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Synthesis and analysis of a fluorinated product analogue as an inhibitor for 1-deoxy-D-xylulose 5-phosphate reductoisomerase

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Abstract—1-Deoxy-D-xylulose 5-phosphate (DXP) reductoisomerase (DXR) is an NADPH-dependent enzyme catalyzing the rearrangement and reduction of DXP to methyl-D-erythritol 4-phosphate (MEP). Two mechanisms for this enzymatic reaction have been proposed, involving either an α-ketol rearrangement or a retroaldol/aldol rearrangement. In this study, a fluorinated product analogue, FCH_2-MEP, was synthesized as a possible mechanism-based inactivator for DXR if the retroaldol/aldol mechanism is operative. FCH_2-MEP was found to be a weak competitive inhibitor, and thus was unable to discriminate between the mechanisms. This result is due to the inability of the targeted enzyme, DXR, to oxidize FCH_2-MEP to the aldehyde intermediate that is common to both mechanisms. While FCH_2-MEP failed to act as a mechanism-based inactivator, the insight gained from this study will assist in the future design of inhibitors of DXR.

Isoprenoids are an important class of compounds that are ubiquitous in nature.[1] Included in this class of natural products are steroids, terpenoids, carotenoids, and ubiquinones, many of which play important roles in living organisms.[2] The basic building blocks for assembling these compounds are two 5-C precursors, isopentenyl diphosphate (IPP, **1**) and dimethylallyl diphosphate (DMAPP, **2**). For many years, it was believed that all organisms use the mevalonate (**3**) pathway for the biosynthesis of IPP, where DMAPP is derived from IPP by the action of IPP isomerase (Scheme 1, pathway A). It was only recently that a new mevalonate-independent pathway (generally referred to as the MEP pathway, see Scheme 1, pathway B) was discovered in eubacteria, archeabacteria, algae, and in the plastids of plants.[3–5] In this pathway, both IPP (**1**) and DMAPP (**2**) are produced directly from 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**8**), which is derived from 2*C*-methyl-D-erythritol 4-phosphate (MEP, **5**). Since the MEP pathway is absent in mammals but is essential for many pathogens, including *Plasmodium falciparum*[6] and *Mycobacterium tuberculosis*,[7] all enzymes in this pathway are potential drug targets.[8]

Much effort has been devoted to exploit this possibility and several leads have been identified.[6,9] A notable example is fosmidomycin (**9**), which has been demonstrated to be an effective treatment for mice infected with malaria.[6] Further analysis showed that fosmidomycin is a slow, tight-binding inhibitor of DXP reductoisomerase (DXR),[10,11] which catalyzes the conversion of 1-deoxy-D-xylulose 5-phosphate (DXP, **4**) to MEP (**5**), the first committed step in the MEP pathway. The design of more specific inhibitors for this enzyme depends on a detailed understanding of its mechanism. Hence, we have initiated mechanistic studies of the DXR catalyzed reaction.[12]

DXR catalyzes the isomerization of DXP (**4**) to methylerythrose phosphate (**10**), followed by the reduction of the aldehyde of methylerythrose phosphate using NADPH to yield MEP (**5**). As shown in Scheme 2, there are two plausible mechanisms for the rearrangement step catalyzed by DXR. The first mechanism involves an α-ketol rearrangement (route A), which is similar to the mechanism catalyzed by ketol acid reductoisomerase, a key enzyme in the biosynthesis of branched chain

