# Enzyme-Assisted Preparation of Isotope-Labeled 1-Deoxy-D-xylulose 5-Phosphate

Stefan Hecht, Klaus Kis,* Wolfgang Eisenreich,* Sabine Amslinger, Juraithip Wungsintaweekul, Stefan Herz, Felix Rohdich, and Adelbert Bacher

*Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany*

*klaus.kis@ch.tum.de*

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Recombinant 1-deoxy-D-xylulose 5-phosphate synthase of *Bacillus subtilis* was used for the preparation of isotope-labeled 1-deoxy-D-xylulose 5-phosphate using isotope-labeled glucose and/or isotope-labeled pyruvate as starting materials. The simple one-pot methods described afford almost every conceivable isotopomer of 1-deoxy-D-xylulose 5-phosphate carrying $^{13}C$ or $^{14}C$ from commercially available precursors with an overall yield around 50%.

## Introduction

1-Deoxy-D-xylulose 5-phosphate (**3**) serves as intermediate in the biosynthetic pathways of thiamine [1] and pyridoxal [2,3] and in the recently discovered non-mevalonate pathway of terpenoid biosynthesis (Figure 1) (for review see refs 4−6). The carbohydrate derivative is biosynthesized from pyruvate (**1**) and D-glyceraldehyde 3-phosphate (**2**) by the catalytic action of 1-deoxy-D-xylulose 5-phosphate synthase.[7,8] The enzyme-catalyzed reaction involves the release of the carboxylic group of pyruvate as carbon dioxide.

The discovery of the alternative, non-mevalonate isoprenoid pathway has triggered an intense burst of research activity. Recently discovered genes, enzymes, and intermediates of the pathway are summarized in Figure 1.[9−12] However, several reaction steps for the conversion of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**5**) to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) remain to be established.

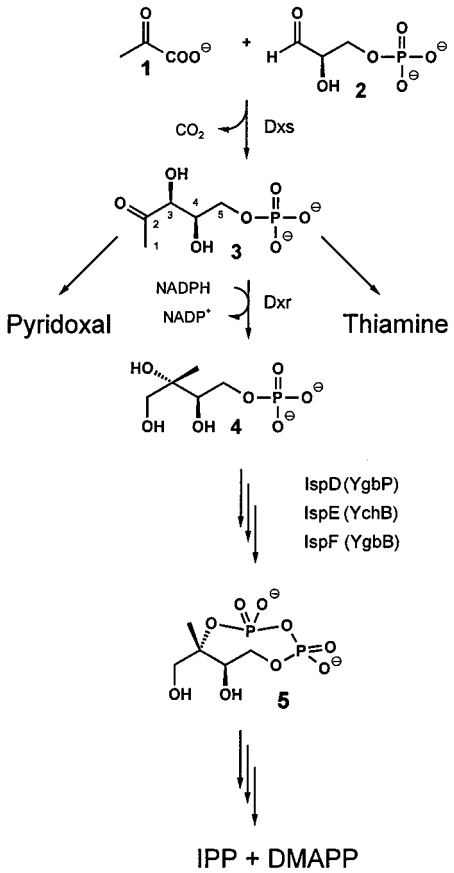


**Figure 1.** 1-Deoxy-D-xylulose 5-phosphate (**3**) at the branching point of the biosynthetic pathways for pyridoxal, thiamine, and terpenoids. Dxs, 1-deoxy-D-xylulose 5-phosphate synthase; Dxr, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; IspD (YgbP), 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase; IspE (YchB), 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase; IspF (YgbB), 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate.

This paper describes the utilization of recombinant 1-deoxy-D-xylulose 5-phosphate synthase from *Bacillus subtilis* for the preparation of a wide variety of 1-deoxy-

* Additional corresponding author email: wolfgang.eisenreich@ch.tum.de.

