Shikimic Acid and Quinic Acid: Replacing Isolation from Plant Sources with Recombinant Microbial Biocatalysis

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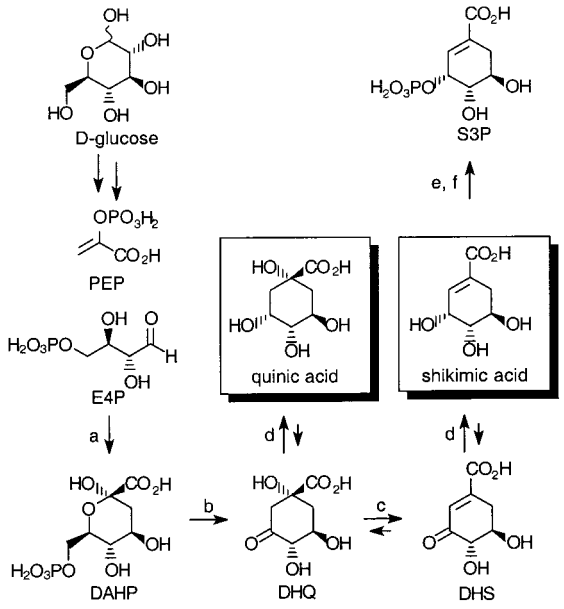
Shikimic acid and quinic acid, hydroaromatics metabolically linked by the common pathway of aromatic amino acid biosynthesis (Scheme 1),[1,2] have emerged as essential chiral starting materials in the synthesis of neuraminidase inhibitors effective in the treatment of influenza.[3] Current isolation of shikimic acid from the fruit of *Illicium* plants[4] precludes its use in kilogram-level syntheses required for clinical evaluation of neuraminidase inhibitors. Quinic acid is more readily available although it is unclear whether its isolation from *Cinchona* bark[5] constitutes a dependable source of the multiton quantities of chiral starting material required for manufacture of a prescription drug. To improve shikimic acid's availability, a shikimate-synthesizing *Escherichia coli* biocatalyst has been constructed. Unexpectedly, quinic acid was synthesized in addition to shikimic acid. Elaborating the mechanism responsible for this contamination has led to the discovery of a new route for quinic acid biosynthesis.

The genomic portion of shikimate-synthesizing SP1.1/pKD12.112 was constructed by insertion of *aroB* into the *serA* locus of *E. coli* and disruption of the *aroL* and *aroK* loci via successive P1 phage-mediated transductions of *aroL478*::Tn*10* and *aroK17*::Cm^R.[6] Shikimic acid accumulates due to the absence of the *aroL*- and *aroK*-encoded isozymes of shikimate kinase while the second copy of *aroB* increases the catalytic activity of rate-limiting 3-dehydroquinate (DHQ) synthase.[7] Disruption of L-serine biosynthesis due to loss of genomic, *serA*-encoded phosphoglycerate dehydrogenase ensures that *serA*-, *aroF*^{FBR}-, and *aroE*-encoding plasmid pKD12.112 is retained by the cell. Carbon flow into the common pathway is increased due to overexpression of *aroF*^{FBR} which encodes a mutant isozyme of DAHP synthase insensitive to feedback inhibition by aromatic amino acids. Overexpression of *aroE*-encoded shikimate dehydrogenase compensates for this enzyme's feedback inhibition by shikimic acid.[7]

Fed-batch fermentor cultivation of SP1.1/pKD12.112 for 42 h resulted in the synthesis of 27.2 g/L of shikimic acid, 12.6 g/L of quinic acid, and 4.4 g/L of 3-dehydroshikimic acid (DHS). DHS accumulation reflected the expected feedback inhibition of shikimate dehydrogenase.[7] By contrast, quinic acid biosynthesis was surprising, given the absence in *E. coli* of quinate dehydrogenase, an oxidoreductase which interconverts 3-dehydroquinic and quinic acids. 3-Dehydroshikimic acid was removed from the fermentation broth by heating followed by adsorbing the resulting protocatechuic acid on activated carbon during decolorization. Unfortunately, quinic acid contamination was in excess of what could be purified away from shikimic acid by crystallization. Quinic acid formation had to be reduced.

