# Synthesis of Gallic Acid and Pyrogallol from Glucose: Replacing Natural Product Isolation with Microbial Catalysis

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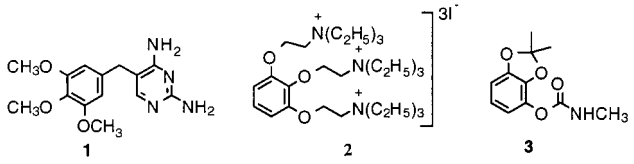
Among a spectrum of uses,[1] gallic acid and pyrogallol are often incorporated into chemical syntheses to provide the trihydroxylated aromatic ring of biologically active molecules such as the antibiotic trimethoprim **1**, the muscle relaxant gallamine triethiodide **2**, and the insecticide bendiocarb **3**. The availability of



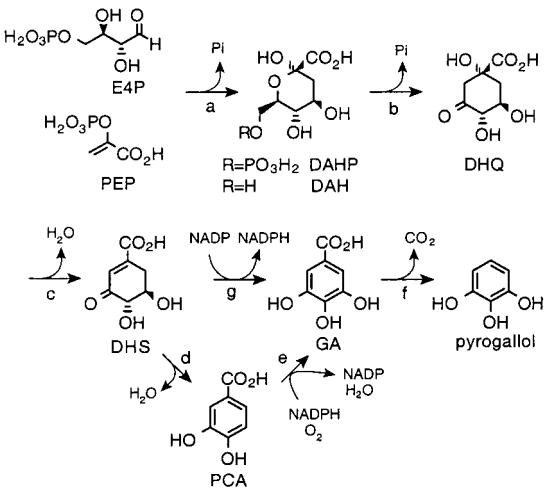
**1** **2** **3**

gallic acid is restricted by its current isolation from insect carapices (gall nuts) harvested in China, and an isolate (tara powder) derived from the ground seed pod of a tree found in Peru.[1] Thermal decarboxylation of gallic acid in copper autoclaves affords pyrogallol.[1] As part of a larger effort[2] to supplant isolation of natural products from scarce natural sources, gallic acid and pyrogallol were targeted for microbe-catalyzed synthesis from abundant glucose. In lieu of an elaborated biosynthetic pathway leading to gallic acid, a pathway had to be created. The result is *Escherichia coli* KL7/pSK6.161, which synthesizes 20 g/L of gallic acid in 12% yield from glucose. *E. coli* RB791*serA*::*aroB*/pSK6.234 then converts gallic acid into pyrogallol in yields of 93−97%.

Biosynthesis[3] of gallic acid has been narrowed to two possible routes. Oxidation (Scheme 1) of 3-dehydroshikimic acid (DHS) to a diketo intermediate followed by spontaneous aromatization might lead to gallic acid. Alternatively, gallic acid may result from the dehydration of DHS followed by hydroxylation (Scheme 1) of the intermediate protocatechuic acid (PCA). Gallic acid has been detected in cultures of *Phycomyces blakesleeanus*,[4] *Pseudomonas fluorescens*,[5] *Entorobacter cloacae*,[5] *Aspergillus terreus*,[6] and recombinant *E. coli*.[7] However, neither DHS dehydrogenase-catalyzed oxidation of DHS (Scheme 1) nor PCA hydroxylase-catalyzed hydroxylation of PCA (Scheme 1) has been detected in these or any other microbes.

We therefore explored recruitment of enzyme activities that, although not associated with gallic acid biosynthesis in their native microbial hosts, are capable of catalyzing Scheme 1 reactions. DHS dehydratase (Scheme 1) has been detected in *Klebsiella*

**Scheme 1**[a]



[a] Enzymes: (a) DAHP synthase (*aroF*); (b) DHQ synthase (*aroB*); (c) DHQ dehydratase (*aroD*); (d) DHS dehydratase (*aroZ*); (e) *p*-hydroxybenzoate hydroxylase (*pobA**); (f) PCA decarboxylase (*aroY*); (g) DHS dehydrogenase. Abbreviations: E4P, D-erythrose 4-phosphate; PEP, phosphoenolpyruvic acid; DAH(P), 3-deoxy-D-*arabino*-heptulosonic acid (7-phosphate); DHQ, 3-dehydroquinic acid; DHS, 3-dehydroshikimic acid; GA, gallic acid; PCA, protocatechuic acid.

