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Preparation, ^{13}C NMR Characterization, and Enzymatic Transformation of [U-^{13}C]Chorismate to [U-^{13}C]Prephenate and [U-^{13}C]Hydroxyphenyl Pyruvate[1]

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Chorismate is the common precursor of the aromatic amino acids and a variety of plant and microbial natural products. [U-^{13}C]Chorismate has been prepared from[U-^{13}C]glucose using *Klebsiella pneumoniae* 62-1. With respect to previously published procedures, our yield represents a threefold improvement. [U-^{13}C]Chorismate was purified directly from the growth medium in a single step by preparative HPLC. The ^{13}C NMR spectrum of [U-^{13}C]chorismic acid was obtained at both 75 MHz and 150 MHz (7.05 and 14.1 T). Near complete spectral assignment was achieved on the basis of chemical shifts, J_{CC}, and ^{13}C–^{1}H coupling patterns. Assignment of C2 and C5 was accomplished via spectral simulation (program courtesy of Dr. Milton Johnston, USF) and verified using homonuclear ^{13}C decoupling. Inspection of the 150-MHz spectrum allowed assignment of additional long range J_{CC}. Partially purified *Escherichia coli* chorismate mutase/prephenate dehydrogenase was used to convert [U-^{13}C]chorismate to [U-^{13}C]prephenate, which in turn was converted to [U-^{13}C] hydroxyphenyl pyruvate by the addition of NAD. ^{13}C NMR of [U-^{13}]prephenate and [U-^{13}C] hydroxyphenyl pyruvate have been assigned based on chemical shifts, J_{CC}, spectral simulation, and homonuclear ^{13}C decoupling.

INTRODUCTION

^{13}C NMR is a valuable tool for studying enzyme transformations. The ^{13}C chemical shifts and carbon–carbon coupling constants (J_{CC}) are exquisitely sensitive to hybridization changes and ^{13}C-labeled precursors can be utilized for monitoring biosynthetic processes (*1–7*). To study enzyme transformations by ^{13}C NMR, one often uses ^{13}C-labeled substrates because of the low natural abundance and sensitivity of ^{13}C. In recent years, the commercial availability of ^{13}C-labeled compounds has led to a proliferation of studies on enzyme mechanisms using ^{13}C NMR, yet many labeled compounds are prohibitively expensive or not commercially available.

In this paper, we describe an improved microbiological production (*8*) and purification of chorismate as [U-^{13}C]chorismate (**I**), the biosynthetic precursor

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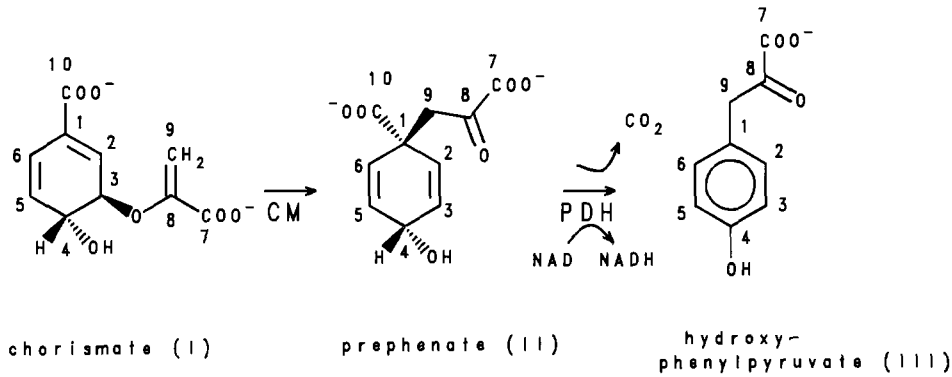

FIG. 1. Enzymatic transformation of chorismate(**I**) to prephenate(**II**) and then to hydroxyphenyl pyruvate(**III**).

of the aromatic amino acids and other natural products. Chorismate mutase-prephenate dehydrogenase (CMPDH)[3] is a bifunctional enzyme present in *Escherichia coli* and some other microorganisms. The CMPDH-catalyzed reactions are illustrated in Fig. 1; chorismate is converted to prephenate (**II**) and in the presence of NAD, prephenate (**II**) is converted to hydroxyphenyl pyruvate (**III**). The conversion of chorismate to prephenate is an unusual enzyme-catalyzed 3,3-sigmatropic electrocyclic rearrangement (*9, 10*); hydroxyphenyl pyruvate (**III**) is an immediate biosynthetic precursor of tyrosine.

