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The Origin of the C-2 Hydroxyl in the Isochorismate Synthase Reaction

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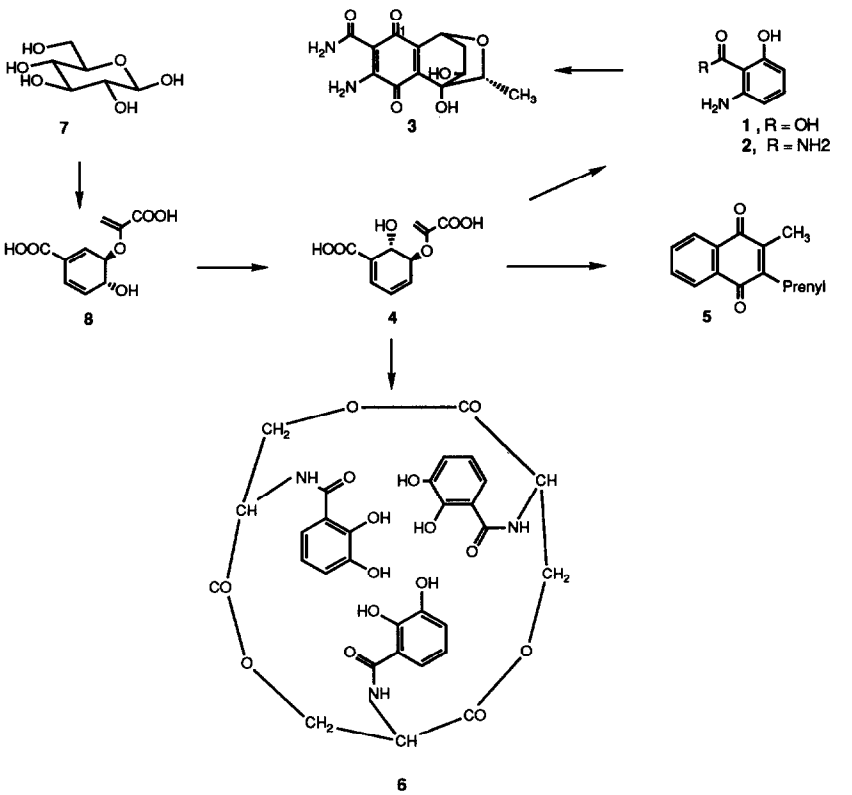
Summary: Isochorismate synthase has been isolated from *Enterobacter aerogenes* 62-1 and partially purified through DEAE cellulose chromatography and ammonium sulfate precipitation. When chorismic acid, **8**, was treated with this preparation in 50% $H_2{}^{18}O$, isochorismic acid **4a**, labeled at the C-2 hydroxyl with ^{18}O, was produced, as demonstrated by ^{13}C NMR spectroscopy. Thus, the enzyme-catalyzed reaction is formally a double S_N2' reaction involving water.

INTRODUCTION

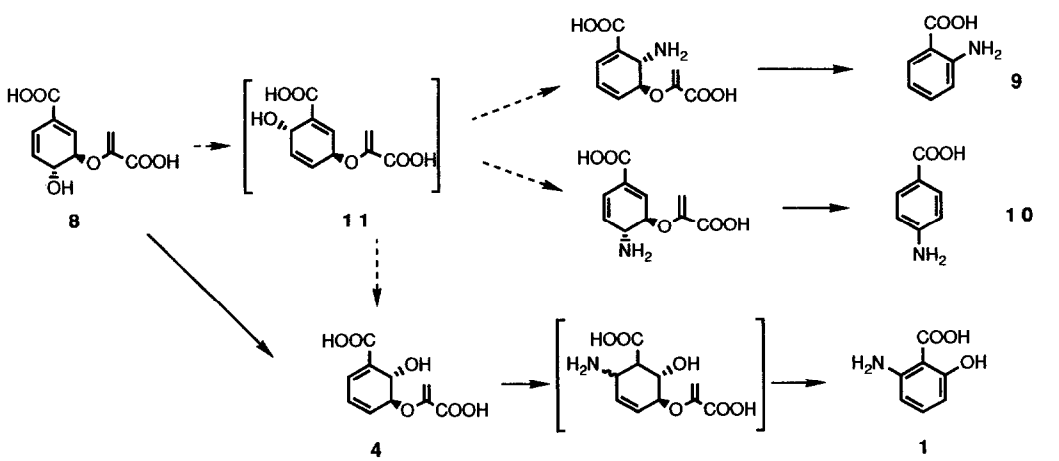
We have demonstrated the intermediacy of a new shikimate pathway product, 6-hydroxyanthranilic acid, **1**,[1] and - as the next step - of 6-hydroxyanthranilamide, **2**,[2] in the biosynthesis of the novel C-glycoside antibiotic sarubicin A, **3**.[1] The oxygen at C-1 had been retained from an organic precursor to **1** from the shikimate pathway. This could be most simply explained by formation of **1** *via* isochorismic acid, **4**, which has been shown to be a precursor to the vitamins K, **5**,[3,4] and of enterobactin **6**.[5,6] These, and the origin of **4** from glucose, **7**, *via* chorismic acid, **8**, are summarized in Scheme 1.

