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Substrate analogs for the investigation of deoxyxylulose 5-phosphate reductoisomerase inhibition: synthesis and evaluation

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Abstract—Deoxyxylulose 5-phosphate (DXP) analogs were synthesized and evaluated as alternative substrates and inhibitors of recombinant *Synechocystis* PCC6803 DXP reductoisomerase (DXR; EC 1.1.1.267). Five of the compounds tested (1,2-dideoxy-D-threo-3-hexulose 6-phosphate, 1-deoxy-L-ribulose 5-phosphate, 2*S*,3*R*-dihydroxybutyramide 4-phosphate, 4*S*-hydroxypentan-2-one 5-phosphate, and 3*S*-hydroxypentan-2-one 5-phosphate) acted as relatively weak competitive inhibitors when compared to fosmidomycin. A sixth compound, 3*R*,4*S*-dihydroxy-5-oxohexylphosphonic acid, served as an alternate substrate, as has recently been reported for the same compound with *Escherichia coli* DXR.

The discovery of the methylerythritol phosphate (MEP) pathway as an alternate pathway for isoprenoid biosynthesis in some organisms including most bacteria, plants, and the malarial parasite *Plasmodium falciparum*, has stimulated extensive research in this area.[1,2] Studies have revealed the potential of finding novel antibacterials, antimalarials, and herbicides from enzyme inhibitors of this pathway.[3] The natural product fosmidomycin has effective antimalarial activity in mice[4] and is currently undergoing clinical trials in humans in Africa and Thailand.[5] Fosmidomycin (**1**) inhibits the second enzyme in the MEP pathway, deoxyxylulose 5-phosphate reductoisomerase (DXR, EC 1.1.1.267),[6] which mediates the conversion of 1-deoxy-D-xylulose 5-phosphate (DXP, **2**) into 2*C*-methyl-D-erythritol 4-phosphate (MEP, **3**) (Fig. 1).

Although fosmidomycin and its analogs are potent inhibitors of DXR,[6] analogs of the DXR substrate, DXP, should also be examined in order to more fully understand the nature of DXP binding and DXR inhibition. Analysis of these compounds as substrates/inhibitors for DXR will provide data that complements the recent X-ray crystal structures of DXR[7–10] and may help in the design of putative DXR inhibitors. The features of

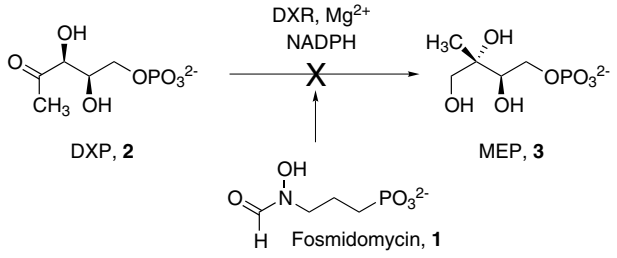

Figure 1. Conversion of DXP to MEP mediated by DXR.

DXP that were considered as potential sites for designing analogs of DXP were the methyl ketone group, the hydroxylated backbone, and the polar phosphate head group. All DXP analogs were synthesized and tested against recombinant DXR from *Synechocystis* PCC6803, which was used for previous studies in our laboratory.[11,12] A trivial name has been provided for each analog that readily distinguishes the structural change.

The compound, 1,2-dideoxy-L-threo-3-hexulose 6-phosphate (1-Me-DXP, **8**) was designed to provide extra steric bulk at the methyl ketone moiety of DXP. It was synthesized by modifying the synthesis of DXP by Blagg and Poulter.[13] The key differences in the synthetic procedure were use of EtMgBr instead of MeMgBr to install the ethyl group and phosphorylation with diphenyl chlorophosphate (Scheme 1). The phosphate protecting groups