Here we present and assign the ^{13}C NMR spectra of [U-^{13}C]chorismate, [U-^{13}C] prephenate, and [U-^{13}C]hydroxyphenyl pyruvate (Fig. 2), the latter two produced via the CMPDH-catalyzed reactions. Using uniformly labeled [^{13}C]glucose (98.9% enrichment) as our starting material, we have been able to assign the one-bond and long range J_{CC}s for these compounds. These studies are preliminary to the further investigation of chorismate metabolism by NMR.

MATERIALS AND METHODS

[U-^{13}C]Glucose (98.9% enriched) was purchased from Cambridge Isotope Laboratories (Woburn, MA). The *Klebsiella pneumoniae* mutant, Kp 62-1, was obtained from the American Type Culture Collection. Kp 62-1 lacks all chorismate mutase activity. HPLC purification of chorismate was carried out on a Waters 600E system equipped with a Waters 994 diode array detector using a Waters PrePak 25 × 10 μBondapac cartridge column. Analytical HPLC runs were carried out on a Waters 8 × 10 μBondapac cartridge column. All chemicals were reagent grade and the solvents used in the chromatographic purification of chorismate were HPLC grade.

[3] Abbreviations used: CMPDH, chorismate mutase-prephenate dehydrogenase; NMR, nuclear magnetic resonance; NAD, nicotinamide adenine dinucleotide; OH-ϕ-pyr, hydroxyphenyl pyruvate.

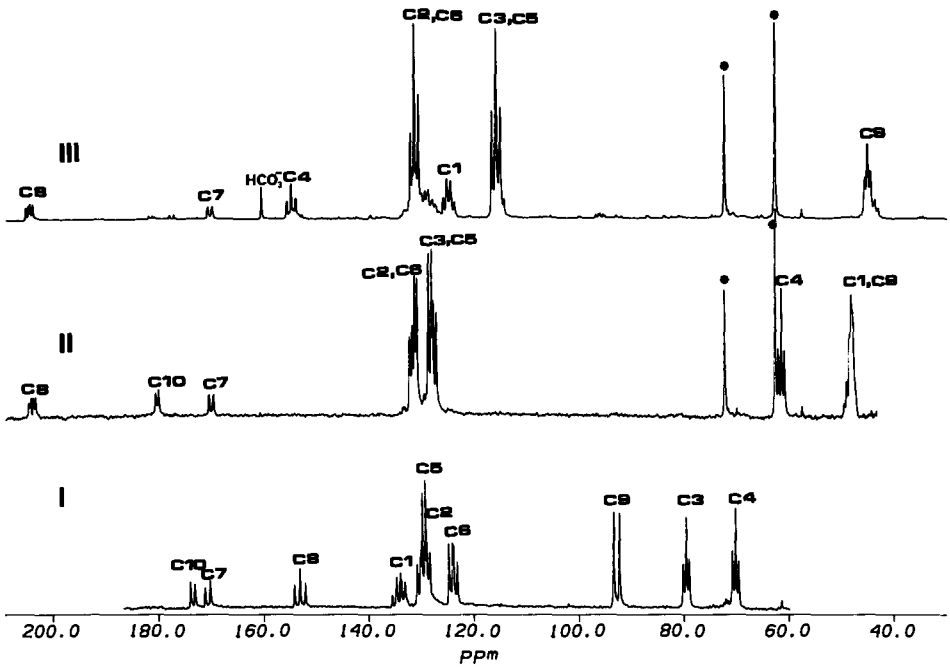

FIG. 2. {^{1}H} ^{13}C NMR spectra of **I, II,** and **III** at neutral pH. *Peaks from glycerol.

E. coli CMPDH was partially purified from an overproducing strain (*11*). Yeast and casamino acids were purchased from Difco.

