Studies on the Non-Mevalonate Pathway − Preparation and Properties of Isotope-Labeled 2*C*-Methyl-D-erythritol 2,4-Cyclodiphosphate

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Recent studies have shown that 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate can be formed from 2*C*-methyl-D-erythritol 4-phosphate by the consecutive action of IspD, IspE and IspF proteins in the non-mevalonate pathway of isoprenoid biosynthesis. We describe here rapid one-pot strategies for the enzyme-assisted preparation of this compound from isotope-labeled pyruvate and glucose that are optimized for the introduction of ^{13}C or ^{14}C and afford a wide variety of isotopomers in mmol quantity. The one-pot reactions involving up to 10 forward reaction steps and up to 15 enzyme catalysts have an overall yield of 50 to 80% of purified product.

Introduction

The discovery of the non-mevalonate or deoxyxylulose phosphate pathway of isoprenoid biosynthesis by Rohmer, Arigoni and their co-workers has triggered an intense burst of research activity.[1−5] The first intermediate of the alternative pathway, 1-deoxy-D-xylulose 5-phosphate (**7**), was shown to be formed by the condensation of D-glyceraldehyde 3-phosphate (**6**) with pyruvate under the catalytic action of 1-deoxy-D-xylulose 5-phosphate synthase (Figure 1).[6−11] The linear pentulose derivative is converted into the branched-chain polyol derivative 2*C*-methyl-D-erythritol 4-phosphate (**8**) by a skeletal rearrangement catalyzed by a reductoisomerase.[12−16] The product is converted into 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**11**) by the consecutive action of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase, 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase, and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase.[17−24] Compound **11** has been isolated earlier as a stress metabolite from bacterial cultures in high yield.[25,26]

The conversion of **11** into the universal terpenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), however, is still to be investigated. In vivo studies with deuterium-labeled precursors suggest that IPP and DMAPP are formed via separate pathways, whereas IPP is the obligatory precursor of DMAPP in the mevalonate pathway.[27,28]

Tracer studies with radioactive and/or stable isotope-labeled precursors have played an important role in the study of the mevalonate as well as the deoxyxylulose phosphate pathway, and it appears likely that isotope-labeled intermediates of the deoxyxylulose phosphate pathway could contribute to the elucidation of the unknown steps as well as to mechanistic studies in the biosynthesis of complex terpenoids.

Results and Discussion

The preparation of isotope-labeled **9** using recombinant enzymes of the deoxyxylulose pathway has already been described.[29] Here we show that all currently known enzymes of the deoxyxylulose phosphate pathway can be used for the preparation of radiolabeled or stable isotope-labeled isotopomers of **11** in high yield. ^{13}C or ^{14}C can be introduced from commercially available glucose and/or pyruvate isotopomers. These precursors are converted into **11** by one-pot reactions involving up to 15 enzyme catalysts (Figure 2). The required enzymes are either commercially available or can be prepared relatively easily from recombinant *Escherichia coli* strains.[6,7,9,12,17,19,21]

The methods described make extensive use of cofactor recycling (Figure 2). Thus, the formation of D-glyceraldehyde 3-phosphate from glucose requires ATP that can be regenerated by pyruvate kinase using phosphoenolpyruvate as a substrate. Similarly, CTP can be recycled by the inclusion of nucleoside monophosphate kinase and nucleoside diphosphate kinase into the reaction mixture. NADPH can be regenerated in situ by reduction of $NADP^+$ with glucose and glucose dehydrogenase. The reutilization of the cofactors can reduce the overall costs quite considerably. For example, the price of NADPH is comparable to that of ^{13}C-labeled glucose or pyruvate. Even more importantly, cofactor recycling reduces the amount of side products, thus facilitating the purification of the reaction products.