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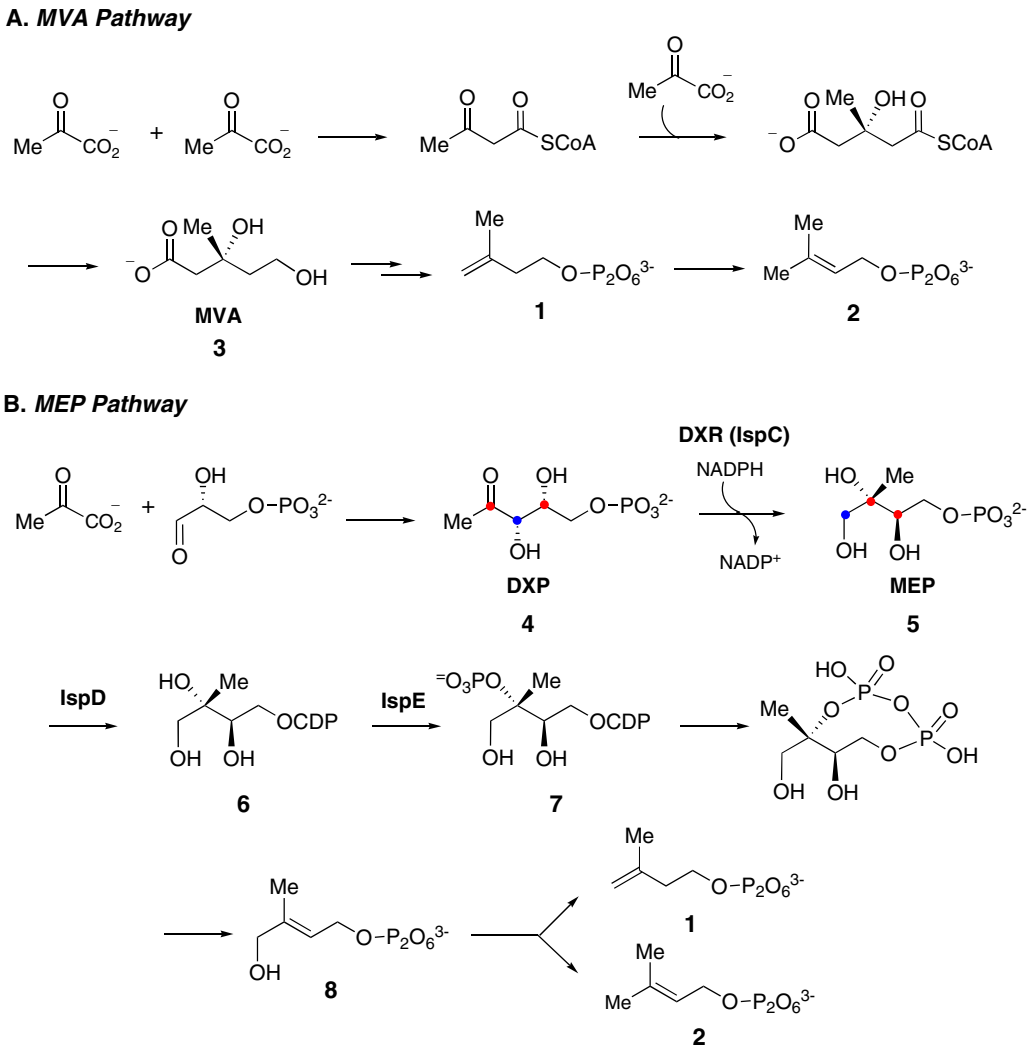
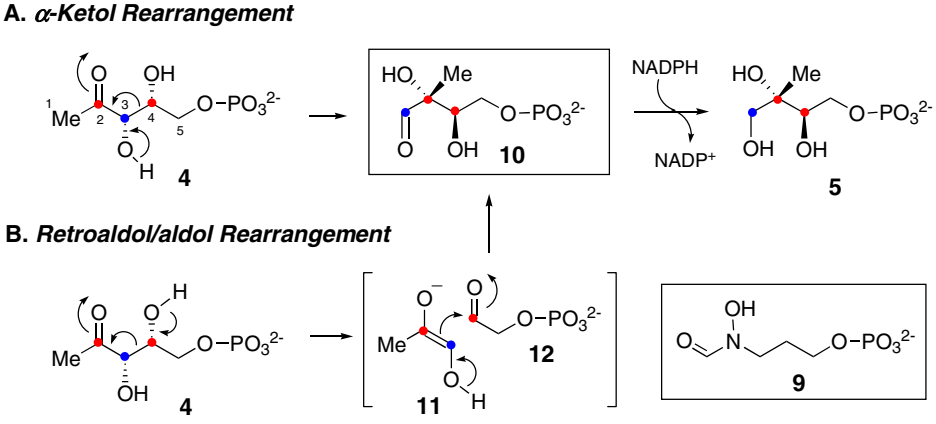


Scheme 1.



Scheme 2.