Isochorismate synthase, which generates **4** from **8**, was first isolated from *Enterobacter aerogenes* 62-1,[7,8] and - in addition to those detailed above - has since been implicated in a number of other pathways.[8-11] *E. aerogenes* 62-1 is a mutant that lacks chorismate mutase and N-phosphoribosylanthranilate synthase activities, and is thus blocked in the biosynthesis of phenylalanine, tyrosine, and tryptophan. As a corollary, *E. aerogenes* 62-1 accumulates chorismate into the growth medium. While continuing investigations of sarubicin biosynthesis, we therefore used this organism to study the enzyme-catalyzed conversion of **8** to **4**, in order that it may be compared with the other enzymes that generate aromatic amino acids from chorismic acid: anthranilic acid (**9**) synthase[12,13] and *p*-aminobenzoic acid (**10**) synthase[14,15] (Scheme 2). Ganem has proposed **11** as a possible common intermediate.[16]

Scheme 1

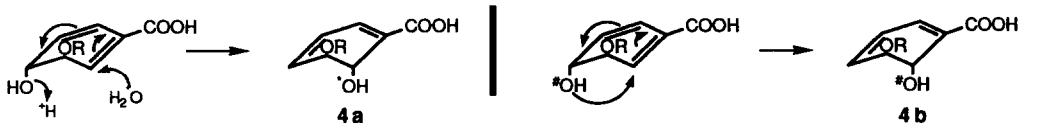


Scheme 2



As shown in Scheme 3, the isochorismate synthase reaction can be viewed as either a double S_N2' reaction with water (perhaps *via* **11**) to give **4a** or a suprafacial 1,5-shift of the C-4 hydroxyl to give **4b**. In order to distinguish between these we planned to carry out the conversion in $H_2{}^{18}O$; if an intermolecular reaction were involved, the C-2 hydroxyl of **4a** would be labeled with ^{18}O, and it was anticipated this could be detected by ^{13}C NMR spectroscopy.[17,18]

Scheme 3



RESULTS AND DISCUSSION

Production of Chorismic Acid. It was found that *E. aerogenes* 62-1 could be conveniently grown in 10-liter volumes, inoculated with 100 mL of a seed culture,[19,20] using a stirred fermentor. After 8 hours at 30°C, the cells were collected and immediately resuspended in the production medium.[19] This was incubated 16 hours and, after removing the cells, the superatant was made alkaline with 10 M sodium hydroxide and chromatographed on Dowex 1X4 to give an oil. From this, 5 grams of crystalline chorismic acid[21] could be obtained which was stable for up to 6 months at -78°C, but which largely rearranged to prephenic acid at -20°C.