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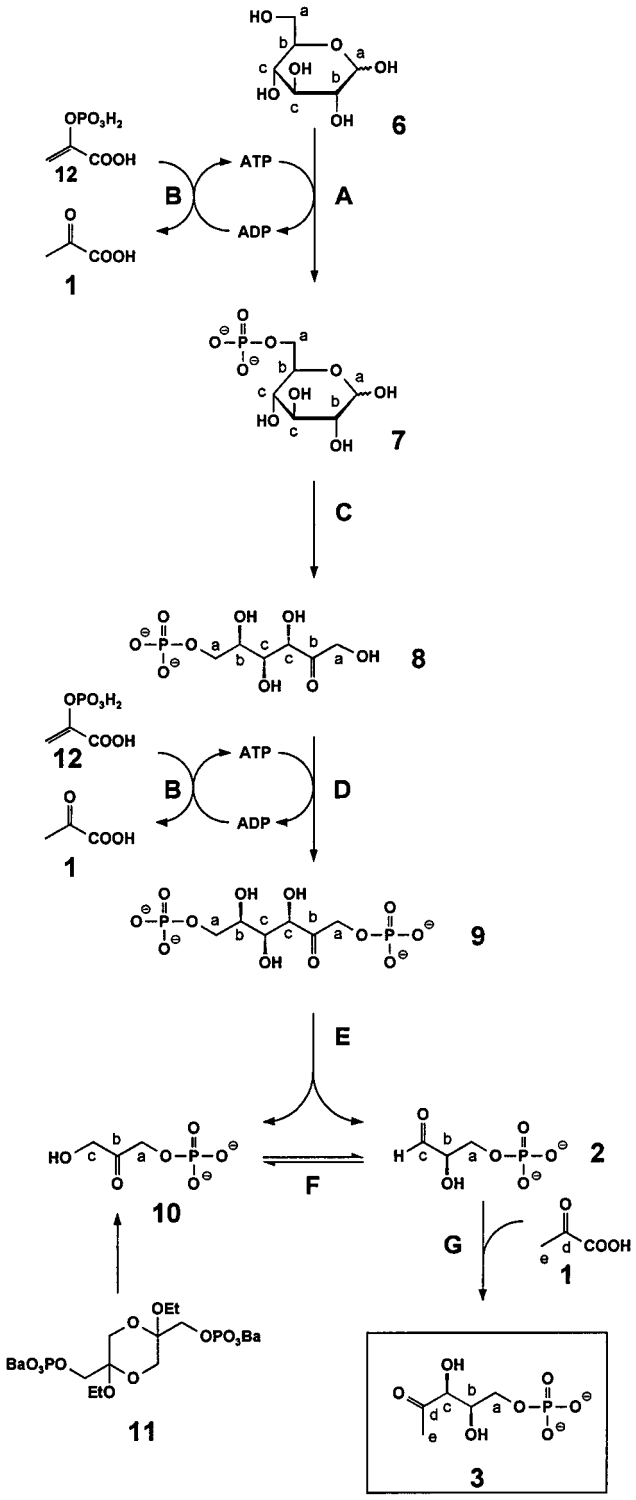


**Figure 2.** Enzymatic synthesis of 1-deoxy-D-xylulose 5-phosphate (**3**). A, hexokinase; B, pyruvate kinase; C, glucose 6-phosphate isomerase; D, fructose 6-phosphate kinase; E, aldolase; F, triose phosphate isomerase; G, 1-deoxy-D-xylulose 5-phosphate synthase.

D-xylulose 5-phosphate isotopomers by simple one-pot reactions with high overall yield.

## Results

The *dxs* gene of *B. subtilis* (formerly designated *yqiE*) was cloned into the expression plasmid pNCO113, which was found to direct the expression of the cognate enzyme to levels around 25% of cell protein in recombinant *Escherichia coli* cells. The recombinant protein was purified to apparent homogeneity by two chromatographic steps. The pure recombinant enzyme had a specific activity of 2.9 $\mu$mol min$^{-1}$ mg$^{-1}$.

