Enzyme Catalysis

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IspH Protein of the Deoxyxylulose Phosphate Pathway: Mechanistic Studies with C_1-Deuterium-Labeled Substrate and Fluorinated Analogue**

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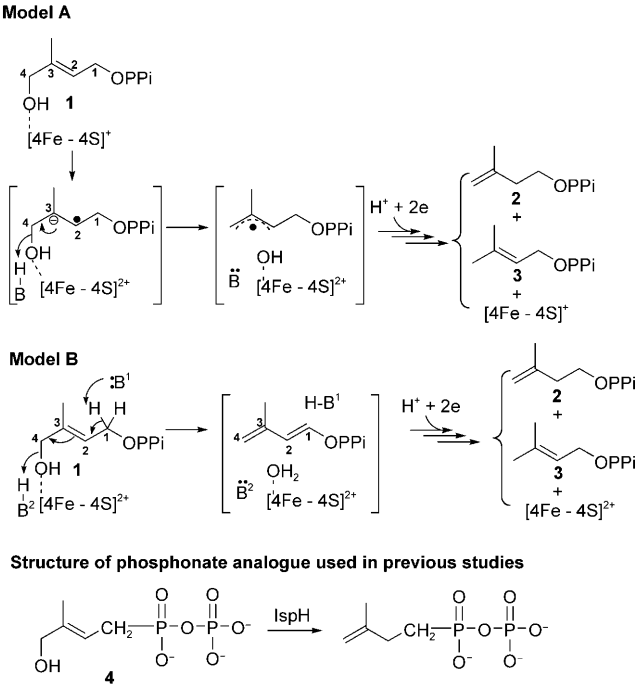
Isoprenoids, such as steroids, terpenoids, carotenoids, and ubiquinones, have important roles in physiological and pathological processes in all organisms, including electron transfer, photosynthesis, membrane stability, and cellular signaling.[1,2] As one of the largest groups of natural products, all isoprenoids are constructed from two precursors, isopentenyl diphosphate (IPP, **2**) and its isomer dimethylallyl diphosphate (DMAPP, **3**; Scheme 1). Two pathways for the biosynthesis of **2** and **3** have been discovered: the deoxyxylulose phosphate (DXP) pathway in green algae, the chloroplasts of higher plants and most eubacteria, and the mevalonic acid (MVA) pathway in animals, fungi, and archaebacteria.[3] The unique distribution of these two biosynthetic pathways among different kingdoms suggests that mechanistic studies on the enzymes in the DXP pathway may lead to the development of mechanism-based inhibitors as antibiotics or herbicides.[4,5] In the MVA pathway, the end product is **2**, which is then isomerized to **3** by isopentenyl pyrophosphate isomerase.[6–10] In contrast, the IspH enzyme in the DXP pathway catalyzes the formation of both **2** and **3** (Scheme 1).[11–18] Several models have been proposed for the IspH-catalyzed reductive dehydration of (*E*)-4-hydroxy-3-methyl-2-butenyl diphosphate (HMBPP, **1**) to form **2** and **3**. In the study reported herein, a substrate analogue and isotopically labeled substrate are utilized as probes to examine the two models in Scheme 1.[19]



Scheme 1. Two IspH mechanistic models and a substrate analogue. $PPi = P_2O_6^{3-}$.

Recently, Rohdich et al. suggested that the IspH-catalyzed transformation is a biological counterpart of the Birch reduction of allylic alcohols with lithium in liquid ammonia (model A in Scheme 1).[13] In model A, it is believed that the conformational restriction at the enzyme-active site favors the C_4 hydroxy group instead of the C_1-diphosphate as the leaving group. In addition, the C_4 hydroxy group is directly ligated to the unique iron site of the iron–sulfur cluster to facilitate the dehydration process. The iron–sulfur cluster is involved in both the dehydration and reduction steps. In model A, all hydrogen atoms in **1** are retained in **2** and **3**, which is consistent with results from feeding experiments using isotopically labeled DXP pathway precursors.[3]

The steady-state kinetic analysis of an IspH substrate analogue, (*E*)-4-hydroxy-3-methyl-2-butenyl diphsophonate (**4**), has revealed that there is a significant decrease in k_{cat} (about 26-fold) relative to that of **1** (Scheme 1).[19] This reduction in k_{cat} could be because **4** is shorter than **1** by one bridging oxygen atom, which results in nonoptimal electron transfer or protonation. It might also be possible that the C_1-position of compound **1** is involved in the reaction, as suggested in model B that follows an aconitase type of dehydration (Scheme 1).[20] Because all hydrogen atoms are retained during the reductive dehydration from **1** to **2** and **3**,[3] the same hydrogen atom removed during the deprotonation step will have to be added back in the subsequent protonation step in model B. Also, the proton abstracted should not readily equilibrate with the solvent. The presence of this type of "sticky proton" has been well documented in the case of aconitase.[20]

