Biosynthesis of terpenoids: 1-deoxy-D-xylulose-5-phosphate reductoisomerase from *Escherichia coli* is a class B dehydrogenase

Tanja Radykewicz[a], Felix Rohdich[a], Juraithip Wungsintaweekul[b], Stefan Herz[a], Klaus Kis[a], Wolfgang Eisenreich[a], Adelbert Bacher[a], Meinhart H. Zenk[b], Duilio Arigoni[c,*]

[a] *Lehrstuhl für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstr. 4, D-85747 Garching, Germany*
[b] *Biozentrum, Martin-Luther-Universität Halle-Wittenberg, D-06099 Halle/Saale, Germany*
[c] *Laboratorium für Organische Chemie, ETH Zürich, Universitätsstr. 16, CH-8092 Zürich, Switzerland*

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Abstract 1-Deoxy-D-xylulose-5-phosphate is converted into 2-*C*-methyl-D-erythritol-4-phosphate by the catalytic action of 1-deoxy-D-xylulose-5-phosphate reductoisomerase (Dxr protein) using NADPH as cofactor. The stereochemical features of this reaction were investigated in in vitro experiments with the recombinant Dxr protein of *Escherichia coli* using (4*R*)- or (4*S*)-[4-$^2H_1$]NADPH as coenzyme. The enzymatically formed 2-*C*-methyl-D-erythritol-4-phosphate was isolated and converted into 1,2:3,4-di-*O*-isopropylidene-2-*C*-methyl-D-erythritol; NMR spectroscopic investigation of this derivative indicated that only (4*S*)-[4-$^2H_1$]NADPH affords 2-*C*-methyl-D-erythritol-4-phosphate labelled exclusively in the H_{Re} position of C-1. Stereospecific transfer of H_{Si} from C-4 of the cofactor identifies the Dxr protein of *E. coli* as a class B dehydrogenase.

Key words: Deoxyxylulose; Methylerythritol; Terpenoid biosynthesis; Stereochemistry; Nuclear magnetic resonance

1. Introduction

The biosynthesis of the terpenoid precursors dimethylallyl pyrophosphate (DMAPP) (**6**, Fig. 1) and isopentenyl pyrophosphate (IPP) (**7**) via mevalonate has been studied in detail (for reviews, see [1–3]). Recently, an alternative mevalonate-independent pathway for the biosynthesis of IPP and DMAPP has been shown to operate in some eubacteria and protozoa, as well as in algae and higher plants (for review, see [4–6]). The first intermediate of this pathway, 1-deoxy-D-xylulose-5-phosphate (**3**), is formed from glyceraldehyde-3-phosphate (**2**) and pyruvate (**1**) by the catalytic action of a synthase specified by the *dxs* gene [7–10]. 1-Deoxy-D-xylulose-5-phosphate is subsequently converted into 2-*C*-methyl-D-erythritol-4-phosphate (**4**) by a NADPH-dependent reductoisomerase specified by the *dxr* gene [11–14]. In the next step, the YgbP protein of *Escherichia coli* catalyzes the formation of 4-diphosphocytidyl-2*C*-methylerythritol (**5**) from 2-*C*-methyl-D-erythritol-4-phosphate and CTP [15].

Recently, we investigated the incorporation of [4-$^2H_1$]1-deoxy-D-xylulose into 2-*C*-methyl-D-erythritol in leaves of *Liriodendrum tulipifera* and found that the ^2H label of the precursor was incorporated stereospecifically into the H_{Si} position at C-1 of 2-*C*-methyl-D-erythritol [16]. We have now extended our studies to the enzymatic level by analyzing the stereochemical details of the related reaction catalyzed by the recombinant 1-deoxy-D-xylulose-5-phosphate reductoisomerase of *E. coli* [7,8].