Purification of Isochorismate Synthase and Isolation of Isochorismic Acid. The original assay[7] for isochorismate synthase activity involved thermal (100°C) decomposition of the product, **4**, to salicylic acid (25%) and 3-carboxyphenylpyruvic acid (75%). The former could be detected by fluorescence spectroscopy; no salicylic acid was formed from **8** under these conditions. This was rather tedious and imprecise. Leistner subsequently developed an HPLC assay using a C_8 reversed-phase column eluting with methanol-water (1:9) containing 0.15 M phosphoric acid, pH 1.9.[3] We have modified this by substituting 0.1% trifluoroacetic acid, pH 2.1, which avoided column degradation and provided a completely volatile solvent for subsequent isolation of **4**. Substrate (retention time, 25.8 min) and product (retention time, 12.7 min) were well-separated and easily quantitated at 278 nm (**8**: $\varepsilon = 2630$;[22] **4** : $\varepsilon = 13{,}000$) by direct injection of a sample from an enzyme incubation (25 μmol Tris-HCl buffer pH 8.0, 5 μmol Mg^{2+}, 10 μmol **8**, variable amounts of enzyme, total volume 1.0 mL).

To obtain isochorismate synthase, *E. aerogenes* 62-1 was grown in the presence of limiting aromatic amino acids (10^{-4} M each), and in the absence of Fe^{2+} to derepress production of the

enzyme.[7] Again, it was found the fermentation could be carried out on a 10-liter scale in a stirred fermentor. The cells could then be collected with a Sharples centrifuge, yielding 76 g (wet weight). Following the published procedure,[7] sonication of the cells in phosphate buffer, centrifugation, and DEAE cellulose chromatography yielded a partially purified enzyme preparation (270 mL) from active fractions identified by the HPLC assay. While this provided a new HPLC peak later shown to be **4** (*vide infra*), further purification was deemed necessary for the anticipated preparative-scale reactions.

The material obtained from the DEAE cellulose column was fractionated with solid ammonium sulfate (AS). Fractions obtained were those from 0-30%, 30-46%, 46-62%, 62-77%, and 77-99% saturation. Each precipitate was dissolved in 0.01 M sodium phosphate buffer, pH 7.0 (5-8 mL), and assayed for isochorismate synthase activity. Most of the activity was found in the fractions of 30-62% saturation, and these were pooled to yield 76 mg of protein. This was repeated with another 68 g of cells, now yielding 108 mg more protein. The purification is given in Table 1.

Table 1 Fractionation of Isochorismate Synthase[a]

Step	Protein conc. (mg/mL)	Total Protein (mg)	%Recovery	Sp. Act.[b]
Cell free extract	7.3	1825	100.0	
DEAE column	0.71	182	10.0	1×10^{-4}
$(NH_4)_2SO_4$	13.5	108	5.9	3×10^{-2}

a. From 68 g cells (wet weight)
b. Specific activity is defined as μmoles product/mg protein/60 min.

Production of **4** was optimized by systematically varying the concentration of **8** and the amount of enzyme preparation. The results are shown in Tables 2 and 3, and indicated that a 1:1 ratio of **8**:protein (w/w) would yield enough **4** for ^{13}C NMR analysis. Isochorismate could not be recovered as efficiently as chorismate. On a preparative scale, **4** could be isolated by lyophilization of the appropriate HPLC fraction. This yielded 3.3 mg of pure **4** from 25 mg of **8** (7.7 mg of this was also recovered by HPLC). The ^1H NMR spectrum of **4** thus obtained was identical to the literature.[7]

Table 2 Effect of Chorismate Concentration on Production of 4

Chorismate (mg)	Peak height ratio (**4** : **8**)
3.0	1.61
5.0	0.74
7.0	0.54
10.0	0.32

Conditions. Volume: 4 mL; protein: 3.4 mg (13.5 mg/mL); Tris-HCl: 50 mM; Mg^{2+}: 5 mM; pH 8.0; Incubation time: 2 hours, at 37 °C. Assay by HPLC

Table 3 Effect of Protein Concentration on Production of 4

Protein (mg)	Peak height ratio (**4** : **8**)
2.0	1.30
5.0	2.12
8.0	2.30
10.0	3.55