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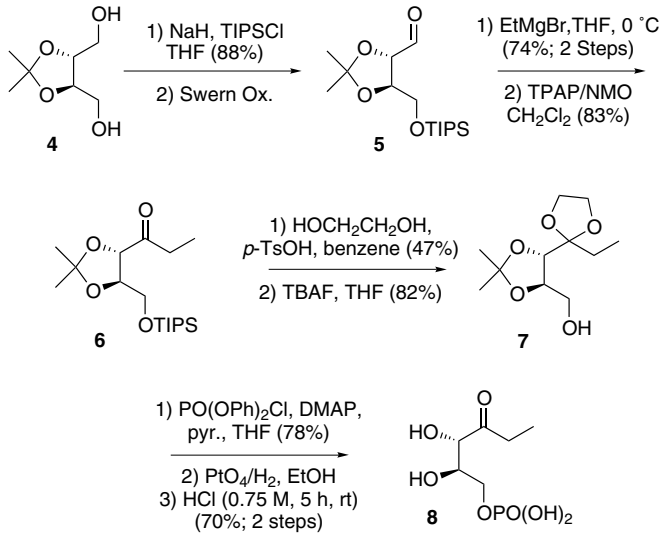
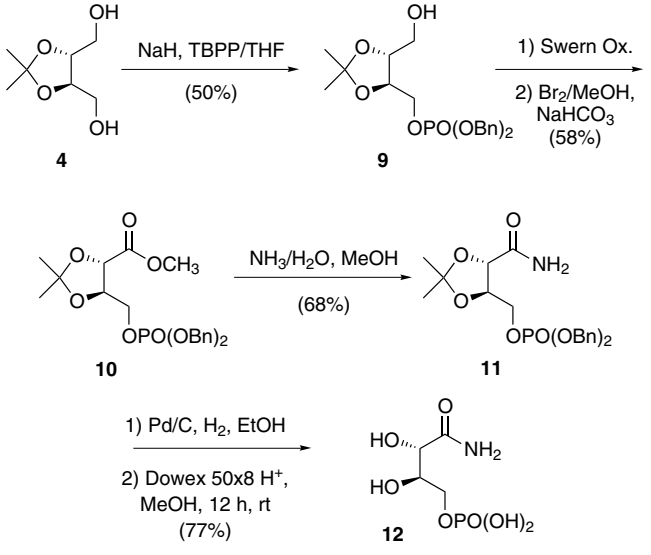


Scheme 1. Synthesis of 1-Methyl-DXP (**8**).

were removed by catalytic hydrogenolysis with Pt(IV) oxide and acidic hydrolysis with aqueous HCl cleaved the acetonide and dioxolane protecting groups to provide **8**.[14] This analog has also been prepared enzymatically in low yield from α-oxobutyrate and glyceraldehyde 3-phosphate.[15]

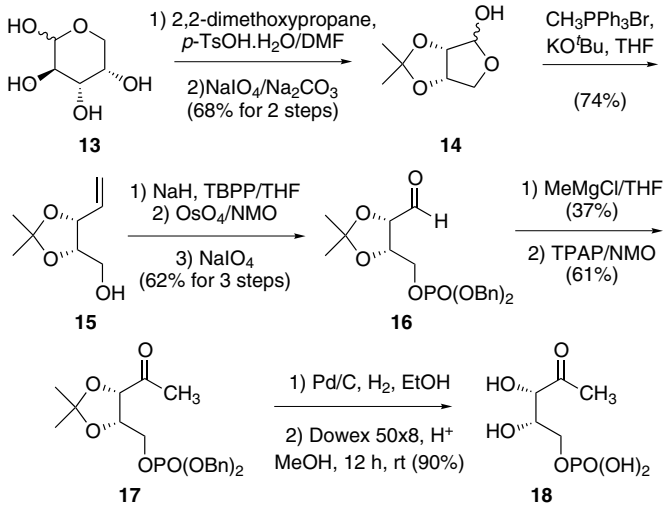
Replacement of the methyl ketone moiety with an amide should provide a compound with similar steric requirements to DXP, but with poorer electrophilicity at the carbonyl moiety compared to the substrate DXP. Therefore, this compound should not be a substrate for DXR and may act as a DXR inhibitor. Scheme 2 shows the procedure for the synthesis of 2*S*,3*R*-dihydroxybutyramide 4-phosphate (DXP-carboxamide, **12**). Monophosphorylation of (−)-2,3-*O*-isopropylidene-D-threitol (**4**; obtained in two steps from diisopropyl-D-tartrate) with tetrabenzyl pyrophosphate provided **9**. Swern oxidation of **9** and oxidation of the resulting aldehyde with bromine in methanol directly afforded the methyl ester **10**. A reaction between the ester **10** and aqueous ammonia gave amide **11**. Finally, catalytic hydrogenolysis and acid hydrolysis of **11** were performed to yield DXP carboxamide (**12**) (12% overall yield, eight steps from diisopropyl-D-tartrate).



Scheme 2. Synthesis of DXP-carboxamide (**12**).