Various isotopomers of D-glyceraldehyde 3-phosphate can be obtained from commercial starting materials. For example, the treatment of [U-$^{13}C_6$]glucose with glycolytic

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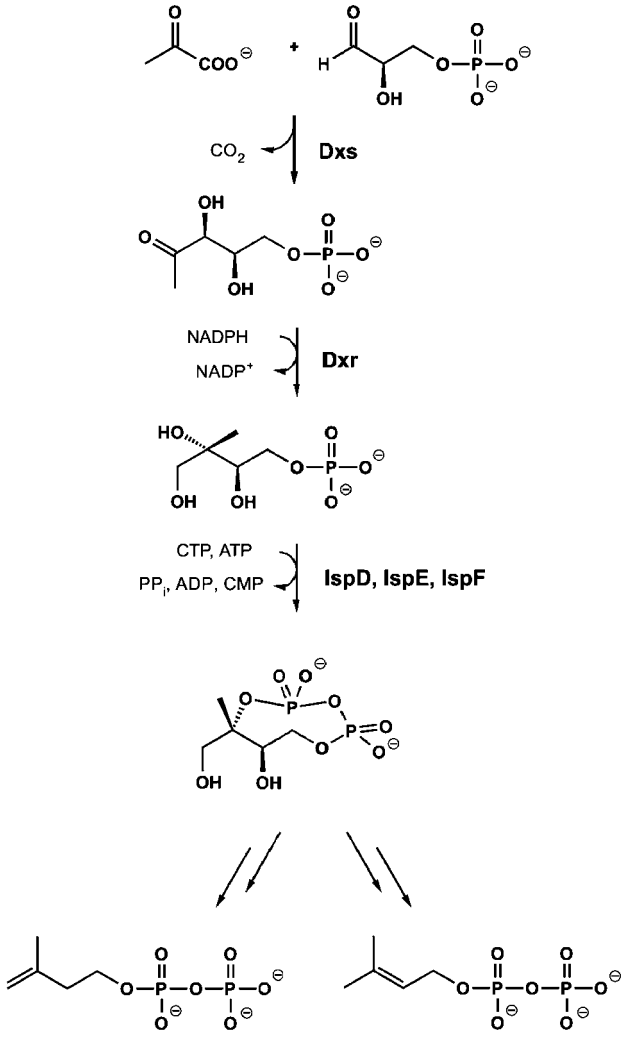

Figure 1. Intermediates and enzymes of the non-mevalonate pathway of terpenoid biosynthesis; 1-deoxy-D-xylulose 5-phosphate synthase (Dxs), reductoisomerase (Dxr), 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase (IspD), 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase (IspE) and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF)

pathway enzymes affords [U-$^{13}C_3$]-D-glyceraldehyde 3-phosphate (Table 1) that can be condensed with pyruvate to form [3,4,5-$^{13}C_3$]-**7**; the subsequent action of the deoxyxylulose phosphate pathway enzymes affords [1,3,4-$^{13}C_3$]-**11**.

The entire reaction sequence from [U-$^{13}C_6$]-glucose (**1**) to [1,3,4-$^{13}C_3$]-**11** can be monitored in real time by ^{13}C NMR spectroscopy (Figure 3) since the sensitivity and selectivity of NMR detection is greatly enhanced by the use of the ^{13}C-labeled starting material. Pyruvate is not required for the synthesis of [1,3,4-$^{13}C_3$]-**11** since it is generated in situ from the phosphoenolpyruvate that is used for the regeneration of ATP. Due to cofactor recycling, only excess pyruvate, pyrophosphate and gluconolactone are formed as side products in stoichiometric amounts, thus affording a crude reaction mixture consisting of a relatively pure primary product (Figure 3). This facilitates purification which is achieved by ion exchange chromatography on Ecteola cellulose. The eluent, triethylammonium acetate, can be removed by lyophilization affording 50% overall yield of [1,3,4-$^{13}C_3$]-**11** based on the isotopically labeled starting material. The ^{13}C NMR spectroscopic data of the purified product (Table 2) show no isotope-labeled components besides the nominal product. Product purity was also established by ^1H NMR spectroscopy and mass spectrometry (Table 3).

[U-$^{13}C_5$]-**11** is similarly obtained from [U-$^{13}C_6$]-**1** in conjunction with [2,3-$^{13}C_2$]-pyruvate. In this case, ATP is added to the reaction mixture in stoichiometric amounts, since ATP regeneration with phosphoenolpyruvate would generate unlabeled pyruvate which would dilute the ^{13}C-labeled pyruvate used in the subsequent formation of **7**.

