

SYNTHESIS OF SHIKIMIC-6-<sup>13</sup>C ACID

Ulrich Hollstein, David L. Mock and Randy R. Sibbitt  
Department of Chemistry, The University of New Mexico  
Albuquerque, New Mexico 87131 U.S.A.

and

Uta Roisch and Franz Lingens  
Institut für Mikrobiologie, Universität Hohenheim,  
7000 Stuttgart 70, Federal Republic of Germany

SUMMARY

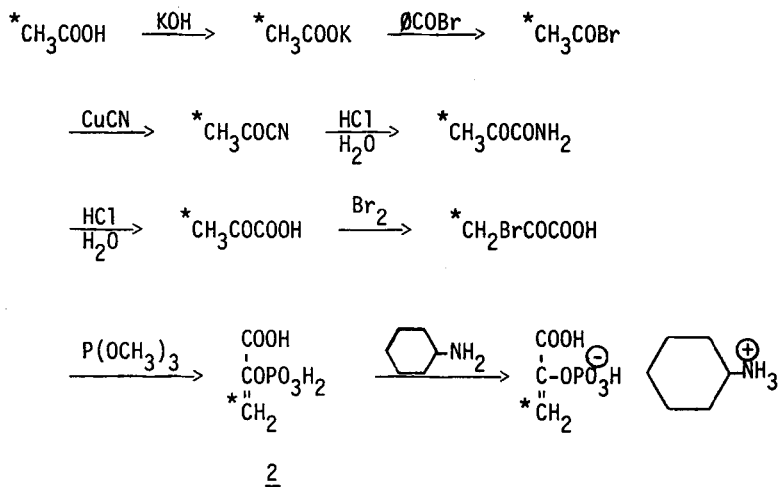
Shikimic-6-<sup>13</sup>C acid,  $43 \pm 5\%$  enriched, was synthesized from 91% enriched acetic-2-<sup>13</sup>C acid via 91% enriched phosphoenolpyruvic-3-<sup>13</sup>C acid. The latter intermediate was condensed with erythrose-4-phosphate by a cell free extract of *E. coli* 83-24.

Key words: Shikimic acid, carbon-13 NMR

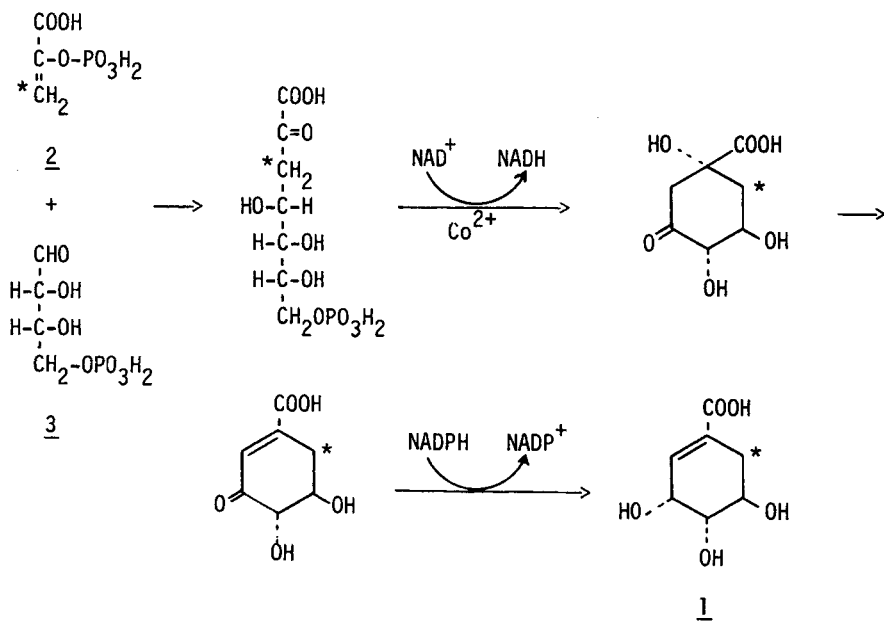
Shikimic acid (1) is an important intermediate along the aromatic biosynthetic pathway. It is, among others, a precursor of phenazine antibiotics, the iodinin representative of which we recently studied. The investigation involved the particular way in which two shikimic acid molecules paired to give the phenazine skeleton<sup>1</sup> and shikimic-6-<sup>13</sup>C acid had to be synthesized for this work.