*pneumoniae* and the encoding *aroZ* gene has been isolated.[8] Mutagenesis of the *pob*A gene, which encodes *p*-hydroxybenzoate hydroxylase in *Pseudomonas aeruginosa*, produced p*obA**, which encodes a mutant enzyme capable of hydroxylating PCA to form gallic acid.[9] Expression of *aroZ* and *pobA** in a DHS-synthesizing microbe thus provided the basis for creating a gallic acid biosynthetic pathway in *E. coli* KL7/pSK6.161.

*E. coli* KL7 carried a mutation in its *aroE* gene and an *aroBaroZ* insert. Plasmid pSK6.161 contained *pobA** and *aroF*$^{FBR}$ inserts. DHS was synthesized by KL7 due to the absence of *aroE*-encoded shikimate dehydrogenase. This DHS was dehydrated by the *K. pneumoniae aroZ* dehydratase to PCA (Scheme 1). Hydroxylation of the PCA by the *P. aeruginosa pobA** hydroxylase then afforded the desired gallic acid (Scheme 1). Since the amount of DHS synthesized is significantly reduced by feedback inhibition of DAHP synthase,[10] a feedback-insensitive mutant enzyme[11] encoded by *aroF*$^{FBR}$ was used. With the *aroBaroZ* insert, *E. coli* KL7 was equipped with two copies of *aroB* to increase DHQ synthase activity and eliminate DAH accumulation (Scheme 1).[12]

Cultivation of *E. coli* KL7/pSK6.161 for 48 h in a fermentor under glucose-rich, nitrogen-rich culture conditions led (Figure 1) to the formation of gallic acid (20 g/L), DAH (8.9 g/L), DHS (11 g/L), PCA (0.90 g/L), and glutamic acid (14 g/L). Gallic acid and PCA were separated from the DAH and glutamic acid upon extraction from cell-free culture supernatants using EtOAc. After concentration of the charcoal-decolorized organic layer, addition

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(1) Leston, G. In *Kirk-Othmer Encyclopedia of Chemical Technology*; Kroschwitz, J. I., Howe-Grant, M., Eds.; Wiley: New York, 1996; Vol. 19, p 778.
(2) (a) Draths, K. M.; Knop, D. R.; Frost, J. W. *J. Am. Chem. Soc.* **1999**, *121*, 1603. (b) Li, K.; Frost, J. W. *J. Am. Chem. Soc.* **1998**, *120*, 10545.
(3) Werner, I.; Bacher, A.; Eisenreich, W. *J. Biol. Chem.* **1997**, *272*, 25474.
(4) (a) Haslam, E.; Haworth, R. D.; Knowles, P. F. *J. Chem. Soc.* **1961**, 1854. (b) Rivero, F.; Cerdá-Olmedo, E. *Mycologia* **1994**, *86*, 781.
(5) Korth, H. *Arch. Mikrobiol.* **1973**, *89*, 67.
(6) Kawakubo, J.; Nishira, H.; Aoki, K.; Shinke, R. *Biosci. Biotech. Biochem.* **1993**, *57*, 1360.
(7) Li, K.; Mikola, M. R.; Draths, K. M.; Worden, R. M.; Frost, J. W. *Biotechnol. Bioeng.* **1999**, *64*, 61.
(8) (a) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1994**, *116*, 399. (b) Draths, K. M.; Frost, J. W. In *Benign by Design*; Anastas, P. T., Farris, C. S., Eds.; ACS Symp. Ser. No. 577; American Chemical Society: Washington, DC, 1994; Chapter 3, p 32. (c) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1995**, *117*, 2395.
(9) (a) Entsch, B.; Palfey, B. A.; Ballou, D. P.; Massey, V. *J. Biol. Chem.* **1991**, *266*, 17341. (b) Eschrich, K.; van der Bolt, F. J. T.; de Kok, A.; van Berkel, W. J. H. *Eur. J. Biochem.* **1993**, *216*, 137.
(10) Ogino, T.; Garner, C.; Markley, J. L.; Herrmann, K. M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5828.
(11) Weaver, L. M.; Herrmann, K. M. *J. Bacteriol.* **1990**, *172*, 6581.
(12) Snell, K. D.; Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1996**, *118*, 5605.

