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Efficient *in Vivo* Synthesis and Rapid Purification of Chorismic Acid Using an Engineered *Escherichia coli* Strain[1]

Corinna Grisostomi,[2] Peter Kast,[2] Rosalino Pulido,[3] Judy Huynh,[4] and Donald Hilvert[2,5]

Departments of Chemistry and Molecular Biology, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

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Chorismate is a key intermediate in the biosynthesis of aromatic amino acids and other natural products in microorganisms and plants. Four chorismate mutase-deficient strains of *Escherichia coli* were evaluated for the large scale microbiological production of this metabolite which is needed for detailed biochemical and structural studies of chorismate-utilizing enzymes. Optimization of culturing and isolation conditions with one of these strains yielded an improved protocol for the production of chorismate in yields that compare favorably with those previously obtained with *Klebsiella pneumoniae* strain 62-1, a Class 2 pathogenic organism. A simple, single-step procedure for the purification of large quantities of chorismic acid by C18 reverse phase silica gel flash chromatography is also described. Chorismic acid obtained through this procedure is 90–98% pure and can be stored as the free acid in crystalline form over several months without decomposition.

INTRODUCTION

(–)-Chorismate (**1,** Fig. 1) is a central metabolite in the biosynthesis of aromatic amino acids and vitamins as well as a variety of other natural products in plants and microorganisms. It is the product of chorismate synthase and a substrate for chorismate mutase, anthranilate synthase, *p*-aminobenzoate synthase, isochorismate synthase, and chorismate lyase (*1*). A great number of molecular genetic, biochemical, and biophysical studies have recently been reported on chorismate-utilizing enzymes (*2–13*) and on corresponding man-made protein catalysts, such as catalytic antibodies with chorismate mutase activity (*14–16*).

Extensive structural and kinetic investigations of these enzymes require the availability of large quantities of chorismic acid. Unfortunately, commercially available chorismate is rather expensive ($460/100 mg) and typically of modest purity. Alter-

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[2] Current address: Department of Chemistry, Swiss Federal Institute of Technology (ETH), Universitätstrasse 16, CH-8092 Zürich, Switzerland.

[3] Current address: Department of Chemistry, Cambridge University, Lensfield Road, Cambridge, CB2 1EW, U.K.

[4] Current address: Molecular Sciences Institute Inc., 9894 Genesee Avenue, La Jolla, CA 92037.

[5] To whom correspondence should be addressed. Fax: +41-1-632-1486. E-mail: hilvert@org.chem.ethz.ch.

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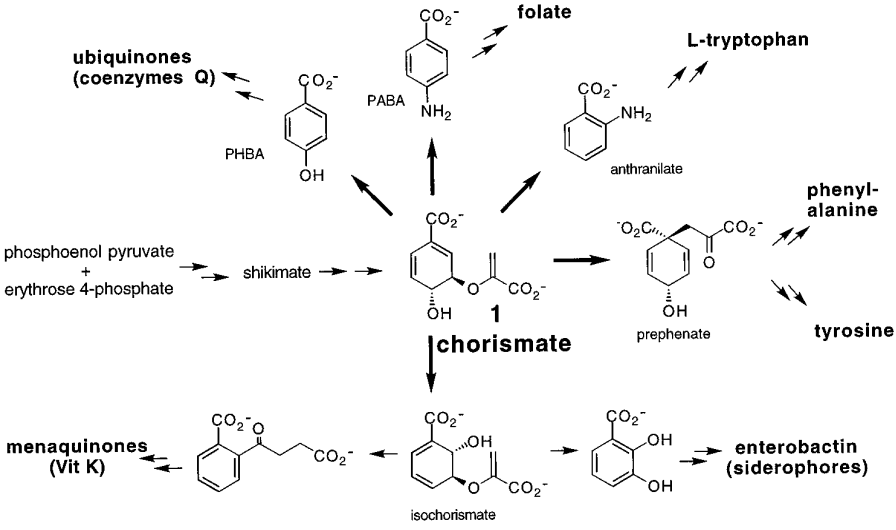

FIG. 1. The importance of (–)-chorismate **1** in *Escherichia coli* metabolism. Two small arrows indicate multiple metabolic steps.