For the preparation of **11** labeled in position 2 and/or 2′, unlabeled **6** can be condensed with isotope-labeled pyruvate, and the resulting **7** can be converted into **11** by a reaction sequence involving the regeneration of ATP and CTP. Again, the entire reaction sequence can be performed as a one-pot reaction with high yield.

Each of the one-pot reactions described above can be scaled down to small reaction volumes well below one milliliter. This allows the synthesis of ^{14}C-labeled **11** from ^{14}C-labeled pyruvate or glucose without dilution of the radioisotope. As an example we prepared [2-^{14}C]-**11** at a specific activity of 15.8 μCi μmol^{-1} with an overall radiochemical yield of 80% based on [2-^{14}C]-pyruvate as starting material.

The chemical synthesis of **11** has not been reported previously to the best of our knowledge. The one-pot reactions described in this paper comprise up to 10 forward reaction steps and involve the catalytic action of up to 15 enzymes, including those used for in situ cofactor recycling. It should be noted that the optimization of these complex reaction sequences was based on real time ^{13}C NMR diagnosis proceeding with enhanced selectivity due to the ^{13}C labeling of the starting materials. It is obvious that the synthetic procedures described can also be used for the preparation of unlabeled **11**.

A variety of other ^{13}C- or ^{14}C-labeled isotopomers can be obtained using the same procedures with pyruvate and/or glucose with appropriate isotope labels as precursors. Notably, it is of course possible to prepare isotopomers carrying ^{13}C at a single position, although the focus of the present work was on multiply labeled products which are particularly useful for biosynthetic studies.

The study shows that complex biochemical reaction sequences comprising numerous reaction steps can be executed in vitro with high efficacy. The optimization of such complex synthetic strategies, however, requires the appropriate analytical tools for online diagnosis. Moreover, it is essential to drive the reaction essentially to completion via the control of the Gibbs free energy difference of the overall reaction. Under favorable conditions, structurally complex natural products can thus be obtained in high yield. The enzyme-based approach provides unique opportunities for the generation of isotopomers with complex labeling patterns. This in turn can provide the basis for the study of the enzyme ligand complexes using a variety of isotope-sensitive techniques such as liquid and solid phase NMR, EPR, and vibronic spectroscopy.

