# Biosynthesis of Terpenoids: Efficient Multistep Biotransformation Procedures Affording Isotope-Labeled 2*C*-Methyl-D-erythritol 4-Phosphate Using Recombinant 2*C*-Methyl-D-erythritol 4-Phosphate Synthase

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This paper describes the recombinant expression of the *ispC* gene of *Escherichia coli* specifying 2*C*-methyl-D-erythritol 4-phosphate synthase in a modified form that can be purified efficiently by metal-chelating chromatography. The enzyme was used for the preparation of isotope-labeled 2*C*-methyl-D-erythritol 4-phosphate employing isotope-labeled glucose and pyruvate as starting materials. The simple one-pot methods described afford numerous isotopomers of 2*C*-methyl-D-erythritol 4-phosphate carrying $^{3}H$, $^{13}C$, or $^{14}C$ from commercially available precursors. The overall yield based on the respective isotope-labeled starting material is approximately 50%.

## Introduction

The biosynthesis of structurally complex natural products in living systems has several unique features in comparison to chemical synthesis in the laboratory or production plant. Chemical synthesis is usually organized in a stepwise fashion, and individual reaction steps are plagued by the formation of undesired side products that must be removed after every step. In contrast, biosynthetic processes occur in very complex cellular environments comprising thousands of different macromolecular and low-molecular-weight components. Nevertheless, individual synthetic processes are characterized by quantitative yields (in other words, without undesired side products) over numerous reaction steps in conjunction with virtually perfect stereocontrol. The main reasons for the superior performance of cellular biochemistry compared to conventional synthetic organic chemistry are (i) the use of virtually perfect catalysts (i.e., enzymes), (ii) the recycling of reagents (i.e., cofactors), and (iii) the efficient management of Gibbs free energy gradients.

In principle, it appears possible to implement these favorable process properties in extracellular synthetic procedures. A wide variety of enzymes are commercially available or can be obtained in high yield using recombinant DNA technology. Cofactors can be recycled in situ using appropriate auxiliary enzymes. In such a biomimetic setting, the central feature is then the control of free energy gradients in the absence of cellular energy production facilities. Sufficiently large free energy gradients can be provided in vitro by enzymatic coupling of the desired biotransformation to exergonic auxiliary reactions.

The implementation of biotransformation processes for synthetic organic chemistry is particularly worthwhile for compounds that are not conveniently accessible by chemical synthesis. Moreover, biotransformation technology enables the introduction of isotope labels at high yield and with rigorous regiochemical and stereochemical control.

As a practical example, we report procedures for the one-pot preparation of the branched polyol, 2*C*-methyl-D-erythritol 4-phosphate, in an isotope-labeled form. The compound is the first committed intermediate in the non-mevalonate pathway of terpenoid biosynthesis,[1−7] which supplies the building blocks for the vast majority of the approximately 30 000 terpenoids known to date (Figure 1).

The methods reported in this paper afford a large variety of different isotopomers of the target compound in high yield by simple and rapid one-pot reactions using commercially available isotope-labeled glucose and/or pyruvate samples as starting materials.

## Results

The *ispC* gene[8] specifying 2*C*-methyl-D-erythritol 4-phosphate synthase, also named 1-deoxy-D-xylulose 5-phosphate reductoisomerase,[9] was amplified by PCR from chromosomal *Escherichia coli* DNA and was placed under the control of a $T_5$ promoter and *lac* operator in the

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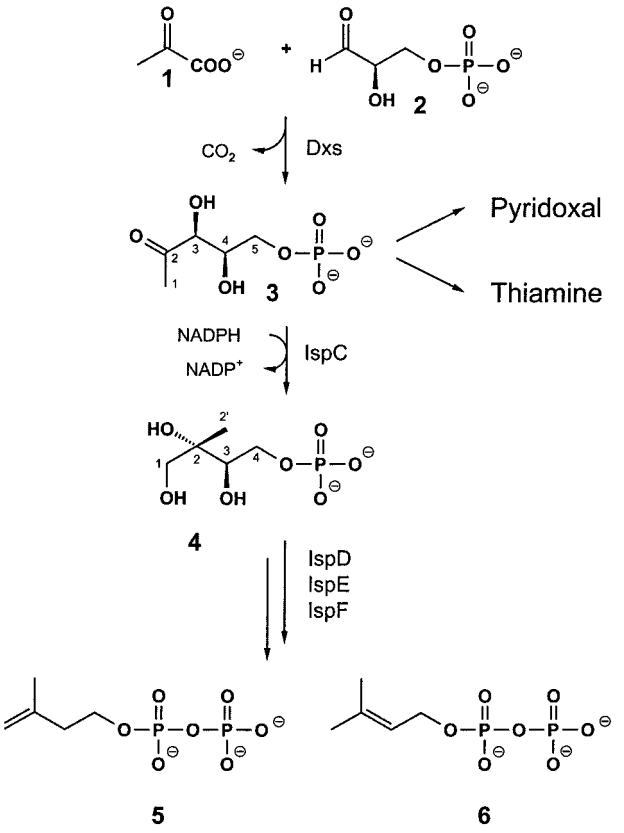


**Figure 1.** Non-mevalonate pathway of isoprenoid biosynthesis. Dxs, 1-deoxy-D-xylulose 5-phosphate synthase; IspC, 2*C*-methyl-D-erythritol 4-phosphate synthase; IspD, 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase; IspE, 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase; IspF, 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase.