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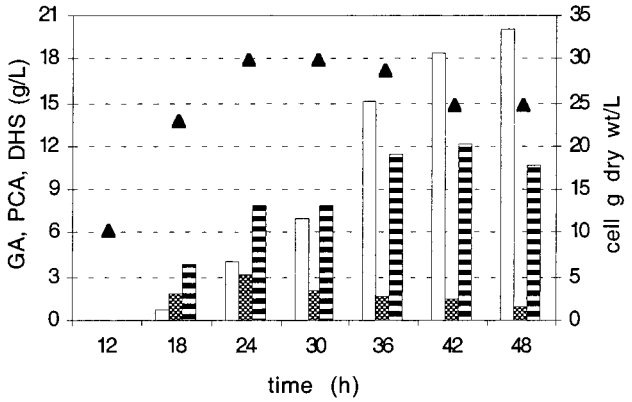


**Figure 1.** Fed-batch, aerobic cultivation of *E. coli* KL7/pSK6.161: ▲, cell growth; open bar, gallic acid (GA); cross-hatched bar, PCA; and horizontally ruled bar, DHS.

of petroleum ether led to precipitation of gallic acid as a white powder free of PCA contamination.

DAH accumulation during gallic acid synthesis was unexpected given that this metabolite has not been observed in *E. coli* strains such as KL7, which possess two copies of *aroB*.[12] We subsequently discovered that PCA competitively inhibits *aroB*-encoded 3-dehydroquinate synthase.[13] The absence of acetic acid accumulation and the formation of substantial amounts of glutamic acid during gallic acid synthesis were also unexpected. Typically, acetic acid is formed during the later stages of *E. coli* cultures when glucose is used as the carbon source.[14] The initial increase and subsequent decrease in PCA concentrations (Figure 1) may indicate that KL7/pSK6.161 can recapture PCA initially exported into the culture medium.

As with the synthesis of gallic acid, we attempted to synthesize pyrogallol directly from glucose. Pyrogallol is typically generated as a catabolic intermediate by microbes using either gallic acid or tannic acid as a sole source of carbon during growth.[15] The most extensively characterized nonoxidative decarboxylase capable of converting gallic acid into pyrogallol has been isolated from *Pantoea agglomerans* T71.[16] Our approach to conversion of gallic acid into pyrogallol was based on the fortuitous discovery that *aroY*-encoded PCA decarboxylase[8] isolated from *K. pneumoniae* also catalyzes the decarboxylation of gallic acid to pyrogallol. PCA decarboxylase has previously been used in microbial syntheses of catechol and adipic acid from glucose.[8]

Synthesis of pyrogallol from glucose was attempted using *E. coli* KL7/pSK6.232. Plasmid pSK6.232 carried an *aroY* insert in addition to *pobA** and *aroF*$^{FBR}$. KL7/pSK6.232 cultured under fermentor conditions failed to grow beyond 18 h of cultivation. Neither gallic acid nor pyrogallol formation could be detected. Only formation of catechol was observed suggesting that the rate of in vivo decarboxylation of PCA was significantly more rapid than PobA*-catalyzed hydroxylation of PCA. Catechol formation and its associated toxicity[8c] toward microbes likely explain the early cessation of growth.

Synthesis of pyrogallol subsequently switched to a strategy where gallic acid solutions containing PCA were added to the culture medium after growth of the *E. coli* catalyst was complete. The microbe, *E. coli*RB791*serA*::*aroB*/pSK6.234, overexpressed only plasmid-localized, *aroY*-encoded decarboxylase activity. This strategy benefited from catechol's attenuated toxicity toward pregrown as opposed to actively growing *E. coli*.[8c] However, it was unclear whether AroY decarboxylase in RB791*serA*::*aroB*/pSK6.234 would have access to the added gallic acid.