To test the C–H bond cleavage at the C_1-position in Scheme 1, the deuterated substrate, (*E*)-4-hydroxy-3-methyl-2-[1,1-$^2H_2$]-butenyl diphosphate ([1,1-$^2H_2$]-**1**), was synthesized and used to measure the primary kinetic isotope effect (KIE)

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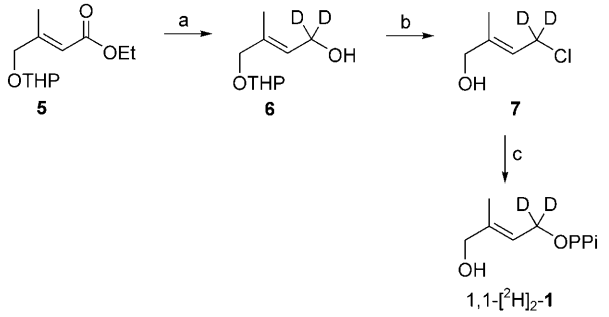
at the C_1-position. To synthesize the desired [1,1-$^2H_2$]-**1**, we modified a reported procedure with $LiAlD_4$ as the reductant to introduce the two deuterium atoms at the C_1-position (Scheme 2).[21] The deuterium-labeled allylic alcohol **6** was



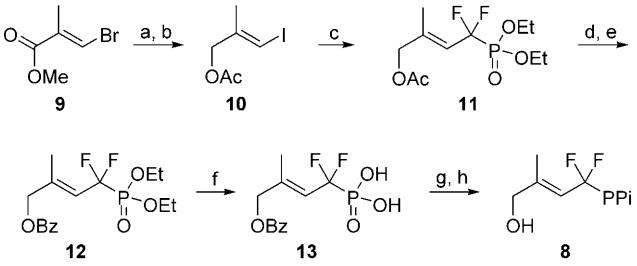
Scheme 2. Synthesis of [1,1-$^2H_2$]-**1**. a) $LiAlD_4$, Et_2O, reflux, 90%; b) 1. *p*-TsCl, DMAP, CH_2Cl_2, RT, 65%; 2. MeOH, TsOH, RT, 82%; c) TBAPP, MeCN, RT, 72%. THP = tetrahydropyran; TsCl = toluenesulfonyl chloride; DMAP = 4-dimethylaminopyridine; TsOH = *p*-toluenesulfonic acid; TBAPP = tris(tetra-*n*-butylammonium)hydrogen pyrophosphate.

converted into the allylic chloride **7** with *p*-TsCl and DMAP in CH_2Cl_2 solvent. Removal of the THP group in compound **6** was achieved by using a catalytic amount of TsOH in MeOH solvent.[22] To simplify the purification process, the diphosphate group was introduced as the last step by using TBAPP in acetonitrile as the pyrophosphorylation reagent. The final product, [1,1-$^2H_2$]-**1**, was purified by cation-exchange chromatography with the ammonium form of Dowex-50 WX8 resin followed by C_{18} reverse-phase chromatography using a two-step solvent system, acetonitrile/10% ammonium hydroxide/H_2O from 10:2.5:0.5 to 6:2.5:0.5, with an overall yield of 34% (see the Supporting Information).

[1,1-$^2H_2$]-**1** was characterized by steady-state kinetics.[19] No primary KIE was observed as the same kinetic parameters were obtained when either **1** or [1,1-$^2H_2$]-**1** was used as a substrate (Figure 2S in the Supporting Information). The deuterium-labeled IPP and DMAPP produced from [1,1-$^2H_2$]-**1** were purified by HPLC and characterized by ^{1}H NMR spectroscopy and mass spectrometry. The ^{1}H NMR spectrum of isolated IPP is consistent with retention of the two deuterium atoms at the C_1-position during catalysis. The ^{1}H NMR signal for the C_2-position hydrogen atoms of IPP produced from [1,1-$^2H_2$]-**1** is a singlet (2.21 ppm), which suggests the retention of the two C_1-position deuterium atoms in IPP and DMAPP (Figure 3S in the Supporting Information). The results from high-resolution mass spectrometry match those of deuterated IPP and DMAPP (Figure 3S in the Supporting Information).