Preparation and Purification of [U-^{13}C]Chorismate

[U-^{13}C]Chorismate was prepared from the culture filtrates of Kp 62-1 containing [U-^{13}C]glucose by modifying the method of Gibson *et al.* (*12*). In view of the cost of the starting material, we chose to optimize the growth variables to maximize the yield of chorismate. Several experimental factors such as cell density, concentration of glucose, degree of aeration, and time of incubation of glucose in Kp 62-1 culture suspension were varied to improve the yield of chorismate. A typical improved preparation of chorismate is as follows: 10 ml of Luria broth in a 25-ml Erlenmeyer is inoculated with a loopful of Kp 62-1 glycerol freeze and shaken at 30 ± 1°C overnight. Growth medium A contains 2.1 g K_2HPO_4, 0.9 g KH_2PO_4, 0.2 g $(NH_4)_2SO_4$, 0.1 g sodium citrate, 0.4 g yeast, 0.4 g casamino acids, 0.4 g glucose, and 8.2 mg tryptophan per 200 ml. Growth medium A is autoclaved prior to addition of filter-sterilized glucose and tryptophan. Medium A is inoculated with 0.8 ml of the starter culture and shaken in a 1000-ml Erlenmeyer for 8–10 h at 30 ± 1°C, at which point the A_{620} of the culture suspension is 1.9–2.1. Medium B contains 5.12 g Na_2HPO_4, 0.54 g KH_2PO_4, 1.08 g NH_4Cl, 8.7 mg $MgCl_2$, and 1.24 mg tryptophan per 400 ml and is not sterilized before use. The culture suspension in medium A is centrifuged at 5K for 20 min; the pellet is suspended in 200 ml of

medium B. This is centrifuged as above and the pellet is resuspended in the remaining 200 ml of medium B to which 1 g of [U-^{13}C]glucose is added. The culture suspension is divided equally into two 1000-ml Erlenmeyer flasks and shaken at 30 ± 1°C for 10–12 h during which time [U-^{13}C]chorismate is excreted from the cells into the medium. The bacteria are centrifuged at 5K for 20 min and the supernatant is filtered through a 0.45 or 0.2-μm nylon membrane. At this point, the crude yield of chorismate, as determined by quantitative chorismate mutase assay (*13*), (282 nm, $\Delta\varepsilon_{(\text{chorismate-prephenate})} = 2.235\ \text{mm}^{-1}\ \text{cm}^{-1}$) is 55–60 mg/l g of glucose.

Chorismate can be directly and quantitatively purified from the spent growth medium using preparative reverse phase HPLC with no preliminary purification. The HPLC procedure is similar to the method Connelly and Siehl used to purify crystalline chorismate (*14*). Throughout the procedure, the sample, solvents, and eluent are kept on ice. After removal of the bacteria, the pH of the spent growth medium (sample) is adjusted to 2.5 and purified in 50-ml aliquots. The sample is loaded on the column at 8 ml/min and washed with 30 ml of 0.1% H_3PO_4 at 3 ml/min followed by a 5-min linear gradient to 15% acetonitrile, 85% of 0.1% H_3PO_4 which continues isocratically for 30 min. The chromatograms were monitored at 274 and 315 nm and the peak corresponding to chorismate (36.5–39.7 min) is collected. The acetonitrile is removed *in vacuo*. The pH of the purified chorismate solution can be adjusted to 7.5, aliquoted, and frozen at −70°C for future use in CMPDH assays. Otherwise, the pH of the aqueous solution is adjusted to 1.9 and chorismate is extracted four times into equal volumes of diethylether with >95% efficiency. The ether layer is dried over anhydrous $MgSO_4$ and rotoevaporated. Chorismate thus obtained is >95% pure as estimated by HPLC. If further purification is necessary, the sample can be loaded back onto the column after completely removing acetonitrile from the fraction corresponding to chorismate. This method uses less than one-third the glucose as in the large scale preparation (*12*) without compromising the yield of chorismate.