2. Materials and methods

2.1. Chemicals and enzymes

D-[1-$^2H_1$]Glucose (98 atom% ^2H) was purchased from Omicron (South Bend, IN, USA), glucose dehydrogenase from *Bacillus megaterium* (EC 1.1.1.47) and alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa were from Sigma. 1-Deoxy-D-xylulose-5-phosphate was prepared enzymatically from pyruvate and glyceraldehyde-3-phosphate using recombinant 1-deoxy-D-xylulose-5-phosphate synthase [7–10] from *Bacillus subtilis*. 1-Deoxy-D-xylulose-5-phosphate reductoisomerase [11–14] was purified from recombinant *E. coli* strain M15 pQEYAEM (S. Herz, F. Rohdich, J. Wungsintaweekul, W. Eisenreich, K. Kis and A. Bacher, unpublished).

2.2. Preparation of [4-$^2H_1$]NADP$^+$

[4-$^2H_1$]NADP$^+$ was prepared by a modification of a known protocol [17]. 100 μmol NADP$^+$ was dissolved in 0.5 ml of D_2O (99.9 atom% ^2H). The pD was adjusted to 7 by addition of 40% deuterated sodium hydroxide (w/v) (99 atom% ^2H). 2 M Potassium cyanide (0.5 ml) was added and the pD was adjusted to 11.3. The reaction was monitored photometrically (260 and 327 nm). After 90 min at room temperature ($OD_{260}/OD_{327} = 3$), the pD was adjusted to 6.5 by addition of 130 mg of deuterated potassium dihydrogen phosphate (95 atom% ^2H) and 150 μl of 37% deuterated HCl (v/v) (99 atom% ^2H). [4-$^2H_1$]NADP$^+$ was precipitated by addition of 3 ml of acetone containing 0.6% phosphoric acid (w/v). The precipitate was lyophilized and stored at −20°C. The deuterium abundance was >95% as analyzed by ^1H NMR spectroscopy [18].

2.3. Preparation of (4R)-[4-$^2H_1$]NADPH [17]

A solution containing 30 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 56 mM D-glucose, 100 μmol [4-$^2H_1$]NADP$^+$ and 30 U (μmol/min) of glucose dehydrogenase, in a total volume of 20 ml, was incubated for 30 min at 25°C. The reaction was monitored photometrically (340 nm). After 30 min, the solution was cooled to 4°C. The enzyme was removed by ultrafiltration using a 30 kDa membrane (Pall Gelman). The filtrate was used directly in assay with 1-deoxy-D-xylulose-5-phosphate reductoisomerase. According to the ^1H NMR spectrum [18] the deuterium enrichment at the (C-4) H_{Re} position was >95%.

2.4. Preparation of (4S)-[4-$^2H_1$]NADPH [17]

A solution containing 30 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 56 mM D-[1-$^2H_1$]glucose, 100 μmol NADP$^+$, 30 U (μmol/min) of glucose dehydrogenase in a total volume of 20 ml, was incubated for 30 min at 25°C. ^2H-labeled NADPH was obtained as described above. According to the ^1H NMR spectrum [18] the deuterium enrichment at the (C-4) H_{Si} position was >92%.

2.5. Preparation of 2-C-methyl-D-erythritol-4-phosphate

A solution containing 0.15 M Tris–HCl, pH 8.0, 20 mM $MgCl_2$,

*Corresponding author. Fax: (41)-1-632 1154.
E-mail: arigoni@org.chem.ethz.ch

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45 mM 1-deoxy-D-xylulose-5-phosphate, 5 mM (4*S*)-[4-$^2H_1$]NADPH or (4*R*)-[4-$^2H_1$]NADPH and 525 μg of purified recombinant 1-deoxy-D-xylulose-5-phosphate reductoisomerase from *E. coli* in a final volume of 20 ml was incubated for 30 min at 37°C. The reaction was monitored photometrically (340 nm). After 3 h, the enzyme was precipitated by addition of 500 μl of 32% HCl (v/v). The mixture was centrifuged (4800 rpm, 10 min). The pH of the solution was adjusted to 8 by addition of 300 μl of 8 M sodium hydroxide. The solution was placed on top of a Dowex 1X8 column (100–300 mesh; formate form; 2×12 cm). The column was washed with 200 ml of water and eluted with 250 ml of 1 M formic acid. The retention volume of 2-*C*-methyl-D-erythritol-4-phosphate was 60 ml. Fractions were collected, lyophilized, and analyzed by ^1H NMR spectroscopy (Fig. 2).

