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Study of 1-Deoxy-D-xylulose-5-phosphate Reductoisomerase: Synthesis and Evaluation of Fluorinated Substrate Analogues

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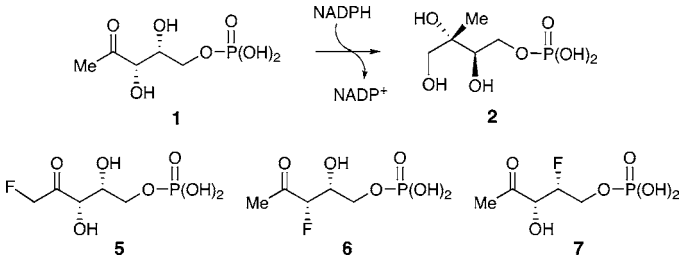
ABSTRACT



1-Deoxy-D-xylulose-5-phosphate (DXP) reductoisomerase is a NADPH-dependent enzyme catalyzing the conversion of DXP to methyl-D-erythritol 4-phosphate (MEP). In this study, each of the hydroxyl groups in DXP and one of its C-1 hydrogen atoms, were separately replaced with a fluorine atom and the effect of the substitution on the catalytic turnover was examined. It was found that the 1-fluoro-DXP is a poor substrate, while both 3- and 4-fluoro-DXP behave as noncompetitive inhibitors.

Terpenoids are a family of secondary metabolites that are widely distributed in nature and are rich in biological activities.[1,2] The terpenoid building block is a five-carbon unit known as isoprene, which has long been established to be derived from acetate via the mevalonate pathway.[3] However, a new isoprene biosynthetic pathway has recently been discovered where the isoprenoid unit is formed from pyruvate and glyceraldehyde-3-phosphate.[4−6] It has been shown that 2-*C*-1-deoxy-D-xylulose-5-phosphate (DXP, **1**) is the immediate product resulting from condensation of these two precursors,[7,8] and conversion of DXP to methyl-D-erythritol 4-phosphate (MEP, **2**) is the first pathway-specific transformation en route to the basic isoprenoid unit.[9] Since this MEP pathway appears to operate in eubacteria, archeabacteria, algae, and plastids of plants, but not in humans, enzymes involved in this pathway could be excellent targets

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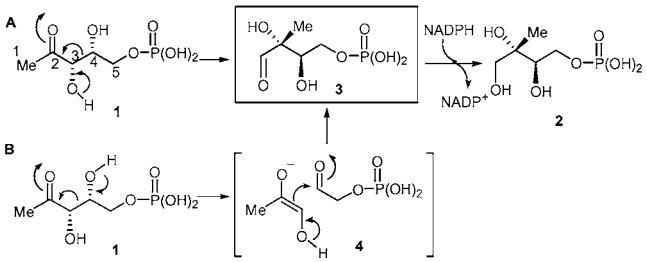
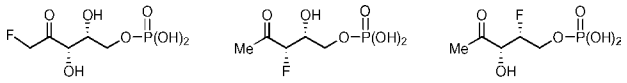
Scheme 1



for the development of new antibiotics.[4−6] In fact, fosmidomycin, which inhibits the conversion of **1** to **2**,[10] has been shown to be effective for the treatment of malaria.[11,12]

The enzyme catalyzing the conversion of DXP (**1**) to MEP (**2**), 1-deoxy-D-xylulose-5-phosphate reducto-isomerase (DXR, also known as MEP synthase), has been isolated from several sources. It is a NADPH-dependent catalyst, which also requires a divalent metal ion for activity.[9,13−15] Two possible mechanisms for the conversion of DXP (**1**) to MEP (**2**) have been proposed. As shown in Scheme 1, the first mechanism involves an α-ketol rearrangement to yield methylerythrose phosphate (**3**) as an intermediate, followed by the reduction of the aldehyde group in **3** by NADPH to drive the equilibrium to completion (route A). A mechanism involving a retroaldolization/aldolization rearrangement is another alternative (route B). This route yields first a bimolecular intermediate (**4**), which is condensed to form **3**. Subsequently, **3** is reduced by NADPH to generate the product **2**.