Conditions: Volume: 4 mL; protein stock: 18.5 mg / mL; chorismate: 10.0 mg; Tris-HCl: 50 mM; Mg^{2+}: 5 mM; pH 8.0; Incubation time: 2 h, at 37 °C. Assay by HPLC

NMR Characterization of Isochorismic Acid. The ^{1}H NMR spectrum of **4** in DMSO-d_6 has been reported. However, in this solvent it has been reported to rearrange to 3'-carboxyphenylpyruvic acid *via* a Claisen rearrangement and elimination of water, but the rate of decomposition has not been reported.[7,23,24] ^{1}H NMR spectra were therefore obtained at 0, 3, 6, 9, 19, 24, 48, 55, and 72 hours at 294 K. Decomposition was followed by comparison of the integration of the H-5 resonance (δ 6.99) and that of a new aromatic resonance (δ 7.45). Under these conditions, ca. 40% decomposition had occurred after 72 hours.

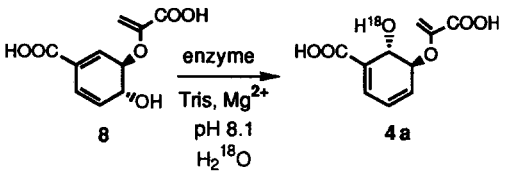
A ^{13}C NMR spectrum of **4** in DMSO-d_6 was obtained between 9 and 19 hours. While decomposition had been minor, the line widths were 2-4 Hz, which would be unacceptable for detection ^{13}C-^{18}O isotope shifts of only 1-3 Hz (anticipated for a secondary alcohol). DMF-d_7 proved to be a generally superior solvent for **4**: decomposition was slower and ^{13}C line widths were narrower. The H-2 and H-3 proton resonances that were well-resolved in DMSO-d_6 (δ 4.46 and 4.95, respectively) now overlapped in DMF-d_7 (δ 4.72).[25] However, since the ^{13}C chemical shifts were nearly identical in both solvents - and the critical C-2 and C-3 resonances were well resolved, the ^{13}C resonances in DMF-d_7 could be confidently assigned via those obtained in DMSO-d_6. The earlier proton assignments were confirmed by a COSY experiment and the critical ^{13}C assignments were then made from an HETCOR experiment. The assignments are given in Table 4.

Table 4 ^{1}H- and ^{13}C NMR Data for Isochorismic Acid

	^{1}H NMR				^{13}C NMR[a]	
Position	DMSO-*d6*	J (Hz)	DMF-*d7*	J (Hz)	DMSO-*d6*	DMF-*d7*
1					131.35	131.91
2	4.46	br s	4.72	m	62.12	63.83
3	4.52	4.95, 0.81	4.72	m	74.17	75.64
4	6.25	9.40, 4.91	6.34	9.22, 5.60	127.77	128.54
5	6.37	9.55, 5.61	6.45	9.40, 5.61	126.34	126.89
6	6.99	5.56	7.10	5.35	130.53	131.79
7					167.50	168.23
8 a	5.33	2.65	5.44	2.33	95.72	95.85
8 b	5.00	2.65	5.09	2.43	95.72	95.85
9					149.41	150.83
10					163.87	164.62

a. Quaternary carbon assignments from comparison to LR-HETCOSY[26] - derived assignments for chorismic acid.