**Table 1. Bacterial Strains and Plasmids Used in This Study**

| strain or plasmid | genotype or relevant characteristic | reference or source |
|---|---|---|
| *E. coli* K-12 | | |
| M15 [pREP4] | *Lac, ara, gal, mtl, recA*$^+$, *uvr*$^+$, [pREP4, *lacI, kan*$^r$] | 23 |
| XL1-Blue | *RecA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F′, *proAB, lacI*$^q$*ZΔM15, Tn10* (*tet*$^r$)] | 24 |
| plasmids | | |
| pQE30 | high-copy N-terminal His-tag vector | Qiagen |
| pQEYAEMECO | expression construct for the *ispC* gene of *E. coli* | this study |

expression plasmid pQE30 (Table 1). The recombinant gene specified the N-terminal sequence motif MRGSHHHHHHGS followed by amino acid residue 1 of the *E. coli* open reading frame.

Recombinant *E. coli* cells carrying that plasmid expressed the *ispC* gene in very high yield. 2*C*-Methyl-D-erythritol 4-phosphate synthase represented approximately 50% of the total protein in the recombinant *E. coli* cells after induction with isopropyl thio-D-galactopyranoside (IPTG).

The recombinant protein could be purified very efficiently by affinity chromatography on $Ni^{2+}$-chelating Sepharose affording homogeneous protein. The apparent subunit molecular mass was 43 kDa as shown by SDS−PAGE in agreement with the calculated mass of 43.3 kDa.

**Enzyme Properties.** The recombinant enzyme sediments in the analytical ultracentrifuge at an apparent velocity of 5.0 S (20 °C). Analysis of the sedimentation data using the program ULTRASCAN indicated a slight asymmetry of the moving boundary, which suggested concentration-dependent aggregation.[10,11] A van Holde−Weischet analysis of the data showed a crossover effect that is characteristic for nonideal systems with a concentration dependency of the sedimentation coefficient.[10−12] The data are compatible with a homotetrameric protein that is subject to aggregation under the experimental conditions used.

In agreement with earlier studies, the enzyme requires divalent metal ions for catalytic activity.[1−6] Manganese and magnesium stimulate the activity to a similar degree at concentrations above 5 mM. At concentrations below 1 mM, manganese was more efficient than magnesium.

2*C*-Methyl-D-erythritol 4-phosphate synthase requires NADPH as a reducing agent.[1−6] About 1% of the activity was found when NADPH was replaced by NADH (0.5 mM).

The enzyme activity can be monitored photometrically.[1] The $K_M$ values determined with $Mn^{2+}$ were 171 and 25 $\mu$M for 1-deoxy-D-xylulose 5-phosphate and NADPH, respectively; $v_{max}$ had a value of 18 $\mu$mol min$^{-1}$ mg$^{-1}$ corresponding to a turnover number of 13 s$^{-1}$ per subunit.

**Synthetic Procedures.** The recombinant protein was used for the preparation of 2*C*-methyl-D-erythritol 4-phosphate isotopomers by rapid one-pot methods specifically designed for the efficient incorporation of $^{13}$C or of radioisotopes ($^3$H, $^{14}$C) as labels for biosynthetic and biological studies.

Five in vitro biotransformation strategies were optimized for different isotope labeling patterns. Procedures 1−4, designed for introducing $^{13}$C or $^{14}$C, use D-glyceraldehyde 3-phosphate prepared from dihydroxyacetone phosphate or glucose as the starting material. Procedure 5 enables the incorporation of $^3$H into the 1*R* position of **4** using 1-deoxy-D-xylulose 5-phosphate (**3**) as the starting material.

**Carbon Labels in Positions 2 and/or 2′ (Procedures 1 and 2).** Carbon atoms 2 and 2′ of **4** are derived from C-2 and C-3, respectively, of pyruvate (**1**; Figure 2). Hence, $^{13}$C label can be introduced into one or both of these positions (designated by the letters d and e in Figure 2) using appropriately $^{13}$C-labeled pyruvate. More specifically, [2-$^{13}C_1$]-, [3-$^{13}C_1$]-, and [2,3-$^{13}C_2$]pyruvate afford [2-$^{13}C_1$]-, [2′-$^{13}C_1$]-, and [2,2′-$^{13}C_2$]**4**, respectively (procedure 1). Moreover, $^{14}$C-labeled **4** can be prepared starting with $^{14}$C-labeled pyruvate (procedure 2).

