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MICROBIAL PRODUCTION AND NMR CHARACTERIZATION OF [2,6,9-^{13}C]CHORISMATE AND [1,3,5,8-^{13}C]CHORISMATE

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Abstract: Chorismate is a biosynthetic precursor of the aromatic amino acids and many natural products. [2,6,9-^{13}C]Chorismate and [1,3,5,8-^{13}C]chorismate have been prepared microbially from [1-^{13}C]glucose and [2-^{13}C]glucose, respectively, using the culture filtrates of *Klebsiella pneumonia* strain 62-1. This is an inexpensive, time saving and easy method in comparison to other published methods for the preparation of selectively ^{13}C-labelled chorismate.

Chorismate is a versatile biosynthetic precursor in the shikimic acid pathway in microorganisms and higher plants.[1-4] The carbon skeleton of chorismate derives from one molecule of erythrose-4-phosphate (E4P) and two molecules of phosphoenolpyruvate (PEP) as illustrated in Figure 1. The conversion of chorismate to prephenate, an unusual enzyme-catalyzed 3,3-sigmatropic rearrangement, is the first committed step in the biosynthesis of phenylalanine and tyrosine. Tryptophan is also derived from chorismate via anthranilic acid. These aromatic amino acids in turn lead to the biosynthesis of a variety of natural products such as alkaloids,[5] coumarins, flavanoids,[6] and lignin precursors.[7] Chorismate is also a precursor to p-aminobenzoate and p-hydroxybenzoate which are in turn precursors for folate cofactors and ubiquinone respectively.

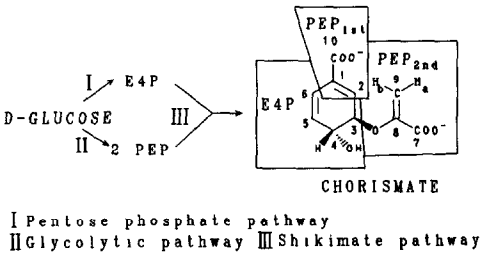


Figure 1: The biosynthesis of chorismate

Chorismic acid has been an immensely popular compound in bioorganic chemistry and yielded itself to total synthesis (as a racemic acid) a decade ago.[8] Unlabelled chorismate is routinely prepared from glucose using the culture filtrates of *Klebsiella pneumoniae* strain 62-1 (Kp62-1), a mutant which lacks chorismate mutase activity, as described by Gibson.[9] A stationary phase culture of Kp62-1 established in rich growth media can be induced to accumulate chorismate when transferred to a defined medium containing glucose as the predominant carbon source with L-tryptophan present to inhibit anthranilate synthase activity. Recently, we have improved this method with a three-fold increase in the yield of chorismate and used [U-^{13}C]glucose in the preparation of [U-^{13}C]chorismate.[10]

^{13}C-labelled compounds are essential to the ^{13}C NMR study of enzyme mechanisms and biosynthetic pathways. Completely ^{13}C-labelled compounds are not often used in such studies because an extensive carbon-carbon coupling network complicates the spectroscopy. Additional limitations are imposed by the commercial availability and cost of ^{13}C-labelled starting materials. In the absence of an appropriate total synthesis for optically pure chorismate, synthetic procedures have been coupled with enzyme-catalyzed reactions in the preparation of selectively isotopically labelled chorismate.[11,12] These procedures rely on the biosynthesis of chorismate from E4P and PEP (Figure 1), neither of which

is commercially available as a ^{13}C-labelled compound. For example, labelled E4P can be prepared by the lead tetraacetate oxidation of labelled glucose-6-phosphate which in turn is prepared enzymatically from labelled glucose using hexokinase.[11] Here we report a novel application of our improved method[10] which uses commercially available mono-^{13}C-labelled glucose in a straightforward microbial preparation of multiply ^{13}C-labelled chorismate. These ^{13}C-labelled compounds may prove useful in studies of chorismate metabolism.