By an improved procedure, adapted from the literature, acetic-2-<sup>13</sup>C acid, 91.92% enriched, was converted via its potassium salt with benzoyl bromide into acetyl-2-<sup>13</sup>C bromide.<sup>2</sup> The acid bromide was treated with cuprous cyanide to give pyruvonitrile-3-<sup>13</sup>C<sup>2</sup> which was hydrolyzed with aqueous hydrochloric acid to pyruvamide-3-<sup>13</sup>C<sup>2</sup> and then to pyruvic-3-<sup>13</sup>C acid. The pyruvic acid was brominated to bromopyruvic-3-<sup>13</sup>C acid<sup>3</sup> and phosphorylated with trimethyl phosphite to yield phosphoenolpyruvic-3-<sup>13</sup>C acid<sup>4</sup> (2), which was converted

to its analytically pure monocyclohexylammonium salt in 20-30% overall yield from AcOH. Its  $^{13}\text{C}$ -NMR spectrum confirmed this structure and the compound showed 91.9% enrichment.



Srinivasan, et al.<sup>5</sup> had shown that cell-free extracts of *Escherichia coli* mutant 83-24 (blocked after shikimic acid) converted erythrose-4-phosphate (3) and phosphoenolpyruvate (2) to shikimate (1).



Phosphoenolpyruvic-3-<sup>13</sup>C acid (2) was condensed with unlabeled erythrose-4-phosphate with a cell-free extract of *E. coli* 83-24 in a two hour incubation according to the method of Scharf and Zenk<sup>6</sup>. The enzymatic reaction was scaled up 45 times. The resulting shikimic-6-<sup>13</sup>C acid was isolated by column chromatography and thin layer chromatography. The overall yields varied considerably from incubation to incubation. Usually about 40-60% of pure material was isolated based on phosphoenolpyruvic-3-<sup>13</sup>C acid.

During the synthesis the <sup>13</sup>C enrichment in shikimic acid had dropped to about 50%. This loss of label could be explained by reactions possible in an aged cell-free extract of *E. coli*. Competing unlabeled phosphoenolpyruvate may be synthesized (1) by the glucose-6-phosphate degradation, and (2) by phosphorylation of unlabeled pyruvate (a main product of the metabolic pool) carried out by pyruvate orthophosphate dikinase:



Also there could be some loss of labeled phosphoenolpyruvate by the pyruvate kinase reaction:



The actual reason for this loss of label was not examined.

#### EXPERIMENTAL

Phosphoenolpyruvic-3-<sup>13</sup>C acid -- Potassium acetate-2-<sup>13</sup>C, 6.89 g (70 mmol), 91.92% enriched, was mixed with 8.55 g (70 mmol) benzoic acid, pulverized, and placed in a distillation flask. Benzoyl bromide, 35 ml (297 mmol) was added and a glass wool plug was placed in the side arm. The flask was slowly heated, whereupon a slow and steady evolution of HBr and distillation of acetyl bromide-2-<sup>13</sup>C occurred at 72-76°C. Yield 6.47 to 7.66 g (75-90%). Dry cuprous cyanide, 11.14 g (124 mmol), was placed in a 125 ml Erlenmeyer flask and 7.64 g (62 mmol) freshly distilled acetyl-2-<sup>13</sup>C bromide was added and enough dry hexane was added to cover the now turquoise cuprous cyanide. The flask was filled with N<sub>2</sub> and sealed. A few minutes rotation ensured mixing of the contents

and the flask was left in the dark for three days at r.t., whereupon the contents were filtered through a fine fritted funnel and washed with absolute ether. Although this ethereal solution of pyruvitrile-3- $^{13}\text{C}$  was used in the next step, in blank runs pyruvitrile was isolated by distillation at  $92^\circ\text{C}$ , yielding 2.14 to 2.57 g (50-60%).

The approximately 110 ml of the above solution of pyruvitrile-3- $^{13}\text{C}$  in ether was saturated at  $0^\circ\text{C}$  with hydrogen chloride. Assuming 80% of pyruvitrile-3- $^{13}\text{C}$  in the previous reaction (50 mmol), one ml (55 mmol) of distilled water was added slowly and saturation with hydrogen chloride at  $0^\circ\text{C}$  was continued for another 30 min, whereupon white crystals of pyruvamide-3- $^{13}\text{C}$  formed. The crystals were collected on a coarse fritted funnel and washed with 75 ml of a chilled mixture of ether/cyclohexane 5:1, saturated with hydrogen chloride. The crystals were freed from hydrogen chloride by a stream of nitrogen. Yield 2.97-3.78 g (55-70%), mp  $110-114^\circ\text{C}$ , after recrystallization from ethyl acetate 2.16-2.70 g (40-50%), mp  $127-128^\circ\text{C}$  (lit<sup>2</sup>:  $127^\circ\text{C}$ ). The crude pyruvamide, 3.34 g (38 mmol), which is not stable, was immediately partially dissolved in 30 ml water, 40 ml of  $\text{N HCl}$  was added and the mixture was heated on a steam bath for 2 hr. Pyruvic-3- $^{13}\text{C}$  acid was continuously extracted with ether. After evaporation of the ether colorless pyruvic-3- $^{13}\text{C}$  acid was distilled in vacuo under nitrogen. Yield 2.19-2.87 g (65-85%), mp  $13.6-13.8^\circ\text{C}$ . Freshly distilled pyruvic-3- $^{13}\text{C}$  acid, 3 g (34 mmol) was heated to  $50^\circ\text{C}$  under exclusion of moisture and 5.45 g (34 mmol) dry bromine was added dropwise with stirring, keeping the temperature at  $50^\circ\text{C}$ . The fuming syrup was immediately washed into a crystallizing dish with the aid of a little hot benzene. The dish was placed in a vacuum desiccator with KOH pellets, whereupon the bromopyruvic-3- $^{13}\text{C}$  acid crystallized. Yield 5.41 g (95%), mp  $70^\circ\text{C}$ . Recrystallization from chloroform (1 ml per g) gave 4.27-4.73 g (75-83%) of hexagonal prisms, mp  $74^\circ\text{C}$ .