The unlabeled glyceraldehyde 3-phosphate required as the starting material can be obtained by hydrolysis of **7** followed by treatment with commercially available triose phosphate isomerase. NADPH is recycled using glucose dehydrogenase with glucose as the substrate.

The reaction sequence starting with **7** can be conveniently carried out as a one-pot reaction involving four enzymes. The reaction progress can be monitored in real time by $^{13}$C NMR analysis. Due to the specific $^{13}$C labeling, the NMR analysis selectively detects the reac-

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**Table 2. NMR Data of 2*C*-Methyl-D-erythritol 4-Phosphate Isotopomers (10% $D_2O$)**

| | chemical shifts (ppm) | | | coupling constants (Hz) | | | | | | high-intensity $^{13}C$ signals[d] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| position | $^1H$ | $^{13}C$ | $^{31}P$ | C1 | C2 | C3 | C4 | C2′ | P | [2-$^{13}$C]-**4** | [3,4,5-$^{13}C_3$]-**4** | [U-$^{13}C_5$]-**4** |
| 1 | 3.50 | 66.2 | | 143.0[a] | 11.7[a] | | | | | s | s | s |
| 2 | | 74.1 | | | | | | 39.6[b] | | | | m |
| 2′ | 1.04 | 18.4 | | | 3.9,[a] 39.6[b] | | | 127.0[a] | | | | d |
| 3 | 3.64 | 73.6 | | | | 135.4[a] | 42.5[b] | | 6.5[c] | | dd | m |
| 4 | 3.77 | 64.5 | | | | 42.5[b] | 141.3[a] | | 4.4[c] | | dd | m |
| | 4.01 | | | | | | 146.2[a] | | | | | |
| P | | | 7.0 | | | | | | | s | m | m |

[a] $^1H-^{13}C$ coupling constants. [b] $^{13}C-^{13}C$ coupling constants. [c] $^{13}C-^{31}P$ coupling constants. [d] s, singlet; d, doublet; dd, double doublet; ddd, double double doublet; m, multiplet.

tants (pyruvate, intermediates, and product), whereas the unlabeled components such as cofactors, buffer components, and proteins do not contribute appreciably to the NMR spectrum. The product, **4**, can be purified by chromatography on ECTEOLA cellulose. The enzyme-mediated biotransformation is essentially quantitative, but the workup procedure used is only capable of recovering about 50% of the product in highly purified form.

**Carbon Labels in Positions 1, 2, 2′, 3, and/or 4 (Procedure 3).** 2*C*-Methyl-D-erythritol 4-phosphate with carbon labels in positions 1, 2, 2′, 3, and/or 4 can be obtained from $^{13}C$-labeled glucose in conjunction with $^{13}C$-labeled pyruvate (Figure 2). The fate of different precursor atoms is indicated in Figure 2 by lowercase letters (a−e). For any desired labeling pattern, the appropriately labeled starting materials can be selected easily on the basis of Figure 2. The reaction is performed in two sequential steps without isolation of intermediate **3**. NADPH is recycled in situ using glucose dehydrogenase. To avoid isotopic dilution by the glucose used as the substrate for NADPH regeneration, the glycolytic enzymes (A and C−F) and 1-deoxy-D-xylulose 5-phosphate synthase (G) operative in the first reaction step must be deactivated by heat treatment after the first reaction phase. As an example, the preparation of [U-$^{13}C_5$]**4** is reported in detail in the Experimental Section.

**Carbon Labels in Positions 1, 3, and/or 4 (Procedure 4).** 2*C*-Methyl-D-erythritol 4-phosphate with carbon labels in position 1, 3, or 4 can be obtained from appropriately labeled glucose as the starting material. ATP consumed in the glycolysis sequence can be regenerated using phosphoenolpyruvate and pyruvate kinase (Figure 2). Pyruvate generated in situ from phosphoenolpyruvate in the ATP-recycling step can serve as endogeneously generated substrate for 1-deoxy-D-xylulose 5-phosphate synthase.

The reaction sequence is performed in two sequential steps without isolation of intermediates. Initially, labeled glucose (**9**) is converted into 1-deoxy-D-xylulose 5-phosphate (**3**). Only 2 equiv of phosphoenolpyruvate, just sufficient for the complete conversion of labeled **9** into **2**, should be used in this step. In the subsequent reduction and isomerization step, a recycling system comprising unlabeled glucose and glucose dehydrogenase is used for NADPH regeneration. If phosphoenolpyruvate (from the first step) is still present at this reaction stage, unlabeled glucose will enter the glycolytic pathway and thus will cause an isotopic dilution in the product. The product **4** is stable under the reaction conditions. $^{13}C$ NMR spectra recorded at several stages of the one-pot reaction are shown in Figure 3, which indicates the near-quantitative conversion of $^{13}C$-labeled **9** into the final product, [1,3,4-$^{13}C_3$]**4**.

