A Facile Enzymatic Synthesis of Isotopically Labeled 2-Methyl-D-erythritol 2,4-Cyclodiphosphate by Spinach Chloroplast Stroma

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Dedicated, on the occasion of his 75th birthday, to *Duilio Arigoni*, the unparalleled master of terpenoid research

For the investigation of the ultimate and penultimate steps of the deoxyxylulose phosphate pathway in plants and microorganisms, and to solve intracellular transport problems, we have developed a facile enzymatic preparation of 2-methyl-D-erythritol 2,4-cyclodiphosphate in highly radioactive form. Use has been made of spinach chloroplast stroma, as well as the stroma of *Capsicum annuum* and *Narcissus pseudonarcissus* chromoplasts, which were shown to transform differently labeled 1-deoxy-D-xylulose 5-phosphate quantitatively into that cyclic diphosphate in the presence of cofactors. This method can also be exploited to synthesize milligram quantities of ^{13}C-labeled cyclic diphosphate. It is shown that recombinant *Escherichia coli* deoxyxylulose phosphate synthase is catalytically active and can be used to synthesize the labeled starting material for further enzymatic work, as well as for feeding of intact plants. The pH and temperature stability of the deoxyxylulose 5-phosphate and the cyclic diphosphate have been determined. Uniformly ^{13}C- or ^{14}C-labeled pyruvate, and uniformly ^{13}C- or ^{14}C-labeled D-glyceraldehyde 3-phosphate were synthesized in almost quantitative yields from uniformly ^{13}C- or ^{14}C-labeled D-glucose *en route* to deoxyxylulose 5-phosphate.

1. Introduction. – A novel pathway leading to terpenoids was discovered through labeling experiments conducted with plants and microorganisms in the laboratories of *Arigoni* [1][2], and *Rohmer*, *Sahm* and co-workers [3]. It is now clearly established that, in higher plants, terpenoids are formed in parallel, both by the classical mevalonate pathway as well as by the pathway starting from deoxyxylulose 5-phosphate (DXP; **3**). After the discovery of the first enzyme (DXP synthase; DXS) opening this pathway [4], within five years, all seven enzymes and genes of this novel metabolic grid were rapidly discovered [5–9]. The common precursors of both the mevalonate and the DXP pathway leading to all terpenoids found in nature are isopentenyl pyrophosphate (IPP; **9**) and dimethylallyl pyrophosphate (DMAPP; **10**). While the mevalonate pathway is assumed to be located in the cytosol, the DXP-pathway enzymes seem to be located in the plastidic compartment of the plant cell, *i.e.*, mainly in chloroplasts and chromoplasts [10–12]. In previous experiments, it had been shown that chromoplasts from both *Narcissus pseudonarcissus* and *Capsicum annuum* are able to convert 2-methyl-D-erythritol 4-phosphate (MEP; **4**) to 2-methyl-D-erythritol 2,4-cyclodiphosphate (cMEPP; **7**) and further to the carotenoids phytoene and β-carotene on an analytical scale *in vitro* [13]. Protein extracts from *C. annuum* chromoplasts containing neither thylakoids nor chromophores converted MEP to a diphosphate, but incorporation into the carotenoid fraction was not observed. The diphosphate isolated from these extracts was unequivocally shown to be the expected cMEPP by ^{13}C-NMR spectroscopy [13]. This result clearly demonstrates that, in

chromoplast, soluble protein extracts, the stroma fraction, the three enzymes encoded by *ispD*, *ispE*, and *ispF* (*Scheme*) are present and catalytically active.

In the bacterium *Escherichia coli*, the reduction of cMEPP (**7**) by the enzyme GcpE (encoded by *ispG*) to yield (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP; **8**) [14–19], as well as the subsequent dehydroxylation of HMBPP to IPP (**9**) and DMAPP (**10**) by the LytB (encoded by *ispH*) enzyme [19–22] seems to be clarified. In the chromoplast system of *C. annuum*, ^{14}C-HMBPP is transformed to phytoene and β-carotene [23]. The functional characterization of the gene products of *ispG* and *ispH* in plants is still an open question. To tackle these as well as mechanistic questions, a facile preparation of highly radioactively labeled (^{14}C or ^{3}H) **7** is necessary. This latter compound plays obviously also the role of a metabolic sink in the DXP pathway as demonstrated in microorganisms and higher plants [24][25]. This observation makes it desirable also to conduct transport experiments in plants, for which the availability of radioactive cMEPP and other intermediates in the DXP pathway are required. The preparation of ^{13}C- or ^{14}C-labeled **7** has been described in detail [26] in 50–80% yield in a one-pot reaction involving 15 enzymes. While some of these enzymes are commercially available, all of the enzymes involved in the DXP pathway had to be cloned and purified from recombinant *E. coli* strains, a time-consuming and expensive task.