The catalysis by the *Escherichia coli* enzyme has been shown to proceed via an ordered mechanism in which NADPH binds before the substrate, DXP, and $NADP^+$ is released after the discharge of MEP.[14,15] Interestingly, a random mechanism has recently been determined for the *Mycobacterium tuberculosis* enzyme.[16] However, due to the significant differences in K_m of the substrates in the forward and reverse reaction, a preferred order of binding exists in the latter case rendering its mechanism effectively similar to that of the *E. coli* enzyme. Early studies have also established that the C-1 *pro-S* hydrogen in MEP (**2**) is derived from H-3 of DXP (**1**) and the hydride transfer from NADPH is *pro-S* specific.[17,18] In addition, the crystal structures of DXR from *E. coli*[19] as well as a complex of DXR with NADPH have been determined.[20] Despite the advances made so far in our understanding of the catalytic properties of DXR, it remains elusive whether catalysis is better described by route A or route B. In an attempt to gain more insight into the mechanism of this intriguing enzyme, we prepared three fluorinated substrate analogues **5**−**7** and examined their competence as substrates or inhibitors upon incubation with DXR.



If the hydroxyl substituent(s) of DXP indeed play(s) a crucial role in DXR catalysis, its replacement with a chemically inert fluorine will effect the catalytic turnover. We therefore replaced each of these hydroxyl groups with an inert fluorine atom and examined the effect of the substitution on the catalytic turnover. In route A, deprotonation of the C-3 hydroxyl group followed by a 1,2-migration to yield an aldehyde intermediate is the key step in the mechanism. Since the C-4 hydroxyl group is not directly involved in catalysis, compound **7** is likely to be a substrate, while compound **6** would not be a substrate if the reaction proceeds via an α-ketol rearrangement. The catalytic rate for **7** might be retarded, however, due to the increased electronegativity of fluorine. In the case of a retroaldolization mechanism, while the C-4 hydroxyl group is involved in the first step of the reaction, the C-3 hydroxyl group participates in the aldolization step such that a new bond forms between carbons derived from C-2 and C-4 of DXP to yield the aldehyde intermediate. Thus, it is expected that if the reaction proceeds via route B, both compounds **6** and **7** will not be turned over by DXR. In contrast, compound **5** should always be recognized by DXR as a substrate, since C-1 is not directly involved in catalysis. However, the electron-withdrawing nature of the fluorine substituent at C-1 will render the 2-keto group more electrophilic and thus facilitate the α-ketol rearrangement. Although a similar argument holds for the retroaldol bond cleavage step in route B, stabilization of the resulting enolate anion by the fluorine substituent at C-1 may compromise its capability as a nucleophile and slow the second half of the reaction. The effect on the overall catalysis will depend on which step is more rate limiting.

To test these predictions, we developed methodology to prepare this series of molecules.[21] The reaction sequence used to synthesize the 1-fluoro analogue **5** from **8**[22] is shown in Scheme 2. Fluorination at C-5 followed by hydrolysis, benzylation, and reductive ring opening of **10** led to the key intermediate **11**, which upon benzoylation and Swern oxidation gave **13** as the product. Subsequent ketalization and de-

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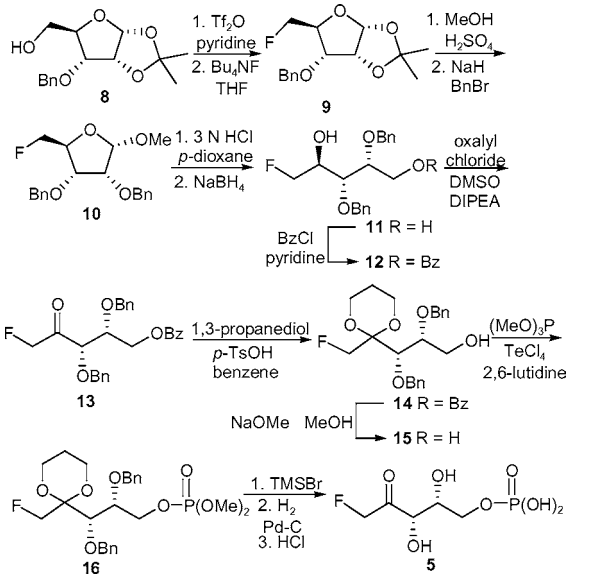
Scheme 2