Enzymatic Transformation of [U-^{13}C]Chorismate to [U-^{13}C]Prephenate and [U-^{13}C]Hydroxyphenyl Pyruvate

[U-^{13}C]Chorismate, 5 mg in 20 mM KP_i buffer (1.8 ml, 10% D_2O) containing 0.5 mM EDTA and 2.5 mM β-mercaptoethanol was converted to [U-^{13}C]prephenate using 0.5 units of partially purified CMPDH (sp act 2.3 μmol/min/mg) (*11*). The chorismate sample containing CMPDH was incubated at 30°C for 18 min. The NMR sample contained 2.6% of glycerol derived from the buffer containing CMPDH. Based on ^{13}C spectra chorismate was quantitatively converted to prephenate. Conversion of prephenate to hydroxyphenyl pyruvate was achieved by adding a 10% molar excess of β-NAD and incubating the NMR sample at 30°C for 40 min. The sample remained at 4°C for around 10.5 h before spectral acquisition.

^{13}C NMR Studies of [U-^{13}C]Chorismate, [U-^{13}C]Prephenate and [U-^{13}C]Hydroxyphenyl Pyruvate

All the {^{1}H} ^{13}C (at 75 or 150 MHz) and {^{1}H, ^{13}C} ^{13}C NMR (at 75 MHz) spectra were recorded using Bruker AM series spectrometers at 15°C. Initial ^{13}C NMR

TABLE 1

^{13}C Chemical Shifts and J_{CC}s of Chorismate (**I**), Prephenate (**II**), and Hydroxyphenyl Pyruvate (**III**)

Compound	Chemical shift (ppm)									
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
I[a]	129.5	134.0	79.4	69.6	131.2	121.9	167.0	148.8	97.7	168.5
I[b]	134.5	129.2	79.7	70.3	130.2	124.1	170.8	153.2	93.0	173.6
II	48.6 (48.7)	131.3	127.7	61.5	127.7	131.3	169.7	203.5	47.9 (48.1)	179.9
III	124.8	131.3	115.8	154.8	115.8	131.3	170.3	204.6	45.1	160.5[c]

[a] [U-^{13}]Chorismic acid at 15°C.
[b] [U-^{13}C]Chorismate in 20 mM kP_i buffer (pH 7.0) containing 0.5 mM EDTA and 2.5 mM β-mercaptoethanol.
[c] Assigned to bicarbonate released from oxidative decarboxylation of prephenate (see text).

studies on chorismate were performed in its acid form because it is more stable than its conjugate base. [U-^{13}C]Chorismic acid (8.6 mg) was dissolved in 1.8 ml water containing 10% D_2O and the spectra were obtained in an 8-mm NMR tube. Samples **I, II,** and **III** at neutral pH were prepared as above. Chemical shifts are reported using dioxane as external reference at 67.4 ppm.

^{13}C NMR of chorismate was obtained at 75 MHz with a spectral width of 9615 Hz, pulse width of 7 μs, 0.59 Hz/point resolution, and 128 scans. ^{13}C NMR spectra prephenate and hydroxyphenyl pyruvate were recorded with widths of 12,500 and 15,625 Hz and a spectral resolution of 0.76 Hz/point and 0.95 Hz/point, respectively. The number of scans varied from 120 to 1000 scans depending on the desired signal to noise ratio. Data was processed on a VAX computer using the FTNMR program of Hare Research Inc.

RESULTS AND DISCUSSION

Assignment of the ^{13}C Spectrum of [U-^{13}C]Chorismic Acid

The initial ^{13}C NMR characterization of [U-^{13}C]chorismate used the acid form because of its greater stability relative to the conjugate base. Spectra were assigned on the basis of ^{13}C–^{1}H coupling patterns, ^{13}C chemical shifts, and ^{13}C–^{13}C coupling constants (see Table 1). All resonances of [U-^{13}C]-chorismic acid could be assigned with the exception of C2 and C5 because of their nearly identical coupling constants to C1 and C5, respectively. To assign C2 and C5 we simulated the ^{13}C NMR spectrum using an IBM PC basic program, courtesy of Milton Johnston, University of Southern Florida. To carry out the simulation we used the coupling constants obtained from the spectrum and alternated the chemical shifts of C2 and C5. As shown in Fig. 3, only one set of chemical shifts, i.e., 134.0 ppm for C2 and 131.2 ppm for C5, not vice versa) match the observed spectral pattern. The assignments were confirmed using {^{1}H, ^{13}C} ^{13}C triple resonance studies. As shown in Fig. 4, decoupling C4 affects the C3 and C5 resonances, which are strongly coupled to