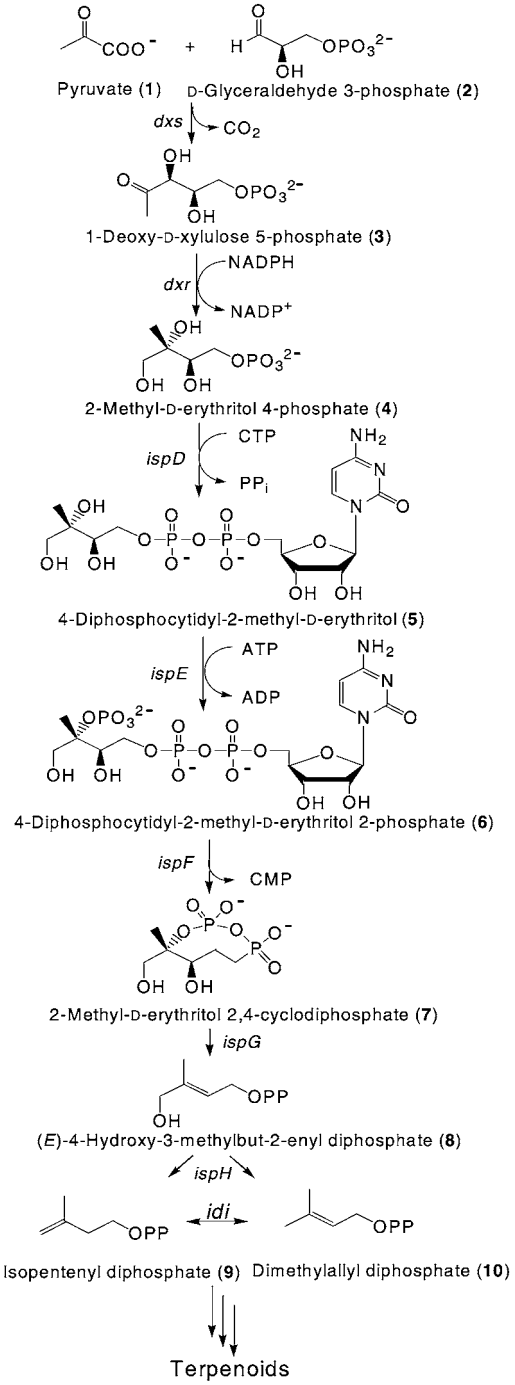
Chloroplasts of spinach (*Spinacea oleracea*) have been used for decades in photosynthesis research, and this plant is usually commercially available all year round. We, therefore, decided to explore this model plant for the synthesis of labeled **7** and congeners, since in this chloroplast compartment all of the DXP pathway enzymes should also be present. D-[*U*-$^{14}C_6$]glucose was chosen as a reasonably priced starting material. The precursors of the first committed intermediate DXP (**3**), namely pyruvate (**1**) and D-glyceraldehyde 3-phosphate (D-GAP; **2**), were synthesized.

2. Results and Discussion. – Early in our considerations for the synthesis of the DXP-pathway intermediates, it was decided to choose a two-stage synthesis. First, labeled **3** should be synthesized, which could be used either for further enzymatic transformations or for application to intact plant systems. Both **1** and **2** had to be synthesized in labeled form from glucose.

The availability of a recombinant *E. coli* strain, which quantitatively converts D-glucose into pyruvate and secretes the pyruvate thus formed into the culture medium, made it possible to synthesize radioactively as well as heavy isotope-labeled pyruvate. Pyruvate labeled in the 1- to 3-positions was obtained in excellent yields. Starting with uniformly ^{14}C-labeled glucose, the subsequent loss of the labeled carboxy group of pyruvate ($^{1}/_{3}$ of total label) in the condensation reaction with D-glyceraldehyde 3-phosphate by action of the DXS enzyme was tolerable due to the ease of preparation and the relatively low cost of glucose. The transformation of D-[*U*-$^{14}C_6$]glucose into [1,2,3-$^{14}C_3$]pyruvate ([1,2,3-$^{14}C_3$]-**1**) catalyzed by the recombinant *E. coli* enzyme was monitored by HPLC analysis and is shown in *Fig. 1*, *a* and *b*. The pyruvate thus formed was used without further purification for the synthesis of DXP.