There are at least two different explanations for the lack of primary KIE and deuterium washout when [1,1-$^2H_2$]-**1** was used as a substrate. All of these results are consistent with model A (Scheme 1) because of the lack of involvement of the C_1-position. Alternatively, if the step involving the C_1-position in model B (Scheme 1) is not the rate-limiting step, no primary KIE for [1,1-$^2H_2$]-**1** will be observed either. To differentiate between these two options, (*E*)-4-hydroxy-3-methyl-2-butenyl-1,1-difluoro diphosphonate (**8**), fluorinated at the C_1-position, was synthesized and evaluated as a substrate analogue. Because two fluorine atoms are used to replace the two hydrogen atoms at the C_1-position, compound **8** is an inhibitor and no catalytic turnover is expected if the reaction follows model B. However, according to model A, IspH will make use of compound **8** as a substrate.

The synthesis of compound **8** is outlined in Scheme 3. Starting from commercially available methyl methacrylate, (*E*)-3-bromo-2-methylacrylate (**9**) was readily synthesized



Scheme 3. Synthesis of **8**. a) CuI, KI, DMF, 140°C, 6 h, 82%; b) 1. DIBAL-H, CH_2Cl_2, −78°C, 95%; 2. Ac_2O, pyridine, DMAP, RT, 94%; c) $(EtO)_2P(O)CF_2ZnBr$, CuBr, THF, RT, 84%; d) 7 N NH_3 in MeOH, RT, 86%; e) BzCl, Et_3N, CH_2Cl_2, DMAP, RT, 98%; f) TMSBr, CH_3CN, RT, quantitative yield; g) 1. Bu_3N, DMF, RT; 2. CDI, DMF, RT; 3. MeOH, DMF, RT; 4. $Bu_3NH_3PO_4$, DMF, RT, 64%; h) 30% $NH_3{\cdot}H_2O$, 30°C, 88%. DIBAL-H = diisobutylaluminum hydride; Bz = benzoyl; TMSBr = bromotrimethylsilane; CDI = 1,1-carbonyldiimidazole.

according to a reported procedure.[23] The synthesis of (*E*)-3-iodo-2-methylallyl acetate (**10**) from **9** involved several steps. The *trans* halogenation was achieved by Cu(I)-assisted halogen exchange to produce a more reactive vinyl iodide.[24] After *trans* halogenation, the ester group was reduced to produce an alcohol, which was then acetylated to form **10**. The coupling between the iodoalkene **10** and the organometallic reagent $(EtO)_2P(O)CF_2Cu{\cdot}ZnBr_2$ was achieved in 84% yield by using a Shibuya–Yokomatsu coupling to form the key intermediate, a difluorinated phosphonate, (*E*)-4-(diethylphosphono)-4,4-difluoro-2-methylbut-2-enyl acetate (**11**).[25] The acetate protecting group in **11** was converted into a more stable benzoyl protecting group to generate (*E*)-4-(diethylphosphono)-4,4-difluoro-2-methylbut-2-enyl benzoate (**12**) because removal of the ethyl groups with TMSBr caused partial acetate ester hydrolysis when **11** was used directly. Once (*E*)-4-(benzoyloxy)-1,1-difluoro-3-methylbut-2-enylphosphonic acid (**13**) was produced by deprotection, the diphosphonate **8** was obtained following a coupling procedure reported by us recently in the synthesis of compound **4**, with 30% total yield over nine steps (see the Supporting Information).[19]

Once diphosphonate **8** was in hand, it was characterized by steady-state kinetics by using our recently reported nicotinamide adenine dinucleotide phosphate (NADPH) consumption assay.[19] IspH can utilize **8** as a substrate. However, compound **8** is an extremely poor substrate with a k_{cat} of 0.022 min^{-1}, which is more than 500-fold lower than that of the natural substrate **1**.[19] To characterize products from the turnover of **8**, a single-turnover experiment was

carried out using IspH (300 μM) with NADPH, flavodoxin (FldA), and flavodoxin reductase (Fpr) as the reducing system. When the reaction was monitored by ^{19}F NMR spectroscopy (Figure 1), in addition to the signals from the