**Table 1. Preparation of 1-Deoxy-D-xylulose 5-Phosphate Isotopomers from Commercially Available Glucose and Pyruvate Isotopomers**

| glucose | pyruvate | 1-deoxy-D-xylulose 5-phosphate | procedure |
|---|---|---|---|
| unlabeled | unlabeled | unlabeled | 1 |
| unlabeled | [2-$^{13}$C] | [2-$^{13}$C] | 5 |
| unlabeled | [2,3-$^{13}C_2$] | [1,2-$^{13}C_2$] | 2 |
| [U-$^{13}C_6$] | unlabeled | [3,4,5-$^{13}C_3$] | 3 |
| [U-$^{13}C_6$] | [2,3-$^{13}C_2$] | [U-$^{13}C_5$] | 4 |
| unlabeled | [2-$^{14}$C] | [2-$^{14}$C] | 5 |

The recombinant enzyme was used to prepare $^{13}$C- or $^{14}$C-labeled isotopomers of 1-deoxy-D-xylulose 5-phosphate (**3**) by condensation of appropriately isotope-labeled pyruvate (**1**) and glyceraldehyde 3-phosphate (**2**). Unlabeled **2** can be prepared at low cost from dihydroxyacetone phosphate (**10**) or from 2,5-diethoxy-1,4-dioxane-2,5-di(methyl phosphate) (**11**) using triose phosphate isomerase. Isotope-labeled **2** can be prepared in situ from commercially available $^{13}$C-labeled glucose (**6**) using commercially available glycolytic enzymes as shown in Figure 2. Various isotopomers of **1** are commercially available.

All isotopomers of **3** described in this paper can be obtained from these respective starting materials by simple one-pot reactions. The introduction of $^{13}$C into positions 1 and/or 2 requires appropriately $^{13}$C-labeled **1** and unlabeled **2**.

Starting from appropriately labeled glucose samples, $^{13}$C can be introduced into positions 3, 4, and/or 5 of **3** by procedure 3 (Table 1). ATP required for phosphorylation of **6** and fructose 6-phosphate (**8**) can be recycled using pyruvate kinase and phosphoenol pyruvate (**12**) as phosphate donor. As an example we showed the preparation of [3,4,5-$^{13}C_3$]-**3** starting with [U-$^{13}C_6$]-**6**.

[U-$^{13}C_5$]-**3** is easily obtained by a one-pot reaction (procedure 4) using [U-$^{13}C_6$]-**6** and [2,3-$^{13}C_2$]-**1**. In this case, ATP must be used in stoichiometric amount and cannot be recycled by pyruvate kinase because **1** generated from **12** in the ATP recycling reaction would dilute the isotope-labeled starting material.

[1,2-$^{13}C_2$]-**3** was prepared from [2,3-$^{13}C_2$]-**1** and unlabeled **2** in a convenient one-pot reaction (procedure 2) that makes use of commercially available dimeric dihydroxyacetone phosphate (**11**), which can be converted very easily to **2** with triose phosphate isomerase.

The one-pot reactions described above can be scaled down linearly to very small reaction volumes suitable for the preparation of $^{14}$C-labeled **3** with high specific radioactivity. As an example, we describe the conversion of [2-$^{14}$C]-**1** to [2-$^{14}$C]-**3**.

The reaction products can be purified by ion exchange chromatography using a volatile buffer such as triethylammonium acetate.

The isotopomeric purity of **3** obtained by our methods is very high (at least 95%). Thus, $^{13}$C NMR analysis of [U-$^{13}C_5$]1-deoxy-D-xylulose 5-phosphate (see Figure 3) shows no evidence for the presence of any unlabeled material.

