generate iminoE4P. The *A. mediterranei rifH* insert encoding aminoDAHP synthase was provided with an *E. coli* promoter and ribosomal binding site.

For the conversion of kanosamine into aminoshikimic acid, *E. coli* enzymes catalyzed the transport and phosphorylation of kanosamine, isomerization of kanosamine 6-phosphate, and fragmentation of aminoF6P to form iminoE4P. Condensation of phosphoenolpyruvate with iminoE4P catalyzed by aminoDAHP synthase was the only *A. mediterranei* enzyme activity expressed in *E. coli*. AminoDAHP was converted into aminoshikimic acid via the sequential action of the *E. coli* shikimate pathway enzymes 3-dehydroquinate synthase, 3-dehydroquinate dehydratase, and shikimate dehydrogenase.

As a control, *E. coli* SP1.1/pJG5.166A was cultured in the absence of added kanosamine under fermentor-controlled conditions. The 2.1 g/L of shikimic acid (entry 2, Table 1) synthesized by *E. coli* SP1.1/pJG5.166A over 48 h reflects the small amount of native DAHP synthase activity that escaped feedback inhibition by the aromatic amino acid supplements added to the culture medium. Repetition of these culture conditions, but with a total of 7.5 g/L of kanosamine added in equal (2.5 g) increments at 18, 24, and 30 h, resulted in the synthesis of 0.81 g/L of aminoshikimic acid in 16% yield from kanosamine and in an overall 4% yield from glucose (entry 3, Table 1) at 33 °C.

Phosphorylation of kanosamine could not be detected by enzyme assay in cell-free lysate prepared from *E. coli* SP1.1/pJG5.166A. As a consequence, plasmid pJG6.181B was constructed with a *Zymomonas mobilis glk* insert[12] encoding glucokinase. This modification was introduced to explore whether increased kanosamine kinase activity and an attendant increase in the rate of kanosamine phosphorylation might result in an increase in the concentration and yield of synthesized aminoshikimic acid. The assayed specific activity for phosphorylation of kanosamine to kanosamine 6-phosphate in *E. coli* SP1.1/pJG6.181B was 0.02 units/mg. Cultivation of *E. coli* SP1.1/pJG6.181B for 48 h (Figure 2)

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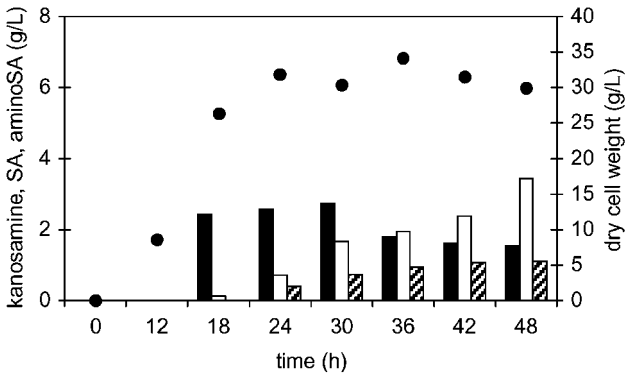

Figure 2. Synthesis of aminoshikimic acid by *E. coli* SP1.1/pJG6.181B under fermentor-controlled conditions. Legend: kanosamine, filled columns; shikimic acid (SA), open columns; aminoshikimic acid (aminoSA), dashed columns; dry cell weight, closed circles.

under fermentor-controlled conditions at 33 °C led to the synthesis of 1.1 g/L of aminoshikimic acid in 19% yield from kanosamine and in an overall 5% yield from glucose (entry 4, Table 1). A cation-exchange resin was again used for the straightforward separation of aminoshikimic acid from unreacted kanosamine and the other components of the culture medium of *E. coli* SP1.1/pJG6.181B.

More shikimic acid was synthesized when kanosamine was added to cultures (entries 3 and 4 vs entry 2, Table 1). This increase can be accounted for by hydrolysis of iminoE4P to form D-erythrose 4-phosphate. Condensation of this kanosamine-derived D-erythrose 4-phosphate with phosphoenolpyruvate catalyzed by the residual, native DAHP synthase activity would lead to formation of DAHP. Further processing of this DAHP by shikimate pathway enzymes would lead to shikimic acid. The concentrations of shikimic acid derived from kanosamine (entries 3 and 4, Table 1) actually exceeded the concentrations of synthesized aminoshikimic acid. Gaining an understanding of how hydrolysis of iminoE4P is minimized in *A. mediterranei* will clearly be essential to improving synthesis of aminoshikimic acid from kanosamine in *E. coli*.

The microbial syntheses of aminoshikimic acid detailed in this report can be compared with the previously reported microbial synthesis of the aminoshikimate pathway product AHBA (Scheme 1) and with microbial synthesis of shikimic acid. AHBA was synthesized with an *E. coli* construct expressing multiple *A. mediterranei* genes including (Scheme 1) *rifL*, *rifK*, *rifM*, *rifN*, *rifH*, *asm47* (a *rifG* homologue), and *asm23* (a *rifJ* homologue).[13] Cultivation of this construct at 13 °C for 48 h led to the synthesis of 0.0031 g/L of AHBA. By contrast, microbial synthesis of aminoshikimic acid from glucose is well removed from the 84 g/L concentration and 33% yield of shikimic acid that can be microbially synthesized from glucose.[11c]

Higher concentrations and yields of aminoshikimic acid are likely to be synthesized as new systems become available for expressing genes in *A. mediterranei* as well as for expressing *A. mediterranei* genes in other microbes. Synthesis of aminoshikimic acid from glucose by *A. mediterranei* ATCC 21789/pJG8.219A demonstrates that a 2.5-fold increase in the expression of aminoshikimate dehydrogenase can already compete with AHBA synthase for in vivo supplies of aminoDHS. The concentration and yield of the aminoshikimate pathway precursor kanosamine synthesized from glucose by *B. pumilus* ATCC 21143 is particularly striking. This result provides a measure of the substantial carbon flux that, in theory, can be directed into biosynthesis of aminoshikimic acid. Finally, there is the synthesis of aminoshikimic acid from kanosamine catalyzed by *E. coli* SP1.1/pJG6.181B. This synthesis, where only a single *A. mediterranei* gene is utilized, suggests that wholesale heterologous expression of *A. mediterranei* genes is not required for the synthesis of aminoshikimic acid.

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Supporting Information Available: Experimental details for plasmid construction, fermentor-controlled cultivation, and product isolation. This material is available free of charge via the Internet at http://pubs.acs.org.

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