Glyceraldehyde-3-phosphate (**2**) was generated *in situ* from labeled D-glucose essentially as described in [26] by using commercially available enzymes. This intermediate was transformed without isolation in the presence of unlabeled **1** and

Scheme *Intermediates and Enzymes of the Deoxyxylulose Phosphate Pathway*



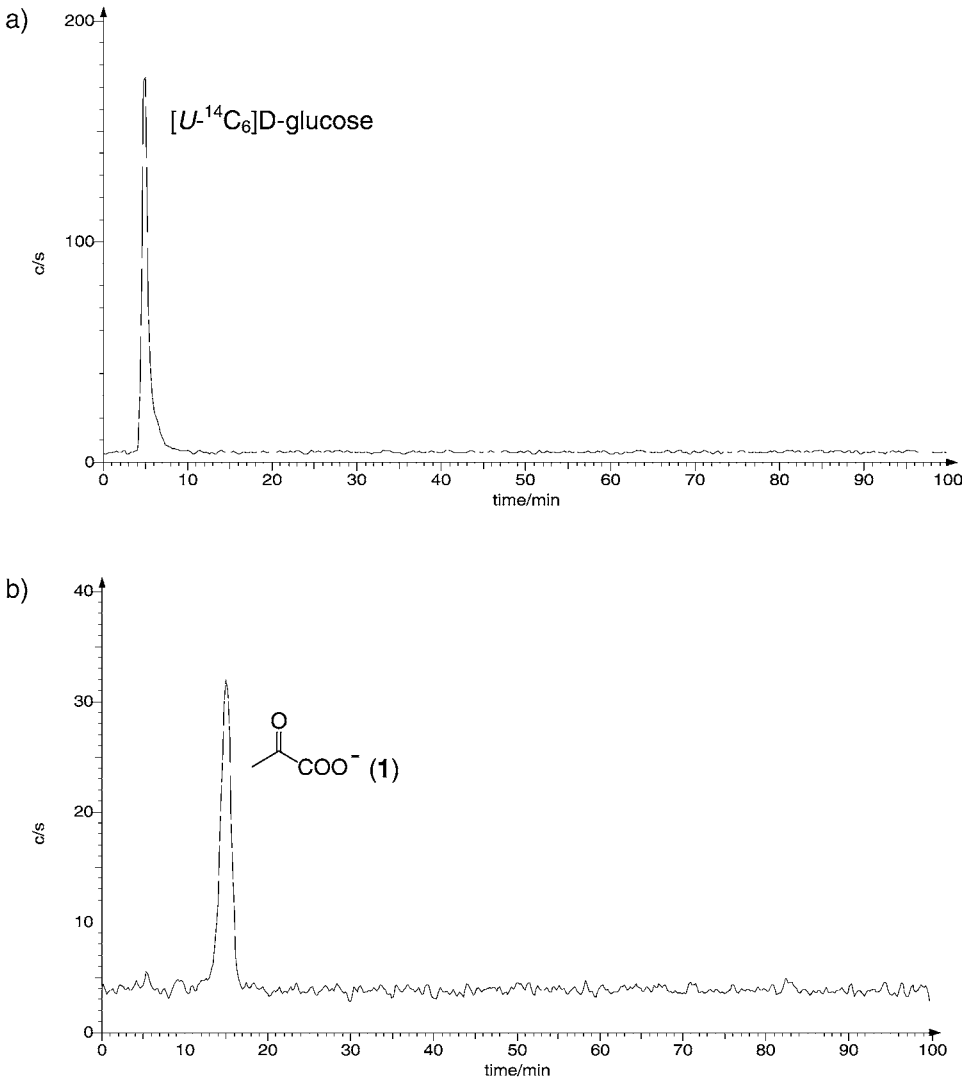


Fig. 1. *Radio-HPLC quantitiation of the transformation of [*U-$^{14}C_6$*]*-D-*glucose to [1,2,3-*$^{14}C_3$*]pyruvate* (**1**) *by* E. coli *YYC202 at 37° and 175 rpm:* a) *at time zero;* b) *after 5 h of incubation*

recombinant DXS directly to **3**. Starting with D-[*U*-$^{14}C_6$]glucose, [3,4,5-$^{14}C_3$]DXP ([3,4,5-$^{14}C_3$]-**3**) was generated. Purification by *DEAE Sephadex* and *Dowex 50 W* × *8* ion-exchange chromatography resulted in the desired compound **3** in $>80\%$ yield.