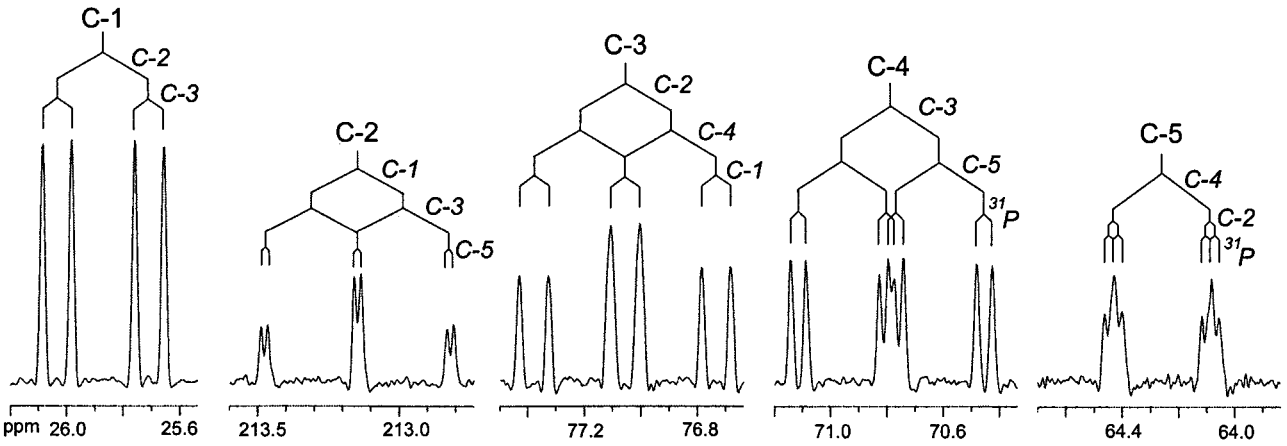


**Figure 3.** $^{13}C$ NMR spectrum of [U-$^{13}C_5$]1-deoxy-D-xylulose 5-phosphate (**3**) (125 MHz, 10% $D_2O$, pH 8).

**Table 2. NMR Data of 1-deoxy-D-xylulose 5-phosphate Isotopomers**

| | chemical shifts (ppm) | | | coupling constants (Hz)[a] | | | | | | high intensity $^{13}C$ signals[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| position | $^1H$ | $^{13}C$ | $^{31}P$ | C1 | C2 | C3 | C4 | C5 | P | [2-$^{13}C$]-**3** | [1,2-$^{13}C_2$]-**3** | [3,4,5-$^{13}C_3$]-**3** | [U-$^{13}C_5$]-**3** |
| 1 | 2.4 | 25.9 | | (128.6) | 41.2 (6.0) | 12.8 | | | | | d | | dd |
| 2 | | 213.1 | | | | 41.3 | | 3.1 | | s | d | | ddd |
| 3 | 4.6 | 77.0 | | | | (144.2) | 40.2 | | | | | dd | ddd |
| 4 | 4.4 | 70.7 | | | | | (145.3) | 43.2 | 7.2 | | | ddd | ddd |
| 5 | 4.0 | 64.3 | | | | | | (145.2) | 4.7 | | | dd | ddd |
| P | | | 7.0 | | | | | | | s | s | dd | dd |

[a] $^{13}C-^{13}C$ and $^1H-^{13}C$ coupling constants; $^1H-^{13}C$ coupling constants are shown in parentheses. [b] s, singlet; d, doublet; dd, double doublet; ddd, double double doublet.

Using the various isotopomers prepared in the course of this study, we were able to determine all homo- and heteronuclear coupling constants for **3** (Table 2).

## Discussion

At this time, the sequence of reactions conducive to isopentenyl pyrophosphate and dimethylallyl pyrophosphate via 1-deoxy-D-xylulose 5-phosphate is still in part unknown. This generates an urgent need for optimized technology to prepare isotope-labeled pathway intermediates that might help to solve the problem. Moreover, the convenient access to a virtually unlimited variety of isotopomers of **3** can serve as basis for studies on the mechanism of formation of a wide variety of terpenoids and of the vitamins thiamine and pyridoxal, as well as for NMR and IR studies of enzymes involved in the respective biosynthetic pathways.