The compound 1-deoxy-L-ribulose 5-phosphate (4-*epi*-DXP, **18**) has the opposite stereochemistry at the C-4 position relative to DXP and will give information about how crucial the stereochemistry of the hydroxyl functional group is for binding to DXR and catalysis. The synthesis of 4-*epi*-DXP began with L-arabinose (**13**, Scheme 3). Selective isopropylidene formation and oxidative cleavage of **13** generated a diastereomeric mixture of **14**.[16] A Wittig reaction of **14** with methyltriphenylphosphonium bromide/potassium *tert*-butoxide yielded the alkene **15**. Phosphorylation of **15** was accomplished using tetrabenzyl pyrophosphate (TBPP). Dihydroxylation and periodate cleavage provided the aldehyde **16** in 62% overall yield for three steps. A single diastereomer was obtained from the methyl Grignard addition to the aldehyde **16**, albeit in low yield. Oxidation of the secondary alcohol using TPAP and NMO provided ketone **17**. Finally, 4-*epi*-DXP (**18**, 5% overall yield, 10 steps from L-arabinose) was obtained by catalytic hydrogenolysis and acid hydrolysis of **17**.

The three remaining compounds that were synthesized have also been prepared and tested against the *E. coli* DXR.[17,18] The 3*S*-hydroxypentan-2-one 5-phosphate (4-deoxy-DXP, **19**) and 4*S*-hydroxypentan-2-one 5-phosphate (3-deoxy-DXP, **20**) compounds were designed to test the importance of the hydroxyl groups at the C3 and C4 positions for binding and for catalysis. The phosphonate analog of DXP, 3*R*,4*S*-dihydroxy-5-oxohexylphosphonic acid (DX-phosphonate, **21**), was prepared to determine the effect of changing from a phosphate to a phosphonate functional group. Our synthetic approaches to 3-deoxy-DXP and 4-deoxy-DXP were similar to the routes previously published.[17] The



Scheme 3. Synthesis of 4-*epi*-DXP (**18**).

synthesis of the DX-phosphonate and analysis as an alternate substrate for *E. coli* DXR were recently reported.[18] Our synthetic approach to DX-phosphonate also started with a tartrate derivative, but involved a different series of steps to provide the final phosphonate analog. The details of the syntheses of all three of these compounds are provided elsewhere.[19]

All six of the DXP analogs were tested as alternate substrates for the *Synechocystis* DXR.[20] All five phosphate analogs of DXP showed no sign of being an alternate substrate for DXR when tested at concentrations up to 30 mM. However, DX-phosphonate (**21**) did show activity as an alternate substrate. This compound has a $K_m = 690\,\mu M$, which is fourfold higher than the $K_{m(DXP)}$ (170 μM).[12] This is the same general result observed for this phosphonate analog with the *E. coli* DXR, although the K_m with the *Synechocystis* DXR is significantly higher than that measured with the *E. coli* DXR.[18] The K_m value of DX-phosphonate (120 μM) with *E. coli* DXR was also fourfold higher than the reported $K_{m(DXP)}$ (30 μM). The change from a phosphate group to a phosphonate leads to a decrease in binding, but because this structural change is distant from the portion of the molecule undergoing transformation, turnover is still possible.

The five DXP analogs that were not alternate substrates were tested as potential inhibitors. All five phosphate analogs were found to be competitive inhibitors of DXR with respect to DXP (Fig. 2). The inhibition by all of the compounds is rather weak, especially when compared to the slow, tight-binding inhibitor fosmidomycin ($K_i = 21$ nM).[21]

The 1-Me-DXP analog, which has an ethyl ketone group rather than the methyl ketone moiety of DXP, has a K_i about three and a half-fold higher than the $K_{m(DXP)}$ The competitive nature of the inhibition indicates that 1-Me-DXP binds at the same site as DXP, although less tightly, but the added steric bulk of the ethyl group prevents successful turnover of this compound. As the addition of the methyl group does not alter the electronic characteristics of the molecule, the lack of turnover may be due to improper alignment of the substrate in the active site or a steric interaction arising upon rearrangement.

Although the amide group of DXP carboxamide and the methyl ketone of DXP are isosteric, the amide is much less electrophilic than the ketone, which likely explains the lack of turnover of this compound. The K_i value is about half of the K_m for DXP, suggesting slightly more

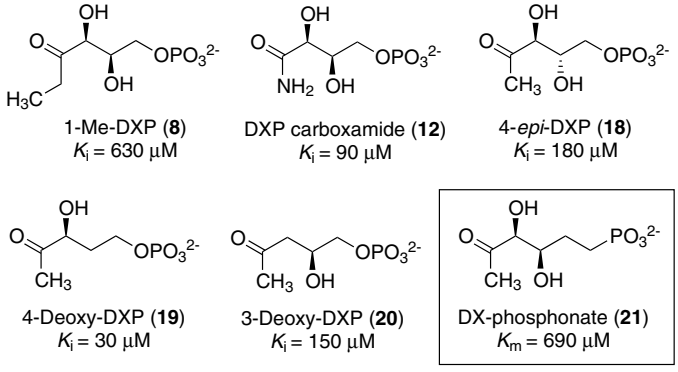


Figure 2. DXP analogs and inhibition (K_i) or Michaelis (K_m) constants. The alternate substrate DX-phosphonate is indicated by a box.

favorable interactions for the amide derivative, possibly through additional hydrogen bonds.

