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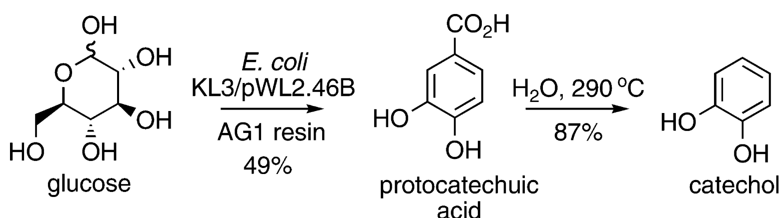

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Benzene-Free Synthesis of Catechol: Interfacing Microbial and Chemical Catalysis

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Abstract: The toxicity of aromatics frequently limits the yields of their microbial synthesis. For example, the 5% yield of catechol synthesized from glucose by *Escherichia coli* WN1/pWL1.290A under fermentor-controlled conditions reflects catechol's microbial toxicity. Use of in situ resin-based extraction to reduce catechol's concentration in culture medium and thereby its microbial toxicity during its synthesis from glucose by *E. coli* WN1/pWL1.290A led to a 7% yield of catechol. Interfacing microbial with chemical synthesis was then explored where glucose was microbially converted into a nontoxic intermediate followed by chemical conversion of this intermediate into catechol. Intermediates examined include 3-dehydroquinate, 3-dehydroshikimate, and protocatechuate. 3-Dehydroquinate and 3-dehydroshikimate synthesized, respectively, by *E. coli* QP1.1/pJY1.216A and *E. coli* KL3/pJY1.216A from glucose were extracted and then reacted in water heated at 290 °C to afford catechol in overall yields from glucose of 10% and 26%, respectively. The problematic extraction of these catechol precursors from culture medium was subsequently circumvented by high-yielding chemical dehydration of 3-dehydroquinate and 3-dehydroshikimate in culture medium followed by extraction of the resulting protocatechuate. After reaction of protocatechuate in water heated at 290 °C, the overall yields of catechol synthesized from glucose via chemical dehydration of 3-dehydroquinate and chemical dehydration of 3-dehydroshikimate were, respectively, 25% and 30%. Direct synthesis of protocatechuate from glucose using *E. coli* KL3/pWL2.46B followed by its extraction and chemical decarboxylation in water gave a 24% overall yield of catechol from glucose. In situ resin-based extraction of protocatechaute synthesized by *E. coli* KL3/pWL2.46B followed by chemical decarboxylation of this catechol percursor was then examined. This employment of both strategies for dealing with the microbial toxicity of aromatic products led to the highest overall yield with catechol synthesized in 43% overall yield from glucose.

As a pseudocommodity chemical produced in volumes of approximately 2.5×10^7 kg/year, catechol is a versatile organic building block for the chemical industry.[1] Hydroxylation of phenol using H_2O_2 leads to a mixture of hydroquinone and catechol, which is separated by distillation.[1] Phenol, in turn, is obtained by Hock oxidation of benzene-derived cumene.[2] Catechol is thus part of the spectrum of petrochemicals synthesized from benzene. An alternative route has been elaborated where catechol is synthesized from glucose using microbial catalysis.[3] Glucose is nontoxic, nonvolatile, and obtained from renewable starch and cellulose. Benzene is carcinogenic,[4] volatile, and primarily derived from nonrenewable petroleum. Longer-term reliance on petroleum must also contend with declining availability[5a] and geopolitical issues[5b] associated with reliance on this carbon source. Despite the numerous problems associated with synthesis of aromatics such as catechol from benzene, it is the yield of catechol synthesized from glucose that will be an essential determinant of this route's viability for large-scale chemical manufacture.

Catechol is microbially synthesized (Scheme 1) by the introduction of *Klebsiella pneumoniae aroZ* and *aroY* genes encoding 3-dehydroshikimate dehydratase and protocatechuate decarboxylase, respectively, into 3-dehydroshikimate-synthesizing *Escherichia coli* constructs.[3] Carbon flow directed into the shikimate pathway is thus diverted at 3-dehydroshikimate into synthesis of catechol (Scheme 1). Microbial synthesis of catechol was first reported under shake flask cultivation conditions.[3]

(1) (a) Hamamoto, T.; Umemura, S. *Ullmann's Encylcopedia of Industrial Chemistry*; Wiley: New York, 2002−2004; www.mrw.interscience.wiley.com/ueic/articles/a19_313/sect2.html. (b) Krumenacker, L.; Costantini, M.; Pontal, P.; Sentenzc, J. *Kirk-Othmer Encyclopedia of Chemical Technology*; Wiley: New York, 1995−2004; www.mrw.interscience.wiley.com/kirk/articles/hydrkrum.a01/abstract-fs.html. (c) Szmant, H. H. *Organic Building Blocks of Chemical Industry*; Wiley: New York, 1989; pp 512−519.
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(5) (a) Campbell, C. J.; Laherrère, J. H. *Sci. Am.* **1998**, *278* (3), 78. (b) Klare, M. T. *Curr. Hist.* **2002**, *101* (659), 414.

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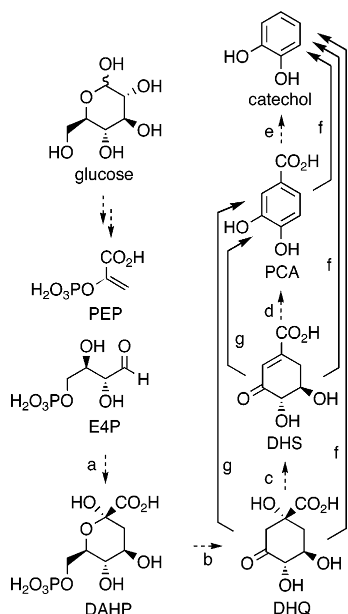
Scheme 1 [a,b,c]



[a] Abbreviations: PEP, phosphoenolpyruvate; E4P, D-erythrose 4-phosphate; DAHP, 3-deoxy-D-*arabino*-heptulosonate (DAH) 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; PCA, protocatechuate. [b] Enzymes and encoding genes (dashed arrows): (a) DAHP synthase (*aroF*^{FBR}); (b) DHQ synthase (*aroB*); (c) DHQ dehydratase (*aroD*); (d) DHS dehydratase (*aroZ*); (e) PCA decarboxylase (*aroY*). [c] Chemical reactions (solid arrows): (f) H_2O, 290 °C; (g) culture medium, reflux.