**Tritium in Position 1 (Procedure 5).** Tritium can be introduced into position 1 of **4** by reduction of **3** with tritium-labeled NADPH, which can be obtained in situ using glucose dehydrogenase and [1-$^3$H]D-glucose as the tritium source. As shown recently, the hydrogen of NADPH is introduced stereospecifically into the 1*R* position of **4**.[3,4] Any $^{13}C$ isotopomer of **3** can be converted into the corresponding 2*C*-methyl-D-erythritol 4-phosphate isotopomer containing tritium in position 1.

## Discussion

2*C*-Methyl-D-erythritol 4-phosphate synthase of *E. coli* had been reported earlier to have a specific activity of 0.5 $s^{-1}$ with $Co^{2+}$ as the cofactor.[6] The N-terminally modified enzyme described in this study has a ca. 26-fold higher catalytic activity with $Mg^{2+}$ or $Mn^{2+}$ as the cofactor. $Co^{2+}$ was a poor activator of enzyme activity with our enzyme. The different enzyme properties might be related to the N-terminal modification.

Isotope-labeled precursors have played a central role in the exploration of the non-mevalonate pathway of terpenoid biosynthesis and have the potential to contribute still further to the elucidation of the missing reaction steps and their mechanisms.[7,13−17]

Due to the versatility of our synthetic procedures, a wide variety of different isotopomers of 2*C*-methyl-D-erythritol 4-phosphate can be prepared from commercially available isotope-labeled glucose and pyruvate as starting materials. Our one-pot procedures make use of cofactor-recycling systems, which facilitates workup and product purification. The procedures can be scaled up for the preparation of gram amounts of the desired isotopomer. Furthermore, for the preparation of **4** labeled with $^{14}C$ or $^3H$ at high specific activity, the procedures can be scaled down linearly to very small volumes.

The yields of the enzyme-catalyzed reaction sequences are almost quantitative as shown by the series of NMR spectra in Figure 3 (although significant losses occur during workup and purification). The driving forces of the reaction are (i) the release of carbon dioxide in the

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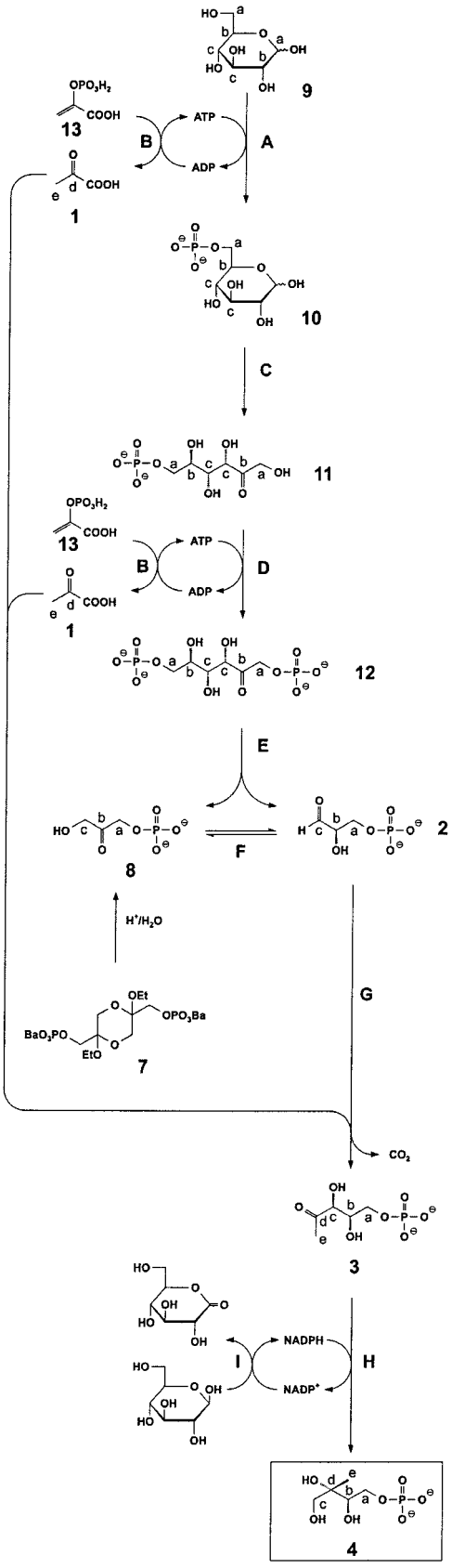


**Figure 2.** Synthesis of $^{13}$C-labeled 2*C*-methyl-D-erythritol 4-phosphates. A, hexokinase; B, pyruvate kinase; C, glucose 6-phosphate isomerase; D, fructose 6-phosphate kinase; E, aldolase; F, triose phosphate isomerase; G, 1-deoxy-D-xylulose 5-phosphate synthase; H, 2*C*-methyl-D-erythritol 4-phosphate synthase; I, glucose dehydrogenase; the transfer of $^{13}$C or $^{14}$C labels from starting material to product is indicated by lowercase letters (a−e).