None of the three analogs that have changes in the hydroxylation pattern of DXP were alternate substrates, indicating the importance of the presence and stereochemistry of both hydroxyls for catalysis. The K_i for 4-*epi*-DXP and the K_m for DXP are similar, suggesting that the stereochemistry of the hydroxyl at C4 of the substrate is not critical for binding, but it is essential for catalysis. The absence of the C4 hydroxyl in 4-deoxy-DXP reinforces the idea that the C4 hydroxyl is not critical for binding because the K_i for this compound (30 μM) is about sixfold lower than the $K_{m(DXP)}$. The K_i for 3-deoxy-DXP is similar to the $K_{m(DXP)}$ indicating that the C3 hydroxyl also is not critical for binding, but essential for catalysis. When tested with the *E. coli* DXR, 3-deoxy-DXP and 4-deoxy-DXP were found to be mixed type inhibitors, with K_i values of 800 and 120 μM, respectively. Our results differ slightly from the *E. coli* results in that both compounds were competitive inhibitors for the *Synechocystis* DXR, rather than mixed type inhibitors. The K_i values were also lower, although in both cases, the K_i value for 4-deoxy-DXP was 5–6-fold lower than for 3-deoxy-DXP. These results may reflect slight differences in the active sites of the *E. coli* and *Synechocystis* enzymes.

The biochemical analysis of these DXP analogs has provided further information about the interactions of the substrate DXP with DXR. The importance of the hydroxyl groups has been clearly demonstrated, as well as the steric limitations near the ketone group of DXR. These data should be helpful, when used in concert with the current X-ray crystal structures of the *E. coli*[7–9] and *Zymomonas*[10] DXR, for designing potent inhibitors of this important enzyme.

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14. All compounds were characterized by ^{1}H, ^{13}C, and ^{31}P NMR as well as HRMS. 1-Me-DXP, **8**, Na^+ salt: $[\alpha]_D^{25} - 1.7$ (*c* 3.0, H_2O); ^{1}H NMR (300 MHz, D_2O): δ 4.53 (d, $J = 1.8$ Hz, 1H, H4), 4.33 (dt, $J = 1.8$, 6.5 Hz, 1H, H5), 3.88 (dd, $J = 7.1$, 6.7 Hz, 2H, H6), 2.71 (m, 2H, H2a, H2b), 1.07 (t, $J = 7.2$ Hz, 3H, H1); ^{13}C NMR (75 MHz, D_2O): δ 216.98 (C3), 76.78 (C4), 70.96 (d, $J = 6.5$ Hz, C5), 65.56 (d, $J = 3.8$ Hz, C6), 32.31 (C2), 7.13 (C1); ^{31}P NMR (121 MHz, D_2O): δ 1.05; HRMS-FAB calcd for $C_6H_{13}O_7P$: 228.0399; found 228.0392. DXP-Carboxamide, **12**, Na^+ salt: $[\alpha]_D^{25} - 26$ (*c* 2.0, H_2O); ^{1}H NMR (300 MHz, D_2O): δ 4.28 (d, $J = 2.1$ Hz, 1H, H2), 4.19 (dt, $J = 2.0$, 6.8 Hz, 1H, H3), 4.00 (m, 2H, H4); ^{13}C NMR (75 MHz, D_2O): δ 178.75 (C1), 71.80 (C2), 71.34 (d, $J = 8.3$ Hz, C3), 67.18 (d, $J = 4.0$ Hz, C4); ^{31}P NMR (121 MHz, D_2O): δ 0.62; HRMS-FAB calcd for $C_4H_9NO_7P$: 214.0117; found 214.0129. 4-*epi*-DXP, **18**, Na^+ salt: $[\alpha]_D^{25} - 4$ (*c* 0.7, H_2O); ^{1}H NMR (300 MHz, D_2O): δ 4.26 (d, $J = 5.0$ Hz, 1H, H3), 4.09 (dt, $J = 5.3$, 5.6 Hz, 1H, H4), 3.90 (dd, $J = 6.0$, 5.8 Hz, 2H, H5), 2.18 (s, 3H, H1); ^{13}C NMR (75 MHz, D_2O): δ 213.93 (C2), 78.31 (C3), 71.50 (d, $J = 8.8$ Hz, C4), 66.57 (d, $J = 5.3$ Hz, C5), 27.84 (C1); ^{31}P NMR (121 MHz, D_2O): δ 0.54; HRMS-FAB calcd for $C_5H_{10}O_7P$: 213.0164; found 213.0167.
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