Enzyme Mechanisms

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The Chemical Mechanism of D-1-Deoxyxylulose-5-phosphate Reductoisomerase from *Escherichia coli***

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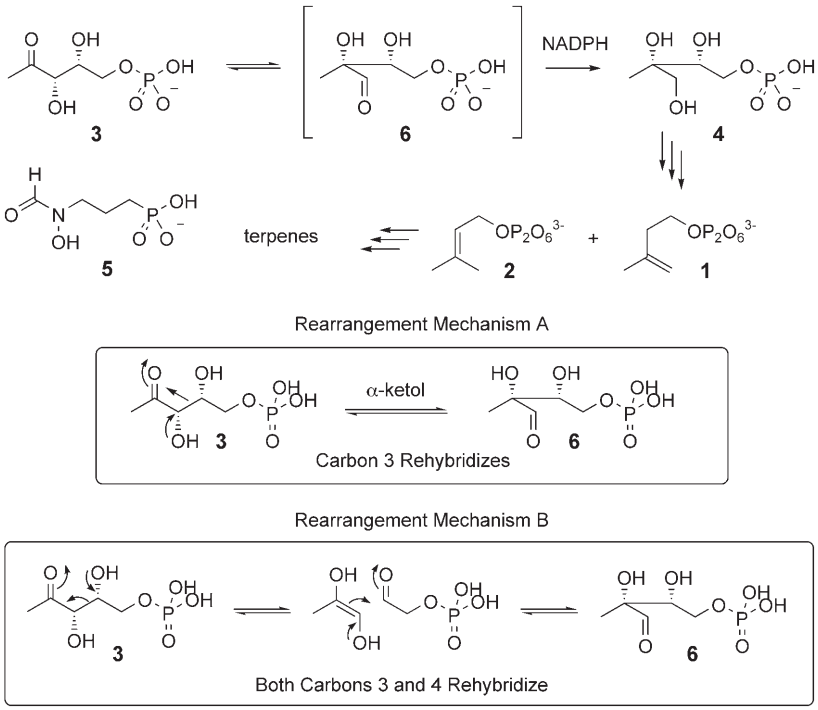
The enzyme D-1-deoxyxylulose-5-phosphate reductoisomerase (DXR, EC 1.1.1.267) catalyzes the key isomerization reaction during the biosynthesis of the terpene precursors isopentenyl diphosphate **1** and dimethylallyl diphosphate **2** through the non-mevalonate pathway in plants and bacteria.[1] DXR catalyzes the interconversion of D-1-deoxyxylulose-5-phosphate (DXP) **3** and D-methylerythritol phosphate (MEP) **4**, utilizing nicotinamide adenine dinucleotide phosphate (NADPH) as the reductant (Scheme 1).[2] Fosmidomycin **5** selectively and potently inhibits DXR, and **5** and its close analogues show useful antimalarial activity both in vitro and in vivo against *Plasmodium falciparum*. Inhibition of DXR thus offers a new and, as yet, little-exploited mechanism for the development of new antimicrobial agents. Although the mechanism of inhibition of DXR by **5** is well understood both kinetically[3,4] and crystallographically,[5–7] little progress has been made in elucidating the chemical mechanism of substrate processing by DXR. The structural and biochemical results reported to date show that DXR is a homodimer, with one active site per subunit, and support a kinetic mechanism in which NADPH binds before DXP **3**.[4] The first reaction catalyzed is an apparent concerted α-ketol rearrangement (mechanism A, Scheme 1) or a step-wise fragmentation–reassembly mechanism through retro-aldol/aldol steps (mechanism B, Scheme 1). The resulting aldehyde **6** is then reduced by using NADPH.



Scheme 1. Reactions catalyzed by DXR and the two possible mechanisms for the rearrangement step.