Upon moving to the higher cell densities associated with fermentor-controlled cultivation, increases in the yield and concentration of catechol were not achieved. Catechol's toxicity toward the producing *E. coli* construct was apparently limiting the yield of catechol synthesized from glucose.[3] Such toxicity of the aromatic product toward the producing microbe is a central challenge in elaborating benzene-free syntheses of aromatic chemicals. Two distinct strategies have been employed to deal with this challenge. In situ resin-based extraction of the product can be used during the course of the microbial synthesis.[6] This reduces the concentration of the product in the culture medium and as a consequence, limits exposure of the producing microbe to the toxic aromatic product. Alternatively, microbial and chemical synthesis can be interfaced. A microbe converts glucose into an intermediate with reduced microbial toxicity followed by chemical conversion of the nontoxic intermediate into the aromatic product.[7] The initial focus of this account was to determine which of these two basic strategies would lead to the highest yields of catechol synthesized from glucose.

In situ resin-based product removal was examined with the direct microbial synthesis of catechol from glucose under fermentor-controlled conditions. Interfacing of microbial with chemical synthesis was examined with microbial synthesis of 3-dehydroshikimate, 3-dehydroquinate, and protocatechuate from glucose under fermentor-controlled conditions followed by chemical synthesis of catechol from these intermediates in near-critical water (Scheme 1). 3-Dehydroshikimate and 3-dehydroquinate are nontoxic toward *E. coli* constructs while protocatechuate displays a lower level of toxicity toward *E. coli* than that observed for catechol. However, while protocatechuate can be easily extracted from culture medium, extraction of 3-dehydroshikimate and 3-dehydroquinate is more difficult. This led to the examination of the chemical dehydration of 3-dehydroshikimate and 3-dehydroquinate in cell-free, protein-free culture medium (Scheme 1). Straightforward, quantitative extraction of the resulting protocatechuate was then followed by its chemical decarboxylation in near-critical water to form catechol (Scheme 1). As a final option, use of both strategies for dealing with the microbial toxicity of aromatic chemicals was examined with employment of in situ resin-based extraction during microbial synthesis of protocatechuate from glucose followed by chemical decarboxylation of this catechol precursor in near-critical water.

(6) (a) Johnson, B. F.; Amaratunga, M.; Lobos, J. H. U.S. Patent 6,114,157, 2000. (b) González-Vara y R.; Vaccari, G.; Dosi, E.; Trilli, A.; Rossi, M.; Matteuzzi, D. *Biotechnol. Bioeng.* **2000**, *67*, 147. (c) Anderson, B. A.; Hansen, M. M.; Harkness, A. R.; Henry, C. L.; Vicenzi, J. T.; Zmijewski, M. J. *J. Am. Chem. Soc.* **1995**, *117*, 12358.

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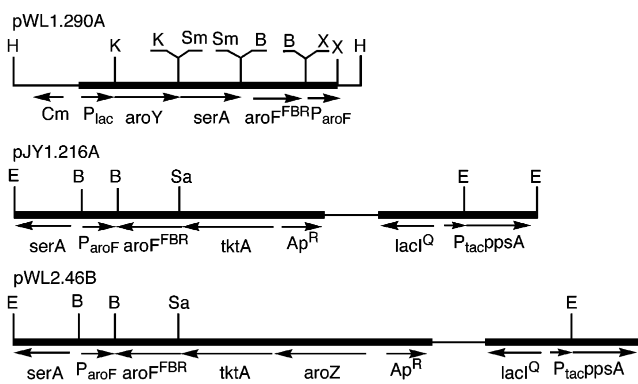
Scheme 2 [a]



[a] Restriction enzyme sites are abbreviated as follows: B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, K = *Kpn*I, Sa = *Sal*I, Sm = *Sma*I, X = *Xba*I. Thin solid line, vector DNA; bold solid line, insert DNA.

Results

Direct Microbial Synthesis of Catechol. *E. coli* AB2834/pKD136/pKD9.069A, which was used in the previous synthesis of catechol from glucose under shake flask cultivation, carried two plasmids maintained by selection pressure provided by inserts encoding resistance to two different antibiotics.[3] The *E. coli* WN1/pWL1.290A construct (Scheme 2) employed for evaluation of catechol synthesis under fermentor-controlled conditions, by contrast, carried a single plasmid and did not rely on selection pressure provided by antibiotics to maintain its plasmid. Host strain *E. coli* WN1 lacked shikimate dehydrogenase activity due to a mutation in its *aroE* locus and carried two chromosomal inserts consisting of a *tktAaroZ* cassette in its *lacZ* locus and an *aroBaroZ* cassette in its *serA* locus.[8] Expression of the two chromosomal *aroZ* inserts encoding 3-dehydroshikimate dehydratase and the plasmid-localized *aroY* insert encoding protocatechuate decarboxylase led to the enzyme-catalyzed conversion of 3-dehydroshikimate into catechol via protocatechuate intermediacy (Scheme 1).

Microbial synthesis of 3-dehydroshikimate was increased as a consequence of the amplified expression of transketolase due to the two genomic copies of *tktA* and amplified expression of a feedback-insensitive isozyme of 3-deoxy-D-*arabino*-

(8) Niu, W.; Draths, K. M.; Frost, J. W. *Biotechnol. Prog.* **2002**, *18*, 201.

Table 1. Resin-Based Extraction of Catechol and Protocatechuate (PCA) from Aqueous Solution

resin	% removed	
	catechol[a]	PCA[b]
AG-1 X8	86	91
Dowex-1 X8	79	75
Dowex-1 X4	73	87
Amberlite IRA400	80	88
Amberlite XAD-2	5	nd[c]
Amberlite XAD-4	33	nd
Amberlite XAD-16HP	11	nd
Amberlite XAD-1180	9	nd
Amberlite XAD-16	42	nd
Sepabead SP850	60	nd
Diaion SP207	44	nd
DiaionHP20SS	24	nd
Diaion HP2MG	36	nd
MCI Gel CHP20P	44	nd

[a] $[\text{catechol}]_{\text{initial}} - [\text{catechol}]_{\text{final}}/[\text{catechol}]_{\text{initial}}$ after incubation with resin. [b] $[\text{protocatechuate}]_{\text{initial}} - [\text{protocatchuate}]_{\text{final}}/[\text{protocatechuate}]_{\text{initial}}$ after incubation with resin. [c] Not determined.

heptulosonate 7-phosphate (DAHP, Scheme 1) synthase resulting from plasmid localization (Scheme 2) of the *aroF*$^{\text{FBR}}$ locus and the P_{aroF} promoter.[9a] The second chromosomal copy of *aroB* inserted into the *serA* locus of *E. coli* WN1 increased 3-dehydroquinate synthase expression so that 3-deoxy-D-*arabino*-heptulosonate (DAH, Scheme 1) did not accumulate when carbon flow directed into the shikimate pathway was increased.[9b] A *serA* insert in plasmid pWL1.290A complemented the inactivated chromosomal *serA* locus in *E. coli* WN1. Growth in minimal salts medium lacking L-serine supplementation depended on expression of plasmid-localized *serA*.[9a] This nutritional pressure was the basis for plasmid maintenance during cultivation of *E. coli* WN1/pWL1.290A.