The preparation of isotope-labeled compounds by chemical synthesis is usually laborious and requires the implementation of specific synthetic strategies for each respective isotopomer. The present study illustrates the power of recombinant enzyme technology for the preparation of isotope-labeled 1-deoxy-D-xylulose 5-phosphate specimens that are not easily obtained by other approaches.

Sahm and co-workers demonstrated in 1998 the suitability of an enzyme-based approach for the preparation of 1-deoxy-D-xylulose 5-phosphate starting with fructose 1,6-diphosphate and pyruvate.[13] However, for the preparation of singly or multiply labeled **2** the use of fructose 1,6-diphosphate is not suitable. It is more convenient to start with appropriately labeled and commercially available glucose isotopomers for the preparation of $^{13}C$-labeled **2**. Furthermore, under the conditions of our improved methods all the glycolysis products have only short lifetimes, which minimizes problems resulting from decomposition products or enzyme inhibition.

Three enzyme-based methods reported in this paper (procedures 2−4) are sufficient for the preparation of virtually every $^{13}C$- or $^{14}C$-substituted isotopomer of **3**; the multistep sequences can be carried out as one-pot reactions with a minimum of effort. Selection of the appropriate product isotopomer is achieved in the simplest possible way by selecting the appropriate isotope-labeled starting material (cf. letters a−e in Figure 2). Compound **3** is a useful substrate for cell-free systems. However, for experiments with whole organisms or cell cultures unphosphorylated **3** is a better substrate. Unphosphorylated **3** can be prepared very easily by dephosphorylation of **3** using alkaline phosphatase, which recently has been demonstrated by our group.[14]

## Experimental Section

**General Methods.** $^1H$ NMR and $^1H$ decoupled $^{13}C$ NMR spectra were recorded using an AVANCE DRX 500 spectrometer from Bruker Instruments, Karlsruhe, Germany. The transmitter frequencies were 500.1 and 125.6 MHz for $^1H$ and $^{13}C$, respectively. The chemical shifts were referenced to external trimethylsilylpropane sulfonate. $^{31}P$ NMR spectra were recorded using an AC250 spectrometer from Bruker at a frequency of 101.3 MHz. $^{31}P$ Chemical shifts were referenced to external 85% $H_3PO_4$. 1-Deoxy-D-xylulose 5-phosphate and

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1-deoxy-D-xylulose were recorded in 90% $H_2O$/10% $D_2O$ (pH 8, uncorrected glass electrode reading).

**Materials.** [U-$^{13}C_6$]glucose, [2-$^{13}C_1$]pyruvate, and [2,3-$^{13}C_2$]-pyruvate were obtained from Omicron, South Bend, IN. [2-$^{14}$C]-pyruvate was obtained from Amersham, Braunschweig, Germany. Sodium pyruvate, dihydroxyacetone phosphate (dilithium salt), D,L-glyceraldehyde 3-phosphoric acid, thiamine pyrophosphate, ATP (disodium salt), phosphoenol pyruvate (potassium salt, PEP), NADPH, 2,5-diethoxy-1,4-dioxane-2,5-di-(methyl phosphate) dibarium salt (dimeric dihydroxyacetone phosphate acetal), and glycolytic enzymes (hexokinase, glucose 6-phosphate isomerase, phosphofructokinase, aldolase, triose phosphate isomerase), and pyruvate kinase were purchased from Sigma Chemicals, Deisenhofen, Germany. Oligonucleotides were custom-synthesized by MWG Biotech, Ebersberg, Germany. T4 ligase was obtained from Gibco-BRL, Eggenstein, Germany. Sepharose QFF and restriction enzymes were purchased from Amersham Pharmacia Biotech, Freiburg, Germany. DNase I was purchased from Roche Diagnostics, Mannheim, Germany. Taq polymerase and isopropyl-$\beta$-D-thiogalactopyranoside were supplied by Eurogentec, Seraing, Belgium. RNase A and silica gel N-HR plates were from Macherey-Nagel, Düren, Germany. ECTEOLA 23 cellulose was obtained from Fluka, Deisenhofen, Germany. Macroprep hydroxyapatite columns, 40 $\mu$m, were supplied from Biorad, Munich, Germany. The preparation of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase will be published elsewhere.