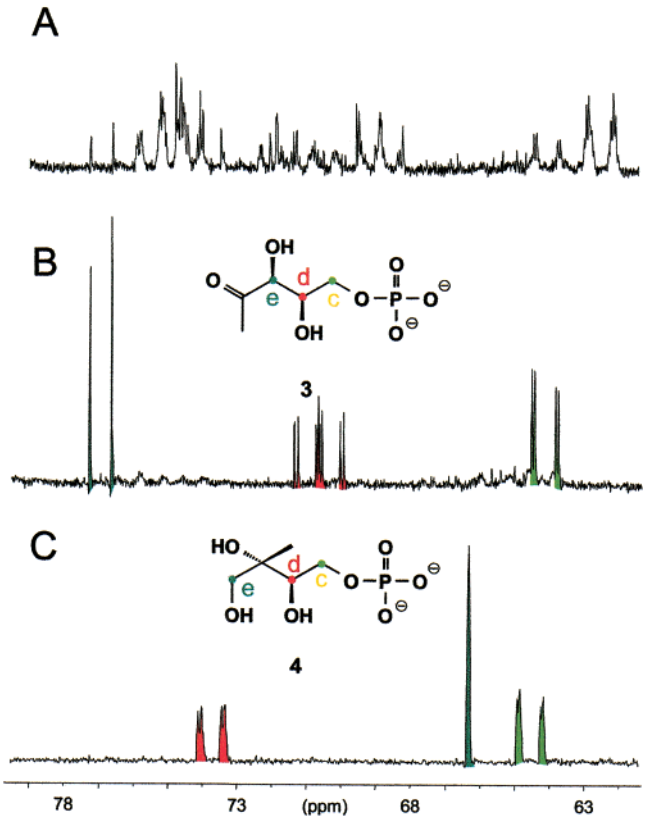


**Figure 3.** Real-time $^{13}$C NMR analysis of a reaction mixture (procedure 4). The detailed reaction conditions are given in the Experimental Section. $^{1}$H-Decoupled $^{13}$C NMR spectra were measured at the beginning of the reaction (A), after conversion of precursors to [3,4,5-$^{13}C_3$]**3** at 12 h after the start of the reaction (B), and at the end of the reaction sequence (C). Color codes of signals refer to the respective reaction component indicated.

formation of **3**, (ii) the recycling of NADPH, and (iii) under certain conditions (procedure 4), the consumption of the pyruvate generated in the ATP regeneration reaction by the 1-deoxy-D-xylulose 5-phosphate synthase reaction. Jointly, these exergonic partial reactions afford a Gibbs free energy gradient that is large enough to drive the main reaction virtually to completion.

By judicious selection of the auxiliary reactions, it is also possible to streamline the composition of the final reaction mixture with regard to product purification. In the reaction sequences described above, the only side products formed in stoichiometric amounts are gluconate, pyruvate, and bicarbonate. Moreover, ethanol is formed stoichiometrically in procedure 1, and 2 equiv of ADP is formed in procedure 3 where the recycling of ADP would result in the dilution of the isotope-labeled pyruvate used as the substrate.

It is obvious that all reaction sequences described in this article can be performed with nonlabeled precursors. Procedures 1 and 4 are best suited for the preparation of unlabeled **4**.

We have previously reported the chemical synthesis of **4** that was comprised of 14 reaction steps with an overall yield of 9%.[18] The method had been designed to allow the incorporation of tritium into position 1 of **4**.

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The resulting [1-$^3$H]**4** played a crucial role in the identification of the *ispD* gene of the non-mevalonate pathway.[15] Two more synthetic procedures for the preparation of **4** have been published so far that allow the introduction of stable isotope labels.[9,19] However, in general, chemical syntheses of multiply labeled compounds need specific strategies and numerous reaction and isolation steps in contrast to the universality of our enzymatic approach.

In comparison to chemical syntheses, the procedures in this paper have the combined advantages of (i) a high yield, (ii) short reaction times, (iii) access to a virtually unlimited variety of $^{13}$C- or $^{14}$C-isotopomers from commercially available precursors, (iv) virtually perfect stereocontrol, (v) the simplicity of the one-pot reaction conditions in aqueous solution, (vi) the possibility of real-time monitoring of the reaction progress by NMR, (vi) the use of commercially available enzymes for all but two reaction steps, and (vii) the exclusive use of starting materials that are commercially available.