2.6. Preparation of 1,2:3,4-di-O-isopropylidene-2-C-methyl-D-erythritol (**8**, Fig. 3)

2-*C*-Methyl-D-erythritol-4-phosphate (**4**, 15 mg) was dissolved in 500 μl of a solution containing 50 U (μmol/min) of alkaline phosphatase, 100 mM Tris–HCl, pH 9.6, 2.5 mM $MgCl_2$, and 2.5 mM $ZnSO_4$. The mixture was incubated for 2 h at 37°C and subsequently lyophilized. The dry substance was dissolved in 200 μl of H_2O and centrifuged. 2-*C*-Methyl-D-erythritol was purified at 65°C by HPLC on a column of Rezex Phenomenex (300×7.8 mm) using H_2O as eluent and a refractometer as detector. The retention volume of 2-*C*-methyl-D-erythritol was 17 ml. 2-*C*-Methyl-*D*-erythritol (7 mg) was dissolved in 9 ml of dry acetone. Dichloromethane (2 ml) containing 1 M $ZnCl_2{\cdot}Et_2O$ were added in one portion at room temperature [19]. After 5 h, 50 ml of chloroform was added and the solution was washed three times with 10 ml of 5% $NaHCO_3$ (w/v) and subsequently

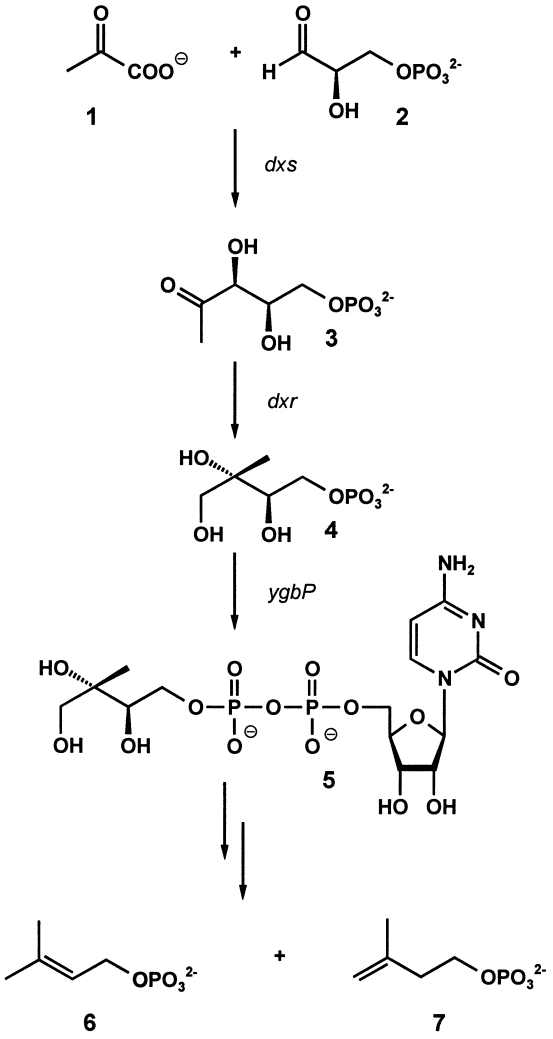

Fig. 1. 1-Deoxyxylulose phosphate pathway to terpenoids [4–6,15].