Tentative support for mechanism B has been gathered by Fox and Poulter[8] and by Lui and co-workers[9] by using fluorinated substrate analogues. However, replacement of hydroxy groups by fluorine atoms could alter substrate binding, and formation of α-fluoroketones affects carbonyl pK_a values and electrophilicity, making interpretation of results difficult. Thus, no conclusive evidence in favor of, or ruling out, either of the possible rearrangement mechanisms has been found despite this subject being of intense interest.[4,8–11] Determination of the chemical mechanism of DXR could allow the development of new classes of inhibitors with potentially useful pharmacological properties.

To distinguish between the two possible rearrangement mechanisms for DXR, we decided to synthesize selectively deuterated substrates for use in kinetic assays. We have already described a short synthesis of enantiomerically pure **3** that allows the incorporation of isotopic labels and the development of a kinetic assay for DXR by using recombinant protein obtained from *Escherichia coli*.[12] Incorporation of ^{2}H selectively at C3 and C4 of DXP would provide substrates that could allow the observation of secondary kinetic isotope effects (KIE) and potentially distinguish the α-ketol mechanism from the retro-aldol/aldol mechanism.

To synthesize the desired isotopically labeled substrates, we modified our previous synthetic route.[12,13] Thus propargyl alcohol **7** was protected with *tert*-butyldimethylsilyl

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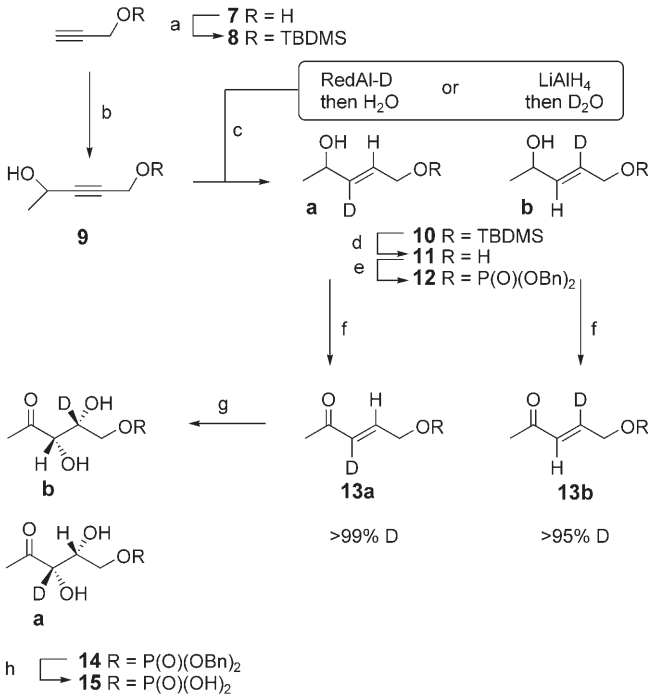
(TBDMS), then deprotonated and treated with acetaldehyde (Scheme 2). The resulting alcohol **9** was treated with RedAl-D followed by a workup with H_2O to give deuterated olefin **10a**.



Scheme 2. Synthesis of stereospecifically ^{2}H-labeled substrates. a) TBDMSCl, imidazole, CH_2Cl_2, 64%; b) EtMgBr, then CH_3CHO, 85%; c) $LiAlD_4$, $MeOCH_2CH_2OH$, toluene then H_2O workup, 81% or $LiAlH_4$, THF, then D_2O workup, 79%; d) TBAF, 0°C, **a** 83%, **b** 44%; e) P(OBn)$_3$, I_2, **a** 71%, **b** 44%; f) Dess–Martin periodinane, CH_2Cl_2, RT, **a** 70%, **b** 95%; g) OsO_4, CH_2Cl_2, −40°C, alkaloid, then MeOH, HCl, **a** 90%, **b** 91%; h) H_2, Pd/C, MeOH, RT, **a** 95%, **b** 91%. TBAF = tetra-*n*-butylammonium fluoride.