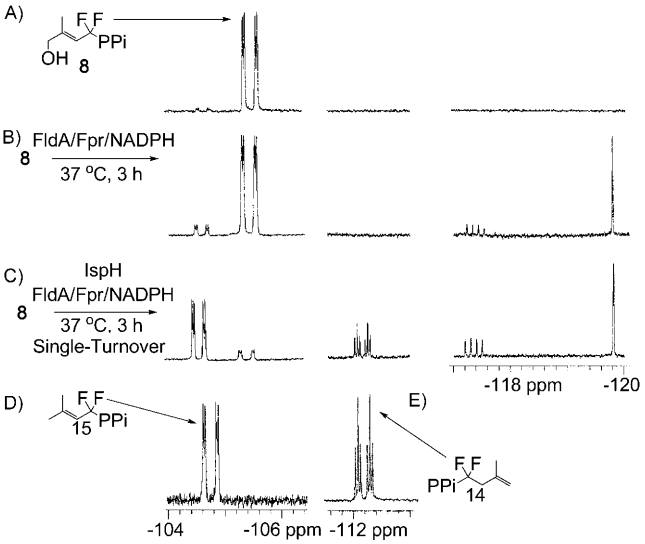
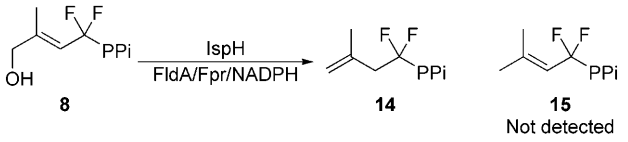


Figure 1. ^{19}F NMR spectra of the substrate (**8**), the reaction mixture, and the products. A) Pure **8**. B) Control reaction without IspH, in which compound **8** was not stable as indicated by the formation of decomposition products. The reaction mixture contained 2.0 mM NADPH, 60 μM flavodoxin (FldA), 26 μM flavodoxin reductase (Fpr), and 0.3 mM **8** in 100 mM Tris-HCl, pH 8.0, with a total volume of 1.5 mL at 37 °C for 3 h. C) Single turnover of IspH reaction with compound **8** (same conditions as (B) with the addition of 0.3 mM IspH). D) Synthetic standard **15**. E) Synthetic standard **14**. By comparing the ^{19}F NMR results in (C–E), it is clear that **14** is the enzymatic product of **8**.

substrate ($\delta = 105.51$ ppm, Figure 1 A) and the decomposition products from **8** ($\delta = -104.60$, -117.38, -119.87 ppm, Figure 1 B; the decomposition products have not been characterized yet), there is clearly another double triplet ($\delta = -112.51$ ppm), which is produced only when IspH is present (Figure 1 C). This product was purified by HPLC methods and analyzed by ^{1}H NMR spectroscopy and mass spectrometry.

To guide the structural assignment of the products, two potential turnover products, 1,1-difluoro-3-methyl-3-butenyl diphosphonate (**14**, Figure 1 E) and 1,1-difluoro-3-methyl-2-butenyl diphosphonate (**15**, Figure 1 D), were synthesized chemically (see the Supporting Information). The ^{1}H NMR, ^{19}F NMR, and mass spectrometry results of the isolated product are all consistent with the production of **14** from **8** as the IspH turnover product (Figure 1 and Figure 4S in the Supporting Information). The production of **14** has also been confirmed by high-resolution ESI mass spectrometry in the negative mode, which gives a major signal at m/z 264.9851 (calculated $[M-H]^-$ for **14** is m/z 264.9848; Figure 4 S-b). Compound **15** was not detected (Figure 1 C and D, Figure 4S in the Supporting Information). Instead of two products (**2** and **3**) from **1**, there is only one detectable product (**14**) produced from **8** (Scheme 4). Similar results were reported by



Scheme 4. IspH reaction using **8** as a substrate.

us when diphosphonate **4** was utilized as the substrate, which produced only the IPP analogue (Figure 1 S-b in the Supporting Information).[19]

Although compound **8** is a poor substrate, with an activity about 20-fold lower than that of **4**, the utilization of **8** by IspH as a substrate is mechanistically important. The production of **14** from **8** by IspH suggests that C_1-deprotonation/protonation may not be part of the IspH-catalyzed reaction. Neither the kinetic studies with isotopically labeled substrate nor the results from use of the fluorinated substrate analogue support the involvement of the C_1-position in IspH-catalyzed reaction.

A radical-mediated reaction finds a precedent in the radical-mediated dehydration reactions of hydroxylacyl-coenzyme A (CoA) to enoyl-CoA.[26,27] In that case, because of the extremely weak acidity of the β position, a complicated radical mechanism is invoked. It is believed that the reduction of a thioester carbonyl group by one electron leads to the production of a radical anion, which is then used to mediate the dehydration reaction to produce a ketyl radical. Recently, the presence of an allylic ketyl radical has been directly observed.[28] A similar model is proposed in model A (Scheme 1) for the IspH-catalyzed reaction, in which the reduction of an alkene instead of thioester is used to form a radical anion. The radical anion produced is then used to mediate a dehydration reaction to form an allylic radical intermediate.

In summary, our studies with a deuterium-labeled substrate and fluorinated analogue suggest no significant influence of the C_1-position in the IspH-catalyzed reaction unless a different mechanism is employed when the substrate analogue is utilized. Future work will be directed towards characterization of the proposed allylic radical intermediate.

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