**Microorganism.** The bacterial strain BR151,[15] the high copy expression vector pNCO113 (PTA-852, American type culture collection, unrestricted patent deposit; see also ref 16) and the expression vector construct for the *dxs* gene of *B. subtilis*, PNCODXSBACSU were used in this study.

**Construction of an Expression Plasmid for the *dxs* gene of *B. subtilis*.** The *dxs* gene of *B. subtilis* (accession number dbj D84432, base pair 193991−195892) was amplified by PCR using chromosomal *B. subtilis* DNA as template and the oligonucleotides 5′-tgatccgccatggatcttttatcaatacagg-3′ and 5′-ttgaatagaggatccccgcc-3′ as primers. The primer sequence was conducive to the exchange of the TTG start codon by ATG. The amplificate was purified, treated with the restriction endonucleases *Nco*I and *BamH*I, and ligated into the plasmid vector pNCO113, which had been treated with the same enzymes. The ligation mixture was electroporated into the recombinant *E. coli* XL1-Blue cells affording the recombinant strain XL1-pNCODXSBACSU.

**Growth of Recombinant *E. coli* Cells.** The recombinant *E. coli* strain XL1-pNCODXSBACSU was grown in 500 mL of Luria-Bertani (LB) medium containing ampicillin (150 mg $L^{-1}$) and kanamycin (50 mg $L^{-1}$) in 2-L shake flasks. The flasks were inoculated with an overnight culture at a ratio of 1:50 and were incubated with shaking at 37 °C. At an optical density of 0.6 (600 nm), isopropyl-$\beta$-D-thiogalactopyranoside was added to a final concentration of 2 mM, and incubation was continued for 5 h. Cells were harvested by centrifugation and stored at −20 °C.

**Purification of 1-Deoxy-D-xylulose 5-Phosphate Synthase.** Frozen cell mass of recombinant *E. coli* strain XL1-pNCODXSBACSU (3.7 g) was thawed in 40 mL of 50 mM Tris hydrochloride, pH 7.8, containing 2 mM dithiothreitol and 4 mg of lysozyme. The suspension was incubated at 37 °C for 30 min. Subsequently, it was cooled on ice, subjected to ultrasonic treatment, and centrifuged. The supernatant was applied to a Sepharose QFF column (4 × 7 cm) that had been equilibrated with 20 mM Tris hydrochloride, pH 7.8, containing 2 mM dithiothreitol (buffer A). The column was washed with 60 mL of buffer A and developed with a gradient of 0−1 M potassium chloride in 800 mL of buffer A. Fractions were combined and dialyzed overnight against buffer A. The solution was applied to a column of hydroxyapatite (2.5 × 6.0 cm) that had been equilibrated with buffer A. The column was washed with 30 mL of buffer A, followed by 60 mL of 20 mM potassium phosphate, pH 6.8, and was then developed with a gradient of 0−1 M potassium phosphate, pH 6.8.

**Assay of 1-Deoxy-D-xylulose 5-Phosphate Synthase.** Assay mixtures contained 200 mM Tris hydrochloride, pH 8.0, 1 mM manganese sulfate, 5 mM sodium pyruvate (**1**), 10 mM D,L-glyceraldehyde 3-phosphate (**2**), 1 mM thiamine pyrophosphate, 1 mM dithiothreitol, 0.3 mM NADPH, 42 $\mu$g (1 U) of recombinant 1-deoxy-D-xylulose 5-phosphate reductoisomerase from *E. coli*, and protein in a total volume of 1 mL. The mixture was incubated at 37 °C, and the absorbance at 340 nm was monitored for 10 min.