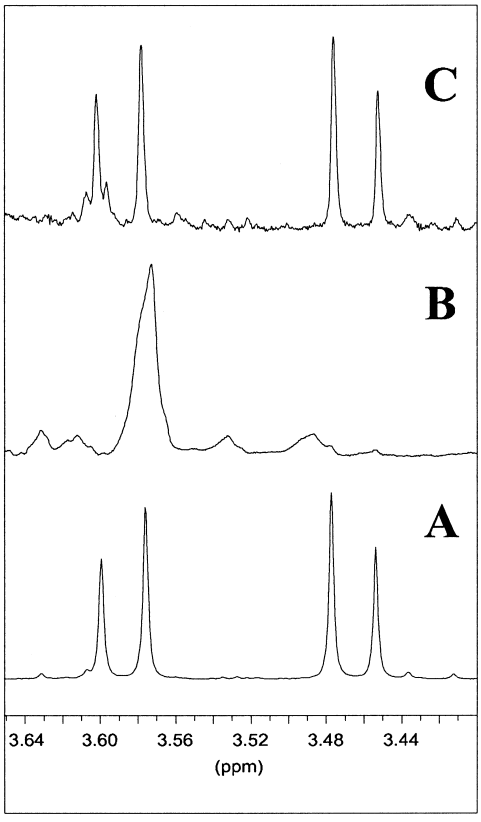

Fig. 2. Partial ^1H NMR spectra (D_2O) of 2-*C*-methyl-D-erythritol-4-phosphate samples. A, Unlabeled reference; B, from the experiment with (4*S*)-[4-$^2H_1$]NADPH; C, from the experiment with (4*R*)-[4-$^2H_1$]NADPH.

with 10 ml of water. The organic solution was dried over $MgSO_4$ and concentrated. The crude product was purified by chromatography on silica (0.5×5 cm) using a mixture of hexane/ethylacetate (3:1, v/v) as eluent. The retention volume of 1,2:3,4-di-*O*-isopropylidene-2-*C*-methyl-D-erythritol was 10–20 ml. Fractions containing the product were pooled and evaporated to dryness.

2.7. NMR spectroscopy

^1H, ^2H, and ^{13}C NMR spectra were measured using a Bruker DRX500 spectrometer. 2-*C*-Methyl-D-erythritol-4-phosphate was measured using D_2O as solvent and 1,2:3,4-di-*O*-isopropylidene-2-*C*-methyl-D-erythritol was measured using $CDCl_3$ as solvent.

3. Results and discussion

In contrast to the in vivo system utilized in a previous study for following the stereochemical course of the conversion of 1-deoxy-D-xylulose into 2-*C*-methyl-D-erythritol in leaves of the tree *L. tulipifera* [16], the availability of the recombinant 1-deoxy-D-xylulose-5-phosphate reductoisomerase from *E. coli* [7,8] provides a welcome opportunity for investigating the stereospecificity of the hydride transfer not only with respect to the substrate but also at the level of the reducing agent. Samples of (4*R*)- or (4*S*)-[4-$^2H_1$]NADPH were prepared by published procedures [17], and their ee values were monitored by NMR spectroscopy [18]. The labelled coenzymes were then used for enzyme assays in conjunction with 1-deoxy-D-xylulose-5-phosphate as substrate and the resulting probes of 2-*C*-methyl-D-erythritol-4-phosphate were analyzed by ^1H and ^2H NMR spectroscopy. The data summarized in Fig. 2 demonstrate that ^2H had been incorporated into 2-*C*-methyl-D-eryth-