The Isochorismate Synthase Reaction in $H_2{}^{18}O$. An incubation of **8** in 50% $H_2{}^{18}O$ with an aliquot of the 30-62% AS fraction of isochorismate synthase was carried out so that if water were involved, both ^{13}C-^{16}O and ^{13}C-^{18}O resonances would be observable, the former providing an internal standard in order to detect the isotope shift. Given the high cost of $H_2{}^{18}O$, the total volume was limited to 4.0 mL. In a test run without ^{18}O, 35 mg of **8** yielded 5.2 mg of pure **4**. These conditions were then used for the key experiment. Thus, Tris base, $MgCl_2 \cdot H_2O$ and **8** were dissolved in 2.0 mL of $H_2{}^{18}O$ (98-99% ^{18}O) and adjusted to pH 8.1 with 1 M NaOH. Enzyme preparation (18.5 mg/mL, 1.89 mL) was added and the volume made up to 4.0 mL. After a two-hour incubation (longer times led to the appearance of decomposition products) work-up afforded 5.5 mg of labeled **4a** after HPLC purification. The sample was analyzed in DMF-*d7* by ^{13}C NMR spectroscopy (Figure **1**) and showed an additional - isotope-shifted resonance for C-2 upfield of the normal resonance ($\Delta\ \delta$ = 1.31 Hz). The two resonances were of approximately equal intensity. Therefore, in the enzyme-catalyzed reaction the C-2 hydroxyl is derived from water by an intermolecular process equivalent to a double S_N2' displacement (overall *syn* geometry). The *Escherichia coli* isochorismate synthase gene (*ent*C) has recently been cloned and overexpressed. The derived enzyme was used in an $H_2{}^{18}O$ experiment; in this case the product was enzymatically converted to 2,3-dihydroxybenzoate, which was analyzed by mass spectrometry. As in our work, it was found that the C-2 hydroxyl of isochorismate was derived from the water.[27]



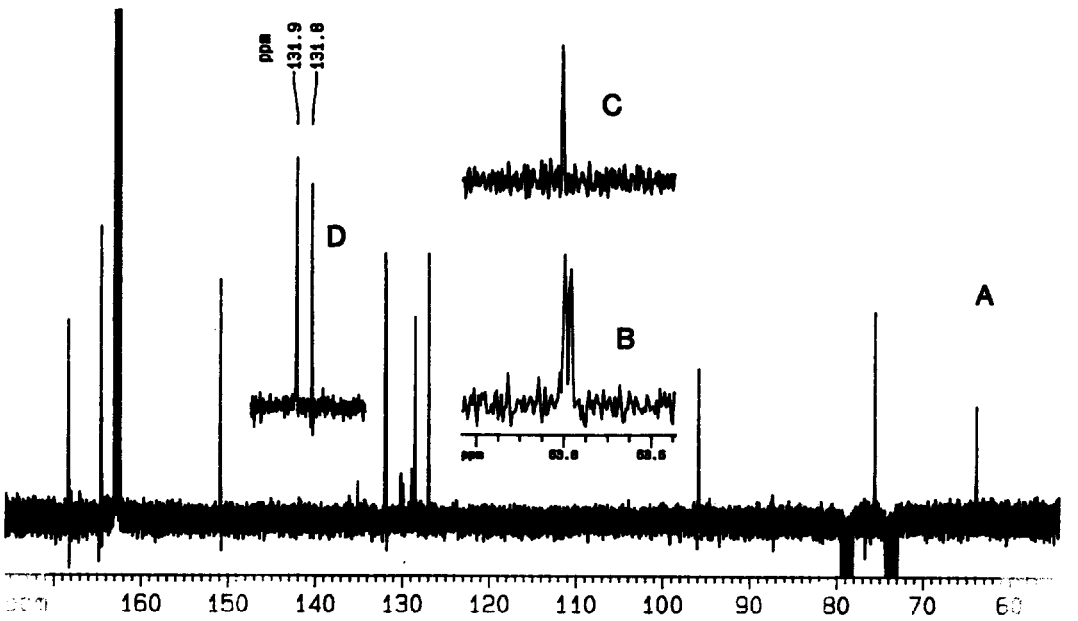


Figure 1. A. ^{13}C NMR Spectrum of **4a** in DMF-d_7. B. Expansion of A showing ^{13}C-^{16}O and ^{13}C-^{18}O resonances for C-2. C. C-2 Resonance of **4** (natural abundance). D. Expansion of 131-132 ppm region in spectrum of **4a**.

Although there are chemical[28] and biochemical[29] precedents for reactions that are formally double S_N2' displacements, the mechanism(s) of these have not yet been established. In the present case the data could accommodate two S_N2' reactions with the intervention of the putative intermediate **11**. It has recently been shown that *p*-aminobenzoic acid is derived from **8** rather than from **4** in both *E. aerogenes* and *Streptomyces* sp.[30] Whether any or all of the pathways from **8** proceed *via* **11** or by concerted processes will be the subject of future investigations.