Similar experiments with [1,2,3-$^{13}C_3$]pyruvate ([1,2,3-$^{13}C_3$]-**1**) to form [1,2-$^{13}C_2$]DXP ([1,2-$^{13}C_2$]-**3**) resulted in minimal 70% yield. The N-terminal histidine extension of the recombinant enzyme did not severely impair the catalytic activity of DXS (0.9 U/mg compared to 0.85 U/mg [4]). The stability of DXP was determined at various temperatures and pH values as shown in *Fig. 2*. It can be seen that DXP is a surprisingly

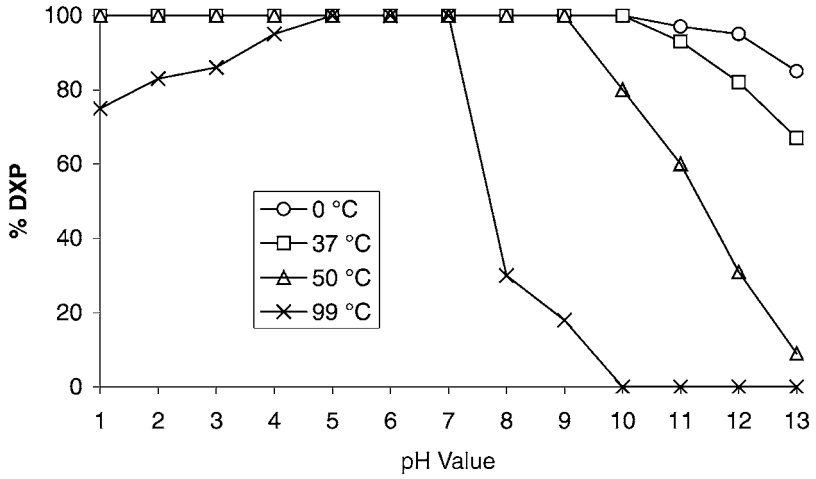


Fig. 2. *Stability of 1-deoxy-*D*-xylulose 5-phosphate* (**3**) *at different pH values and at different temperatures*

stable compound in the temperature range from 0° – 37° and pH range from 1 to 9.5, while, at 99°, the molecule remains intact only from pH 5.0 to 7.0 during a 60-min time period. Thus, the purification of DXP by ion-exchange chromatography and exposing this intermediate briefly to acidic conditions does not pose a serious stability problem.

Variously labeled DXP (**3**) produced by the above procedures was transformed to the cyclic diphosphate by the stroma fraction from spinach chloroplasts. Incubation of, *e.g.*, 50 μCi of [3,4,5-$^{14}C_3$]DXP ([3,4,5-$^{14}C_3$]-**3**; spec. act. 155 μCi/μmol; *Fig. 3*,*a*) in the presence of NADPH, CTP, ATP, Mg^{++}, Mn^{++} ions as cofactors necessary for the pathway enzymes DXR, *ispD*, *ispE* and *ispF*, as well as NaF, an inhibitor of phosphatases at pH 7.6, led to quantitative formation of the desired cyclic diphosphate (*Fig. 3*,*b*). If necessary, the cyclic diphosphate is purified by chromatography on *DEAE Sephadex*. The yield of purified **7** was *ca.* 80%, and the purity as monitored by ion-pair radio-HPLC was 99.9% (*Fig. 3*,*a* and *b*). We also determined the stability of cMEPP at different temperatures and different pH values. As seen in *Fig. 4*, the 2,4-cyclic diphosphate is less stable than DXP (*Fig. 2*). It is stable at a temperature range from 0° to 50° only from pH 3 to 9 for a time period of 60 min. Below pH 2 and above pH 8, a rapid degradation of the molecule is observed. However, for purification by ion-exchange chromatography, the acid milieu generated in this procedure can be tolerated by this compound for short periods of time, especially if conducted at +4°.

The use of ion-exchange chromatography is especially convenient in cases where DXP or other intermediates such as MEPP are not completely transformed to cMEPP by the stroma protein fraction. Since we found that the cyclic diphosphate is rather resistant towards treatment with alkaline phosphatase, the labeled impurities or residual DXP (**3**), MEP (**4**), or other intermediates can be quickly hydrolyzed (30 – 60 min) by an excess of the commercial phosphatase, and the neutral hydrolysis products deoxyxylulose and/or methylerythritol can easily be separated from the stable cyclic phosphate by ion-exchange chromatography yielding pure cMEPP (**7**).