It should be mentioned that these favorable aspects of "long shot" in vitro biotransformation are not in any way limited to the biosynthesis of terpenoids. Similar synthetic strategies could be used for a wide variety of natural products in an isotope-labeled or unlabeled form. The rapidly growing body of genomic data from a wide variety of organisms, the advanced state of recombinant DNA technology, and the specific design of recombinant enzymes for ease of purification provide a steadily improving framework for the generalization of this approach.

## Experimental Section

**Materials.** The following materials were obtained from the sources indicated in parentheses: $^{13}$C-labeled compounds (Isotec, Miamisburg, OH); [1-$^3$H]glucose (Amersham Pharmacia Biotech, Freiburg, Germany); sodium [2-$^{14}$C]pyruvate (NEN, Boston, MA); oligonucleotides (MWG Biotech, Ebersberg, Germany); T4 ligase (Gibco-BRL, Eggenstein, Germany); Sepharose Q FF, Superdex 75 HR 26/60, and restriction enzymes (Amersham Pharmacia Biotech); chemicals and enzymes (Sigma Chemicals, Deisenhofen, Germany); DNase I (Roche Diagnostics, Mannheim, Germany); Taq polymerase and IPTG (Eurogentec, Seraing, Belgium); RNase A and Nucleosil 10SB (16 × 250 mm) (Macherey & Nagel, Düren, Germany); ECTEOLA 23 cellulose (Fluka, Deisenhofen, Germany); TLC plates (Merck, Darmstadt, Germany); and $Ni^{2+}$-chelating Sepharose and Dowex ion exchangers (Amersham Pharmacia, Braunschweig, Germany). 1-Deoxy-D-xylulose 5-phosphate was prepared as described elsewhere.[20]

**Proteins.** The preparation of recombinant 1-deoxy-D-xylulose 5-phosphate synthase from *Bacillus subtilis* has been reported elsewhere.[20]

**Microorganisms and Plasmids.** Bacterial strains and plasmids used in this study are summarized in Table 1.

**Construction of an Expression Plasmid.** The *ispC* gene of *E. coli* coding for 2*C*-methyl-D-erythritol 4-phosphate synthase (GenBank accession no. AE000126) was amplified from base pair position 9887 to 11 083 by PCR using the oligonucleotides ECOYAEM1 (5′-ggaggatccatgaagcaactcacc-3′) and ECOYAEM2 (5′-gcgcgactctctgcagccgg-3′) as primers and chromosomal *E. coli* DNA as the template. The amplificate was digested with the restriction endonucleases *Bam*HI and *Pst*I. The fragment was ligated into the expression plasmid vector pQE30, which had been digested with the same restriction enzymes. The ligation mixture was electroporated into *E. coli* XL1-Blue and M15 [pREP4] cells affording the recombinant strains XL1-pQEYAEMECO and M15-pQEYAEMECO, respectively.

**Sequence Determination.** DNA sequencing was performed by the automated dideoxynucleotide method.[21] N-Terminal peptide sequences were obtained by the pulsed-liquid mode.

**Purification of Recombinant 2*C*-Methyl-D-erythritol 4-Phosphate Synthase from *E. coli*.** Recombinant *E. coli* M15-pQEYAEMECO cells (2 g) were suspended in 25 mL of buffer A (100 mM Tris hydrochloride (pH 8.0), 0.5 M sodium chloride) containing 20 mM imidazole hydrochloride, 25 mg of lysozyme, and 2.5 mg of DNase I. The mixture was incubated at 37 °C for 30 min, cooled on ice, and subjected to ultrasonic treatment. The suspension was centrifuged at 15 000 rpm for 30 min. The supernatant was loaded on a $Ni^{2+}$-chelating Sepharose column (2 × 8 cm) at a flow rate of 3 mL/min, which had been equilibrated with 20 mM imidazole in buffer A. The column was developed with a linear gradient of 20−500 mM imidazole in buffer A (total volume = 300 mL). Fractions were combined and concentrated by ultrafiltration.

**Assay of 2*C*-Methyl-D-erythritol 4-Phosphate Synthase.** 2*C*-Methyl-D-erythritol 4-phosphate synthase was assayed according to Takahashi et al.[1]

**Analytical Ultracentrifugation.** The concentrated protein from the purification step described above was loaded on top of a Superdex 75 HR 26/60 column, which had been equilibrated with 100 mM Tris hydrochloride (pH 8.0) containing 1 mM dithiothreitol, 0.02% sodium azide, and 50 mM sodium chloride at a flow rate of 3 mL/min. 2*C*-Methyl-D-erythritol 4-phosphate synthase was eluted to a volume of 132 mL. The peak fraction with an absorbance of about 1 OD at 280 nm was used as a sample for analytical ultracentrifugation.