Treatment of the alcohol **9** with $LiAlH_4$, followed by D_2O workup gave the isomeric deuterated olefin **10b**. Deprotection of the primary alcohols gave the diols **11a** and **11b**, and selective phosphorylation afforded the alcohols **12a** and **12b**. Dess–Martin oxidation then gave the ketones **13a** and **13b** and asymmetric dihydroxylation, by using stoichiometric OsO_4 according to our previously published procedures,[12] gave the protected DXR isotopomers **14a** and **14b**. Deprotection was achieved by hydrogenolysis to afford the substrate isotopomers **15a** and **15b**. ^{13}C NMR spectroscopic analysis indicated that **15a** incorporated greater than 98% ^{2}H, whereas **15b** incorporated greater than 92% ^{2}H. No ^{2}H was observed at the undesired position in either case.

Previous investigations have indicated that the dihydroxylation step affords the diol with an e.r. value of 92:8.[12,13] In this case, the e.r. ratio was determined by reacting the deprotected ^{2}H substrates with NADPH (catalyzed by DXR). Because L-DXP is not a substrate, the extent of reaction, found by measuring the final consumption of NADPH, indicates the proportion of D-DXP. The e.r. value of **15a** was 92:8, whereas the e.r. value of **15b** was 92.5:7.5.

We and others have previously determined reaction conditions for the kinetic assay of DXR.[2–4,12] DXR requires a metal ion, usually Mg^{2+} or Mn^{2+} ions. In the presence of the Mn^{2+} ion (1 mM), DXP, and DXR (40 nM), NADPH (0.4 mM) is consumed and the reaction can be followed spectrophotometrically at 340 nm. By using this assay, the kinetic parameters of the reaction catalyzed by DXR were measured for the three substrates (Table 1). Comparison of the (V_H/K_{MH})/(V_D/K_{MD}) values for the two ^{2}H-labeled substrates show an inverse isotope effect for both substrate isotopomers. This must be a secondary KIE as C–H bonds remain intact throughout the reaction.

Table 1: Kinetic parameters measured for *E. coli* DXR.

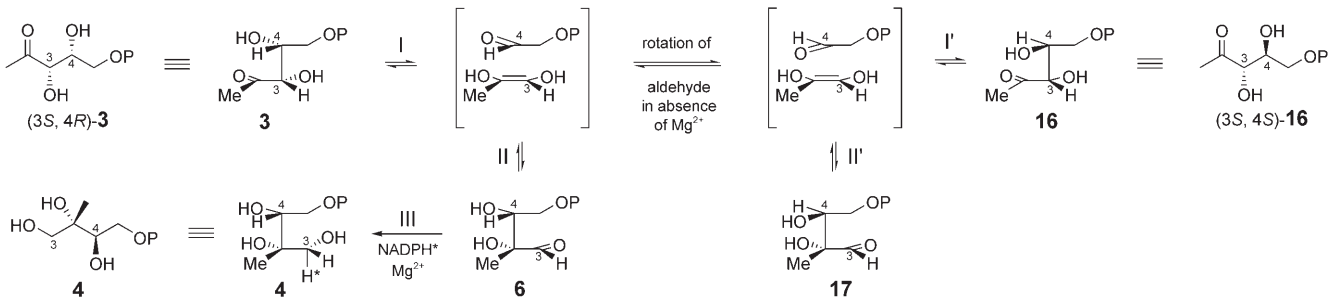
Substrate	K_M [mM]	V_{max} [OD min^{-1}][a]	V_{max}/K_M	k_H/k_D
3	$3.10\times10^{-1}\pm4.6\times10^{-2}$	$7.38\times10^{-3}\pm3.0\times10^{-4}$	0.024	1.0
15a	$5.37\times10^{-1}\pm4.8\times10^{-2}$	$1.32\times10^{-2}\pm5.0\times10^{-4}$	0.026	0.92
15b	$4.23\times10^{-1}\pm3.0\times10^{-2}$	$1.19\times10^{-2}\pm3.2\times10^{-4}$	0.028	0.86

[a] OD = optical density.