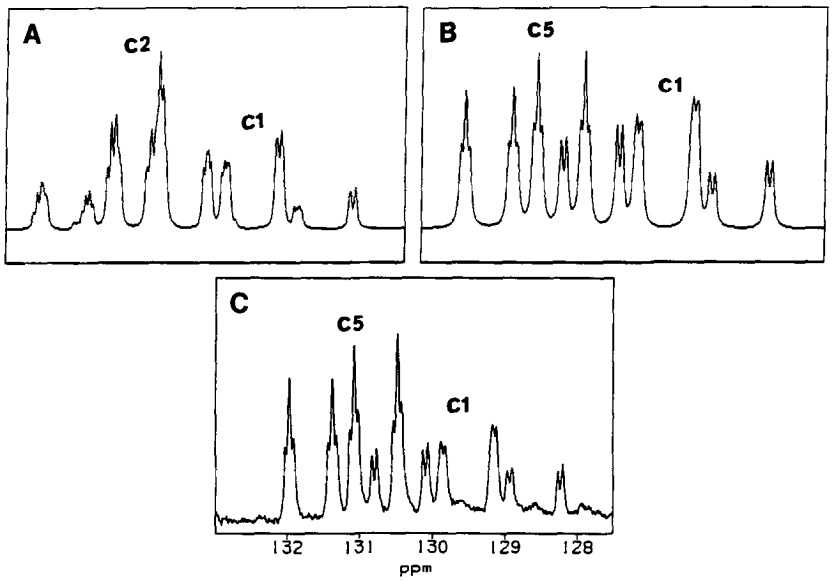

FIG. 3. Simulation of $\{^1H\}$ ^{13}C NMR spectrum between 127.5 and 132.5 ppm. Spectral parameters of: (A) Chemical shifts of C1 = 129.5 ppm, C2 = 131.2 ppm, and C5 = 134.0 ppm. (B) Chemical shifts of C1 = 129.5 ppm, C2 = 134.0 ppm, and C5 = 131.2 ppm. The other nuclear spins included in the simulation are C3, C4, C6, and C10 and J_{CC}'s from Table 1 are used. (C) Observed $\{^1H\}$ ^{13}C spectrum.

C4 and the fine coupling to C1 collapses. This also confirms the assignment of C2 and C5.

Assignment of the ^{13}C Spectrum of [U-^{13}C]Chorismate at Neutral pH

The ^{13}C NMR spectrum of chorismate (**I**) at pH 7.0 at 75 MHz (Fig. 2) has been assigned on the basis of chemical shifts and J_{CC}s and the assignments confirmed using $\{^1H, ^{13}C\}$ ^{13}C triple resonance experiments. ^{13}C NMR chemical shifts of [U-^{13}C]chorismate are sensitive to pH changes as shown in Table 1 and therefore resonances must be assigned with caution. For example, assignments of C1 and C2 resonances at acidic pH are almost reversed at neutral pH. The ^{13}C resonances of α and β (unconjugated) carbons to ionizable carboxylic acid groups shift downfield and conjugated β carbon resonances shift upfield. The chemical shifts of C7 and C10 show an expected downfield chemical shift change (*15*). The one-bond coupling constants ($^1J_{CC}$s) (Table 2) remain virtually unchanged at neutral pH compared to acidic pH, except for $^1J_{C1,C10}$ and $^1J_{C7,C8}$; the latter decrease by ~7.0 Hz due to ionization of carboxylic acids (*15*). By comparison, ionization of acetic acid decreases the J_{CC} by 5.1 Hz.

Assignment of the ^{13}C Spectrum of [U-^{13}C]Prephenate

Partially purified *E. coli* CMPDH was utilized in the conversion of [U-^{13}C]chorismate to [U-^{13}C]prephenate (**II**) (see Materials and Methods). Assignments of ^{13}C spectra were done using the above-mentioned techniques. Zamir *et al.* have as-