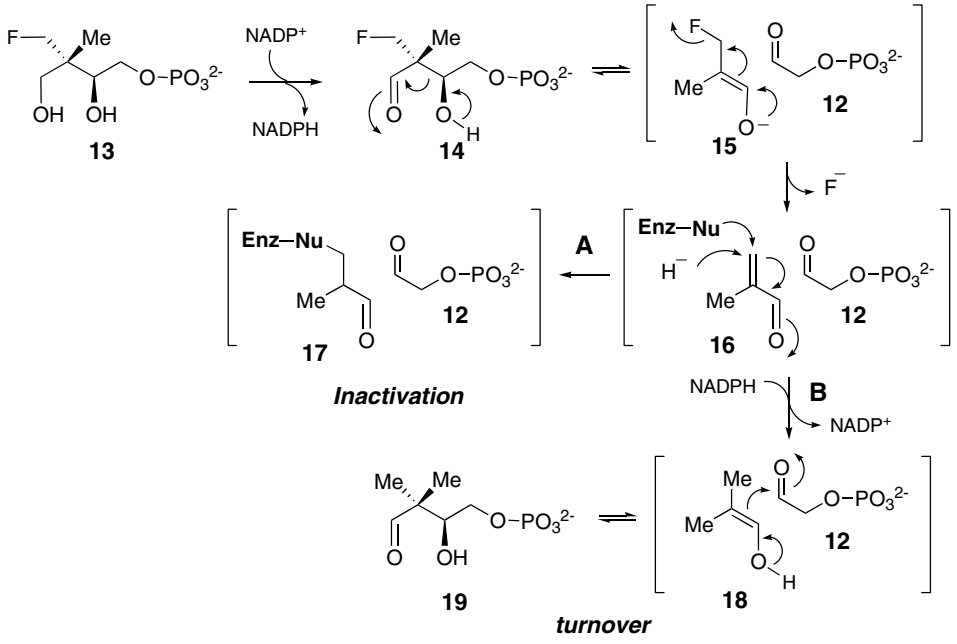
amino acids.[13] This mechanism is initiated by the deprotonation of the C-3 hydroxyl group followed by a 1,2-migration to yield the aldehyde intermediate (**10**). The second mechanism proceeds with a retroaldol/aldol rearrangement to produce the same intermediate, methylerythrose phosphate (**10**, Scheme 2, route B). Here the enzyme first catalyzes the cleavage of the C3–C4 bond through a retroaldol mechanism to yield a three-carbon (**11**) and a two-carbon phosphate (**12**) intermediate. These intermediates then condense through an aldol reaction to form a new C–C bond in **10**. Reduction of **10** by NADPH drives the equilibrium toward **5**.

We envisioned that a fluoromethyl analogue of MEP, a phosphate mono-((2*S*,3*S*)-3-fluoromethyl-2,4-dihydroxy-3-methyl-butyl) ester (FCH_2-MEP, **13**), may be a DXR inactivator whose mode of action could shed light on the catalytic mechanism of DXR. Thus far, all of the inhibitors that have been tested for DXR are either DXP (**4**) or fosmidomycin (**9**) analogues.[11,12,14–20] Interestingly, while the DXR reaction is reversible,[11] there are no reports where MEP (**5**) analogues are exploited as inhibitors for this enzyme. There are also no known examples of compounds that irreversibly inactivate DXR in a mechanistically relevant manner. In view of this void, compound **13** was designed as a possible MEP-based suicide inhibitor. If DXR proceeds via the α-ketol rearrangement mechanism (Scheme 2, route A), compound **13** may act as a competitive inhibitor against MEP (**5**) when the DXR reaction is carried out in the reverse direction (**5** → **4**). In contrast, if a retroaldol/aldol mechanism (Scheme 2, route B) is operative, compound **13** may covalently modify DXR and irreversibly inactivate the enzyme.