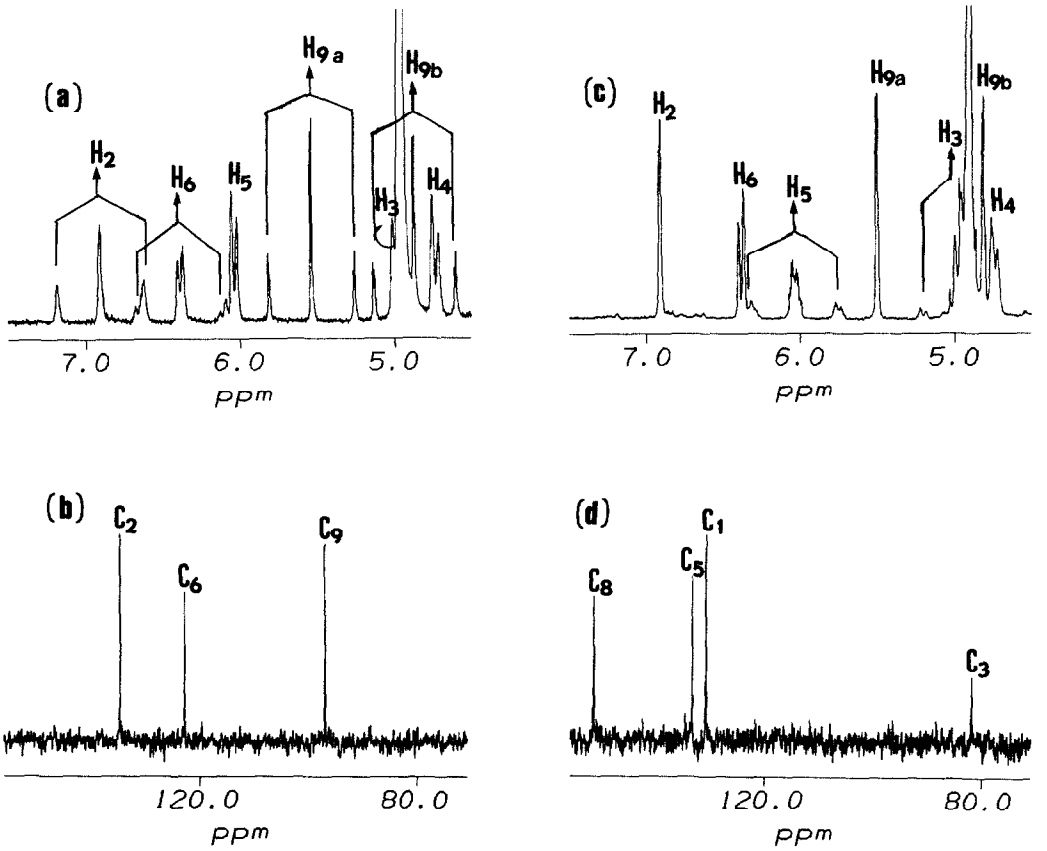
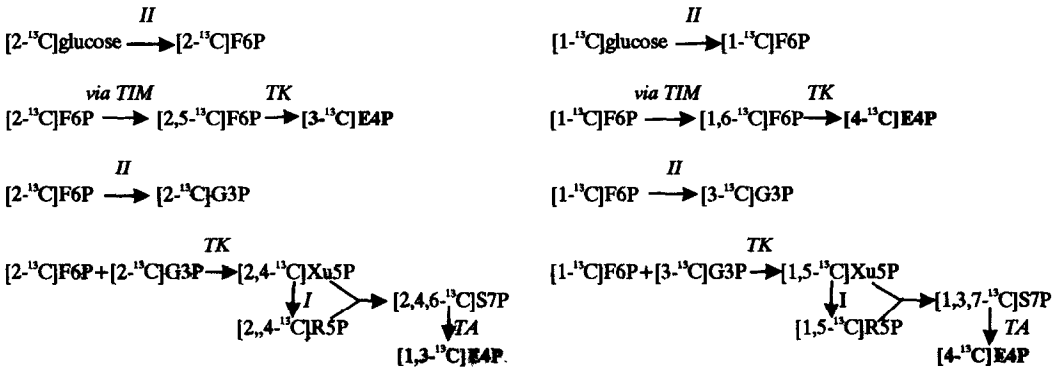


Figure 2: NMR spectra of [2,6,9-^{13}C]chorismate (a=^{1}H, b=^{13}C) and [1,3,5,8-^{13}C]chorismate (c=^{1}H, d=^{13}C)