The two proposed rearrangement mechanisms suggested for DXR differ in terms of bond-breaking, bond-making, and rehybridization events (Scheme 1). Secondary kinetic isotope effects are particularly useful for examining changes in hybridization at a carbon atom.[14] In the case of the α-ketol rearrangement (mechanism A, Scheme 1), C2 and C3 rehybridize during the reaction, whereas C4 remains unchanged. For the retro-aldol/aldol reaction (mechanism B, Scheme 1), C3 and C4 both rehybridize. The observation of kinetic isotope effects for substrates bearing ^{2}H at C3 and C4 strongly supports the operation of mechanism B involving rehybridization at both secondary alcohol carbon atoms during reaction. These results are not consistent with the α-ketol mechanism, which would be expected to show a KIE for the 3-[^{2}H] substrate but not the 4-[^{2}H] substrate.

The observation of kinetic isotope effects for both ^{2}H-labeled substrates rules out the possibility that the reduction step (Scheme 3, III) is the rate-limiting step (RLS) of the DXR-catalyzed reaction—it would be highly unlikely that 4-[^{2}H] could exert a kinetic influence over this reaction (although ^{2}H at C3 would be expected to exert such an influence if the reduction were the RLS). The RLS must therefore be one of the preceding steps. An inverse KIE is indicative of a RLS involving a rehybridization of carbon from sp^2 to sp^3, thus it is likely that it is the recombination step (Scheme 3, step II) that is rate limiting for DXR.

These results are supported by other observations from the literature. Oefner and co-workers recently reported the X-ray crystal structure of *E. coli* DXR in complex with different substrates.[6] To prevent turnover (i.e. formation of MEP **4**), divalent metal ions were omitted from the crystallization. One structure, obtained in the presence of enantiopure D-DXP **3** and NADPH, showed the presence of both DXP **3** and its 4-epimer L-1-deoxyribulose-5-phosphate **16** in the active site. Oefner and co-workers were not able to explain this observation. We investigated this reaction by incubating **3** with DXR in the absence of both NADPH and

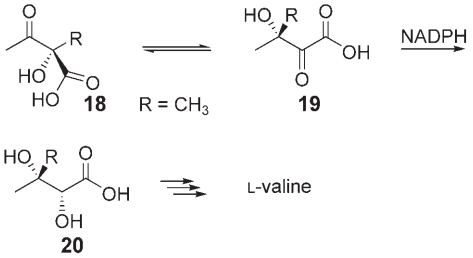


Scheme 3. Detailed consideration of the retro-aldol/aldol rearrangement.

metal ions in D_2O/Tris-HCl buffer solution (Tris = tris(hydroxymethyl)aminomethane) in an NMR tube for 12 h. Under these conditions, a low concentration of **16** (approximately 5%) was detected by ^{13}C NMR—in particular C4 of **16** was observed at $\delta = 71.6$ ppm, showing the characteristic $^3J_{CP}$ coupling.[15] This compound was not formed in the absence of purified enzyme. The formation of **16** is further evidence in favor of the proposed retro-aldol/aldol mechanistic sequence as this compound could not be formed by an α-ketol-type process. Diastereomer **16** has previously been reported as a weak competitive inhibitor of DXR from *Synechocystis* PCC6803, indicating that it can bind at the active site. However, isomerization assays in the absence of divalent metal ions were not reported for this compound.[15]