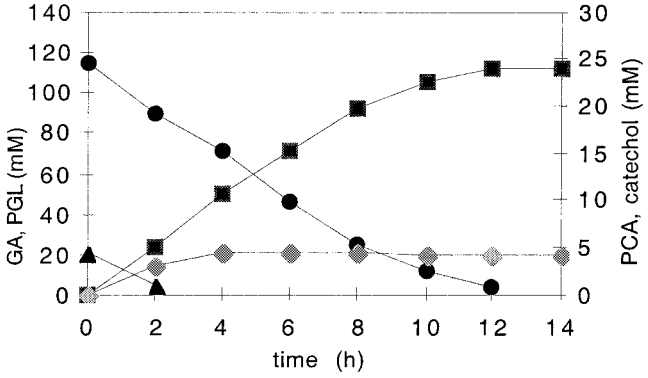


**Figure 2.** Anaerobic decarboxylations catalyzed by *E. coli* RB791*serA*::*aroB*/pSK6.234: circle, gallic acid (GA); square, pyrogallol (PGL); triangle, PCA; and tilted square, catechol.

After RB791*serA*::*aroB*/pSK6.234 completed its growth on glucose in a fermentor, gallic acid and PCA were added to give concentrations of 115 and 4.5 mM, respectively. Glucose addition and aeration were then terminated, and the fermentor was sparged with a continuous flow of $N_2$. Within 14 h (Figure 2), gallic acid and PCA were converted into a solution containing 112 mM pyrogallol (97%) and 4 mM catechol. Repetition of the decarboxylation with culture supernatant produced by gallate-synthesizing KL7/pSK6.161 resulted in a 93% yield of pyrogallol. The concentrations of DHS, DAH, and glutamic acid remained unchanged. Pyrogallol was isolated from cell-free culture supernatants by extraction with EtOAc. Removal of solvent from the charcoal-decolorized organic layer followed by heating under vacuum to remove catechol contamination afforded pyrogallol as a white solid.

Significant improvements in KL7/pSK6.161 remain to be elaborated before the current 12% yield for synthesis of gallic acid from glucose reaches the maximum theoretical[8c,17] 43% yield. Increasing the in vivo activities of AroB, AroZ, and PobA* along with suppression of glutamic acid synthesis are obvious (albeit nontrivial) avenues that warrant exploration in the future. While the synthesis of gallic acid from glucose requires controlled expression of multiple genes, microbe-catalyzed decarboxylation of gallic acid benefits from being a much simpler biocatalytic conversion. The high-yielding decarboxylation of gallic acid catalyzed by RB791*serA*::*aroB*/pSK6.234 and the ease of purifying product pyrogallol are already at the stage where this biocatalytic route is an attractive alternative to currently employed[1] chemical decarboxylation of gallic acid.

Microbe-catalyzed syntheses of natural products are typically based on pathways where the intermediates, enzymes, and encoding genes have been identified and characterized.[2a] Microbe-catalyzed synthesis of gallic acid, by contrast, is based on a hypothetical biosynthetic pathway. By exploiting modified enzyme function (PobA*) and the diversity of enzyme function (AroZ and AroY), gallic acid and pyrogallol have been successfully synthesized from glucose. In the process, another example[2b] is provided for how chemistry can reach beyond known biosynthetic pathways to create new catalytic function appropriate for natural product synthesis.

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

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(13) Chandran, S.; Frost, J. W. Unpublished results.
(14) Konstantinov, K. B.; Nishio, N.; Seki, T.; Yoshida, T. *J. Ferm. Bioeng.* **1991**, *71*, 350.
(15) Armstrong, S. M.; Patel, T. R. *J. Basic Microbiol.* **1994**, *34*, 123.
(16) Zeida, M.; Wieser, M.; Yoshida, T.; Sugio, T.; Nagasawa, T. *Appl. Environ. Micriobiol.* **1998**, *64*, 4743.
(17) Förberg, C.; Eliaeson, T.; Häggström, L. *J. Biotechnol.* **1988**, *7*, 319.