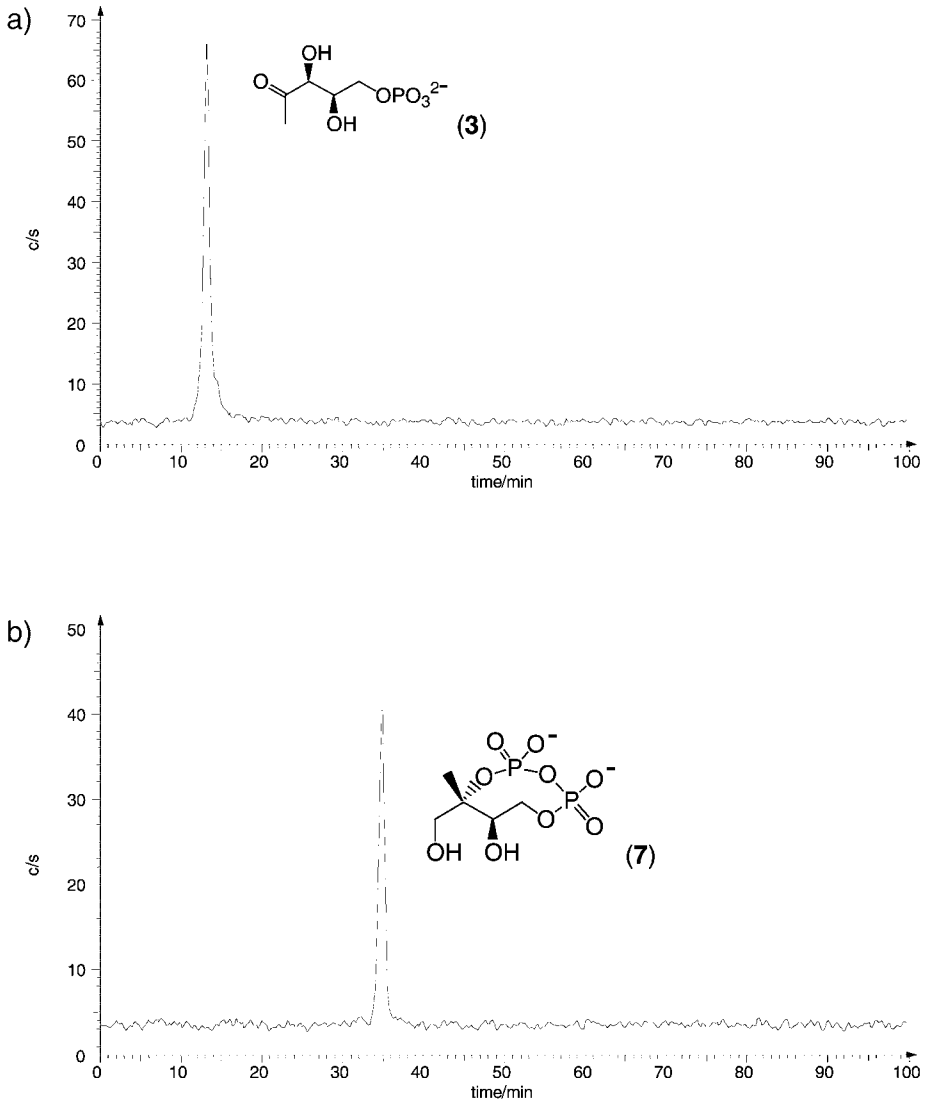


Fig. 3. *Radio-HPLC quantitation of the transformation of [3,4,5-$^{14}C_3$]-1-deoxy-*D*-xylulose 5-phosphate* (**3**) *to [1,3,4-$^{14}C_3$]-2-methyl-*D*-erythritol 2,4-cyclodiphosphate* (**7**) *by stroma derived from spinach chloroplasts in presence of NADPH, CTP, ATP, Mg^{++}, and Mn^{++} ions as cofactors and NaF as inhibitor of phosphatases:* a) *at time zero;* b) *after 20 h of incubation*

The recycling of cofactors as used by *Schuhr et al.* [26] for the preparation of **7** by the recombinant DXP-pathway enzymes is not necessary for the synthesis of radioactive cMEPP of high specific activity, but is of advantage for the preparation of ^{13}C-labeled cyclic diphosphate by the procedure given here.