Hydrodynamic studies were performed with an analytical ultracentrifuge Optima XL-1 (Beckman Coulter) equipped with UV and interference optics. Experiments were performed with double-sector cells equipped with aluminum centerpieces and sapphire windows. Partial specific volumes and buffer densities were estimated according to published procedures.[22]

**Preparation of [2-$^{13}$C$_1$]2*C*-Methyl-D-erythritol 4-Phosphate (Procedure 1).** A solution containing 440 mg (660 μmol) of dimeric dihydroxyacetone phosphate acetal (**7**) in 10 mL of water was added to a suspension of Dowex 50 WX8 (5 mL, $H^+$ form). The mixture was incubated at 65 °C for 2 h. The solid was filtered off and washed with water. The combined solution was lyophilized. Dihydroxyacetone phosphate glass (242 mg, containing about 92 mg (0.33 mmol) of **8**) was dissolved in 2.5 mL of water, and the pH was adjusted to 8.0 by the addition of 8 M sodium hydroxide. A solution (1.5 mL) containing 450 mM Tris hydrochloride (pH 8.0), 30 mM magnesium chloride, 8.5 mg (20 μmol) of thiamine diphosphate, 1.5 mg (10 μmol) of dithiothreitol, and 363 mg (330 μmol) of [2-$^{13}$C$_1$]pyruvate (**1**, sodium salt) was added. The pH was adjusted to 8.0, and 88 units (15 μg) of triose phosphate isomerase and 9.6 units (3.0 mg) of 1-deoxy-D-xylulose 5-phosphate synthase were added. The mixture was incubated at 37 °C for 11 h. NADPH (317 mg, 350 μmol) and 2*C*-methyl-D-erythritol 4-phosphate synthase (1 mg) were added, and incubation was continued at 37 °C for 2 h. The product was purified by chromatography on ECTEOLA cellulose as described below, yielding 43.6 mg (201 μmol, 61%).

**Purification of 2*C*-Methyl-D-erythritol 4-Phosphate (General Procedure).** Crude reaction mixtures obtained as described above were lyophilized. The crude product was

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dissolved in water (1 g/100 mL). The solution was placed on a column of ECTEOLA 23 cellulose (5 × 30 cm), which was developed with a linear gradient of 0 to 0.3 M triethylammonium acetate (total volume = 4 L) at a flow rate of 1.5 mL/min. Fractions (10 mL) were collected and analyzed by TLC using Silica NH−Sil plates, which were developed with a mixture of 2-propanol/ethyl acetate/water (6:1:3, v/v). The product was identified as a brown spot after staining with a mixture of vanillin/sulfuric acid (10 g/L). Fractions were combined and lyophilized. The chemical purity of 2*C*-methyl-D-erythritol 4-phosphate (triethylammonium salt) was approximately 90%, as estimated by $^1$H NMR spectroscopy.

**Preparation of [2-$^{14}$C]2*C*-Methyl-D-erythritol 4-Phosphate (Procedure 2).** A solution containing 260 mM Tris hydrochloride (pH 8.0), 10 mM magnesium chloride, 80 μg (0.50 μmol) of dithiothreitol, 0.4 mg (0.9 μmol) of thiamine diphosphate, 1.0 mg (4.8 μmol) of dihydroxyacetone phosphate (lithium salt), 0.5 mg (4.8 μmol) of [2-$^{14}$C]pyruvate (sodium salt, 10.4 μCi/μmol), and 250 units (30 μg) of triose phosphate isomerase in a total volume of 450 μL was incubated at 37 °C for 15 min. A solution of 0.5 units (0.2 mg) of 1-deoxy-D-xylulose 5-phosphate synthase from *B. subtilis* in 50 μL of 100 mM Tris hydrochloride (pH 8.0) was added, and the mixture was incubated for 2 h. Subsequently, a solution containing 100 mM Tris hydrochloride (pH 8.0), 10 mM magnesium chloride, 1.8 mg of NADPH (2.0 μmol), and 0.1 mg (0.9 units) of recombinant 2*C*-methyl-D-erythritol 4-phosphate synthase in a total volume of 200 μL was added. The mixture was incubated at 37 °C for 30 min. Aliquots (10 μL) were applied to an HPLC column of Nucleosil 10SB (4.6 × 250 mm) that was developed with 10 mM ammonium formate in 0.2 M formic acid at a flow rate of 1 mL/min. The effluent was monitored by a solid-state scintillation counter. [2-$^{14}$C]2*C*-Methyl-D-erythritol 4-phosphate was eluted at 16 mL. Fractions were combined and lyophilized affording 20 μCi of [2-$^{14}$C]2*C*-methyl-D-erythritol 4-phosphate (radiochemical yield = 40%).