**Unlabeled 1-Deoxy-D-xylulose 5-Phosphate (3) (Procedure 1).** A solution of D,L-glyceraldehyde 3-phosphoric acid (**2**) (51 mg, 300 $\mu$mol) in 1 mL of water was neutralized with 1 M sodium hydroxide and added to a solution containing 150 mM Tris hydrochloride, pH 8.0, 10 mM magnesium chloride, 0.50 mg (3.2 $\mu$mol) of dithiothreitol, 2.7 mg (6.4 $\mu$mol) of thiamine pyrophosphate, 16.5 mg (150 $\mu$mol) of sodium pyruvate (**1**), and 2 U (0.6 mg) of recombinant 1-deoxy-D-xylulose 5-phosphate synthase from *B. subtilis* in a total volume of 3.2 mL. The mixture was incubated at 37 °C for 6 h.

**Purification of 1-Deoxy-D-xylulose 5-Phosphate (3).** Typically, 1 g of lyophilized reaction mixture was dissolved in 15 mL of water and applied to a column of ECTEOLA 23 cellulose (55 × 4 cm) at 4 °C. The column was developed with a linear gradient of 0−0.3 M triethylammonium acetate (pH 6.0; total volume, 4.1 L; flow rate, 2 mL $min^{-1}$). Fractions of 25 mL were collected and analyzed by thin-layer chromatography using silica gel N-HR plates, which were developed in *n*-propanol/ethyl acetate/water (6:1:3, v/v). The plates were dried, sprayed with anisaldehyde−sulfuric acid reagent,[17] and heated to 100 °C. The product was identified as blue-green spots with a $R_f$ value of about 0.5. Fractions were combined and lyophilized repeatedly to remove triethylammonium acetate. Yield, 27.5 mg (67.5 $\mu$mol, 46%) as triethylammonium salt.

**[1,2-$^{13}C_2$]1-Deoxy-D-xylulose 5-Phosphate ([1,2-$^{13}C_2$]-3) (Procedure 2).** A solution containing 2.0 g (3.0 mmol) of dimeric dihydroxyacetone phosphate acetal (**11**) in 50 mL of water was added to a suspension of Dowex 50 WX8 (30 mL, $H^+$ form). The mixture was incubated at 65 °C for 4 h. The solid was filtered off and washed with water. The combined solution was lyophilized. Dihydroxyacetone phosphate glass (1.1 g, containing about 0.42 g (1.5 mmol) of **10**) was dissolved in 12 mL of water, and the pH was adjusted to 6.0 by the addition of 8 M sodium hydroxide. A solution (6.6 mL) containing 450 mM Tris hydrochloride, pH 8.0, 30 mM magnesium chloride, 31 mg (73 $\mu$mol) of thiamine pyrophosphate, 0.50 mg (3.3 $\mu$mol) of dithiothreitol, and 0.17 g (1.5 mmol) of [2,3-$^{13}C_2$]pyruvate (sodium salt) (**1**) was added. The pH was adjusted to 8.0, and 400 U (0.07 mg) of triose phosphate isomerase and 9.6 U (3.0 mg) of 1-deoxy-D-xylulose 5-phosphate synthase were added. The mixture was incubated at 37 °C for 11 h. The product was purified as described above. Yield, 0.25 g (0.60 mmol, 40%) as triethylammonium salt.