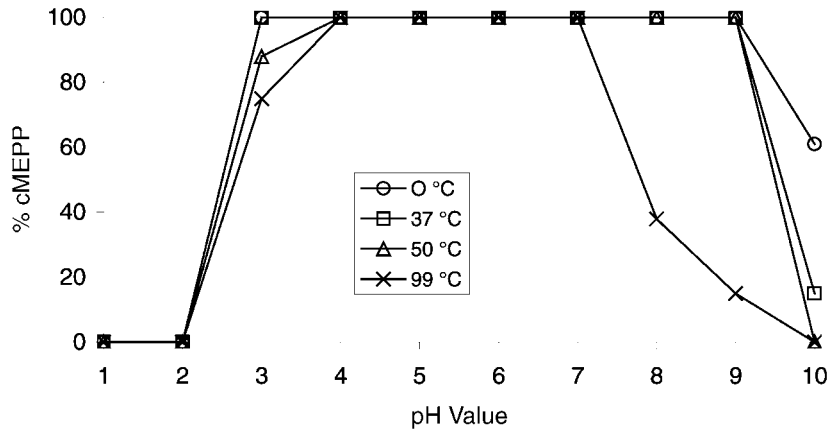


Fig. 4. *Stability of 2-methyl-*D*-erythritol 2,4-cyclodiphosphate* (**7**) *at different pH values and at different temperatures*

Stroma from both *Narcissus pseudonarcissus* flowers and *Capsicum annuum* fruit chromoplasts [13] can be used equally well for the synthesis of radioactive cMEPP from DXP with high specific activity. The disadvantage of these systems, however, is the seasonal availability.

While the method of *Schuhr et al.* [26] for the synthesis of larger quantities (>50 mg) of **7** labeled with ^{13}C is certainly advantageous, the procedure given here involving the stroma fraction of chloro- and chromoplasts is of considerable advantage for the facile preparation of sufficient radioactive **7** labeled with ^{14}C, ^{3}H, and $^{32/33}P$ for biochemical and enzymatic studies.

Experimental Part

Plant Material. Spinach (*Spinacea oleracea cultivar 'Matador'*) was grown in an experimental garden or purchased locally. *Capsicum annuum* fruits as well as *Narcissus pseudonarcissus* freshly opened flowers were obtained at local markets.

Buffers. Buffer *A* used for preparation of chromo- and chloroplasts contained 50 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM DTE, and 0.4M sucrose. The buffer *B*, pH 7.6, used for preparation of chromo- and chloroplasts contained 50 mM HEPES and 1 mM DTE. Buffer *C* used for preparation of *E. coli* YYC202 contained 50 mM MOPS, pH 7.0.

Medium. Medium *A* used for cultivating *E. coli* YYC202 [27] contained 33.7 mM Na_2HPO_4, 20.0 mM KH_2PO_4, 8.6 mM NaCl, 7.6 mM NH_4Cl, 1.0 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM glucose, and 2 mM AcONa.

Preparation of Chloroplasts and Stroma. Young developing spinach leaves (250 g) were cut in small pieces and crushed at full speed for three times three seconds in a *Braun* kitchen mixer in 500 ml of ice-cold buffer A. The homogenate was pressed through four layers of *Nylon* gauze (50 μm), and the filtrate was centrifuged at $3{,}300 \times g$ for 10 min at 4°. The resulting green pellet was carefully resuspended in the above buffer and centrifuged again as described above. The pellet was resuspended in buffer *B*, filtered through one layer of *Nylon* gauze. The resulting chloroplast suspension (16 ml) contained *ca.* 5 mg chlorophyll per ml, measured as given in [28].

The washed chloroplasts were carefully resuspended in buffer *B*, stored on ice for 30 min, and then centrifuged at $110{,}000 \times g$ for 60 min at 4°. The supernatant (*ca.* 36 ml) was concentrated by *YM-10* centriprep columns (*Amicon*) to 7.5 ml. The concentrate was passed in 2.5-ml portions through *Sephadex G-25* columns (*Amersham Biosciences*), which were previously equilibrated with buffer *B*. Elution of the protein fractions was

achieved with 3.5 ml of buffer *B*. The total volume of stroma soln. was 7 ml containing 58 mg protein. Protein concentrations were measured as described in [29]. The chromoplasts from *N. pseudonarcissus* and *C. annuum* were isolated as described in [13], and the stroma fractions isolated as given above.

Enzymes. Alkaline phosphatase, hexokinase, glucose-6-phosphate isomerase, fructose-6-phosphate kinase, aldolase, and triosephosphate isomerase were obtained from *Sigma–Aldrich* (Taufkirchen), 1-deoxy-D-xylulose 5-phosphate synthase [4] containing an hexahistidine extension was constructed in the *pCR®*T7/NT-*TOPO®* vector (*Invitrogen*) and transformed in *E. coli* BL21 (DE3) strain. The expressed protein was purified by metal-chelate (Co^{2+}) affinity chromatography (*Talon*; *BD Biosciences/Clontech*). The recombinant DXS had a specific activity of 0.9 U/mg.