Scheme 1[a]



[a] (a) 3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthase (*aroF*^{FBR}); (b) 3-dehydroquinate synthase (*aroB*); (c) 3-dehydroquinate dehydratase (*aroD*); (d) shikimate dehydrogenase (*aroE*); (e,f) shikimate kinase (*aroK*, *aroL*).

Quinic acid biosynthesis, while widespread in plants, has only been observed in a single microbe, *E. coli* AB2848*aroD*/pKD136/pTW8090A.[8] This heterologous construct expresses quinate dehydrogenase encoded by the *qad* locus isolated from *Klebsiella pneumoniae*.[8] Quinate dehydrogenase-catalyzed oxidation of quinic acid is driven by catabolic consumption of the resulting 3-dehydroquinic acid via the β-ketoadipate pathway in *K. pneumoniae* and other microbes. Reduction of 3-dehydroquinic acid by quinate dehydrogenase dominates in *E. coli* AB2848*aroD*/pKD136/pTW8090A because of the absence of 3-dehydroquinic acid catabolism. Quinic acid synthesis in *E. coli* SP1.1/pKD12.112 thus implicates the existence of an oxidoreductase which reduces 3-dehydroquinic acid.

Because of structural similarities between 3-dehydroshikimic and 3-dehydroquinic acids (Scheme 1), purified *E. coli* shikimate dehydrogenase was incubated with 3-dehydroquinic acid. Quinic acid formation was observed. The Michaelis constant, $K_m = 1.2$ mM, and maximum velocity, $v_{max} = 0.096$ mmol L^{-1} min^{-1}, for shikimate dehydrogenase-catalyzed reduction of 3-dehydroquinic acid to quinic acid compares with $K_m = 0.11$ mM and $v_{max} = 0.11$ mmol L^{-1} min^{-1} for shikimate dehydrogenase-catalyzed reduction of 3-dehydroshikimic acid to shikimic acid. To further explore the role of *aroE*-encoded shikimate dehydrogenase in the formation of quinic acid in an intact microbe, *aroB* was site-specifically inserted into the *serA* locus of an *E. coli aroD* mutant lacking 3-dehydroquinate dehydratase. The resulting *E. coli* QP1.1 was then transformed with plasmid pKD12.112. With the use of fed-batch fermentor conditions identical to those used for SP1.1/pKD12.112, QP1.1/pKD12.112 synthesized 60 g/L of quinic acid along with 2.6 g of 3-dehydroquinic acid (Figure 1) in 60 h.

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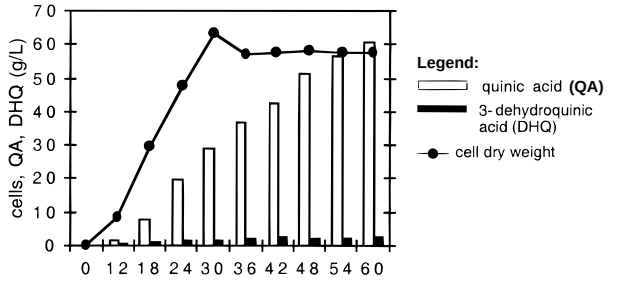

Figure 1. Cultivation of QP1.1/pKD12.112 under fed-batch fermentor conditions.

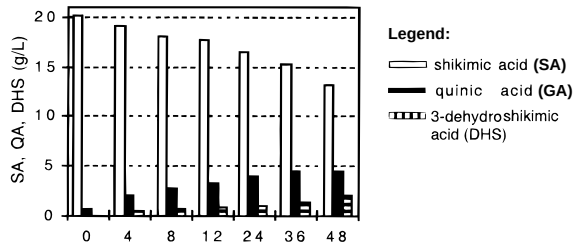

Figure 2. Equilibration of shikimic acid and quinic acid catalyzed by SP1.1/pKD12.112.

Minimizing the cytosolic concentration of 3-dehydroquinic acid appeared to be a reasonable strategy for reducing quinic acid contamination of the shikimic acid synthesized by *E. coli* SP1.1/pKD12.112. The *aroD* gene encoding 3-dehydroquinate dehydratase was consequently localized on plasmid pKD12.152A along with *aroE*, *aroF*^{FBR}, and *serA*. However, attendant amplified expression of 3-dehydroquinate dehydratase did not reduce the levels of quinic acid contamination in the shikimic acid synthesized by SP1.1/pKD12.152A under fed-batch fermentor conditions identical to those employed for SP1.1/pKD12.112. This suggested that quinic acid formation may not result from de novo biosynthesis but rather from equilibration of initially synthesized shikimic acid.