EXPERIMENTAL

General. ^1H NMR and ^{13}C NMR spectra were taken on a Bruker AM 400 or AC 300 spectrometer with samples in 5 mm tubes. ^{13}C NMR spectra were Waltz decoupled.

Fermentations were carried out in a Lab-Line model 3595 incubator shaker or a New Brunswick MicroFerm™ stirred fermenter. Sterile transfers were carried out in an EdgeGUARD™ hood manufactured by the Baker Company, Inc.(Sanford, ME). Centrifugations were run in an IEC model B20A refrigerated centrifuge, and sonications were done using a Heat Systems-Ultrasonics, Inc., model W-225R cell disrupter.

Dowex 1X4 (100-200 mesh) ion exchange resin was purchased from Sigma Chemical Company, and converted to the necessary ionic form according to the manufacturer's recommendation. DEAE cellulose (DE52) was purchased pre-swollen from Whatman Inc., and was equilibrated according to the manufacturer's instructions. All chemicals were enzyme grade.

HPLC separations on a Lichrosorb C-8 reversed phase column (4.6x250 mm, 5mm) from Alltech were carried out with a Waters Assoc. 6000A pump, a Linear UV200 UV/vis detector and a Hewlett-Packard 3396A integrator. DEAE cellulose chromatography was monitored with a Pharmacia UV2 detector.

Enterobacter aerogenes 62-1 has been described by Gibson and Gibson,[20] and was kindly provided by Professor G.A. Berchtold.

Production of Chorismic Acid

The procedure used is a modification of the reported conditions.[20]

Growth Medium. A salts base was prepared which consisted of: $MgSO_4 \cdot 7H_2O$ (10 g), citric acid monohydrate (100 g), KH_2PO_4 (500 g), $NaNH_4HPO_4$ (175 g) dissolved in water (670 mL). To a solution of the salts base (200 mL) was added Difco yeast extract (20 g), Difco casamino acids (20 g), and DL-tryptophan (410 mg) and made up to 10 L with water and the solution autoclaved at 121 °C for 45 min. After cooling, a sterile 16% glucose solution (100 mL) was added.

Accumulation Medium. Na_2HPO_4 (128 g), KH_2PO_4 (13.6 g), glucose (180 g), NH_4Cl (27 g) were dissolved in water (10 L) and $MgCl_2$ (0.05 M, 20 mL), and L-tryptophan (0.01 M, 10 mL) added. The mixture was not sterilized.

An aliquot of the growth medium (100 mL) was removed and inoculated with *E. aerogenes* 62-1 by sterile loop transfer and shaken at 30 °C for 8 h at 250 rpm and then transferred to the 10 L growth medium. Cells were grown at 30 °C for 6 h in a Microferm™ 14 L fermentor with stirring at 400 rpm and aeration at a rate of 10 L / min. The cells were collected by centrifugation with a Sharples™ centrifuge, immediately resuspended in the accumulation medium, and incubated for 16 h under the same conditions as for the growth of the cells.

Isolation of Chorismic Acid, 8. After removal of the cells from the accumulation medium via centrifugation, the supernatant was made alkaline by the addition of 10 M NaOH (50 mL) and applied to a Dowex 1X4 column (100-200 mesh, Cl^- form, 5 x 10 cm) at a rate of 50 mL / minute. The operations were carried out at 4 °C. Chorismate was eluted after washing the column with water (200 mL) and then with 1 M NH_4Cl (adjusted to pH 8.5 with conc. NH_4OH). Fractions were collected at 1.5 mL / min and monitored by UV (280 nm). Those containing chorismate were pooled, acidified to pH 1.5 (conc. HCl), extracted eight times with ether, the organic phase dried over $MgSO_4$ and concentrated at 20 °C at aspirator pressure. The faint yellow solid (6.25 g) was crystallized from 1:1:2/ether : CH_2Cl_2 : hexane with cooling to -78 °C[21] to yield a white powder (5.95 g) which was stored at -80 °C: ^1H NMR (DMF-d_7 400 MHz) δ 6.83 (d, J = 1.98 Hz, 1 H), 6.33 (dt, J = 9.99, 1.84 Hz, 1 H), 6.05 (dd, J = 10.08, 3.02 Hz, 1 H), 5.44 (d, J = 2.48 Hz, 1 H), 5.04 (dd, J = 10.44, 2.92 Hz, 1 H), 4.99 (d, J = 2.47 Hz, 1 H), 4.63 (dt, J = 10.43, 2.58 Hz, 1 H); ^{13}C NMR (DMF-d_7 100.6 MHz) δ 166.38, 164.66, 151.07, 133.81, 133.39, 130.18, 121.54, 96.22, 69.84; UV max (c = 4.425 x 10^{-4} M, H_2O) 278 nm (2750).