Polystyrene, quaternary ammonium resins (AG-1 X8, Dowex-1 X8, Dowex-1 X4, Amberlite IRA400) were examined (Table 1) for binding of aromatic solutes in aqueous solutions of catechol and protocatechuate at initial concentrations of 0.05 and 0.1 M, respectively. Polyaromatic resins (Amberlite XAD-2, Amberlite XAD-4, Amberlite XAD-16HP, Amberlite XAD-1180, Amberlite XAD-16, Sepabead SP850, CHP20P) along with the brominated polystyrene Sepabead SP207 and polymethacrylate HP2MG were examined (Table 1) for binding catechol in aqueous solutions containing this aromatic solute at an initial concentration of 0.081 M. Resin AG-1 X8 was determined (Table 1) to be the best strong anion exchanger for removing catechol and protocatechuate from solution. The uncharged resins were only evaluated for binding catechol (Table 1), with Sepabead SP850 removing the most catechol from solution. Sepabead SP850 and AG-1 X8 were then evaluated for in situ resin-based extraction during microbial synthesis of catechol. Although both resins afforded the same yield of catechol synthesized from glucose, approximately 20% higher concentrations of synthesized catechol where achieved using Sepabead SP850. This led to use of Sepabead SP850 for in situ resin-based extraction during synthesis of catechol while resin AG-1 X8 was similarly employed during synthesis of protocatechuate.

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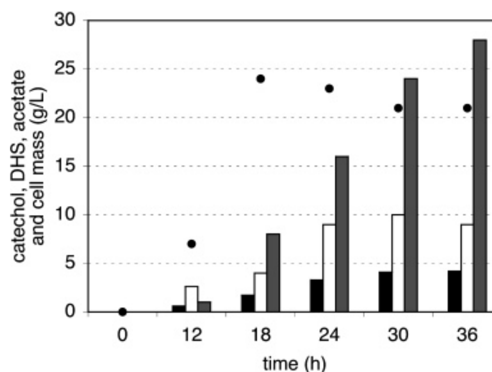

Figure 1. Direct synthesis of catechol from glucose using *E. coli* WN1/pWL1.290A cultured under fermentor-controlled conditions. Legend: black bars, catechol; open bars, 3-dehydroshikimate (DHS); gray bars, acetate; black circles, dry cell mass.

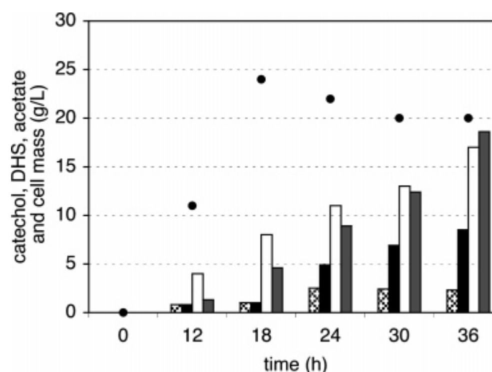

Figure 2. In situ resin-based extraction during synthesis of catechol from glucose using *E. coli* WN1/pWL1.290A cultured under fermentor-controlled conditions. Legend: hashed bars, catechol in culture medium; black bars, culture medium catechol + resin-bound catechol; open bars: 3-dehydroshikimate (DHS); gray bars, acetate; black circles, dry cell mass.

Cultivation of *E. coli* WN1/pWL1.290A under fermentor-controlled, glucose-rich culture conditions for 36 h resulted in the synthesis (Figure 1) of 4.2 g/L of catechol along with substantial concentrations of byproducts including acetate (28 g/L) and 3-dehydroshikimate (9 g/L). After purification by sublimation, the yield of catechol synthesized from glucose was 5% (mol/mol). In situ resin-based extraction was then examined to determine if the concentrations and yields of catechol could be increased. During the cultivation of *E. coli* WN1/pWL1.290A, culture medium was pumped from the fermentor and through a column packed with Sepabead SP850. The rate of flow through the resin was sufficient to create a fluidized bed. With in situ resin-based extraction, the concentration of catechol in the culture medium was maintained at a concentration below approximately 2.5 g/L. Culturing *E. coli* WN1/pWL1.290A under fermentor-controlled, glucose-rich conditions with resin-based extraction of product resulted in the synthesis (Figure 2) of 8.5 g/L of catechol. Once again, substantial concentrations of acetate (19 g/L) and 3-dehydroshikimate (17 g/L) were generated (Figure 2). After purification by sublimation, the yield of catechol synthesized from glucose was 7% (mol/mol).

3-Dehydroshikimate-, 3-Dehydroquinate-, and Protocatechuate-Synthesizing Constructs. *E. coli* KL3/pJY1.216A, which lacked a catalytically active shikimate dehydrogenase due to a mutated chromosomal *aroE* locus, synthesized and exported 3-dehydroshikimate into its culture medium.[10] A mutated chromosomal *aroD* locus and attendant absence of 3-dehydro-

quinate dehydratase activity in *E. coli* QP1.1/pJY1.216A led to synthesis and export of 3-dehydroquinate into its culture medium. The 3-dehydroshikimate dehydratase encoded by the *aroZ* insert in plasmid pWL2.46B catalyzed the dehydration of 3-dehydroshikimate (Scheme 1). The result was the synthesis and export of protocatechuate into the culture medium of *E. coli* KL3/pWL2.46B. *E. coli* KL3/pJY1.216A, *E. coli* QP1.1/pJY1.216A, and *E. coli* KL3/pWL2.46B all carried an *aroB* insert in their *serA* locus and plasmid-localized *serA* for the reasons detailed for catechol-synthesizing *E. coli* WN1/pWL1.290A. Carbon flow directed into the shikimate pathway was increased in each construct by increasing the in vivo activity of DAHP synthase and increasing the availability of this enzyme's substrates.