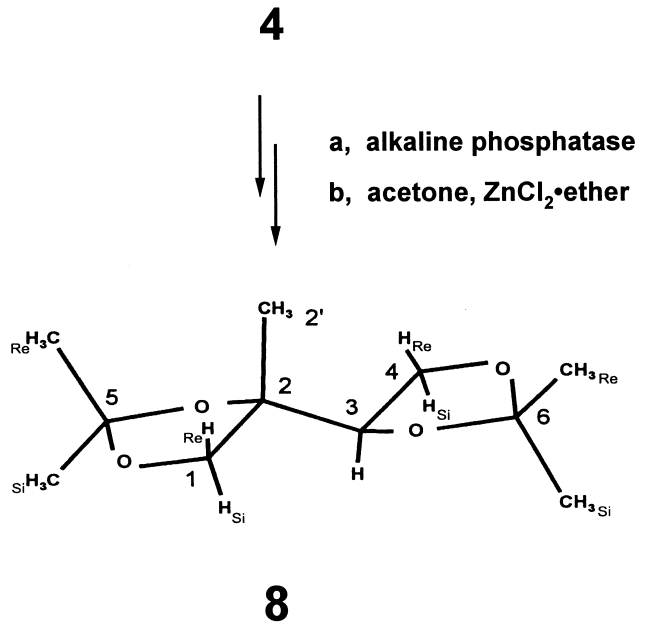

Fig. 3. Preparation of 1,2:3,4-di-*O*-isopropylidene-2-*C*-methyl-D-erythritol (**8**) from 2-*C*-methyl-D-erythritol-4-phosphate (**4**).

ritol-4-phosphate from (4*S*)-[4-$^2H_1$]NADPH but not from (4*R*)-[4-$^2H_1$]NADPH. Accordingly, the Dxr protein of *E. coli* is acting as a class B dehydrogenase.

In order to assess the location of ^2H, the 2-*C*-methyl-D-erythritol-4-phosphate sample obtained from (4*S*)-[4-$^2H_1$]NADPH was dephosphorylated by alkaline phosphatase and 2-*C*-methyl-D-erythritol was then transformed into 1,2:3,4-diisopropylidene-2-*C*-methylerythritol (**8**, Fig. 3). The ^{13}C and ^1H NMR signals of the unlabeled reference compound had been assigned previously on the basis of two-dimensional NMR experiments [16]. Monodeuteration at C-1 in the specimen from (4*S*)-[4-$^2H_1$]NADPH was revealed by the appearance in the ^{13}C NMR spectrum of a triplet (J_{CD} = 22.6 Hz) with an upfield shift of −355 ppb from the normal singlet at 73.01 ppm (Fig. 4). Under ^2H decoupling the triplet collapsed to a singlet with an intensity of 96% relative to the overall intensity of the C-1 signal. In addition, in the ^1H NMR spectrum of the compound the intensity of the (C-1) H_{Re} doublet at 3.68 ppm was strongly decreased (more than 90%) while the signal of the corresponding H_{Si} at 3.99 ppm had collapsed to an α-shifted (−20 ppb) singlet; in keeping with this only one singlet at 3.7 ppm was detected in the ^2H NMR spectrum of the material (Fig. 5). As a consequence of the chemical correlation between the labeled bisacetonide (**8**) and its 2-*C*-methyl-D-erythritol-4-phosphate progenitor the doublet at 3.59 ppm in the ^1H NMR spectrum of the latter (cf. Fig. 2) can now be assigned to the H_{Re} ligand at C-1.

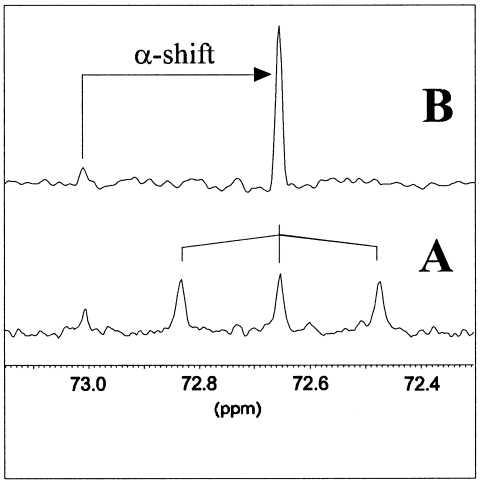

Fig. 4. ^{13}C NMR signal for C-1 of the bisacetonide (**8**) from the experiment with (4*S*)-[4-$^2H_1$]NADPH. A, Proton decoupled; B, proton and deuterium decoupled.