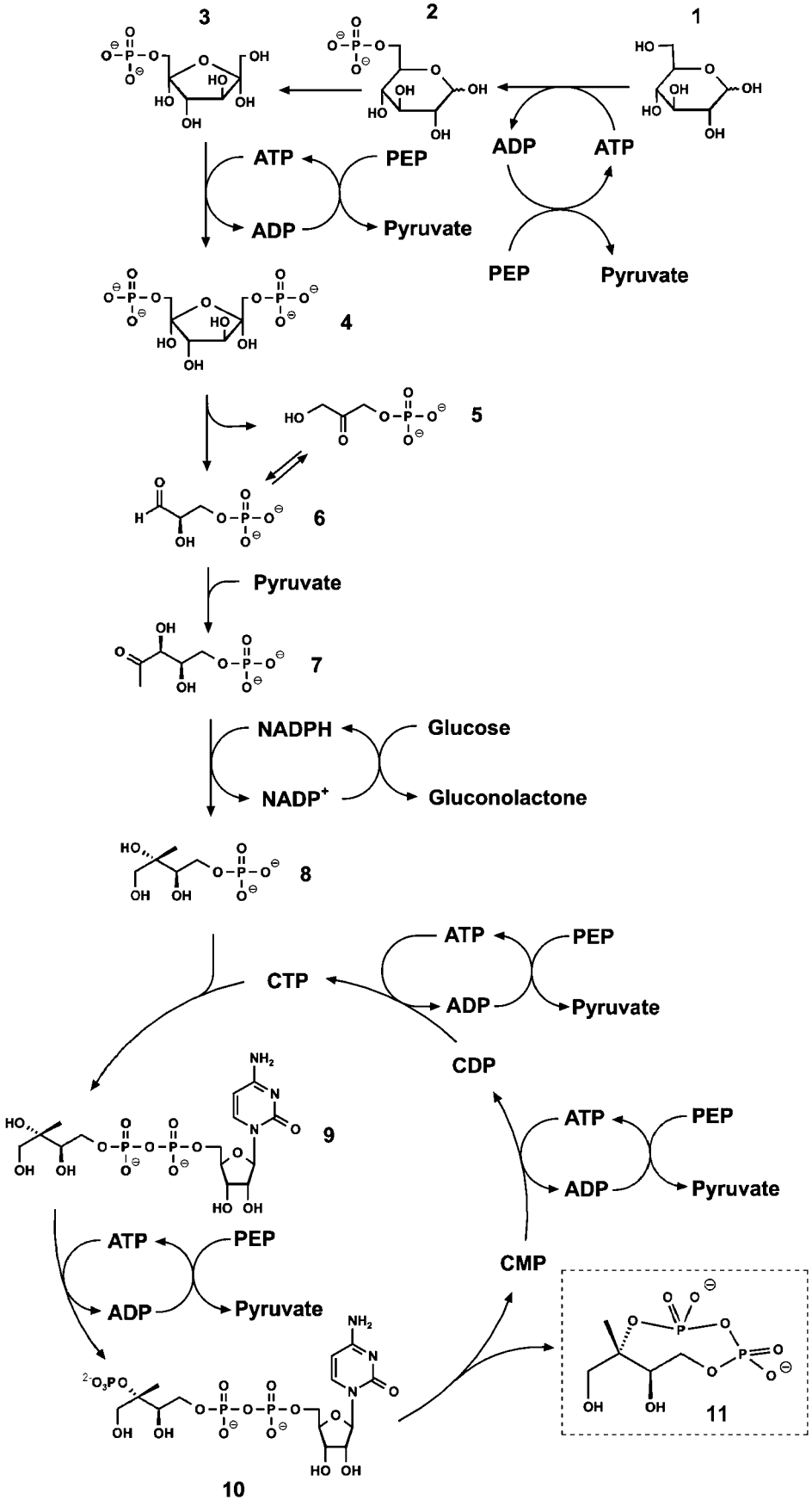

Figure 2. Synthetic route to 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate (**11**); the cofactor recycling enzymes used are also shown

Table 1. Preparation of ^{13}C-labeled **11** from pyruvate, glucose (**1**) and dihydroxyacetone phosphate (**5**)

C_2 precursor	C_3 precursor	Product
[2,3-$^{13}C_2$]pyruvate	[U-$^{13}C_6$]-**1**	[U-$^{13}C_5$]-**11**
pyruvate	[U-$^{13}C_6$]-**1**	[1,3,4-$^{13}C_3$]-**11**
[2-^{13}C]pyruvate	**5**	[2-^{13}C]-**11**
[2-^{14}C]pyruvate	**5**	[2-^{14}C]-**11**