Plasmids pJY1.216A and pWL2.46B both carried P_{aroF} promoter and *aroF*^{FBR} inserts in order to amplify expression of an isozyme of DAHP synthase that was insensitive to feedback inhibition (Scheme 2). Plasmid localization of the P_{aroF} promoter separated from its *aroF* open reading frame served to bind Tyr repressor protein. The result was transcriptional derepression and amplified expression of plasmid-localized *aroF*^{FBR}, which was under the control of its native P_{aroF} promoter. Due to the absence of complete shikimate pathways in *E. coli* KL3/pJY1.216A, *E. coli* QP1.1/pJY1.216A, and *E. coli* KL3/pWL2.46B, aromatic amino acids needed to be added to cultures. Use of the isozyme of DAHP synthase that was insensitive to feedback inhibition by aromatic amino acids was thus important to ensure that carbon flow continue to be directed into the shikimate pathway upon addition of these supplements to cultures.

Increasing the availability of D-erythrose 4-phosphate and phosphoenolpyruvate (Scheme 1), which are the substrates for DAHP synthase, followed from expression of the *tktA* and *ppsA* inserts (Scheme 2) in both plasmids pJY1.216A and pWL2.46B. The *tktA* insert, which encodes transketolase, was under the transcriptional control of its native promoter. Resulting amplified expression of *tktA*-encoded transketolase increases the availability of D-erythrose 4-phosphate (Scheme 1).[11] Expression of *ppsA*, which encodes phosphoenolpyruvate synthase, was under the transcriptional control of a P_{tac} promoter. Transcription of *ppsA* was repressed by the *lac* repressor encoded by the plasmid-localized *lacI*^{Q} insert and induced upon addition of isopropyl-β-thiogalactopyranoside (IPTG) to the culture medium. Increased expression of *ppsA*-encoded phosphoenolpyruvate synthase served to increase phosphoenolpyruvate (Scheme 1) availability derived from cellular supplies of pyruvate.[10,12]

Microbial Synthesis, Isolation, and Reactivity of Protocatechuate Precursors. Cultivation of *E. coli* KL3/pJY1.216A under glucose-rich, fermentor-controlled conditions led to the synthesis of 61 g/L of 3-dehydroshikimate in 36% yield (mol/mol). Removal of cells and protein was followed by liquid−liquid continuous extraction using EtOAc.[13] Of the 3-dehydroshikimate originally in the culture medium, 80% was extracted. *E. coli* QP1.1/pJY1.216A synthesized 58 g/L of 3-dehydroquinate in 28% yield (mol/mol) under glucose-rich, fermentor-controlled conditions. Again, cells and protein were removed from the culture medium. Liquid−liquid continuous extraction using EtOAc resulted in isolation of 67% of the 3-dehydroquinate originally in the culture medium.

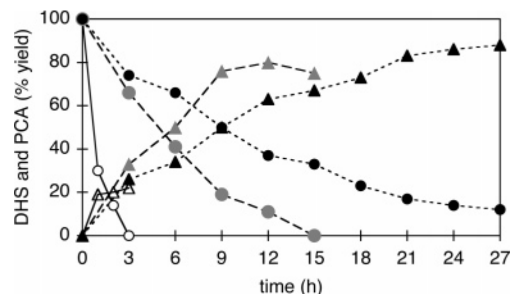

Figure 3. Protocatechuate synthesized from 3-dehydroshikimate in refluxing aqueous salts solution as a function of time and pH.[a,b,c,d] [a]Abbreviations: DHS, 3-dehydroshikimate; PCA, protocatechuate. [b](mol unreacted DHS or product PCA/mol starting DHS) × 100%. [c]All reactions were heated to reflux under N_2. [d]Legend: (a) solid lines, pH 7; open circles, DHS; open triangles, PCA; (b) dashed lines, pH 2.5; gray circles, DHS; gray triangles, PCA; (c) dotted lines, pH 2.2; black circles, DHS; black triangles, PCA.

Given the tedious extraction required to isolate 3-dehydroshikimate and 3-dehydroquinate from culture medium and the significant loss of these intermediates observed during this process, chemical dehydration of 3-dehydroshikimate and 3-dehydroquinate in culture medium followed by extraction of the resulting protocatechuate was examined. This chemical dehydration was first examined using purified 3-dehydroshikimate dissolved in an aqueous salts solution intended to mimic the culture medium used during microbial synthesis (Figure 3). When refluxed at pH 7 under N_2 atmosphere for 3 h, a 22% yield of protocatechuate was obtained (Figure 3a). Acidification of the reaction medium to pH 2.5 using concentrated H_2SO_4 followed by refluxing under an N_2 atmosphere for 15 h led to a 75% yield of protocatechuate (Figure 3b). Acidification to pH 2.5 using H_2SO_4 addition followed by elution through Dowex 50 (H^+ form) gave a pH 2.2 reaction solution. Subsequent refluxing under an N_2 atmosphere for 27 h gave an 88% yield of protocatechuate along with a 12% yield of unreacted 3-dehydroshikimate (Figure 3c). These results reveal a pronounced tradeoff between reaction rate and reaction yield.

The strategy for isolation of 3-dehydroshikimate and 3-dehydroquinate by dehydration and extraction of the resulting protocatechuate was then examined in actual culture medium. After centrifugation to remove *E. coli* KL3/pJY1.216A cells from cultures containing 3-dehydroshikimate, the standardized procedure for removal of proteins involved acidification of the culture supernatant to pH 2.5 with concentrated H_2SO_4. Precipitated protein was removed by centrifugation, and the resulting protein-free supernatant eluted through Dowex 50 (H^+ form). Refluxing the resulting pH 2.2 solution containing 3-dehydroshikimate under N_2 for 27 h afforded protocatechuate in 93% isolated yield (mol/mol). The same procedure was then applied to *E. coli* QP1.1/pJY1.216A cultures containing 3-dehydroquinate. Cell-free, protein-free culture supernatant from *E. coli* QP1.1/pJY1.216A was eluted through Dowex 50 (H^+ form) and the resulting pH 2.2 solution refluxed under N_2 for 27 h. Protocatechuate generated by this dehydration of 3-dehydroquinate was isolated in 100% yield (mol/mol).