The mechanism presented in Scheme 3 rationalizes the formation of **16**. In the absence of Mg^{2+} or Mn^{2+} ions, the retro-aldol step (Scheme 3, reaction I) is still operative, giving aldehyde and enediol intermediates in the active site. In the absence of Mg^{2+} or Mn^{2+} ions, the aldehyde can rotate by 180° and recombine with the enediol (Scheme 3, reaction I′), slowly forming **16**. Presumably, the presence of the divalent metal ion during normal catalysis prevents aldehyde rotation and formation of **16**. However, metal ions are evidently not required for reactions I and I′. There was also no evidence for the formation of **6** or **17** in the active site, so either Mg^{2+} ions are required for steps II and II′, or the concentration of **6** and **17** is too low to detect. It appears unlikely that Mg^{2+} ions would be required for step II but not step I, so it is probable that the equilibrium favors compounds **3** and **16** in the absence of Mg^{2+} ions. This is supported by DFT calculations, which show **3** as being intrinsically 5.05 kcal mol^{-1} lower in energy than **6** (B3LYP/6-31G(d)*//MMFF). It is known that Mg^{2+} or Mn^{2+} ions are required for step III, presumably for coordination to the carbonyl group, facilitating hydride transfer from NADPH to the *Re* face of the aldehyde.[10,16]

The enzyme ketolacid reductoisomerase (KARI, EC 1.1.1.86) catalyzes a similar sequence of reactions to DXR.[17] In this case, the substrate is (*S*)-2-acetolactate **18**, which is isomerized to 3-hydroxy-3-methyl-2-ketobutyrate **19**, which is in turn reduced by NADPH to give (*R*)-2,3-dihydroxy-3-methylbutyrate **20** (Scheme 4). The branched product **20** is a precursor of L-valine. Recent mechanistic and crystallographic investigations of KARI have revealed that two Mg^{2+} ions are required at the active site.[18] The mechanism of the rearrangement catalyzed by KARI is generally accepted as being a concerted α-ketol reaction as



Scheme 4. Reactions catalyzed by KARI in the formation of L-valine.

there is no possibility of a retro-aldol/aldol sequence. An Mg^{2+} ion is required for this isomerization step. The equilibrium between **18** and **19** lies heavily in favor of **18**, and this is why KARI must combine the isomerization and reduction in a single active site.

Both the mechanism of KARI and its requirement for Mg^{2+} ions differ from DXR, which appears not to require metal catalysis for the retro-aldol/aldol rearrangement. However, KARI is similar to DXR in requiring Mg^{2+} ions for the reduction step. Peptide-sequence analysis shows that there is very limited sequence similarity between DXR and KARI. For example, there is only 10% sequence identity between DXR and KARI from *E. coli*, and no sequence conservation at the active sites. Thus the resemblance between DXR and KARI appears to be superficial, reflecting neither mechanism, active-site structure, or peptide sequence.

The mechanism proposed for DXR shown in Scheme 3 closely resembles the retro-aldol/aldol mechanism of L-ribulose-5-phosphate-4-epimerase.[19] This enzyme interconverts L-ribulose-5-phosphate and D-xylulose-5-phosphate, but requires a divalent metal ion. DXR appears to catalyze a very similar reaction, albeit slowly, in the absence of a metal. However, DXR and L-ribulose-5-phosphate-4-epimerase from *E. coli* share only 11% sequence identity.

In conclusion, the observation of inverse secondary kinetic isotope effects for both 3-[^{2}H] and 4-[^{2}H]-D-1-deoxyxylulose-5-phosphate during DXR-catalyzed isomerization indicates that DXR operates through a retro-aldol/aldol mechanism. This is supported by the observed formation of L-deoxyribulose-5-phosphate **16** in the absence of metal ions. Thus, the isomerization reaction differs from the enzyme KARI, which uses an α-ketol mechanism. The isomerization equilibrium catalyzed by DXR strongly favors the starting material **3** over the branched aldehyde product **6**, and the

formation of **6** appears to be the rate-limiting step. The reaction is driven to completion, however, by coupling to the oxidation of NADPH. In the case of KARI, knowledge of its chemical mechanism has led to the understanding of inhibition by compounds based on transition-state structures.[20] The determination of the mechanism of DXR should contribute in a similar way.

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