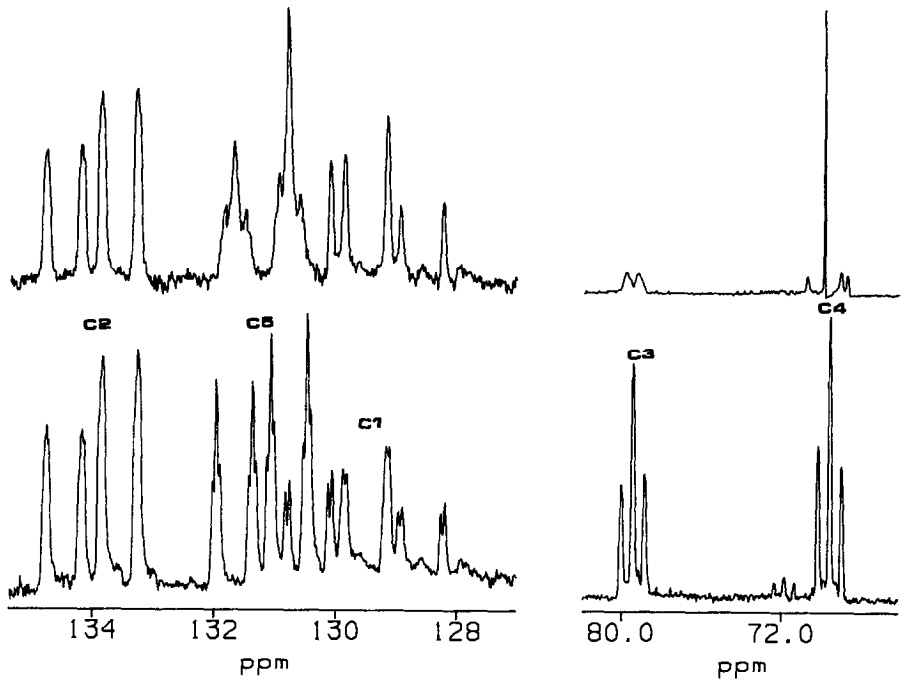

FIG. 4. {^{1}H, ^{13}C} ^{13}C NMR spectrum of chorismic acid. Bottom: {^{1}H} ^{13}C spectrum of chorismic acid showing C1, C2, C3, C4, and C5 resonances. Top: Irradiation of C4 affects strongly coupled C3 and C5 resonances and long range coupled C1 resonance.

signed the ^{13}C NMR spectrum of unlabeled disodium prephenate by comparison with the ^{13}C NMR spectrum of arogenate (*16*). Based on {^{1}H, ^{13}C} ^{13}C triple resonance studies on ^{13}C-labeled prephenate their assignments of C2(C6) and C3(C5) have to be reversed. In the case of prephenate, there is a heavy overlap between the C1 and the C9 resonances and the spectral pattern is complicated by the fact that C1 and C9 are strongly coupled. Moreover, C1 is coupled to three other carbons and long range coupled to C4. The spectral simulation program we used is limited to seven nuclear spins. Therefore, only approximate values for chemical shift of C1 and $J_{C1,C9}$ are included. However, C1 and C9 chemical shifts of unlabeled prephenate are shown in brackets. Although the coupling constants could not be measured, there is very fine long range coupling between C2(C6) and C10, C2(C6) and C4 and C3(C5) and C1, in addition to the ones shown in Table 2 as deduced from {^{1}H, ^{13}C} ^{13}C triple resonance studies.

Assignment of the ^{13}C Spectrum of Hydroxyphenyl Pyruvate

All the ^{13}C resonances of [U-^{13}C]hydroxyphenyl pyruvate could be assigned on the basis of chemical shifts and coupling constants. C2 and C6 resonances are degenerate as are those of C3 and C5, and fall within the expected chemical shift ranges (*15*). As seen in the case of prephenate $^3J_{C7,C9}$ is high because of the electron-

TABLE 2

J_{CC}s of Chorismate (**I**), Prephenate (**II**), and Hydroxyphenyl Pyruvate (**III**)