Direct Microbial Synthesis of Protocatechuate. *E. coli* KL3/pWL2.46B provided an opportunity to directly synthesize

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(11) (a) Draths, K. M.; Frost, J. W. *J. Am. Chem. Soc.* **1990**, *112*, 1657. (b) Draths, K. M.; Pompliano, D. L.; Conley, D. L.; Frost, J. W.; Berry, A.; Disbrow, G.; Starversky, R. J.; Lievense, J. *J. Am. Chem. Soc.* **1992**, *114*, 3956.
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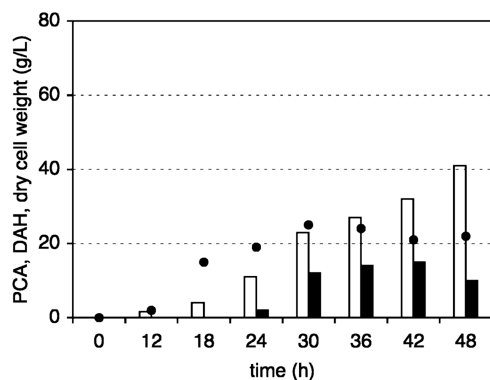

Figure 4. Direct synthesis of protocatechuate (PCA) from glucose using *E. coli* KL3/pWL2.46B cultured under fermentor-controlled conditions. Legend: open bars, protocatechuate; black bars, 3-deoxy-D-*arabino*-heptulosonate (DAH); black circles, dry cell weight.

protocatechuate from glucose rather than using microbial synthesis of 3-dehydroshikimate or 3-dehydroquinate from glucose followed by chemical dehydration of these hydroaromatics. The key variable was the toxicity of protocatechuate to *E. coli* KL3/pWL2.46B. While 3-dehydroshikimate and 3-dehydroquinate are not toxic to the *E. coli* constructs used for their synthesis, protocatechuate is known to display toxicity toward *E. coli* albeit at a level substantially lower than that observed for catechol.[3] *E. coli* KL3/pWL2.46B cultured under glucose-rich, fermentor-controlled conditions synthesized 41 g/L of protocatechuate in 48 h in 26% (mol/mol) yield (Figure 4). In addition to protocatechuate, *E. coli* KL3/pWL2.46B synthesized 15 g/L of DAH (Scheme 1) by 42 h of cultivation (Figure 4). Formation of DAH arises from inhibition of 3-dehydroquinate synthase by protocatechuate.[14]

To further increase the yield and concentration of protocatechuate synthesized by *E. coli* KL3/pWL2.46B, in situ product recovery employing resin-based extraction was explored. As with the synthesis of catechol by *E. coli* WN1/pWL1.290A, in situ product recovery using resin-based extraction during cultivation of *E. coli* KL3/pWL2.46B under glucose-rich, fermentor-controlled conditions was designed to reduce the concentration of protocatechuate in the culture medium. During cultivation of *E. coli* KL3/pWL2.46B, culture medium from the fermentor was pumped through a column packed with AG-1 X8. The concentrations of protocatechuate in the culture medium did not exceed 13 g/L over the course of the cultivation (Figure 5). Based on the protocatechuate eluted from the AG1 X8 resin and the protocatechuate extracted from the culture medium, *E. coli* KL3/pWL2.46B synthesized the equivalent of 71 g/L of protocatechuate in 49% (mol/mol) yield (Figure 5). Byproduct DAH was synthesized in concentrations of 16 g/L after 36 h of cultivation (Figure 5).

Chemical Conversion of 3-Dehydroquinate, 3-Dehydroshikimate, and Protocatechuate into Catechol. The reactivities of 3-dehydroquinate (Table 2), 3-dehydroshikimate (Table 3), and protocatechuate (Table 4) in water were examined at temperatures ranging from 190 °C to 310 °C. Although protocatechuate was the dominant product, a low yield of catechol could be detected when aqueous 3-dehydroquinate was reacted in water heated at 190 °C (Table 2). The yield of protocatechuate then declined while the yield of catechol

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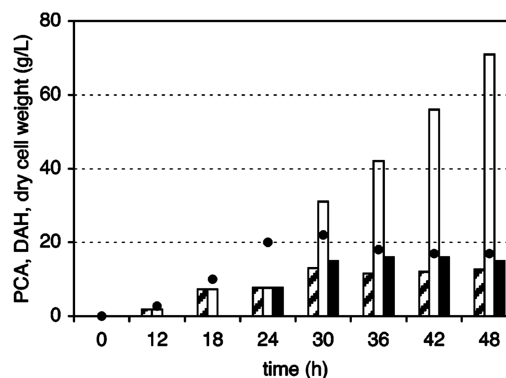

Figure 5. In situ resin-based extraction of protocatechuate (PCA) from glucose using *E. coli* KL3/pWL2.46B cultured under fermentor-controlled conditions. Legend: hashed bars, culture medium PCA; open bars, culture medium PCA + resin-bound PCA; black bars, 3-deoxy-D-*arabino*-heptulosonate (DAH); black circles, dry cell weight.

Table 2. Percent Yield of Catechol Synthesized from 3-Dehydroquinate in Water as a Function of Temperature

	T (°C)						
	190	210	230	250	270	290	310
PCA[a,b]	68	55	29	28	11	0	0
catechol[a,b]	4	11	34	40	44	54	54

[a] Abbreviation: PCA, protocatechuate.

Table 3. Percent Yield of Catechol Synthesized from 3-Dehydroshikimate in Water as a Function of Temperature

	T (°C)						
	190	210	230	250	270	290	310
DHS[a,b]	13	6	0	0	0	0	0
PCA[a,b]	82	78	71	18	0	0	0
catechol[a,b]	3	5	21	86	87	90	90

[a] Abbreviations: DHS, 3-dehydroshikimate; PCA, protocatechuate. [b] (mol product/mol DHS) × 100%.

Table 4. Percent Yield of Catechol Synthesized from Protocatechuate in Water as a Function of Temperature

	T (°C)						
	190	210	230	250	270	290	310
PCA[a,b]	99	80	75	13	5	0	0
catechol[a,b]	1	20	25	81	89	94	92

[a] Abbreviation: PCA, protocatechuate. [b] (mol product/mol PCA) × 100%.

increased as the reaction temperature was increased (Table 2) from 190 °C to 270 °C. Protocatechuate could no longer be detected upon increasing the reaction temperature to 290 °C. The 54% yield of catechol when 3-dehydroquinate was heated at 290 °C did not improve when the reaction temperature was increased to 310 °C.