[2,6,9-^{13}C]chorismate was prepared from 99% [1-^{13}C]glucose using Kp62-1 and purified directly from the growth medium by HPLC according to our published method.[10] The ^{1}H and ^{13}C NMR spectra of the product were obtained on a 7T spectrometer (Bruker AM300) and are presented in Figures 2a and 2b.[13,14] The satellites in the proton spectrum (which correspond to a proton attached to ^{13}C) were integrated with respect to the center resonance (which corresponds to a proton attached to ^{12}C) to calculate the absolute ^{13}C enrichment (see Figure 2a). The H_{9a} resonance could be integrated accurately, because it does not overlap with other resonances; C_9 has 34% absolute enrichment. Inverse gated decoupling was used to quantify the ^{13}C NMR resonances[15] (Figure 2b); the relative ^{13}C enrichment of C_2, C_6, and C_9 is 1.5:1.0:1.4. Therefore, the absolute ^{13}C enrichments at C_2 and C_6 are 36% and 24% respectively.

[1,3,5,8-^{13}C]chorismate was prepared analogously from [2-^{13}C]glucose. The proton NMR spectrum of [1,3,5,8-^{13}C]chorismate is shown in Figure 2c.[16] The absolute ^{13}C enrichment of C_5 as calculated by integration of the upfield H_5 satellite is 32%. One of the ^{13}C satellites of the H_3 resonance is visible, whereas the ^{12}C-H_3 resonance falls too close to the water signal to be accurately integrated. The ^{13}C NMR spectrum of [1,3,5,8-^{13}C]chorismate,[15] as illustrated in Figure 2d, shows the four resonances from C_1, C_3, C_5 and C_8 in a ratio of 1.1:0.3:0.7:1.0.[17] Therefore, the absolute enrichment at C_1, C_3, C_5, and C_8 are 50%, 14%, 32%, and 46% respectively.

The formation of [1,3,5,8-^{13}C] chorismate from 99% [2-^{13}C]glucose was initially surprising due to the significant enrichment at C_3 and C_5. Based on the pentose phosphate pathway, C_1-C_4 of E4P arises from C_3-C_6 of glucose and thus C_3-C_6 of chorismate would not be predicted to contain any label (see Figure 1). On the other hand, the glycolytic pathway divides each [2-^{13}C]glucose into two equivalent PEP molecules, each expected to contain ~50% ^{13}C label at C_2. Therefore, a straightforward prediction is that [2-^{13}C]glucose would result in the formation of [1,8-^{13}C]chorismate containing 50% ^{13}C-label at C_1 and C_8. To rationalize the existence of ^{13}C at C_3 and C_5, the ^{13}C must derive from [1-^{13}C]E4P and [3-^{13}C]E4P and must arise through the combined triosephosphate isomerase [TIM], transketolase [TK], and transaldolase [TA] catalyzed reactions as illustrated in Scheme 1. By a similar analysis, a straightforward prediction is that ~50% [2,9-^{13}C]chorismate would arise from 99% [1-^{13}C]glucose. The observed ^{13}C label at C_6 must come from [4-^{13}C]E4P whose production is rationalized in Scheme 1. By analogy [3-^{13}C]glucose is predicted to yield [3,4,7,10-^{13}C]chorismate.



F6P, fructose-6-P; G3P, glyceraldehyde-3-P; E4P, erythrose-4-P; Xu5P, xylulose-5-P; R5P, ribose-5-P; S7P, sedoheptulose-7-P; *I*, pentose phosphate pathway; *II*, glycolysis; *TIM*, triose phosphate isomerase; *TA*, transaldolase; *TK*, transketolase.

Scheme 1

One probable application of ^{13}C-labelled chorismate is the preparation of its ^{13}C-labelled metabolites. For example, we have used [2,6,9-^{13}C]chorismate in the preparation of [2,6,9-^{13}C]prephenate via the chorismate mutase-catalyzed reaction[11] as shown in Figures 3a and 3b.[18] The H_2 and H_6 resonances of prephenate are degenerate; the absolute ^{13}C enrichment on C_2/C_6 is 60% and C_9 is 40% based on the ^{1}H proton spectrum.