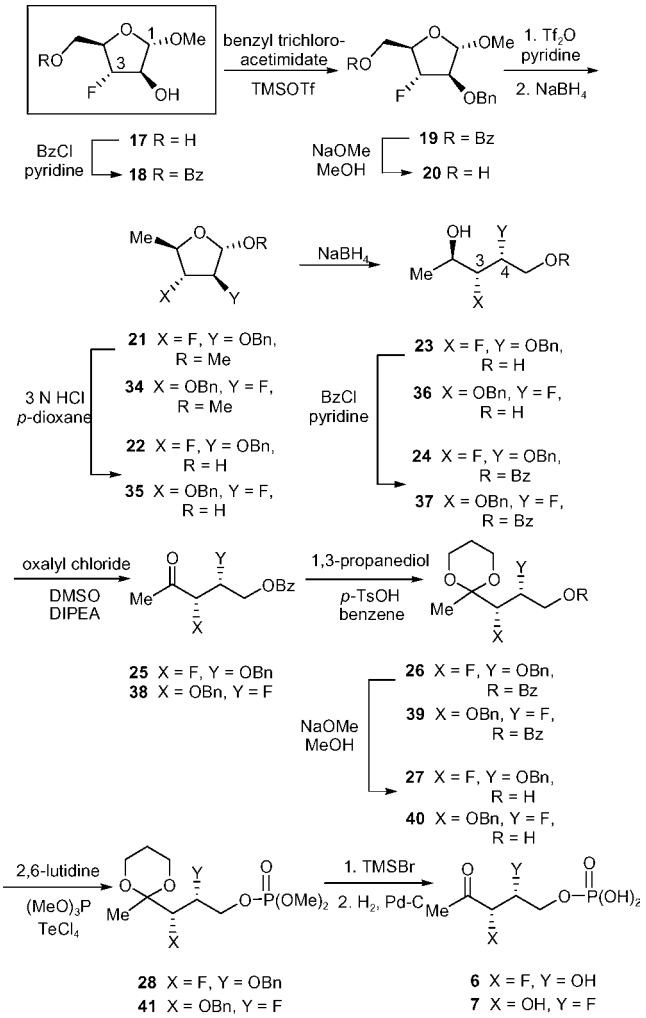


esterification afforded **15**. This compound was then subjected to treatment of trimethyl phosphite, 2,6-lutidine and tellurium tetrachloride to phosphorylate the 5-OH group.[23] The final conversion was achieved by successive treatment of **16** with trimethylsilyl bromide and palladium on carbon. The ketal group was hydrolyzed during the hydrogenolysis reaction, presumably due to either the residual acid from the TMS−Br treatment or the slightly acidic nature of the Pd−C catalyst. The desired product **5** was purified using cellulose chromatography.

The 3- and 4-fluoro analogues, **6** and **7**, were synthesized using a similar strategy except for the initial steps. As shown in Scheme 3, synthesis of **6** from **17**[24,25] involved selective protection/deprotection of the C-2 and C-5 hydroxyl groups followed by C-5 deoxygenation to give **21**. The same intermediate bearing a fluorine substituent at C-2 (**34**) was prepared, as shown in Scheme 4, from **29**[26] via a sequence of C-5 deoxygenation, methanolysis, and fluorination reactions.[27] Since the product of methanolysis of **31**, was obtained as a mixture of β/α isomers in a 6:1 ratio, acetylation to give **32** was necessary to facilitate their separation by silica gel chromatography. Subsequent transformations of **21** and **34** to **6** and **7**, respectively, followed the procedures used to make compound **5**.

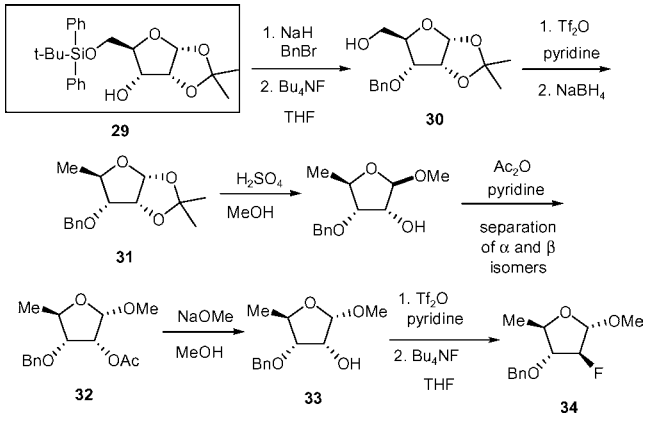
When compounds **6** and **7** (0.5−5 mM) were incubated in the presence of DXP (**1**, 50−100 μM), Mg^{2+}, and NADPH with heterologously expressed DXR from *E. coli*,[21] neither one was turned over by DXR. Instead, both compounds were found to be inhibitors. A Dixon plot[28] of the reciprocal

