Stereochemistry of the Reduction Step Mediated by Recombinant 1-Deoxy-D-xylulose 5-Phosphate Isomeroreductase
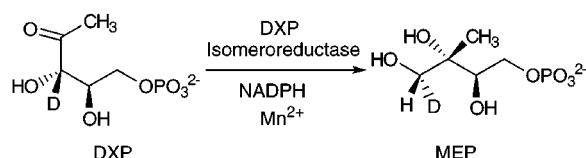
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ABSTRACT

The stereochemistry of the 1-deoxy-D-xylulose 5-phosphate (DXP) isomeroreductase reduction step has been examined using the recombinant enzyme from *Synechocystis* sp. PCC6803. Using [3-^{2}H]DXP and [4S-^{2}H]NADPH, it has been determined that the C1 *pro-S* hydrogen in the 2-C-methyl-D-erythritol 4-phosphate product derives from C3 of DXP, indicating that hydride attack occurs on the *re* face of the intermediate aldehyde. The 4S-hydride from NADPH is delivered, assigning this enzyme as a class B dehydrogenase.

The methylerythritol phosphate (MEP) pathway[1] to isoprenoids has been the focus of intense research since the first report of this biosynthetic route in 1993.[2] The genes for the first two enzymes in the pathway have now been reported,[3,4] with the gene encoding the 1-deoxy-D-xylulose 5-phosphate isomeroreductase (DXR)[5] the most recently identified.[4] This enzyme converts 1-deoxy-D-xylulose 5-phosphate (DXP, **1**) to 2-C-methyl-D-erythritol 4-phosphate (MEP, **2**) by catalyzing a rearrangement of the carbon backbone and a NADPH-dependent reduction at C1 of the

proposed intermediate 2-C-methylerythrose 4-phosphate (Figure 1). The stereochemistry of this reductive step is the focus of this Letter.
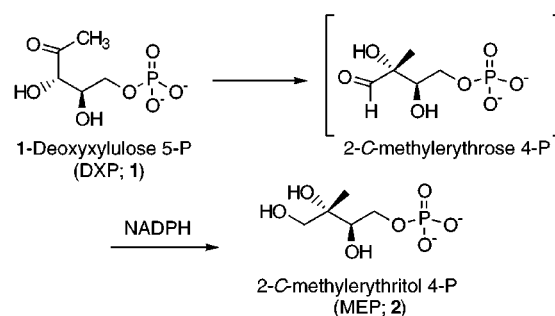

Figure 1. Conversion of DXP to MEP by DXP isomeroreductase.

The stereochemical issues that need to be addressed are the following: (1) Which hydrogen at C1 of 2-C-methyl-

(1) The methylerythritol phosphate pathway, or MEP pathway, was suggested as a standardized name at the 4th European Symposium on Plant Isoprenoids, Barcelona, Spain, April 21−23, 1999. Prior names have included non-mevalonate pathway, mevalonate-independent pathway, triosephosphate/pyruvate pathway, deoxyxylulose pathway.

(2) Rohmer, M.; Knani, M.; Simonin, P.; Sutter, B.; Sahm, H. *Biochem. J.* **1993**, *295*, 517−524.

(3) Sprenger, G. A.; Schörken, U.; Wiegert, T.; Grolle, S.; DeGraaf, A. A.; Taylor, S. V.; Begley, T. P.; Bringer-Meyer, S.; Sahm, H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12857−12862.

(4) Takahashi, S.; Kuzuyama, T.; Watanabe, H.; Seto, H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9879−9884.

(5) DXP isomeroreductase describes the order of enzymatic transformations, an isomerization, followed by a reduction. This name is presented as an alternative to DXP reductoisomerase.[4]

erythritol phosphate originates from C3 of DXP and which derives from NADPH? (2) Delivery of hydride from NADPH occurs on which face of the intermediate aldehyde—*re* or *si*? (3) Which hydride from NADPH is delivered in the reduction (class A or class B dehydrogenase)?