Protocatechuate was the dominant product formed along with a small amount of catechol when 3-dehydroshikimate was heated at 190 °C and 210 °C (Table 3). Unreacted 3-dehydroshikimate was also detected at these temperatures. Catechol became the dominant product when the reaction temperature was increased to 250 °C, and protocatechuate formation was no longer observed when the reaction temperature was increased above 250 °C. Increasing the reaction temperature beyond 250 °C led to only small increases in the yield of catechol (Table 3) with the highest yield of 90% being realized at a reaction temperature

Table 5. Routes and Associated Yields for Syntheses of Catechol from Glucose

entry	Step 1[a] microbial synthesis	% crude yield[b]	% isol. yield[b,c]	step 2[a] reactant → product	% isol. yield[b,c]	step 3[a] reactant → product	% isol. yield[b,c]	% total yield[d]
1	glucose → catechol	6	5					5
2[e]	glucose → catechol	9	7					7
3	glucose → DHQ	28	19	DHQ → catechol	54			10
4	glucose → DHS	36	29	DHS → catechol	90			26
5	glucose → DHQ	28		DHQ → PCA	100	PCA → catechol	87	25
6	glucose → DHS	36		DHS → PCA	93	PCA → catechol	89	30
7	glucose → PCA		26	PCA → catechol	91			24
8[e]	glucose → PCA		49	PCA → catechol	87			43

[a] Abbreviations: DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; PCA, protocatechuate. [b] (mol product/mol glucose or reactant) × 100%. [c] Isolated yields of catechol are based on product after sublimation. [d] (mol sublimed catechol)/(mol glucose) × 100%. [e] In situ, resin-based extraction.

of 290 °C. Unlike the reactivity observed for 3-dehydroquinate, a high percentage of the reacted 3-dehydroshikimate could be accounted for by the formation of protocatechuate and catechol over the entire temperature range examined (Table 3).

Increasing the reaction temperature of aqueous solutions of protocatechuate above 190 °C also resulted in a steady increase in the yield of catechol (Table 4). Catechol became the dominant product upon reacting aqueous protocatechuate at 250 °C. As with 3-dehydroquinate and 3-dehydroshikimate, the highest yield of catechol was obtained at a reaction temperature of 290 °C. Reaction in water heated at 290 °C thus became the standard for conversion of aqueous solutions of 3-dehydroquinate, 3-dehydroshikimate, and protocatechuate into catechol. Commercially available protocatechuate was employed for the reactions summarized in Table 4. Reaction in water heated at 290 °C of protocatechuate chemically derived from 3-dehydroquinate, protocatechuate chemically derived from 3-dehydroshikimate, protocatechuate microbially synthesized directly from glucose, and protocatechuate microbially synthesized directly from glucose using in situ resin-based extraction afforded catechol in yields of 87%, 89%, 91%, and 87%, respectively.

Discussion

Syntheses of aromatics from glucose are designed to avoid use of petroleum-based benzene as a starting material. However, the aromatic product in benzene-free syntheses is frequently toxic to the microbes employed as catalysts. In addition to catechol, benzene-free synthesis of phenol,[7b] hydroquinone,[7a] and *p*-hydroxybenzoate[6a,15] have had to contend with the toxicity of these aromatic products toward the microbes constructed for their synthesis. One option that has been successfully employed entails microbial synthesis of a nontoxic intermediate followed by single-step chemical conversion of this intermediate into the desired aromatic product. The microbial catalyst is thus completely separated from exposure to the toxic aromatic product. This interfacing of microbial and chemical synthesis has been previously employed successfully. In the synthesis of phenol, microbial synthesis of shikimic acid from glucose is followed by conversion of this nontoxic hydroaromatic into phenol in near-critical water.[7b] Hydroquinone has been synthesized by initial microbe-catalyzed conversion of glucose into quinic acid.[7a] A number of different chemical oxidations can then be used to convert this nontoxic hydroaromatic into hydroquinone.[7a]

In situ resin-based extractive fermentation has also been utilized in benzene-free syntheses of aromatics from glucose. As an example, substantially higher concentrations of *p*-hydroxybenzoate are synthesized when in situ resin-based extraction is used during the microbial synthesis of this aromatic under fermentor-controlled conditions.[6a] At the end of the microbial synthesis, absorbed *p*-hydroxybenzoate is simply eluted from the resin. Resin-based extraction thus serves two purposes. Absorption to the resin lowers the concentration of the aromatic product such as *p*-hydroxybenzoate in the culture medium thereby reducing its toxic impact on the microbe's metabolism. Simultaneously, resin-based extraction provides for convenient isolation and purification of aromatic products such as *p*-hydroxybenzoate from the microbial culture medium. Direct synthesis of an aromatic product from a carbohydrate starting material using resin-based aromatic product extraction has yet an additional advantage in the overall reduction in the number of steps required for synthesis of aromatics. By contrast, interfacing microbial and chemical synthesis of aromatics typically requires isolation of the intermediate from the culture medium, chemical reaction of the intermediate, and a final isolation of the desired aromatic product from the chemical reaction mixture.

Catechol has been microbially synthesized from phenol,[16] benzoate,[17] aniline,[18] toluene,[19b] and benzene.[19] In addition to their derivation from petroleum feedstocks, these previously employed starting materials are toxic toward microbes. By contrast, glucose is not toxic to microbes. This difference in toxicity in addition to the anticipated difference in resin absorption of the aromatic product relative to the carbohydrate starting material made in situ resin-based extraction a particularly attractive option for the direct microbial synthesis of catechol from glucose. Unfortunately, in situ, resin-based extraction resulted in only a yield of 7% for the synthesis of catechol from glucose (entry 2, Table 5). The reduction in the concentration of catechol in the culture medium attendant with its absorption to Sepabead SP850 was apparently not sufficient

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to reduce the toxic impact of catechol on *E. coli* WN1/pWL1.290A metabolism.

Attention then turned to the use of a microbial synthesis interfaced with chemical synthesis as a route for the synthesis of catechol. A related strategy has been previously employed.[20] Hexokinase-catalyzed phosphorylation of glucose was followed by cyclization of the resulting glucose 6-phosphate catalyzed by 2-deoxy-*scyllo*-inosose synthase. The 2-deoxy-*scyllo*-inosose obtained from this in vitro, coupled enzymatic reaction was then converted into catechol upon reaction with HI and HOAc. The overall yield of catechol from glucose was 22%.[20] Synthesis of 2-deoxy-*scyllo*-inosose from glucose using an intact microbe remains to be established.

Near-critical water has been previously employed for the benzene-free synthesis of phenol from glucose.[7b] Microbe-synthesized shikimic acid was the key intermediate in this conversion. The successful conversion of shikimic acid into phenol in near-critical water suggested that these reaction conditions might be applicable to the conversion of 3-dehydroquinate, 3-dehydroshikimate, and protocatechuate into catechol. Accordingly, reaction in degassed water heated at 290 °C led to the synthesis of catechol from 3-dehydroquinate, 3-dehydroshikimate and protocatechuate in yields, respectively, of 54%, 90%, and 94% (Table 2, 3 and 4). By contrast, heating 3-dehydroshikimate in aqueous 12 M HCl to reflux for 24 h led to the formation of protocatechuate in 90% yield with no detectable formation of catechol. Protocatechuate was formed in 99% yield with no detectable formation of catechol when 3-dehydroshikimate was heated to reflux for 24 h in acetic acid that was 12 M in H_2SO_4. Heating of the catechol precursors dissolved in water at 290 °C thus proved to be a unique set of reaction conditions. This may reflect the pronounced increase in dissociation constant and decrease in dielectric constant associated with water heated to its critical point.[21] Use of near-critical water avoids the waste streams generated from employment of mineral acids as catalysts. At the same time, the energy input required to heat water to 290 °C is quite significant and will need to be taken into consideration in future economic and life-cycle analyses of the synthesis of catechol from glucose.