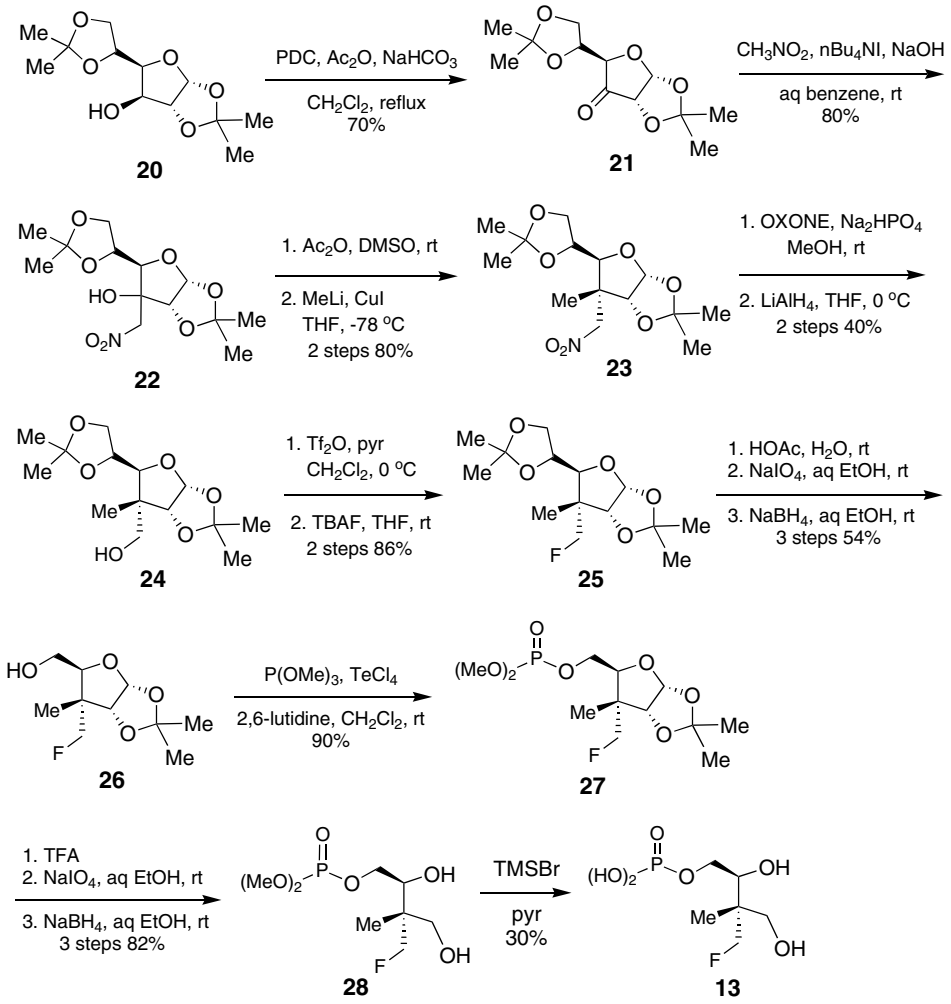
The possible scenarios for inactivation of DXR by **13** are depicted in Scheme 3. When the reaction is run in the direction of DXP (**4**) formation, FCH_2-MEP (**13**) will be oxidized to the corresponding aldehyde (**14**), which then proceeds through the same retroaldol mechanism, as MEP would, to cleave the C2–C3 bond. Because the subsequent aldol condensation cannot occur in this case, the resulting 4-C fragment **15** may instead undergo fluoride elimination to yield the Michael acceptor **16**.[21] As shown in path A, this intermediate could trap a nearby active site nucleophile resulting in the inactive, covalently modified enzyme, **17**.[22] Alternatively, 1,4-reduction of **16** by NADPH (generated *in situ*) to give **18** followed by aldol condensation with **12** could generate **19**(path B),[23] which, in equilibrium with **18** and **12**, may be released as the turnover product(s). Clearly, the outcome of incubation of **13** with DXR could be mechanistically informative.

The synthesis of compound **13** was carried out by the reactions shown in Scheme 4 starting with the commercially available diisopropylidene protected glucose (**20**). Based on a literature procedure,[24] the preparation was initiated by the oxidation of **20** using PDC to generate the 3-keto product (**21**) in 70% yield. Condensation of **21** with nitromethane gave **22**, which underwent dehydration upon treatment with acetic anhydride in DMSO to generate a nitroalkene intermediate. Stereoselective nucleophilic addition of lithium dimethylcuprate on the *si* face of the nitroalkene group afforded **23**, having the desired configuration at the quaternary C-3 center. Oxidation of the nitronate **23** using Oxone®, followed by lithium aluminum hydride reduction, yielded **24**, which was then converted to **25**.[24] Selective hydrolysis of the 5,6-*O*-isopropylidene group, treatment of the resulting diol with sodium metaperiodate, followed by sodium borohydride reduction, produced alcohol **26**. After investigating various phosphorylation reagents, trimethyl phosphine and $TeCl_4$ were found to efficiently phosphorylate the primary alcohol[12] of **26** in high yield (90%) to provide **27**. The 1,2-*O*-isopropylidene protecting group was then removed with aqueous trifluoroacetic acid, and the resulting diol was reacted with $NaIO_4/NaBH_4$ to shorten the chain by one carbon unit to give **28**. Upon treatment with trimethylsilyl bromide followed by aqueous hydrolysis, the phosphotriester **28** was converted to **13**. The crude product was purified by cellulose chromatography under basic conditions (6:3:1 *i*-propanol/H_2O/NH_4OH).[25]