Bromopyruvic-3- $^{13}\text{C}$  acid, 5.6 g (34 mmol), which is unstable, was immediately added slowly to 4.3 g (35 mmol) trimethyl phosphite in 100 ml absolute ether. A brisk reaction occurred immediately and after 5 min the solvent was evaporated at r.t. A solution of 3.4 g (34 mmol) cyclohexylamine in 40 ml water was added and after 73 hr water was evaporated at reduced pressure at  $40^\circ\text{C}$ .

The solid cyclohexylammonium salt was dissolved in 45 ml warm methanol and upon addition of an equal volume of ether to the warm solution the monocyclohexylammonium salt of phosphoenolpyruvate-3-<sup>13</sup>C crystallized. After standing at 0°C overnight the yield was 4.3 g (50%), mp 130-142°C (dec). Anal. Calcd C<sub>9</sub>H<sub>18</sub>NO<sub>6</sub>P: C, 40.45; H, 6.79; N, 5.24. Found: C, 40.72; H, 6.99; N, 5.39. The proton decoupled <sup>13</sup>C NMR spectrum was recorded on a Bruker WP-60 instrument, operating at 22.63 MHz, using the pulse Fourier transform technique. Table 1 shows the observed peaks.

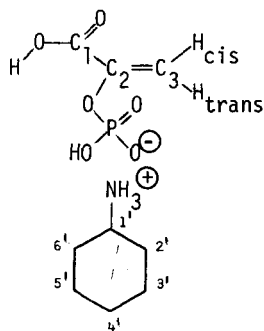


Table 1. Proton decoupled <sup>13</sup>C-NMR of the cyclohexylammonium salt of phosphoenolpyruvic acid. Concentration 0.4 M, solvent and lock compound D<sub>2</sub>O, pH 2.5, 35°C, standard external TMS.

ppm (rel. to TMS)	J <sub>CP</sub> (Hz)	Assignment
168.3	7.5	C-1
146.4	7.5	C-2
111.0	4.5	C-3*
52.6	--	C-1'
32.7	--	C-2'/C-6'
26.7	--	C-4'
26.2	--	C-3'/C-5'

\* In the <sup>13</sup>C-enriched compound the intensity ratio of this peak to other peaks, as compared to corresponding intensity ratios in the unenriched compound, showed at least 90% enrichment.

The fine structure of the peak at 111.0 ppm consisting of 8 lines in the proton coupled spectrum was analyzed to give J<sub>CH<sub>trans</sub></sub> = 165.0 Hz, J<sub>CH<sub>cis</sub></sub> = 162.5 Hz and J<sub>CP</sub> = 4.5 Hz. The <sup>31</sup>P-NMR-FT spectrum, measured at 36.4 MHz, gave a doublet at 0 ppm (H<sub>3</sub>PO<sub>4</sub> standard) with J<sub>PC</sub> = 4.5 Hz.

Shikimic-6-<sup>13</sup>C acid -- E. coli 83-24, kindly supplied by B. D. Davis, was grown on minimal medium<sup>7</sup> supplemented with 50  $\mu\text{mol}$  each of L-phenylalanine, L-tyrosine, and L-tryptophan and 0.5  $\mu\text{mol}$  each of *p*-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, and *p*-aminobenzoic acid with aeration at 30°C. When the cells reached an optical density at 578 nm of about 2.5 they were harvested by centrifugation for 10 min at 10,000 x g, washed once with chilled 0.9% NaCl and stored at -25°C for up to several months.

For crude extract fraction, cell-free extracts were prepared by grinding frozen cells with 1.5 times the weight of alumina powder (Alcoa 305) in a mortar. The suspension was taken up in a 3-fold volume of 0.03 M potassium phosphate buffer pH 7.4. All operations were carried out at 0-4°C. Cell-free extracts, obtained by centrifugation at 25,000 x g for 10 min, were dialyzed for 6-8 hr against 0.03 M phosphate buffer pH 7.4. The dialyzed extract, which usually had a protein concentration of 25 mg/ml, was aged five days at 0°C before use.