Synthesis of Labeled Pyruvate from D-*Glucose.* Medium A (300 ml) and 10 ml of an overnight *E. coli* YYC202 culture [27] were shaken at 37° and 175 rpm for 24 h. The bacteria were harvested by centrifugation (1,900 × *g*, 10 min, 4°), and the bacterial pellet was resuspended in *ca.* 2.5 ml buffer *C*. A *Warburg* vessel was charged in the outer well with 100 μCi D-[*U*-$^{14}C_6$]glucose (311 μCi/μmol, 500 μl; *Amersham Biosciences*) in 200 μl of buffer *C* and *ca.* 100 μl of the above bacterial suspension ($OD_{600} = 2.0$). The inner well contained 0.2 ml of 2N KOH. The vessel was shaken at 37° and 175 rpm for 5 h. The content of the outer vessel was transferred to an *Eppendorf* tube, centrifuged (1,900 × *g*, 10 min, 4°), and the bacterial pellet was washed twice with 50 μl of H_2O. The supernatant and washing solns. were combined, and adjusted to pH 7.5 with 0.5N KOH. Aliquots were subjected to radio-HPLC [13] and paper chromatography (AcOEt/AcOH/H_2O 3 : 3 : 1; R_f (pyruvate) 0.68). The yield of labeled **1** was usually more than 90% (spec. act.: 155.5 μCi/μmol). The preparation did not contain any radioactive impurity.

Scaling up this procedure to 50-mg quantities of D-[*U*-$^{13}C_6$]glucose or more, [1,2,3-$^{13}C_3$]pyruvate could be obtained in more than 90% yield. ^{1}H-decoupled ^{13}C-NMR data of [1,2,3-$^{13}C_3$]-**1** (300 MHz, H_2O): 205.5 (*dd, J* = 62.2, 39.7, C(2)); 170.8 (*dd, J* = 62.2, 13.3, C(1)); 27.3 (*dd, J* = 39.7, 13.3, C(3)).

*Synthesis of 1-Deoxy-*D-*[1,2-$^{14}C_2$]xylulose 5-Phosphate* ([1,2-$^{14}C_2$]-**3**). The labeled [1,2,3-$^{14}C_3$]pyruvate ([1,2,3-$^{14}C_3$]-**1**; 0.57 μmol, 90 μCi) was incubated with 1.2 μmol of **2**, 5 μmol of $MgCl_2$, 0.5 μmol of thiamine diphosphate, and 0.03 U recombinant DXS in a total volume of 1.0 ml. The mixture was incubated for 3–4 h at 37°. The formation of DXP was monitored by radio-HPLC [13]. After completion, the mixture was applied to a *DEAE Sephadex* (formate form) column (1.0 × 8 cm) with a linear gradient of 0.06 to 0.56M ammonium formate at pH 8.0. The flow rate was 1.1 ml/min, and the eluate was collected in 8-ml fractions. The retention volume of radioactive DXP was *ca.* 80 ml. The fractions containing the labeled DXP were pooled and applied to a column (1.2 × 20 cm) containing 9 ml of *Dowex 50 W* × *8* resin (H^+ form). The acidic eluate was collected, adjusted to pH 7.5 with 0.5N KOH, and evaporated. The yield was 78%, and the specific activity was 103 μCi/μmol. Radio-HPLC showed no radioactive contamination.

The position of the labeled C-atoms in ^{14}C-DXP was determined by ^{13}C-NMR spectroscopy of [1,2-$^{13}C_2$]DXP ([1,2-$^{13}C_2$]-**3**) prepared from [1,2,3-$^{13}C_3$]-**1** and D-GAP (**2**). ^{1}H-Decoupled ^{13}C-NMR data of [1,2-$^{13}C_2$]-**3** (300 MHz, H_2O): 213.5 (*d, J* = 41.2, C(2)); 26.4 (*d, J* = 41.2, C(1)).