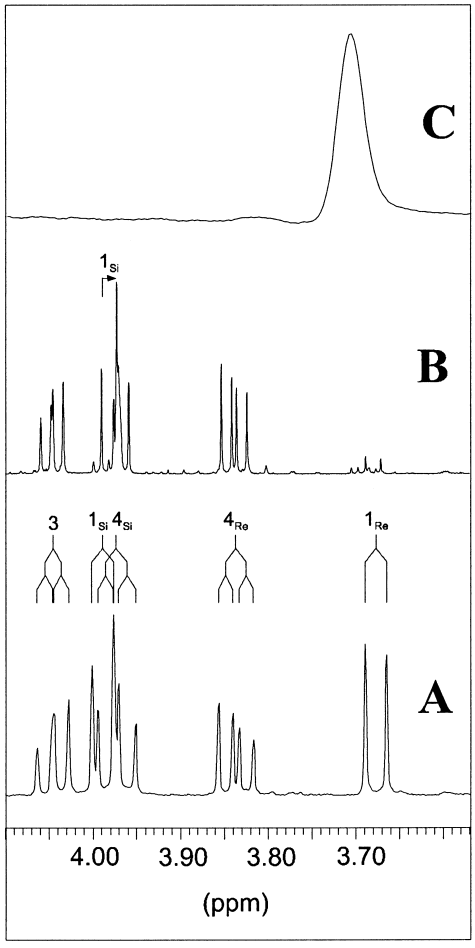

Fig. 5. Partial spectra ($CDCl_3$) of bisacetonide (**8**). A, ^1H NMR of unlabeled reference; B, ^1H NMR of sample from the experiment with (4*S*)-[4-$^2H_1$]NADPH; C, ^2H NMR of the same sample as in B.

Thus, in the reaction catalyzed by the recombinant 1-deoxy-D-xylulose-5-phosphate reductoisomerase from *E. coli* H_{Re} at C-1 of the product 2-*C*-methyl-D-erythritol-4-phosphate is derived specifically from H_{Si} at C-4 of the cofactor (cf. H• in Fig. 6). At the substrate level this matches the results previously secured for the biosynthesis of 2-*C*-methyl-D-erythritol in leaves of *L. tulipifera* [16]. In an article published after completion of the present work it was shown that the same stereochemical picture also applies to the reaction mediated by the homologous reductoisomerase from *Synechocystis* [20].

Specific delivery of H_{si} from NADPH in the reaction mediated by the homologous Dxr protein from the cyanobacterium *Synechocystis* had been invoked by Proteau in an attempt to

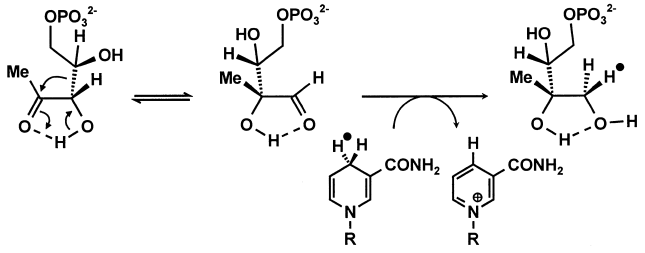

Fig. 6. Stereochemical course of the reaction catalyzed by the 1-deoxy-D-xylulose-5-phosphate reductoisomerase from *E. coli*.

rationalize the outcome of in vivo incubation experiments with [1-$^2H_1$]glucose, in which part of the label was transferred to all positions of phytol corresponding to C-1 of 2-*C*-methyl-D-erythritol-4-phosphate [21]. The underlying argument that such a transfer is possible only if the two dehydrogenases involved display the same cofactor stereospecificity rests, however, on a fallacy; what is obviously correct for a set of two successive stoichiometric reactions ceases to be valid when applied to a biological situation in which the cofactor acts as a catalyst undergoing continuous recycling. It is easy to convince oneself that in such a case transfer of the label is possible even if the stereospecificities of the two reactions do not match; of course, in the first turnover the wrong hydrogen (i.e. the one already present in the oxidized form of the cofactor) will be transferred, but this leaves behind a deuterated cofactor which, from there on, will act as a channel for the continuous flux of the label from the first substrate.

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