Production and Isolation of Isochorismate Synthase

Growth of Cells. In 9.5 L of water was dissolved KH_2PO_4 (136 g), $(NH_4)_2SO_4$ (20 g), $MgSO_4 \cdot H_2O$ (2.0 g), $CaCl_2$ (0.10 g), L-phenylalanine (0.33 g), L-tyrosine (0.36 g), L-tryptophan (0.41 g), and the solution adjusted to pH 7.4 (2.5 M KOH). After autoclaving the solution at 121 °C for 45 min, a sterile glucose solution (50 g in 500 mL water) was added. An aliquot (100 mL) was removed and inoculated with *E. aerogenes* 62-1 via sterile loop transfer, and then incubated at 37 °C with shaking at 250 rpm for 8 h. The seed culture was transferred to the 10 L volume and the cells grown at 37 °C with stirring at 400 rpm and aeration at 10 L/min for 15 h. At this point, the cells were collected by centrifugation and washed with 0.05 M sodium phosphate buffer, pH 7.0 to yield 76 g of cells. After suspension in 0.01 M phosphate buffer (250 mL), the cells were sonicated 20 times for 30 s each at 70 watts, with 2-3 min cooling in an ice bath between sonications, and the cell debris removed by centrifugation (0 °C, 45 min at 38400 x g) to yield the crude cell-free extract (250 mL, 7.3 mg / mL)

DEAE Chromatography. A DEAE cellulose column (Whatman DE 52 pre-swollen, 160 g, 5 x 20 cm) was prepared and equilibrated with 0.01 M sodium phosphate buffer, and the crude cell-free extract, which was diluted with 350 mL phosphate buffer, was applied to the DEAE column at 4 mL/min. The column was washed with additional buffer (150 mL), and isochorismate synthase eluted with 0.01 M phosphate buffer which was 0.1 M in NaCl. Column fractions collected (16 mL each) were monitored by UV (254, and 280 nm), and those containing protein were assayed for isochorismate as described below. Fractions containing isochorismate synthase activity were pooled (255 mL, 0.71 mg / mL). Protein concentration was determined by the method of Lowry.[31]

$(NH_4)_2SO_4$ Precipitation. Protein obtained from the DEAE column was fractionally precipitated with solid $(NH_4)_2SO_4$ in five fractions. Thus, the DEAE eluant (200 mL) was brought to

30% saturation with $(NH_4)_2SO_4$ and the precipitate centrifuged (47500 x g, 45 min). Fractions at 46%, 62%, 77%, and 99% $(NH_4)_2SO_4$ saturation were similarly obtained. Assay by HPLC revealed most of the activity in the 30-46% and 46-62% fractions. These were dissolved in phosphate buffer (0.01 M) and pooled (8.0 mL, 13.5 mg / mL). The preparation was used without any further purification.

Assay for Isochorismate Synthase Activity

Assay mixtures consisted of a final volume of 200 μL containing 10 μmol Tris, 1 μmol Mg^{2+}, 0.5 μmol chorismate, and 25 μL of protein solution (final pH 8.0) which were incubated at 37 °C for 1 to 2 h. Samples were assayed by direct injection of 5-10 μL of the assay mixture.