In an attempt to avoid extraction of 3-dehydroshikimate from culture medium, conversion of this hydroaromatic into catechol in cell-free, protein-free culture medium was examined. Unfortunately, heating 3-dehydroshikimate-containing cell-free, protein-free culture medium at 290 °C resulted in the formation of catechol in less than 1% yield. A similar phenomenon had previously been observed during attempts to synthesize phenol from cell-free, protein-free culture medium containing shikimate that was heated to near-critical temperatures.[22] The need to extract 3-dehydroquinate and 3-dehydroshikimate from culture medium prior to their chemical conversion to catechol thus emerged as a critical variable in the overall yields achieved for the conversion of glucose into catechol. For example, 3-dehydroquinate was extracted from acidified cell-free, protein-free culture medium less efficiently than 3-dehydroshikimate (entry 3 versus entry 4, Table 5). The subsequent modest conversion of 3-dehydroquinate into catechol resulted in a 10% overall yield for the synthesis of catechol from glucose (entry 3, Table 5). This overall yield is approximately the same as that realized for the synthesis of catechol directly from glucose using in situ resin-based extraction (entry 3 versus entry 2, Table 5). The 26% overall yield for synthesis of catechol from glucose via intermediacy of isolated 3-dehydroshikimate reflected the high-yielding conversion of 3-dehydroshikimate into catechol as well as the ability to extract 3-dehydroshikimate from culture medium more efficiently than 3-dehydroquinate (entry 4, Table 5).

The need to use continuous liquid−liquid extraction for the isolation of 3-dehydroquinate and 3-dehydroshikimate raised questions as to whether this type of extraction could be scaled to the volumes associated with producing a pseudocommodity chemical like catechol. An alternative strategy was therefore pursued, which entailed the chemical conversion of 3-dehydroquinate and 3-dehydroshikimate in culture medium into protocatechuate followed by straightforward and quantitative extraction of this aromatic. Such a strategy takes further advantage of the high-yielding conversions that were observed for the conversion of protocatechuate into catechol when reacted in water heated at 290 °C. Proceeding with isolated protocatechuate as a route to catechol required that the conversion of 3-dehydroquinate and 3-dehydroshikimate into protocatechuate be high yielding. Fortunately, chemical dehydration of 3-dehydroshikimate to form protocatchuate in cell-free, protein-free culture medium was a high-yielding reaction (entry 6, Table 5). The reaction conditions leading to high yields of protocatechuate from 3-dehydroshikimate also proved to be directly applicable to chemical dehydration of 3-dehydroquinate (entry 5, Table 5). The requirement that the syntheses of protocatechuate be run at acidic pH was advantageous since cell-free culture medium containing 3-dehydroshikimate and 3-dehydroquinate was acidified to pH 2.5 in order to precipitate proteins.

The 25% yield for the overall conversion of glucose into catechol via intermediacy of protocatechuate chemically synthesized from 3-dehydroquinate (entry 5, Table 5) was a substantial improvement over the 10% yield for the overall conversion of glucose into catechol via isolated 3-dehydroquinate (entry 3, Table 5). This improvement reflects the quantitative chemical dehydration of 3-dehydroquinate and quantitative extraction of the resulting protocatechuate from cell-free, protein-free culture medium. The higher conversion of protocatechuate into catechol in water heated at 290 °C relative to the modest conversion of 3-dehydroquinate into catechol under similar reaction conditions also contributes to the overall yield improvement. The 30% yield for the overall conversion of glucose into catechol via intermediacy of protocatechuate chemically synthesized from 3-dehydroshikimate (entry 6, Table 5) was significantly improved over the 26% overall yield for the conversion of glucose into catechol via isolated 3-dehydroshikimate (entry 4, Table 5). This yield improvement was primarily due to the high-yielding chemical dehydration of 3-dehydroshikimate in cell-free, protein-free culture medium and quantitative extraction of the resulting protocatechuate. The yields for conversion of protocatechuate into catechol (entry 6, Table 5) and conversion of 3-dehydroshikimate into catechol (entry 4, Table 5) in water heated at 290 °C were approximately the same.

Microbial synthesis of protocatechuate from glucose followed by chemical decarboxylation to form catechol was initially

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examined as a variant on the basic theme of interfacing microbial and chemical synthesis for the conversion of glucose into catechol. We had previously established that protocatechuate was toxic toward *E. coli* constructs, although this toxicity was significantly less than that observed for catechol.[3] Nonetheless, a 26% yield was realized with the microbial synthesis of protocatechuate from glucose (entry 7, Table 5), which was comparable to the yield of 3-dehydroquinate microbially synthesized from glucose (entry 3 and 5, Table 5). Subsequent chemical decarboxylation of this protocatechuate in water heated to 290 °C afforded an overall yield of 24% for the conversion of glucose into catechol (entry 7, Table 5).

In situ resin-based extraction was then included in the direct microbial synthesis of protocatechuate. The resulting yield of protocatechuate microbially synthesized from glucose improved to 49% (entry 8, Table 5), which was a pronounced improvement relative to the 26% yield (entry 7, Table 5) obtained in the absence of in situ resin-based extraction. This yield improvement apparently reflects both the relatively mild toxicity of protocatechuate toward the producing *E. coli* construct and the effectiveness of AG-1 X8 resin in reducing the concentration of protocatechuate in culture medium during its microbial synthesis. Combined with the decarboxylation of protocatechuate in water heated at 290 °C, the direct microbial synthesis of protocatechuate from glucose utilizing in situ resin-based extraction afforded a 43% overall yield for synthesis of catechol from glucose (entry 8, Table 5). Thus in the final analysis, the central finding in the conversion of glucose into catechol was not a determination of whether in situ resin-based extraction of microbe-synthesized product or interfacing microbial and chemical synthesis constituted the best synthetic strategy. Instead, it was the functional integration of both of these methods for reducing and circumventing the toxicity of the aromatic product toward the producing microbial construct that led to the highest overall yield of catechol synthesized from glucose.