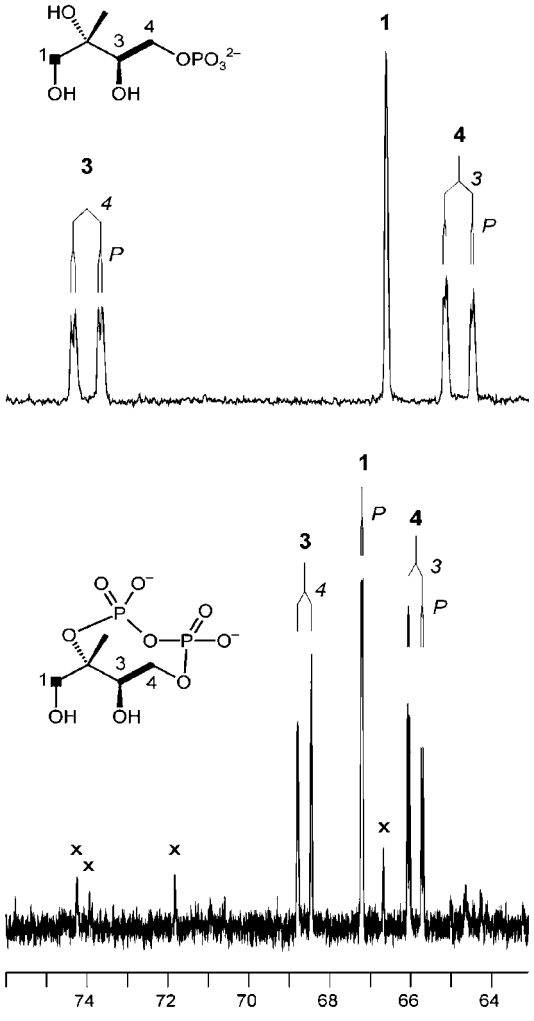

Figure 3. ^{13}C NMR spectra of a crude reaction mixture; top: prior to the addition of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase the dominant ^{13}C-labeled molecular species is [1,3,4-$^{13}C_3$]-**8**; bottom: after termination of the reaction the dominant ^{13}C-labeled species is [1,3,4-$^{13}C_3$]-**11**; X designates impurities

Table 2. ^{13}C NMR spectroscopic data of ^{13}C-labeled **11** using D_2O as solvent

			[U-$^{13}C_5$]-**11**		[1,3,4-$^{13}C_3$]-**11**	
Position	^{13}C (δ)	J_{CH} (Hz)	J_{CC} (Hz)	J_{CP} (Hz)	J_{CC} (Hz)	J_{CP} (Hz)
1	67.9	145	42	[a]		4
2	83.9		40, 43, 41	8		
2-methyl	16.4	148	40	6		
3	68.4	n. d.	43, 43		43	
4	65.5	n. d.	43	6	43	n.d.

[a] Not determined.

Experimental Section

General: ATP, CTP, $NADP^+$, TPP and Ecteola cellulose were purchased from Sigma (Deisenhofen, Germany). [U-$^{13}C_6$]glucose was obtained from Omicron, South Bend, IN, USA. [2,3-$^{13}C_2$]pyruvate and [2-$^{13}C_1$]pyruvate were purchased from Cambridge Isotope Laboratories, Andover, MA, USA. [2-^{14}C]pyruvate was obtained from NEN, Boston, MA, USA. Hexokinase from yeast (EC 2.7.1.1), phosphoglucose isomerase from yeast (EC 1.1.1.49), fructose 6-phosphate kinase from rabbit muscle (EC 2.7.1.11), fructose 1,6-bisphosphate aldolase from rabbit muscle (EC 5.3.1.1), glucose dehydrogenase from *Bacillus megaterium* (EC 1.1.1.47), nucleoside monophosphate kinase from bovine liver (EC 2.7.4.4), and nucleoside diphosphate kinase from baker's yeast (EC 2.7.4.6) were purchased from Sigma (Deisenhofen, Germany). Recombinant 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase, 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate kinase, and 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase from *E. coli* were prepared by published procedures.[17,19,21] The preparation of 1-deoxy-D-xylulose 5-phosphate synthase from *Bacillus subtilis* and reductoisomerase from *E. coli* will be published elsewhere. NMR spectra were obtained using a Bruker AVANCE DRX 500 spectrometer (Karlsruhe, Germany). Chemical shifts were referenced to external trimethylsilylpropane sulfonate or 85% ortho-phosphoric acid, respectively. ESI mass spectra were recorded on a LCQ Finnigan mass spectrometer (San Jose, CA, USA) in the negative and positive ion detection mode. Radioactive TLC spots were detected with a Storm 860 phosphor imager from Molecular Dynamics (Sunnyvale, CA, USA).