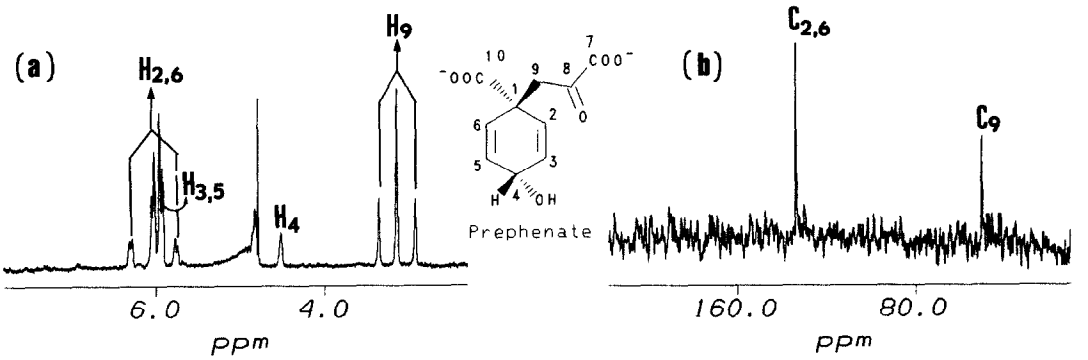


Figure 3: The ^{1}H (a) and ^{13}C (b) spectra of [2,6,9-^{13}C]prephenate

Acknowledgments

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13. The NMR data were processed on a Silicon Graphics 4D-35 using the program Felix 2.0 (Hare Research, Inc.). ^{1}H NMR assignments follow Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1987,** *109,* 5008. ^{13}C NMR assignments follow Rajagopalan et al.[10]
14. [2,6,9-^{13}C]Chorismate ^{1}H NMR chemical shifts (relative to d4-methanol at δ 3.35): H_2 δ 6.91, H_6 δ 6.39, H_5 δ 6.04, H_{9a} δ 5.54, H_{9b} δ 4.87, and H_4 δ 4.74; One bond carbon-proton coupling constants: H_2-C_2 168 Hz, H_6-C_6 162 Hz, H_{9a}-C_9 165 Hz, and H_{9b}-C_9 159 Hz. ^{13}C NMR chemical shifts (relative to d4-methanol at δ 49.0): C_2 δ 134.54 C_6 δ 122.76 C_9 δ 97.14.
15. The amount of ^{13}C labeling was quantitatively estimated using inverse gated decoupling and a relaxation delay of 140 s between pulses, during which the decoupler was gated off, for complete recovery of quarternary carbon signals. The samples were 9 mM in concentration, and a 5 mM broad band probe was used. The other spectral parameters include a spectral width of 15625 Hz, 64 K data points, composite pulse decoupling, and a 90° pulse width of 9 μs.
16. [1,3,5,8-^{13}C]chorismate ^{1}H NMR chemical shifts (relative to d_4-methanol), see ref. 14 and H_3 δ4.98. One bond carbon-proton coupling constants: H_5-C_5 168 Hz; H_3-C_3 138 Hz.
17. [1,3,5,8-^{13}C]chorismate ^{13}C NMR chemical shifts (relative to d_4-methanol at δ 49.0): C_1 δ 130.90 C_3 δ 81.86 C_5 δ 133.45 C_8 δ 151.63.
18. [2,6,9-^{13}C]Chorismate in 50 mM Kpi (D_2O) at pH 8.0 was converted to [2,6,9-^{13}C]prephenate using a catalytic amount of *B. subtilis* chorismate mutase. ^{1}H NMR chemical shifts of [2,6,9-^{13}C]prephenate (relative to external TSP at δ 0.0): H_2/H_6 δ 6.03; H_5 δ 5.93; H_4 δ 4.52; and H_9 δ 3.14; one bond carbon-proton coupling constants: H_2/H_6-C_2/C_6 165 Hz, H_9-C_9 131 Hz. ^{13}C NMR chemical shifts of [2,6,9-^{13}C]prephenate (relative to external TSP at δ 0.0) C_2/C_6 δ 134.04, and C_9 δ 50.62.