Compound	Coupling constants (J_{CC})		
	$^1J_{CC}$[a]	$^2J_{CC}$[b]	$^3J_{CC}$[c]
I[a]	$J_{1,2}$ = 69.4, $J_{1,6}$ = 53.6, $J_{1,10}$ = 72.4, $J_{2,3}$ = 44.6, $J_{3,4}$ = 44.6, $J_{4,5}$ = 44.6, $J_{5,6}$ = 67.2, $J_{7,8}$ = 81.5, $J_{8,9}$ = 81.5	$J_{2,10}$ = 2.6, $J_{6,10}$ = 3.0, $J_{7,9}$ = 6.0	$J_{1,4}$ = 5.3, $J_{2,5}$ = 4.2, $J_{3,6}$ = 3.8, $J_{3,7}$ = 2.6, $J_{3,9}$ = 3.4, $J_{3,10}$ = [c], $J_{4,8}$ = 3.1, $J_{5,10}$ = 4.2
I[b]	$J_{1,2}$ = 69.8, $J_{1,6}$ = 51.7, $J_{1,10}$ = 64.9, $J_{2,3}$ = 44.6, $J_{3,4}$ = 44.2, $J_{4,5}$ = 44.9, $J_{5,6}$ = 67.2, $J_{7,8}$ = 74.3, $J_{8,9}$ = 80.4	$J_{2,10}$ = 2.3, $J_{6,10}$ = 1.9, $J_{7,9}$ = 6.0	$J_{1,4}$ = 4.5, $J_{2,5}$ = 4.4, $J_{3,6}$ = 4.5, $J_{3,7}$ = 4.5, $J_{3,9}$ = 4.5, $J_{3,10}$ = [c], $J_{4,8}$ = 2.3, $J_{5,10}$ = 4.2,
II	$J_{1,2}$ = 38.1, $J_{1,6}$ = 38.1, $J_{1,9}$ = 33.0–37.5, $J_{1,10}$ = 45.3, $J_{2,3}$ = 69.1, $J_{4,5}$ = 44.9, $J_{3,4}$ = 44.9, $J_{5,6}$ = 69.1, $J_{7,8}$ = 61.9, $J_{8,9}$ = 37.4	$J_{7,9}$ = 12.8	$J_{4,1}$ = 4.5
III	$J_{1,2}$ = 57.7, $J_{1,6}$ = 57.7, $J_{1,9}$ = 44.9, $J_{2,3}$ = 57.7, $J_{3,4}$ = 65.3, $J_{4,5}$ = 65.3, $J_{5,6}$ = 57.7, $J_{7,8}$ = 62.6, $J_{8,9}$ = 37.4	$J_{7,9}$ = 12.8	$J_{4,1}$ = 9.1

[a] See footnote to Table 1.
[b] See footnote to Table 1.
[c] C3 and C10 resonances are too broad to obtain $J_{3,10}$.

deficient carbonyl group between C7 and C9. On the basis of the $\{^1H\}$ ^{13}C coupling pattern of C9 and also $\{^1H, ^{13}C\}$ ^{13}C triple resonance studies, C2(C6) and C3(C5) are spin coupled to C9 and the coupling constants range from 3.0 to 3.8 Hz. There are several other small peaks in the spectrum which probably arise from the slow degradation of hydroxyphenyl pyruvate. The resonance at 160.5 is assigned to bicarbonate, released as a byproduct from the enzyme-catalyzed oxidative decarboxylation of prephenate.

The ^1H NMR spectra of chorismate at acidic and neutral pH in water (*17*) suggest that the major conformer in solution is in pseudodiequatorial form; approximately 12–17% is present in the pseudodiaxial form. Molecular orbital calculations by Andrews *et al.* (*18*) show that chorismate is predominantly in the diequatorial form at room temperature; the energy difference between the axial and the equatorial conformers is 7 kcal/mol. $\{^1H\}$ ^{13}C NMR spectra of [U-^{13}C]chorismate at 15°C also suggest the presence of only one form, which is the diequatorial form. If there is a change in conformation one would expect a much larger change in the chemical shifts of C3 and C4 upon going to neutral pH. Table 2 shows that chemical shifts of C3 and C4 of chorismic acid and potassium chorismate (pH 7) do not differ

much. Therefore under physiologic conditions chorismate is most probably in the lower energy diequatorial form.

This work lays the foundation for further NMR studies of the metabolism of chorismate. In bacteria chorismate is further metabolized to *p*-hydroxybenzoate, *p*-aminobenzoate, the isoprenoid quinones, and the folate cofactors (*19*). In fungi and plants the chorismate-derived natural products also include coumarins, flavonoids, betalains, lignin precursors, and a variety of alkaloids and quinones (*20*).

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