[U-$^{13}C_5$]-2*C*-methyl-D-erythritol 2,4-Cyclodiphosphate (11): A reaction mixture containing 120 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 1.31 g (2.20 mmol) of ATP, 0.20 g (1.09 mmol) of [U-$^{13}C_6$]glucose, 0.24 g (2.17 mmol) of [2,3-$^{13}C_2$]pyruvate, 53 mg (0.12 mmol) of thiamine pyrophosphate, 102 U of hexokinase, 62 U of phosphoglucose isomerase, 28 U of fructose 6-phosphate kinase, 15 U of fructose 1,6-bisphosphate aldolase, 167 U of triose phosphate isomerase and 1.8 mg (3.8 U) of 1-deoxy-D-xylulose 5-phosphate synthase in a total volume of 71 mL was incubated at 37 °C. The pH was stabilized at 8.0 by the occasional addition of 2 N

Table 3. ESI mass spectrometry of **11**

Isotopomer	Mass in negative ion detection mode	Mass in positive ion detection mode
[1,3,4-$^{13}C_3$]-**11**	*m/z* = 280.3	[a]
[U-$^{13}C_5$]-**11**	*m/z* = 282.7	*m/z* = 486.1 (triethylammonium salt)

[a] Not determined.

NaOH, and the reaction was monitored at intervals by ^{13}C NMR spectroscopy. The ^{13}C-labeled starting material had disappeared after 22 hours. Unlabeled glucose (0.39 g, 2.16 mmol), 30 mg of $NADP^+$, 1.5 mg (10 U) of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, and 27 U of glucose dehydrogenase were then added. The reaction mixture was incubated at 37 °C. Aliquots were monitored at intervals by ^{13}C NMR spectroscopy. The reaction was virtually complete after 20 h.

Dithiothreitol (1 M, 0.4 mL), 53 mg (0.1 mmol) of CTP, 1.25 g (6.06 mmol) of phosphoenolpyruvate, 11 U of nucleoside monophosphate kinase, 250 U of nucleoside diphosphate kinase, 1.6 mg of pyruvate kinase, 4.2 mg (105 U) of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase, 2.8 mg (92 U) of 4-diphosphocytidyl-2*C*-methyl-D-erythritol 2-phosphate kinase, and 0.6 mg (1.7 U) of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase were added. The solution was incubated at 37 °C. The pH was stabilized at 8.0 by the occasional addition of 2 N HCl. The reaction was terminated after 15 h by lyophilization.

Purification of 11: Typically, 1 g of lyophilized reaction mixture was dissolved in 15 mL of water and applied to a column of Ecteola 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 l; flow rate, 2 mL min^{-1}). Fractions of 25 mL were collected and analyzed by thin layer chromatography using silica gel N-HR (Macherey & Nagel, Düren, Germany) plates which were developed in *n*-propanol/ethyl acetate/water (6:1:3, v/v/v), dried, sprayed with vanilline-sulfuric acid reagent, and heated at 100 °C. The product afforded reddish brown spots (R_f value of 0.42). Chromatograms of radiolabeled samples were monitored using a phosphor imager. The fractions were then combined and lyophilized repeatedly to remove triethylammonium acetate. The product was obtained as the triethylammonium salt (slightly brown solid, yield 1.1 mmol, 50%). The NMR and MS data of [U-$^{13}C_5$]-**11** ($C_5H_{12}O_9P_2$, M_r 283) are summarized in Table 2 and 3, respectively.

[1,3,4-$^{13}C_3$]-2*C*-Methyl-D-erythritol 2,4-Cyclodiphosphate (11): A solution containing 300 mL of 150 mM Tris hydrochloride (pH 8.0), 10 mM $MgCl_2$, 1 g (5.4 mmol) of [U-$^{13}C_6$]-glucose, 231 mg (1.5 mmol) of dithiothreitol, 300 mg (0.7 mmol) of thiamine pyrophosphate, 100 mg (0.2 mmol) of ATP, 2.2 g (10.8 mmol) of phosphoenolpyruvate (potassium salt), 1500 U of hexokinase, 300 U of phosphoglucose isomerase, 105 U of fructose 6-phosphate kinase, 610 U of triose phosphate isomerase, 57 U of fructose 1,6-bisphosphate aldolase, 403 U of pyruvate kinase, and 3.7 mg (8 U) of 1-deoxy-D-xylulose 5-phosphate synthase was incubated at 37 °C for 24 hours. ^{13}C NMR spectroscopy indicated an almost quantitative conversion of glucose to [3,4,5-$^{13}C_3$]-1-deoxy-D-xylulose 5-phosphate.