Equilibration of quinic and shikimic acids has previously been examined in cell-free extracts of *K. pneumoniae*.[9] To be relevant to quinic acid formation in *E. coli* SP1.1/pKD12.112, the common pathway must be able to operate in vivo in the reverse of its normal biosynthetic direction. To test this possibility, SP1.1/pKD12.112 cells collected from the fermentor after 24 h were washed, resuspended in fresh minimal salts medium containing shikimic acid, and then shaken. Formation of quinic acid and 3-dehydroshikimic acid along with a corresponding decrease in shikimic acid concentration (Figure 2) indicated that SP1.1/pKD12.112 can catalyze formation of quinic acid from initially synthesized shikimic acid.

The possible role of shikimic acid transport from the culture medium into the microbial cytoplasm during observed equilibration pointed to a strategy for minimizing quinic acid contamination. Shikimic acid transport[10] in *E. coli* may be an evolutionary vestige of a previous ability to catabolize shikimic and quinic acids as sole sources of carbon for growth and metabolism. Since utilization of non-glucose carbon sources is often subject to catabolite repression, increasing D-glucose availability might repress shikimic acid transport, thereby minimizing formation of quinic acid.

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Table 1. Products Synthesized by *E. coli* SP1.1/pKD12.112 as a Function of Time and D-Glucose Addition Parameters

	$K_c = 0.1$			$K_c = 0.8$		
	SA[a]	QA	DHS	SA	QA	DHS
12 h	1.1	0.0	0.3	1.0	0.0	0.2
18 h	5.3	2.5	1.2	3.1	0.0	0.6
24 h	11.4	5.7	2.2	6.4	0.8	1.2
30 h	17.1	8.3	2.7	10.9	1.3	2.2
36 h	23.1	10.8	4.2	15.7	1.8	3.5
42 h	27.2	12.6	4.4	20.2	1.9	4.6

[a] Concentrations in g/L of shikimic acid (SA), quinic acid (QA), and 3-dehydroshikimic acid (DHS).

The rate of D-glucose addition, and thus D-glucose availability, in all fermentation runs was controlled by the proportional-integral-derivative (PID) setting gain (K_c). For example, synthesis by *E. coli* SP1.1/pKD12.112 of the mixture of shikimic and quinic acids (Table 1) employed a PID setting of $K_c = 0.1$. Increasing D-glucose availability by increasing the PID setting to $K_c = 0.8$ resulted (Table 1) in a drastic reduction in the formation of quinic acid throughout the entire fermentation. After 42 h of cultivation, *E. coli* SP1.1/pKD12.112 synthesized 20.2 g/L of shikimic acid, 4.6 g/L of DHS, and only 1.9 g/L of quinic acid. The decrease in the synthesized titers of shikimic acid is consistent with the known impact of increased D-glucose availability on the concentration and yield of L-phenylalanine synthesized by *E. coli*.[11] More importantly, improvement of the shikimate:quinate molar ratio from 2.4:1 ($K_c = 0.1$) to 11.8:1 ($K_c = 0.8$) allowed quinic acid to be completely removed during crystallization of shikimic acid.

In addition to the neuraminidase inhibitor GS4101, quinic acid has been used in the synthesis of antitumor agent esperamicin-A, immunosuppressant FK506, and a variety of other molecules.[12] Shikimic acid has recently been used as the starting point for synthesis of a large combinatorial library of molecules.[13] Microbial syntheses of shikimic acid and quinic acid reported in this account are already capable of supplanting isolation from plant sources. As for the future, comparison of the theoretical maximum yield[14] (43%) for microbial synthesis of shikimic and quinic acids from D-glucose with the yields achieved thus far for microbial synthesis of shikimic acid (14%) and quinic acid (23%) suggests that sizable increases in yields and titers are possible. Such improvements could lead to a pronounced expansion in the synthetic utilization of the highly functionalized, six-membered carbocyclic rings and multiple asymmetric centers found in shikimic and quinic acids.

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Supporting Information Available: Fermentation and purification methods (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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