**Preparation of [U-$^{13}C_5$]2*C*-Methyl-D-erythritol 4-Phosphate (Procedure 3).** A reaction mixture containing 150 mM Tris hydrochloride (pH 8.0), 10 mM magnesium chloride, 44 mg (0.10 mmol) of thiamine diphosphate, 1.02 g (1.79 mmol) of ATP, 0.17 g (0.89 mmol) of [U-$^{13}C_6$]glucose, 200 mg (1.79 mmol) of [2,3-$^{13}C_2$]pyruvate, 410 units (0.05 mg) of triose phosphate isomerase, 360 units (3.6 mg) of hexokinase, 50 units (0.07 mg) of phosphoglucose isomerase, 20 units (3.2 μg) of fructose 6-phosphate kinase, 35 units (6.0 μg) of aldolase, and 2 units (0.6 mg) of recombinant 1-deoxy-D-xylulose 5-phosphate synthase from *B. subtilis* in a total volume of 58 mL was incubated at 37 °C overnight, while the pH was held at a constant value of 8.0 by the addition of 1 M sodium hydroxide. The reaction was stopped by adding 3 mL of 2 M hydrochloric acid. The precipitate was removed by centrifugation, and the pH of the solution was readjusted to 8.0. A solution (72 mL) containing 0.97 g (5.4 mmol) of glucose, 100 mg (0.1 mmol) of $NADP^+$, 10 units (1.4 mg) of 2*C*-methyl-D-erythritol 4-phosphate synthase, and 120 units (2.2 mg) of glucose dehydrogenase was added. The mixture was incubated at 37 °C overnight. Aliquots (5 mL) were applied to an HPLC column of Nucleosil 10 SB (16 × 250 mm) using 0.5 M formic acid as the eluent at a flow rate of 13 mL/min. The effluent was monitored refractometrically. The product was eluted at 14.5 min. Fractions were combined and lyophilized, yielding 86 mg (0.89 mmol, 50%). The chemical purity of 2*C*-methyl-D-erythritol 4-phosphate was > 97%, as estimated by $^1$H NMR spectroscopy.

**Preparation of [1,3,4-$^{13}C_3$]2*C*-Methyl-D-erythritol 4-Phosphate (Procedure 4).** A solution containing 150 mM Tris hydrochloride (pH 8.0), 10 mM magnesium chloride, 1.0 g (5.4 mol) of [U-$^{13}C_6$]glucose, 0.23 g (1.5 mmol) of dithiothreitol, 0.3 g (0.7 mmol) of thiamine diphosphate, 0.1 g (0.2 mmol) of ATP (disodium salt), and 2.2 g (11 mmol) of potassium phosphoenolpyruvate in a total volume of 300 mL was adjusted to pH 8.0 by the addition of 8 M sodium hydroxide. Hexokinase (1500 units, 10 mg), 300 units (400 μg) of phosphoglucose isomerase, 105 units (1 mg) of fructose 6-phosphate kinase, 610 units (100 μg) of triose phosphate isomerase, 57 units (10 μg) of aldolase, 400 units (2.8 mg) of pyruvate kinase, and 8 units (3 mg) of 1-deoxy-D-xylulose 5-phosphate synthase were added. The solution was incubated at 37 °C for 24 h. Subsequently, 2.0 g (11 mmol) of glucose, 200 mg (200 μmol) of $NADP^+$, 100 units (1.8 mg) of glucose dehydrogenase, and 39 units (5.7 mg) of 2*C*-methyl-D-erythritol 4-phosphate synthase were added, and incubation at 37 °C was continued for 18 h. The product was purified by the general method described above, yielding 1.0 g (4.6 mmol, 41%).

**Preparation of (1*R*)-[1-$^3$H]2*C*-Methyl-D-erythritol 4-Phosphate (Procedure 5).** A solution containing 100 mM Tris hydrochloride (pH 8.0), 10 mM magnesium chloride, 0.16 mg (0.20 μmol) of $NADP^+$, 1 μg (6 nmol) of [1-$^3H_1$]D-glucose (120 μCi), 5.0 μg (23 nmol) of 1-deoxy-D-xylulose 5-phosphate, 2 units (36 μg) of glucose dehydrogenase, and 0.2 units (29 μg) of 2*C*-methyl-D-erythritol 4-phosphate synthase in a total volume of 301 μL was incubated at 37 °C for 1 h. The mixture was applied to a column of Dowex 1X8 (0.5 × 5.5 cm, formate form), which was washed with 6 mL of water followed by 10 mL of 1 M formic acid. The effluent was lyophilized affording 68 μCi of (1*R*)-[1-$^3$H]2*C*-methyl-D-erythritol 4-phosphate (radiochemical yield = 57%).

**NMR.** $^1$H NMR and $^1$H-decoupled $^{13}$C NMR spectra were recorded at 500.1 and 125.6 MHz for $^1$H and $^{13}$C, respectively. The chemical shifts were referenced to external trimethylsilylpropane sulfonate. $^{31}$P NMR spectra were recorded at a frequency of 101.3 MHz. $^{31}$P chemical shifts were referenced to external 85% $H_3PO_4$. 2*C*-Methyl-D-erythritol 4-phosphate spectra were recorded in 90% $H_2O$/10% $D_2O$ (pH 8.0, uncorrected glass electrode reading).

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