Glucose (2 g, 10.8 mmol), 100 U of glucose dehydrogenase, 200 mg (0.2 mmol) of $NADP^+$ and 5.7 mg (39 U) of 1-deoxy-D-xylulose 5-phosphate reductoisomerase were then added. The mixture was incubated at 37 °C for 18 h. ^{13}C NMR spectroscopy showed virtually complete conversion of 1-deoxy-D-xylulose 5-phosphate into [1,3,4-$^{13}C_3$]-2*C*-methyl-D-erythritol 4-phosphate.

Phosphoenolpyruvate (6.8 g, 33 mmol) and CTP (100 mg, 0.2 mmol) were added. The pH was adjusted to 8 by the addition of 8 M NaOH. Nucleoside monophosphate kinase (10 U), 100 U of nucleoside diphosphate kinase, 4.5 mg (110 U) of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase, 17.6 mg (580 U) of 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase, 5 mg (12.6 U) of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase, 720 U of pyruvate kinase, and 0.1 mmol of ATP (50 mg) were added. The mixture was incubated at 37 °C for 30 h. ^{13}C NMR spectroscopy showed the formation of [1,3,4-$^{13}C_3$]-2*C*-methyl-D-erythritol 2,4-cyclodiphosphate accompanied by virtually complete depletion of [1,3,4-$^{13}C_3$]-2*C*-methyl-D-erythritol 4-phosphate. The mixture was centrifuged in order to remove the precipitate, and the supernatant was lyophilized. Purification as described above afforded 5.4 mmol of product (yield, 50%). The NMR and MS data of [1,3,4-$^{13}C_3$]-**11** ($C_5H_{12}O_9P_2$, M_r 281) are summarized in Table 2 and 3, respectively.

[2-^{14}C]-2*C*-Methyl-D-erythritol 2,4-Cyclodiphosphate (11): A solution containing 150 mM Tris hydrochloride, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 6.3 mg (29.9 μmol) of dihydroxyacetone phosphate, 2.4 mg (4 μmol) of ATP, 11.3 mg (19.2 μmol) of CTP, 1.3 mg (2.7 μmol) of thiamine pyrophosphate, 4.0 mg (19.3 μmol) of phosphoenolpyruvate, 21.8 mg (23.0 μmol) of NADPH, triose phosphate isomerase (250 U), 0.14 mg (0.3 U) of 1-deoxy-D-xylulose 5-phosphate synthase, 0.13 mg (0.8 U) of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, 0.07 mg (1.7 U) of 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase (total volume, 1 mL) was added to 1.77 mg (15.8 μmol) of [2-^{14}C]pyruvate (250 μCi, 15.8 μCi $μmol^{-1}$), and the mixture was incubated at 37 °C.

After 5 h, 24 U pyruvate kinase and 0.14 mg (4.6 U) of 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase were added. After another 6.5 h, 0.12 mg (0.3 U) of 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase were added. The reaction was terminated after 8 h.

Aliquots (250 μL) were applied to a HPLC column (Nucleosil 5 SB, 25 × 0.75 cm) which was developed with 100 mM ammonium formate in 40% methanol. The effluent was monitored in real time by solid-phase scintillation counting (Biostep GmbH, Jahnsdorf, Germany). The retention volume was 86 mL. Evaporation of the methanol followed by lyophilization afforded the ammonium salt of [2-^{14}C]-2*C*-methyl-D-erythritol 2,4-cyclodiphosphate as a white solid (12.6 μmol, 200 μCi; 15.8 μCi $μmol^{-1}$; radiochemical yield, 80%).

[2-^{13}C]-2*C*-Methylerythritol 2,4-Cyclodiphosphate (11): The same procedure as described for [2-^{14}C]-**11** afforded [2-^{13}C]-**11** from [2-^{13}C]pyruvate (yield, 12.6 μmol, 80%).

Acknowledgments

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