Our earlier work examining the MEP pathway in the cyanobacterium *Synechocystis*[6] prompted us to examine the cyanobacterial isomeroreductase. The *Synechocystis* protein sequence has 42% identity to the *Escherichia coli* DXR sequence. A cosmid clone containing the *dxr* gene was obtained from the Kazusa DNA Research Institute. The desired gene was subcloned into pUC18, and unique site-elimination mutagenesis was used to introduce *Bam*H I and *Hin*d III sites at the 5′ and 3′ ends of the gene, respectively. The gene was then ligated into a pBAD/His expression vector, and expression upon arabinose induction in *E. coli* provided the recombinant isomeroreductase with a *N*-terminal 6xHis sequence. Partial purification using Ni-NTA spin columns (Qiagen) provided recombinant enzyme for stereochemical studies. The enzyme was assayed by monitoring the decrease in absorbance at 340 nm.[4] The product of the enzymatic reaction was converted to 2-*C*-methylerythritol triacetate (**3**) by treating the crude mixture with alkaline phosphatase, followed by acetylation with acetic anhydride and pyridine (Scheme 1). The purified derivative was analyzed by comparing GC/MS and ^{1}H NMR data with an authentic standard.

Scheme 1[a]
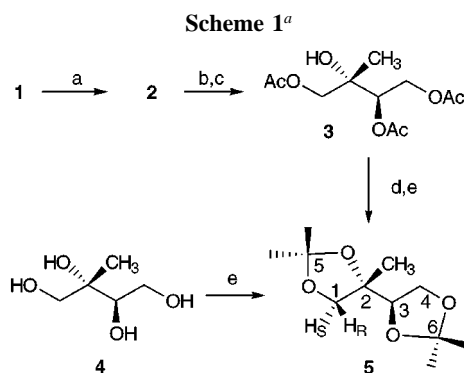
[a] (a) DXR; (b) alkaline phosphatase; (c) Ac₂O, pyr; (d) Amberlite IRA400 (OH⁻); (e) CH₃C(OCH₃)₂CH₃, pTsOH, THF.

To distinguish the *pro-R* and *pro-S* protons at C1 of MEP, a derivative was required in which the two protons could be unambiguously assigned. A derivative that potentially could fulfill this requirement was the bisacetonide derivative.[7] Unlabeled 2-*C*-methylerythritol (**4**) was prepared[8] and converted to the bisacetonide **5** with 2,2-dimethoxypropane and toluenesulfonic acid. A survey of NMR solvents revealed that the ^{1}H NMR spectrum in acetone-d_6 provided resolution

of all five backbone protons, and this solvent was used for further analysis. HSQC and HMBC experiments were used to assign the ^{1}H and ^{13}C resonances.[9] GNOESY and 1D DPFGSE[10] NOE experiments were utilized to assign the diastereotopic groups in the molecule. The C1 hydrogens appeared as a pair of doublets, one centered at 3.71 ppm and the other at 3.95 ppm. Strong NOE correlations between the 3.71 ppm proton, the C2 methyl group, and one of the C5 isopropylidene methyls[11] allowed us to assign all three of these groups to the same face of the dioxolane ring. On the basis of NOE experiments, the *pro-S* proton at C1 has a shift of 3.95 ppm and the *pro-R* proton resonates at 3.71 ppm.

To provide a monodeuterated compound for the stereochemical analysis, it was necessary to synthesize deoxyxylulose phosphate specifically deuterated at C3. This was accomplished by modifying the synthesis of 3-[2]H-deoxyxylulose[12] to allow for synthesis of the 5-phosphate from the known intermediate **6** (Scheme 2). The labeled substrate

Scheme 2[a]
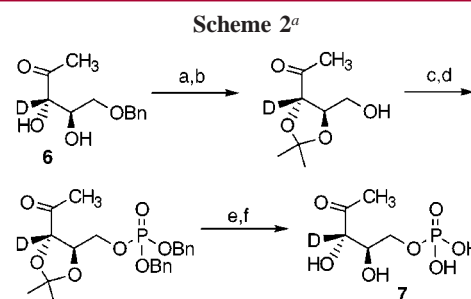
[a] (a) CH₃C(OCH₃)₂CH₃, pTsOH, THF; (b) H₂, Pd/C, EtOH; (c) tetrazole, (BnO)₂PN*i*Pr₂; (d) *t*BuOOH, CH₂Cl₂; (e) H₂, Pd(OH)₂/C; (f) Dowex 50, H₂O.