natively, chorismic acid can be produced microbiologically using the chorismate mutase-deficient *Klebsiella pneumoniae* strain 62-1 (*17–20*). We and others (*21*) have found, however, that the original protocol for production and purification is cumbersome and provides relatively impure material. By optimizing growth conditions for *K. pneumoniae* 62-1 (subsequently referred to as ''Kp'') in small scale cultures, the yield of chorismate per molecule of glucose has been increased threefold, a significant improvement for the preparation of radiolabeled chorismic acid (*22*). Although the use of preparative HPLC has facilitated purification (*21*, *22*), manipulation of large amounts of chorismic acid remains impractical and none of the new procedures increased the yield of chorismate obtainable per liter of culture. Furthermore, *K. pneumoniae* is a Class 2 pathogen (*10*), and its handling requires specific biosafety practices and installations (*23*). In search for an improved method for producing chorismate, we have evaluated several recently constructed mutant strains of *Escherichia coli* lacking chorismate mutase activity as alternatives to Kp. One of these nontoxic *E. coli* strains, KA12, provides good yields of (–)-chorismate under optimized conditions. The compound can be readily purified on a large scale in one step by flash chromatography on a C18-reverse phase silica gel column.

MATERIALS AND METHODS

Analytical Procedures and Materials

Culture supernatants were assayed for chorismic acid by analytical HPLC using a Vydac C18 reverse phase analytical column (injection volume 10 μl; flow rate 1 ml/min; 5 min wash with 0.05% CF_3COOH; followed by a 25-min linear gradient

to 20% acetonitrile for 25 min; monitored at 274 nm; retention time (RT) = 21.5 min). The concentration of chorismic acid was estimated by peak integration and comparison with a standard curve generated with solutions of known concentration; *p*-bromobenzoic acid was used as an internal standard (RT = 31.5 min) (*24*). The purity of final chorismic acid preparations was also analyzed by analytical reverse phase HPLC on a C18 column using a Hewlett Packard 1100 system equipped with a diode array detector (injection volume 10 μl; flow rate 1 ml/min; 10 min wash with 20% acetonitrile in 0.05% aq. CF_3COOH, followed by a 30-min linear gradient from 20 to 40% acetonitrile in 0.05% aq. CF_3COOH, RT = 17.2 min). Nuclear magnetic resonance (NMR) spectra were recorded on Brucker AMX 400 and AX 250 MHz spectrometers. Fast atom bombardment (FAB) mass spectra were recorded on a VG ZAB-2VSe double focusing high resolution mass spectrometer. Analytical thin layer chromatography (TLC) was performed using precoated 0.25-mm C18-reverse phase silica plates from E. Merck and Macherey-Nagel & Co. All TLC plates contained a fluorescent indicator, and UV-active substances were visualized under short-wave UV light. Other compounds were detected by means of an iodine or a diluted potassium permanganate stain. Flash column chromatography was performed on octadecyl-functionalized silica gel from Aldrich, and elution with the desired eluant was carried out under pressure. All chemicals used were reagent grade; solvents for analytical HPLC were HPLC grade. Yeast extract and casamino acids were purchased from Difco.

Bacterial Strains

The bacterial strains used in this study are listed in Table 1. Strain 62-1 of the species *K. pneumoniae* (formerly also known as *Aerobacter aerogenes*) was acquired from the American *Type Culture* Collection (Rockville, MD). *E. coli* strain KA11 was obtained by generalized P1 transduction, as described earlier for KA12 (*10*). Standard microbial techniques and LB medium were according to Miller (*25*). Biosafety Level 2 practices (*23*) were adopted when working with the Class 2 pathogen *K. pneumoniae*.