To investigate whether FCH_2-MEP (**13**) could inactivate DXR, FCH_2-MEP (1.8 mM) was incubated with DXR (500 nM), $NADP^+$ (300 μM), BSA (1 mg/mL), and $MgCl_2$ (2 mM) in 100 mM tris(hydroxymethyl)aminomethane (Tris)HCl buffer (pH 7.6) at room temperature



Scheme 3.



Scheme 4.

for 16 h. An identical incubation mixture without **13** was run as a control. An aliquot (10 μL) of each incubation mixture was assayed for activity by adding it to a solution (190 μL) of NADPH (150 μM), DXP (285 μM), $MgCl_2$ (2 mM), and BSA (1 mg/mL) in 100 mM Tris–HCl buffer (pH 7.6). No loss in activity was observed as compared to the control. Evidently, this compound is not an irreversible inactivator for DXR. TLC analysis of the incubation mixture also failed to detect any new product formation. To determine if DXR could catalyze the elimination of a fluoride ion from **13**, **13** (10 mM) was mixed in a NMR tube with 2 mM $MgCl_2$ and 10 mM $NADP^+$ in 100 mM Tris–HCl buffer in D_2O (pD 7.8). The ^{19}F NMR spectrum of this sample was recorded. DXR was then added to this mixture to a final concentration of 60 μM, and the solution was incubated at room temperature for 15 h. The ^{19}F NMR spectrum of this sample was again recorded at the end of the incubation period. No new peak in the ^{19}F NMR spectrum was detected. It was determined that our failure to observe fluoride elimination was due to its inability to oxidize **13** to **14** using $NADP^+$, and not to the inability of DXR to catalyze the retroaldol reaction on **14**. This conclusion was made based on the absence of NADPH production, monitored at 340 nm, upon mixing **13** with DXR, $NADP^+$, and $MgCl_2$.[26]

The fact that FCH_2-MEP (**13**) is neither a suicide inhibitor nor a substrate for DXR makes it unsuitable to distinguish between the two proposed rearrangement mechanisms for DXR (Scheme 2). The inability of DXR to catalyze the initial oxidation of **13**, which is a prerequisite for both rearrangement mechanisms, may be due to the increased steric bulk of the fluoromethyl group, which may prevent the required preorganization of **13** in the active site for the oxidation to occur. Interestingly, incubation of **13** in the presence of MEP (**5**) revealed that it is a weak inhibitor against DXR.[27] As shown in Figure 1, when the reaction was run in the reverse direction with MEP (96 μM, which is roughly half its K_m value) as the substrate, the addition of excess FCH_2-MEP (2.9 mM) led to 45% inhibition of the reaction.

In summary, in this study, we report the synthesis and analysis of FCH_2-MEP (**13**) as a potential inhibitor of DXR. Although compound **13** was not able to inactivate DXR as originally proposed, it was found to be a weak competitive inhibitor of DXR. The cytidylated form of FCH_2-MEP (equivalent of **6**) could be made synthetically or possibly by IspD (see Scheme 1) and the resulting compound could be a viable inhibitor for IspE, which is also a potential drug target. More impor-