Scheme 3



enzyme activity as a function of inhibitor concentration at different substrate concentrations shows that **6** behaves as a noncompetitive inhibitor with respect to DXP with a K_i value of 444 μM (Figure 1 of the Supporting Information). Similar results were also noted for **7** with a K_i of 733 μM. A possible

Scheme 4



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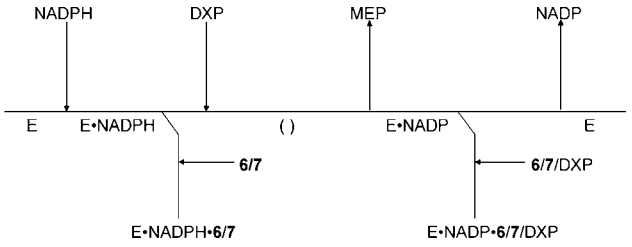
Scheme 5



explanation of the noncompetitive inhibition pattern observed is that **6** and **7**, both as DXP mimics, are capable of binding two distinct forms of the DXR enzyme.[29]

As illustrated in Scheme 5, the first encounter between DXR and **6** (or **7**) is with the E·NADPH complex, taking place before DXP (**1**) binds. The second encounter is with the E·$NADP^+$ complex and occurs after MEP (**2**) is released but prior to the departure of $NADP^+$. The first binding interaction will affect the slope of the double reciprocal plot due to the reversible binding of **6** (or **7**) to the same enzyme form as for DXP. The second binding interaction will affect the intercept of the double reciprocal plot due to the binding of **6** (or **7**) and DXP to two different enzyme forms (E·$NADP^+$ for **6**/**7** and E·NADPH for DXP) that are separated by an irreversible step.[30] When the K_i values for these two interactions are comparable, the inhibition pattern will appear as noncompetitive.

While further experiments to determine the inhibition kinetics are necessary to fully corroborate the proposed mechanism, the above inhibition model is favored in view of the close structural resemblance of **6** and **7** to DXP, which are expected to bind to DXR at the same site. More importantly, the fact that substrate inhibition is observed at high concentrations of DXP (>500 μM), which may be attributed to the formation of an E·$NADP^+$·DXP ternary complex, also supports the proposed model. It should be noted that in the inhibition studies, since the concentration of **6** (or **7**) is much higher than that of DXP in the incubation mixture, the inhibitor (instead of substrate) would dominate the second binding event. Because the inability of DXR to process **6** and **7** is most likely a consequence of having chemically inert functional groups at site of action preventing turnover to the product, the above results may be considered as preliminary evidence implying that the retroaldolization/aldolization mechanism (route B) is operative.

On the basis of the above findings, we anticipated that the 1-fluoro substrate analogue **5** would bind favorably to DXR. Incubation of DXR with **5** showed that this compound is, in fact, a substrate for DXR, with a K_m value of 100 μM and k_{cat} value of 4.5 s^{-1}, albeit only about 13% (k_{cat}/K_m) effective when compared to DXP, which has a K_m value of 61 μM and a k_{cat} value of 21.3 s^{-1} under the same assay conditions.[21] The slightly elevated K_m suggests that the fluorine substitution at C-1 likely affects the electronic as well as the steric properties of the substrate. However, the reduction in catalytic efficiency is mainly the result of decreased k_{cat} of **5**, which is also consistent with a mechanism based on route B.

Clearly, both hydroxyl groups at C-3 and C-4 of DXP are crucial for catalysis. A similar conclusion was also reached in a recent report in which the corresponding 3- and 4-deoxy-DXP were found to be mixed-type inhibitors for DXR.[15] It is possible that these hydroxyl groups are sites for divalent metal chelation, which play an essential role facilitating the proposed α-ketol rearrangement. However, our findings may more appropriately be interpreted in favor of the retroaldolization/aldolization mechanism for the isomerization catalyzed by DXR, yet it is not possible at this time to fully distinguish it from the α-ketol rearrangement. Undoubtedly, more experiments are needed to unravel the details of this intriguing enzymatic reaction. This knowledge is essential for the development of novel therapeutic agents directed against the reactions involved in the formation of isoprenoids in many pathogens.

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Supporting Information Available: Experimental details, kinetic data, and NMR and mass spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(29) An alternative but less favored interpretation of these data is that **6** and **7** cannot directly compete with DXP (**1**) for the same binding site. This phenomenon could be a result of their inability to chelate the divalent metal ion to form a complex, which may be essential for efficient binding to the active site of DXR. Instead, **6** and **7**, in the presence of DXP, may bind DXR at a site away from the active site perhaps causing inhibition through an allosteric effect.

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