Optimized Production of Chorismic Acid by Overproducing Cell Cultures

Culturing and isolation conditions were optimized as described under Results and Discussion. The new protocol for 1-L cell cultures for either *K. pneumoniae* strain 62-1 (Kp) or *E. coli* KA12 involves inoculation of 2 × 5 ml of LB medium with a single colony each and shaking overnight at 37°C. Two 500-ml aliquots of growth medium A (per L: 2 g casamino acids, 2 g yeast extract, 41 mg L-tryptophan, 20 ml 50× Vogel & Bonner salts (*18*)) in 2-L Erlenmeyer flasks were autoclaved, provided with glucose to 0.16% (w/v), and then inoculated with 5 ml preculture. The cultures were incubated at 30°C (220 rpm) for 4–6 h to give A_{620} = 1.9–2.1 and then centrifuged at 2000*g* for 20 min (4°C). The cells were resuspended in nonsterile (2 × 500 ml) accumulation medium B (per L: 12.8 g Na_2HPO_4, 1.36 g KH_2PO_4, 18 g glucose, 2.7 g NH_4Cl, 20.3 mg $MgCl_2 \cdot 6H_2O$, 2 mg L-tryptophan (*18*, *19*). This suspension was shaken at 30°C (220 rpm) for 16 h allowing for production of chorismate and its secretion into the medium. After removing the cells by centrifu-

TABLE 1
Klebsiella pneumoniae and *Escherichia coli* Strains Used in this Study

Strain	Genotype	Relevant phenotype[a]	Source or construction	Ref.
K. pneumoniae 62-1		Phe$^-$, Tyr$^-$, Trp$^-$	ATCC No. 25306	(*17*, *41*)
E. coli KB224	F$^-$, λ^-, Δ(*pheA-tyrA-aroF*), *thi-1*, *endA1*, *hsdR17*, Δ(*argF-lac*)*205*(*U169*), *supE44*	Phe$^-$, Tyr$^-$	K. Backman	(*29*)
E. coli KB357	F$^-$, λ^-, Δ(*pheA-tyrA*), *thi-1*, *endA1*, *hsdR17*, Δ(*argF-lac*)*205*(*U169*), *supE44*	Phe$^-$, Tyr$^-$	K. Backman	(*30*)
E. coli KA11	Same as KB357, but Δ(*srlR-recA*)*306*::Tn*10*	Phe$^-$, Tyr$^-$, Tet^R, RecA$^-$	P1 transduction of KB357 to Tet^R	This work
E. coli KA12	Same as KB224, but Δ(*srlR-recA*)*306*::Tn*10*	Phe$^-$, Tyr$^-$, Tet^R, RecA$^-$	P1 transduction of KB224 to Tet^R	(*10*)

[a] All strains listed are chorismate-mutase deficient, but proficient for anthranilate synthase. To avoid converting a significant fraction of the accumulated chorismate to anthranilate, L-tryptophan is added to the growth and accumulation media to repress the synthesis of (and inhibit) anthranilate synthase (*17*).

gation at 2000*g* for 20 min (4°C), the supernatant was collected, brought to pH 9.0 by the addition of 10 N NaOH, and transferred under pressure to an ion-exchange column (BioRad AG1-X8, 200–400 mesh, Dowex 1-Cl; 6 × 2.4-cm bed size). The column was washed with 80 ml water and chorismate was eluted as the ammonium salt with 1 M NH_4Cl (pH ≈ 8.5). Fifty 6-ml fractions were collected and A_{274} was determined for each at a 1:200 dilution in water. Chorismate-containing fractions were pooled, acidified with concentrated HCl to pH 1.5, and extracted first with dichloromethane (3 × 75 ml) to remove most of the phenylpyruvate and then with ethylacetate (4 × 50 ml). The combined ethylacetate extracts were washed with brine and dried over Na_2SO_4. Removal of the solvent *in vacuo* yielded a yellow oil, which was stored at −78°C to minimize decomposition.

Purification of Chorismic Acid by Reverse Phase Flash Chromatography

Crude chorismate was purified in a single step by flash chromatography on C18 reverse phase silica gel (*26*, *27*). The crude material (150 to 420 mg) was loaded on 220 g C18 reverse phase silica (4.5 × 30 cm bed size) and eluted with 10 mM ammonium acetate (pH ≈ 6) under pressure. Individual fractions (50 × 10 ml), stored on ice, were checked for chorismate by thin layer chromatography on C18 reverse phase silica gel plates (R_f: 0.83, 1 M ammonium acetate). The relevant fractions were pooled and ammonium acetate removed by lyophilization to yield the desired material as the free acid in 90–98% purity. Sample purity was assessed by ^{1}H-NMR and analytical reverse phase HPLC, monitored at 274 and 225 nm.