7 was incubated with recombinant isomeroreductase, providing monodeuterated MEP which was dephosphorylated with alkaline phosphatase. The proteins from the mixture were precipitated with ethanol, and the supernatant was concentrated and then treated with acetic anhydride and pyridine. The conversion of methylerythritol to the triacetate provided a derivative that was readily purified by flash chromatography (40% EtOAc/hexanes). The triacetate was deprotected with a basic ion-exchange resin and then converted to the bisacetonide (Scheme 1). The ^{1}H NMR spectrum of this derivative showed a slightly broadened singlet at 3.69 ppm

(6) Proteau, P. J. *Tetrahedron Lett.* **1998**, *39*, 9373−9376.

(7) Anthonsen, T.; Hagen, S.; Sallam, M. A. E. *Phytochemistry* **1980**, *19*, 2375−2377.

(8) Duvold, T.; Cali, P.; Bravo, J. M.; Rohmer, M. *Tetrahedron Lett.* **1997**, *38*, 6181−6184.
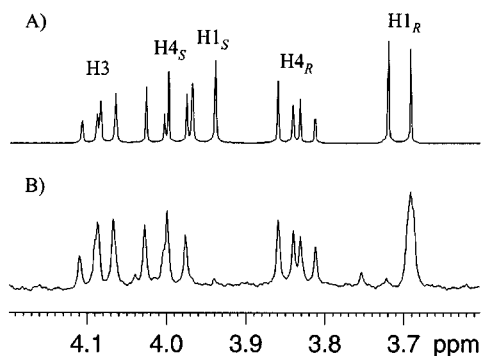
(9) ^{1}H NMR (acetone-d_6): δ 4.09 (dd, *J* = 7.0, 5.8 Hz, H3), 4.00 (dd, *J* = 8.7, 7.0 Hz, H4S), 3.95 (d, *J* = 8.7 Hz, H1S), 3.84 (dd, *J* = 8.7, 5.8 Hz, H4R), 3.71 (d, *J* = 8.7 Hz, H1R), 1.35 (s, C6-CH₃R), 1.310 (s, C5-CH₃S), 1.306 (s, C5-CH₃R), 1.27 (s, C6-CH₃S), 1.21 (s, C2-CH₃). ^{13}C NMR (acetone-d_6): δ 110.11 (C5), 110.00 (C6), 81.83 (C2), 79.44 (C3), 73.49 (C1), 66.00 (C4), 27.58 (C5-CH₃S), 27.02 (C5-CH₃R), 26.60 (C6-CH₃R), 25.04 (C6-CH₃S), 19.80 (C2-CH₃).

(10) GNOESY = Gradient NOESY. Wagner, R.; Berger, S. *J. Magn. Reson.* **1996**, *123A*, 229−232. DPFGSE = double pulsed field gradient spin−echo. Stott, K.; Keeler, J.; Van, Q. N.; Shaka, A. J. *J. Magn. Reson.* **1997**, *125*, 302−324.

(11) The selective excitation and high resolution possible with the DPFGSE experiment allowed us to assign the correlations from the C2 methyl and the 3.71 ppm signal specifically to the methyl at 1.306 ppm.

(12) Giner, J.-L. *Tetrahedron Lett.* **1998**, *39*, 2479−2482.

Figure 2. Partial ^{1}H NMR spectrum of 2-*C*-methylerythritol bisacetonide. (A) Unlabeled standard. (B) Monodeuterated product derived from enzymatic reaction.

while the doublet at 3.95 ppm is not present (Figure 2). This result indicates that the proton from C3 of deoxyxylulose becomes the *pro-S* proton of 2-*C*-methylerythritol.[13] The *pro-R* hydrogen at C1 of MEP, therefore, derives from NADPH. This result also establishes that hydride delivery is to the *re* face of the proposed aldehyde intermediate.

In addition to identifying the *pro-S* and *pro-R* hydrogens in the bisacetonide derivative, the stereochemical assignments for the triacetate methylene protons at C1 were determined. The *pro-S* hydrogen resonates at 4.156 ppm, while the *pro-R* hydrogen signal is at 3.90 ppm.[14] Because the triacetate is a readily accessible derivative from crude methylerythritol, future efforts to determine the stereochemistry at C1 of MEP could utilize the triacetate.