Samples were applied to an Alltech analytical column packed with Lichrosorb C-8 reversed phase material (4.6 x 250 mm, 5 mm) with a C-8 guard column attached (4.6 x 100 mm, 30-40 mm), and eluted with an isocratic solvent system consisting of 9 : 1 water : methanol and 0.1% trifluoroacetic acid. A flow rate of 1.0 mL / min provided **18** at 12.7 min followed by **19** at 25.8 min. Detection was by UV with the detector set at 278 nm.

Enzymatic Synthesis and Isolation of Isochorismic Acid.

Tris base (55.4 mg, 458 μmol), $MgCl_2 \cdot H_2O$ (27.0 mg, 133 μmol), and **8** (35.0 mg, 155 μmol) were dissolved in 2.0 mL H_2O and the pH adjusted to 8.2 (1 M NaOH, 30 μL). Enzyme from the $(NH_4)_2SO_4$ precipitation (1.89 mL, 35.0 mg protein) was added to give a final volume of 4.0 mL, pH 8.0. Incubation of the mixture was performed at 37 °C for 2 h, whereupon the reaction was terminated by the addition of conc HCl (300 μL), and then saturated with NaCl. Extraction of the acidified mixture 5 times with an equal volume of ether and then 3 times with ethyl acetate, followed by evaporation of the combined organic phases at aspirator pressure and 20 °C, yielded a colorless oil. The residue was immediately dissolved in methanol (100 μL), and then diluted with water (800 μL) and filtered through a 5 μm membrane. Separation of **4** from **8** was accomplished with a semi-prep HPLC column (Whatman C-8, 10 mm Partisil™, 10 x 250 mm) eluting with 95 : 5 water : methanol, and 0.1% trifluoroacetic acid at 3.0 mL / min. Collection of **4**, eluting at 8.0 min, followed by **8** eluting at 16.0 min by repeated injections (250 μL) and lyophilization of the respective samples yielded **4** (5.5 mg) and **8** (12.9 mg). Physical data for **4**: ^1H NMR (DMSO-d_6 400 MHz) δ 6.99 (d, J = 5.56 Hz, 1 H), 6.37 (dd, J = 9.55, 5.61 Hz, 1 H), 6.25 (dd, J = 9.40, 4.91 Hz, 1 H), 5.33 (d, J = 2.65 Hz, 1 H), 5.00 (d, J = 2.65 Hz, 1 H), 4.52 (dd, J = 4.95, 0.81 Hz, 1 H), 4.46 (br s, 1 H); ^{13}C NMR (DMSO-d_6 100.6 MHz) δ 167.50, 163.87, 149.41, 131.35, 130.53, 127.77, 126.34, 95.72, 74.17, 62.12; ^1H NMR (DMF-d_7 400 MHz) δ 7.10 (d, J = 5.35 Hz, 1 H), 6.45 (dt, J = 9.40, 5.61 Hz, 1 H), 6.34 (dd, J = 9.22, 5.60 Hz, 1 H), 5.44 (d, J = 2.33 Hz, 1 H), 5.09 (d, J = 2.43 Hz, 1 H), 4.72 (m, 2 H); ^{13}C NMR (DMF-d_7 100.6 MHz) δ 168.23, 164.62, 150.83, 131.91, 131.79, 128.54, 126.89, 95.85, 75.64, 63.83. UV max (c = 4.425 x 10^{-4} M, H_2O) 278 nm (12800).

Enzymatic Synthesis of Isochorismate in $H_2{}^{18}O$

The enzymatic reaction was performed exactly as above except that $H_2{}^{18}O$ (2 mL, 98 - 99% ^{18}O) was used in place of $H_2{}^{16}O$ to give a final ratio of 50% ^{18}O labeled water. Incubation followed by isolation and HPLC purification yielded **8** (11.9 mg) and **4** (4.6 mg). The ^{13}C NMR data for **4** was obtained under the following conditions: SI = TD = 16K; sweep width = 12195 Hz; PW = 30°, RD = 0; AQ = 2.69 s; decoupler offset 8100 Hz; NS, 16323. A Gaussian multiplication was performed on the raw data (LB = -0.50, GB = 0.40) before Fourier transformation.

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