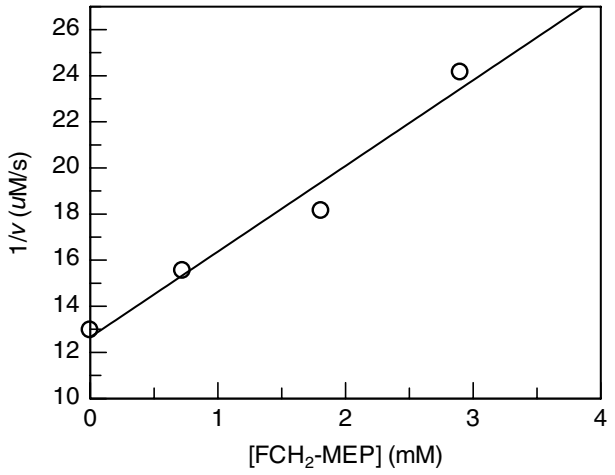


Figure 1. Dixon plot showing the inhibition by FCH_2-MEP (**13**) on the formation of DXP (**4**) catalyzed by DXR. The incubation mixture contained 40 nM DXR, 400 μM $NADP^+$, 2 mM $MgCl_2$, and 96 μM MEP (**5**) in 100 mM Tris–HCl buffer, pH 7.6.

tantly, our results provide useful information to guide the design of future inhibitors of DXR. The inability of DXR to oxidize **13**–**14** and the weak inhibition of **13** toward DXR are most likely due to the steric hindrance caused by the substitution of a fluoromethyl group for a hydroxyl group. This conclusion is consistent with an early observation in which one carbon extension of the backbone of DXP (**4**) rendered the resulting analogue (Et-DXP, (2*R*,3*S*)-2,3-dihydroxy-4-oxohexyl dihydrogen phosphate) a weak inhibitor instead of a substrate for DXR.[19] Thus, future inhibitor design might consider the steric limitations of the active-site of DXR. However, the possibility that replacement of the hydroxyl group with $–FCH_2$ disrupts necessary hydrogen bonding interactions cannot be ruled out. While FCH_2-MEP (**13**) fails to act as a mechanism-based inactivator, MEP-based inhibitors remain one option to modulate the function of DXR. Attempts to resolve the mechanism of DXR-catalyzed reaction and to design effective inhibitors against this enzyme are in progress.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.11.062.

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25. Characterization of FCH_2-MEP (**13**): ^1H NMR (300 MHz, D_2O) δ 0.78 (s, 3H, $J = 2.1$ Hz), 3.46 (ddd, 2H, $J = 1.5$, 11.4, 12.3 Hz), 3.70 (dd, 2H, $J = 5.4$, 6.9 Hz), 3.85 (dd, 1H, $J = 5.4$, 6.6 Hz), 4.31 (d, 1H, $J_{H-F} = 47.4$ Hz), 4.32 (d, 1H, $J_{H-F} = 47.4$ Hz). ^{13}C NMR (75 MHz, D_2O) δ 13.9, 14.0, 42.8, 43.0, 63.55, 63.62, 65.9, 72.63, 72.68, 72.72, 72.77, 85.1, 87.3. ^{19}F NMR (282 MHz, D_2O) δ 2.45 (t, $J_{H-F} = 49.2$ Hz). ^{31}P NMR (121 MHz, D_2O) δ 3.24 (s). HRMS (CI) calcd for $C_6H_{13}FO_6P$ 231.0434; Found: 231.0430.
26. To determine if $NADP^+$ can carry out the initial oxidation of the primary hydroxyl group in **13** to generate the aldehyde intermediate **14**, the formation of NADPH upon mixing **13**, $NADP^+$, $MgCl_2$, and DXR was investigated. A solution of 8.5 mM **13**, 1 mM $NADP^+$, 2 mM $MgCl_2$, and 1 mg/mL BSA in 100 mM Tris–HCl buffer (pH 7.6) was placed in a cuvette, and the absorbance of the solution at 340 nm was determined. DXR, with a final concentration of 77 μM, was then added to the cuvette, and the solution was monitored at 340 nm for the production of NADPH.
27. The assays were run at 25 °C in degassed and N_2 saturated 100 mM Tris–HCl buffer (pH 7.6) containing 2 mM $MgCl_2$, 1 mg/mL BSA, 400 μM $NADP^+$, 96 μM MEP, and varying concentrations of FCH_2-MEP (0–2.9 mM). The reactions were initiated by the addition of enzyme to a final concentration of 50 nM. All reactions were monitored by following the rate of production of NADPH at 340 nm ($\sum_{340} = 6.22\ mM^{-1}\ cm^{-1}$). The concentrations of MEP and DXR were determined as previously described, see Refs. 11 and 12.