*Synthesis of 1-Deoxy-*D-*[3,4,5-$^{14}C_3$]xylulose 5-Phosphate* ([3,4,5-$^{14}C_3$]-**3**). A mixture containing 120 mM of *Tris* · HCl buffer, pH 8.0, 6 mM $MgCl_2$, 1.6 mM ATP, 1.6 mM thiamine diphosphate, 75 μCi D-[*U*-^{14}C]glucose (spec. act. 311 μCi/μmol, 0.4 mM; *Amersham Biosciences*), 1.6 mM sodium pyruvate (**1**), 2 U hexokinase, 1 U glucose-6-phosphate isomerase, 0.3 U fructose-6-phosphate kinase, 0.22 U aldolase, 2 U triosephosphate isomerase, and 0.04 U recombinant DXS, in a total volume of 600 μl, was incubated at 37°. The formation of labeled DXP was monitored by radio-HPLC and was found to be virtually complete after 2–4 h. The mixture was purified as described above with a *DEAE Sephadex* column. The yield of [3,4,5-$^{14}C_3$]DXP ([3,4,5-$^{14}C_3$]-**3**) was 82%. Radio-HPLC showed no radioactive contamination.

The positions of the labeled C-atoms in the labeled product were determined by ^{13}C-NMR spectroscopy of [3,4,5-$^{13}C_3$]DXP ([3,4,5-$^{13}C_3$]-**3**) prepared from pyruvate and D-[1,2,3-$^{13}C_3$]GAP ([1,2,3-$^{13}C_3$]-**2**) derived from [*U*-$^{13}C_6$]glucose as starting material. ^{1}H-Decoupled ^{13}C-NMR data of [3,4,5-$^{13}C_3$]-**3** (300 MHz, H_2O): 77.8 (*d, J* = 39.8, C(3)); 71.2 (*ddd*, *J*(C,C) = 43.6, 39.8, *J*(C,P) = 7.5, C(4)); 66.1 (*dd, J*(C,C) = 43.6, *J*(C,P) = 5.1, C(5)).

*Synthesis of 2-([^{14}C]Methyl)- and 2-([^{13}C]Methyl)-*D-*erythritol 2,4-Cyclodiphosphate from Labeled DXP.* To a final total volume of 700 μl consisting of 100 mM HEPES buffer, pH 7.6, 6 mM $MnCl_2$, 10 mM $MgCl_2$, 7 mM NaF, 6 mM ATP, 2 mM CTP, 3 mM NADPH, together with 355 to 710 μM of labeled DXP was added 2 mg of stroma protein. The mixture was incubated at 30° and monitored by radio-HPLC. After the reaction was found to be complete, or maximally after 20 h, the reaction was terminated. Usually, **3** was cleanly transformed to **7** in quant. yields. In some preparations, either **3** was incompletely transformed, or labeled intermediates such as MEP (**4**) or 4-(diphosphocytidyl)-2-methyl-D-erythritol (**5**) were present to small degrees. In these cases, 1 U

alkaline phosphatase was added, and the reaction was continued for 30 to 60 min. During this time, all noncyclic intermediates were hydrolyzed quantitatively, while cMEPP remained untransformed.

The above mixture (either with or without phosphatase treatment) was subjected to *DEAE Sephadex* column chromatography, applying the same ammonium formate gradient as given above. The retention volume of **7** was *ca.* 120 ml. The fractions containing the cyclic diphosphate were pooled and lyophilized. The yield was *ca.* 80%, no radioactive impurities were seen upon radio-HPLC analysis. ^{13}C-NMR spectroscopy of [*methyl*-$^{13}C_2$]-**7** derived from [1,2-$^{13}C_2$]-**3** provided the positions of the isotopic lable. ^{1}H-Decoupled ^{13}C-NMR data of [*methyl*-$^{13}C_2$]-**7** (300 MHz, H_2O): 84.9 (*dd, J*(C,C) = 39.8, *J*(C,P) = 8.6, C(2)); 17.4 (*dd, J*(C,C) = 39.8, *J*(C,P) = 5.4, Me).

Stability Tests for Labeled DXP and cMEPP. A mixture containing 0.1 mM of ^{14}C-labeled DXP (**3**) or cMEPP (**7**) (0.05 μCi), and the respective unlabeled compound in 0.1M buffer of indicated pH in a total volume of 100 μl was incubated at the indicated temp. for 60 min. The buffers used were: pH 1.0 and 2.0, KCl/HCl buffer; pH 3.0–6.0, citrate/NaOH buffer; pH 7.0 and 8.0, KH_2PO_4/Na_2HPO_4 buffer; pH 9.0–11.0, $Na_2B_4O_7$/HCl/NaOH buffer; pH 12.0 and 13.0, glycine/NaCl/NaOH buffer. After incubation, the solns. were adjusted to neutrality by 0.1N HCl or 0.1N KOH at 0°. An aliquot of 50 μl was removed and directly subjected to radio-HPLC. The residual radioactivity of the original compound was determined, and the results were expressed as % of the starting material.

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