Experimental Section

General Chemistry. ^{1}H NMR spectra were recorded at 300 MHz on a Varian Gemini-300 spectrometer. Chemical shifts for ^{1}H NMR spectra are reported (in parts per million) relative to internal sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP, δ 0.0 ppm) with D_2O as the solvent. ^{13}C NMR spectra were recorded at 75 MHz on a Varian Gemini-300 spectrometer. Chemical shifts of ^{13}C NMR spectra are reported (in parts per million) related to internal acetonitrile (CH_3CN, δ 3.69 ppm) in D_2O. To determine the concentration of 3-dehydroquinate, 3-dehydroshikimate, protocatechuate and catechol in culture medium and reaction solutions, a portion (0.5−1.0 mL) of the solution was concentrated to dryness, concentrated to dryness one additional time from D_2O, and then redissolved in 1 mL D_2O containing 10 mM TSP. Concentrations were determined by comparison of an integrated ^{1}H NMR resonance corresponding to each molecule with the integrated ^{1}H NMR resonance corresponding to TSP (δ 0.0). A standard calibration curve was individually determined for each molecule using solutions of known concentrations prepared from authentic, purified samples. The following resonances were used to quantify each molecule: 3-dehydroquinate (δ 4.35, d, 1 H); 3-dehydroshikimate (δ 4.25, d, 1 H); protocatechuate (δ 6.93, d, 1 H) and catechol (δ 6.98, m, 2 H).

Anion-exchange resin AG-1 X8 (chloride form, 50−100 mesh) was obtained from Bio-Rad while anion-exchange resins Dowex-1 X4 (chloride form, 50−100 mesh), Dowex-1 X8 (chloride form, 50−100 mesh) and Amberlite IRA-400 (chloride form, 20−50 mesh) were obtained from Supelco. All of the anion-exchange resins were converted to their phosphate form by elution with 10 bed volumes of 1 M KH_2PO_4 prior to use. Polyaromatic resins Sepabead SP850 (20−60 mesh), Amberlite XAD-2, XAD-4, XAD-16, XAD-16HP, XAD-1180 (20−60 mesh), Diaion SP207 (20−60 mesh), Diaion HP20SS (75−150 μm), Diaion HP2MG (25−50 mesh), and MCI GEL CHP20P (75−150 μm) were obtained from Supelco.

Chemical Synthesis of Protocatechuate. Chemical dehydration of 3-dehydroshikimate to form protocatechuate was first evaluated in a reaction solution that lacked added D-glucose but otherwise mimicked the culture medium (see Supporting Information) used during microbial synthesis of 3-dehydroquinate and 3-dehydroshikimate under fermentor-controlled conditions. A 0.23 M 3-dehydroshikimate reaction solution was prepared by dissolving 2.0 g of 3-dehydroshikimate in 50 mL aqueous salts solution and was adjusted to pH 2.5 or pH 7.0 by addition of concentrated H_2SO_4 or $NH_3 \cdot H_2O$. The pH 2.2 reaction solution was obtained by elution of a pH 2.5 solution containing 3-dehydroshikimate through a column containing 10 g of Dowex 50 (H^+). The three different solutions were then heated to reflux under N_2. Aliquots were withdrawn and analyzed by ^{1}H NMR every 0.5 h (pH 7.0) or 3 h (pH 2.2 and pH 2.5). The yields of protocatechuate obtained are summarized in Table 2.

Chemical dehydrations to form protocatechuate were then examined in culture medium from fermentor-controlled microbial syntheses. Culture medium (1 L) containing 3-dehydroshikimate synthesized by cultivation of *E. coli* KL3/pJY1.216A was centrifuged (14 000 *g*, 20 min) to remove cells, acidified to pH 2.5 with concentrated H_2SO_4 (3−5 mL) to precipitate protein and centrifuged again (14 000 *g*, 20 min). The resulting cell-free, protein-free culture medium (1 L) was eluted through a column containing 200 g Dowex 50 (H^+) and then heated to reflux under N_2 for 24 h. Culture medium (1 L) from the synthesis of 3-dehydroquinate by *E. coli* QP1.1/pJY1.216A was treated in the same way as 3-dehydroshikimate-containing culture medium to remove cells and proteins. After elution through 200 g Dowex 50 (H^+), the resulting cell-free, protein-free culture medium containing 3-dehydroquinate was refluxed under N_2 for 24 h. For the individual chemical dehydrations of 3-dehydroshikimate and 3-dehydroquinate in cell-free, protein-free culture medium, product was extracted with EtOAc (3 × 400 mL), dried over Na_2SO_4 and concentrated to afford a 93% yield of protocatechuate from 3-dehydroshikimate and a 100% yield from 3-dehydroquinate. The ^{1}H NMR and ^{13}C NMR spectra of the isolated protocatechuate were identical with those of authentic protocatechuate.

Chemical Synthesis of Catechol. Aqueous solutions of 3-dehydroquinate, 3-dehydroshikimate or protocatechuate were heated to 190 °C, 210 °C, 230 °C, 250 °C, 270 °C, 290 °C, and 310 °C in a Parr (Model No. 4742) high-pressure, stainless steel reaction vessel with a working volume of 21 mL. The reactor was submerged in a sand bath contained in a heating mantle. Heating was controlled by a TEMP−O-Trol thermo-control unit (Model TOT-VOVC). For the initial experiments designed to optimize reaction temperatures, 3-dehydroquinate and 3-dehydroshikimate were isolated from the culture supernatants of microbial syntheses (see Supporting Information). Protocatechuate was purchased from Aldrich. Distilled, deionized water was used for all reactions.

3-Dehydroquinate (2.11 g, 11.0 mmol), 3-dehydroshikimate (1.89 g, 11.0 mmol), or protocatechuate (1.69 g, 11.0 mmol) was dissolved in 6 mL of water. These solutions were then degassed by a subsurface feed of Ar for 15 min followed by a subsurface feed of CO_2 for another 15 min. After flushing the headspace with Ar, the high-pressure reaction vessel was sealed according to the manufacturer's specifications and submerged in a sand bath. A heating rate of 1.5 °C/min was maintained until the desired final temperature was attained. The final temperature was maintained (±4 °C) for 30 min. Subsequently, the vessel was removed from the sand bath and cooled to room temperature by placing under a cooling fan. The reaction mixture was then extracted with EtOAc (5 × 20 mL). The vessel interior was also thoroughly rinsed

with EtOAc. The organic layers were combined, washed with brine, dried over Na_2SO_4, and concentrated to afford a residue containing catechol and protocatechuate (if present). Kugelrohr distillation of the residue under reduced pressure afforded catechol as white crystals in the receiving vessel. Protocatechuate remained in the distillation vessel.

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Supporting Information Available: Culture medium, selection of resins, fermentor-controlled microbial syntheses, isolation of microbe-synthesized products, and genetic manipulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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