**[3,4,5-$^{13}C_3$]1-Deoxy-D-xylulose 5-Phosphate ([3,4,5-$^{13}C_3$]-3) (Procedure 3).** A solution containing 150 mM Tris hydrochloride, 10 mM magnesium chloride, 1.0 g (5.4 mmol) of [U-$^{13}C_6$]glucose (**6**), 0.23 g (1.5 mmol) of dithiothreitol, 0.3 g (0.7 mmol) of thiamine pyrophosphate, 0.1 g (0.2 mmol) of ATP (disodium salt), and 2.2 g (11 mmol) of phosphoenol pyruvate (**12**) (potassium salt) in a total volume of 300 mL was adjusted to pH 8 by the addition of 8 M sodium hydroxide. A solution containing 1,500 U (10 mg) of hexokinase, 300 U (0.4 mg) of glucose 6-phosphate isomerase, 105 U (1 mg) of fructose 6-phosphate kinase, 610 U (0.1 mg) of triose phosphate isomerase, 57 U (0.01 mg) of aldolase, 403 U (2.8 mg) of pyruvate kinase, and 8 U (2.5 mg) of 1-deoxy-D-xylulose 5-phosphate synthase in 4 mL of Tris hydrochloride, pH 8.0,

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were added. The mixture was incubated at 37 °C for 24 h. The product was purified as described above. Yield, 2.3 g (5.7 mmol, 53%) as triethylammonium salt.

**[U-$^{13}C_5$]1-Deoxy-D-xylulose 5-Phosphate ([U-$^{13}C_5$]-3) (Procedure 4).** A solution containing 150 mM Tris hydrochloride, pH 8, 6 mM magnesium chloride, 0.17 g (0.89 mmol) of [U-$^{13}C_6$]D-glucose (**6**), 200 mg (1.79 mmol) of [2,3-$^{13}C_2$]-pyruvate (sodium salt) (**1**), 44 mg (55 $\mu$mol) of thiamine pyrophosphate, 1.02 g (1.79 mmol) of ATP (disodium salt), 84 U (1.4 mg) of hexokinase, 51 U (0.5 mg) of glucose 6-phosphate isomerase, 18 U (0.30 mg) of phosphofructokinase, 34 U (2.6 mg) of aldolase, 412 U (0.07 mg) of triose phosphate isomerase, and 2 U (0.6 mg) of 1-deoxy-D-xylulose 5-phosphate synthase in a total volume of 58 mL was incubated at 37 °C for 12 h. The product was purified as described above. Yield, 0.34 g (0.84 mmol, 47%) as triethylammonium salt.

**[2-$^{13}C_1$]1-Deoxy-D-xylulose 5-Phosphate ([2-$^{13}C_1$]-3) (Procedure 5).** A solution containing 260 mM Tris hydrochloride pH 8.0, 10 mM magnesium chloride, 77 $\mu$g (0.50 $\mu$mol) of dithiothreitol, 0.4 mg (1 $\mu$mol) of thiamine pyrophosphate, 2.5 mg (13 $\mu$mol) of dihydroxyacetone phosphate (dilithium salt, hydrate form) (**10**), 1.4 mg (13 $\mu$mol) of sodium [2-$^{13}$C]pyruvate (**1**), 250 U (0.03 mg) triose phosphate isomerase, and 2 U (0.6 mg) 1-deoxy-D-xylulose 5-phosphate synthase from *B. subtilis* in a total volume of 0.5 mL was incubated at 37 °C for 1 h. The product was purified as described above. Yield, 3.1 mg (7.7 $\mu$mol, 61%) as triethylammonium salt.

**[2-$^{14}$C]1-Deoxy-D-xylulose 5-Phosphate ([2-$^{14}$C]-3) (Procedure 5).** A solution containing 150 mM Tris hydrochloride, pH 8.0, 10 mM magnesium chloride, 1.77 mg (15.8 $\mu$mol, 250 $\mu$Ci) of [2-$^{14}$C]pyruvate (sodium salt), 3.25 mg (15.8 $\mu$mol) of dihydroxyacetone phosphate (dilithium salt), 12 mg (25 $\mu$mol) of thiamine pyrophosphate, 7.7 mg (50 $\mu$mol) of dithiothreitol, and 0.4 U (0.1 mg) of 1-deoxy-D-xylulose 5-phosphate synthase in a total volume of 1 mL was incubated at 37 °C for 2 h. The product was purified as described above. Yield, 183 $\mu$Ci (73%); radiochemical purity, 100%.

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