The remaining stereochemical issue involved in this transformation is which hydride is delivered from NADPH. On the basis of incubation experiments with [1-^{2}H]glucose in the cyanobacterium *Synechocystis* sp., we have proposed that the 4*S*-hydride of NADPH is delivered.[6]

Stereospecifically deuterated [4*S*-^{2}H]NADPH was generated from NADP$^+$ using glucose dehydrogenase (NADP$^+$-dependent, from *Cryptococcus uniguttulatus* EC 1.1.1.119) and [1-^{2}H]glucose.[15] The [4*R*-^{2}H]NADPH was prepared from

[^{2}H$_8$]-2-propanol and alcohol dehydrogenase (NADP$^+$-dependent, from *Thermoanaerobium brockii* EC 1.1.1.2).[16] The deuterated NADPH samples were purified by HPLC.[16] Independent incubations of these labeled NADPH samples with unlabeled DXP and recombinant DXR provided MEP that was subsequently dephosphorylated and converted to the triacetate derivative for GC/EIMS analysis. An unlabeled triacetate standard showed three key fragment ions at *m/z* 159, 129, and 117. The product from the [4*S*-^{2}H]NADPH incubation displayed fragment ions at 160, 129, and 118, while the product from the 4*R* experiment had a mass spectrum typical for the unlabeled standard. The increase in mass by one unit for the *m/z* 159 and 117 ions demonstrates the incorporation of a deuterium atom only when the [4*S*-^{2}H]NADPH is used. Transfer of the 4*S*-hydride classifies DXP isomeroreductase as a class B dehydrogenase. Ketol-acid reductoisomerase, a mechanistically related enzyme involved in valine and isoleucine biosynthesis, is also a class B dehydrogenase.[17]

These experiments have demonstrated for the deoxy-xylulose phosphate isomeroreductase from *Synechocystis* that the C1 *pro-S* hydrogen derives from C3 of DXP. The *pro-R* hydrogen, therefore, arises from NADPH. Delivery of hydride is to the *re* face of the aldehyde with the *pro-S* hydride from NADPH being transferred. (Figure 3).
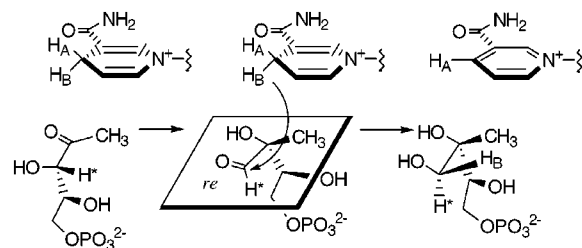

Figure 3. Stereochemical course for DXR-mediated conversion of DXP to MEP.

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(13) During the preparation of this manuscript, an article was published in which a similar approach was used to address the stereochemistry of the reduction step in the formation of 2-*C*-methylerythritol in higher plants: Arigoni, D.; Giner, J.-L.; Sagner, S.; Wungsintaweekul, J.; Zenk, M. H.; Kis, K.; Bacher, A.; Eisenreich, W. *Chem. Commun.* **1999**, 1127−1128.

(14) ^{1}H NMR (CDCl$_3$): δ 5.18 (dd, *J* = 8.0, 2.8 Hz, H3), 4.56 (dd, *J* = 11.8, 2.8 Hz, H4a), 4.163 (dd, *J* = 11.8, 8.0 Hz, H4b), 4.156 (d, *J* = 11.7 Hz, H1*S*), 3.90 (d, *J* = 11.7 Hz, H1*R*), 2.11 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H), 1.24 (s, C2-CH$_3$).

(15) Mostad, S. B.; Helming, H. L.; Groom, C.; Glasfeld, A. *Biochem. Biophys. Res. Commun.* **1997**, *233*, 681−686.

(16) Jeong, S.-S.; Gready, J. E. *Anal. Biochem.* **1994**, *221*, 273−277.

(17) You, K.-s.; Arnold, L. J.; Allison, W. S.; Kaplan, N. O. *TIBS* **1978**, *3*, 265−268.