The enzymatic synthesis of the shikimic acid was carried out by the method described by Scharf and Zenk<sup>6</sup>. The reaction mixture for the conversion of phosphoenolpyruvic acid to shikimic acid contained in a volume of 160 ml: potassium phosphate buffer pH 7.4 14 mmol,  $\text{CoCl}_2$  20  $\mu\text{mol}$ , NAD 135  $\mu\text{mol}$ , NADPH 36  $\mu\text{mol}$ , erythrose-4-phosphate (prepared from glucose-6-phosphate according to the method of Ballou, *et al.*<sup>8</sup>) 400  $\mu\text{mol}$ , phosphoenolpyruvic-3-<sup>13</sup>C acid 400  $\mu\text{mol}$ , glucose-6-phosphate 500  $\mu\text{mol}$ , glucose-6-phosphate dehydrogenase (from yeast) 50 units, and aged cell-free extract E. coli 83-24 corresponding to 1 g of protein.

The mixture was incubated at 37°C for 2 hr. The protein was precipitated with 45 ml of 3 M perchloric acid, removed by centrifugation and washed twice with water. The combined supernatants were adjusted to pH 5 with a 3 N KOH and chromatographed on a column (2x16 cm) of Amberlite CG-400, acetate form. Elution was done with water, then with a linear gradient of 0 to 3 N acetic acid (2x300 ml). Aliquots of individual fractions were tested by the oxidation of shikimic acid by periodic acid described by the method of Gaitonde and

Gordon<sup>9</sup>. Fractions containing shikimic acid were combined and evaporated to dryness under reduced pressure, dissolved twice in ethanol and the solvent was evaporated *in vacuo*. The residue was dissolved in 50% ethanol and spotted on thin layer plates prepared with Cellulose MN 300. The plates were developed in the solvent system butanol-acetic acid-water (4:1:5 organic phase). The shikimic acid bands were located by spraying the reference shikimic acid spotted alongside the <sup>13</sup>C-material with the periodate benzidine reagent prepared according to Cifonelli and Smith<sup>10</sup>. The shikimic acid bands were eluted with water and lyophilized. The yield was 41.8 mg (240 μmol).

The <sup>13</sup>C enrichment was determined from its <sup>13</sup>C-NMR spectrum which was recorded on a Varian FT-80A instrument, operating at 20 MHz. Although this spectrum did not match that of unlabeled shikimic acid, it could be explained if (1) a trace of a paramagnetic ion were present, making C-7 and C-1 unobservable because of relaxation induced line broadening, and if (2) the solution were essentially neutral due to dilution. It could be shown that the

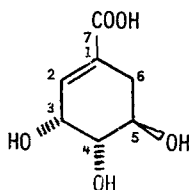


Table 2. <sup>13</sup>C-NMR of shikimic acid-6-<sup>13</sup>C and unenriched shikimic acid in D<sub>2</sub>O, δ-values downfield from TMS=0.

Assignment	Enriched sample, ~2% in D <sub>2</sub> O, 35°C, 5 mm tube, external TMS, Varian FT-80 A <sup>a</sup>	Unenriched sample, ~18% in D <sub>2</sub> O, 35°C, 5 mm tube, external TMS, Varian FT-80 A <sup>b</sup>	
	pH ~ 7.0	pH ~ 2.0	pH ~ 7.0
C-7	unobservable	170.4	175.8
C-2	130.4	137.8	130.9
C-1	unobservable	130.3	136.8
C-3	71.9	71.8	72.7
C-5	66.7	67.2	67.5
C-4	66.1	66.4	66.9
C-6	32.4	31.0	33.4

<sup>a</sup>Recorded at Varian/Darmstadt, West Germany

<sup>b</sup>Recorded at The University of New Mexico

presence of a mole fraction  $\text{Cu}^{++}$  of about  $2 \times 10^{-3}$  obliterated C-7 and C-1 entirely in unlabeled shikimic acid and that in going from pH  $\sim 2$  (18% shikimic acid in  $\text{D}_2\text{O}$ ) to pH  $\sim 7$  the positions of C-1 and C-2 essentially interchanged, with downfield shifts of C-7 and C-6 (Table 2). In the enriched sample homonuclear coupling between the enriched C-6 and its unenriched neighbor C-5 is observed (C-1 and  $J_{\text{C}_1-\text{C}_6}$  being unobservable). The coupling constant is  $37.7 \pm 0.7$  Hz, well within the literature range of  $J \approx 35-38$  Hz for one bond  $\text{sp}^3-\text{sp}^3$  carbon coupling<sup>11</sup>. From the height of C-5 and its satellites an enrichment of  $43 \pm 5\%$  at C-6 is calculated.

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