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Synthesis of [1-^{13}C] and stereo-specifically [1-^{2}H] labeled fluorinated substrate analogues of IspH enzyme in the deoxyxylulose phosphate pathway

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ABSTRACT

IspH in the deoxyxylulose phosphate (DXP) pathway catalyzes the reductive dehydration of (*E*)-4-hydroxy-3-methyl-2-butenyl diphosphate (HMBPP) to isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which are the starting materials for the synthesis of thousands of isoprenoids. Several models have been proposed in the literature to account for this unique transformation, and most of them involve the formation of an allylic radical intermediate. To facilitate trapping and characterizing the proposed intermediates in the IspH-catalyzed reactions, in the present work, we report the synthesis of four isotopically labeled IspH substrate analogues. These isotopically labeled mechanistic probes will be utilized in the future for characterizing the proposed IspH reaction intermediates by the combination of bioorganic and biophysical approaches.

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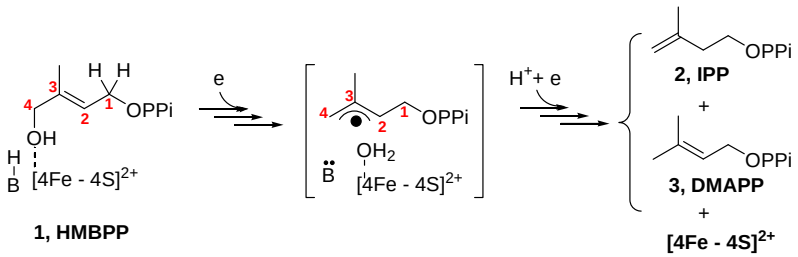
Isoprenoids are one of the largest classes of natural products with diverse structures including acyclic, monocyclic, and polycyclic compounds.[1,2] As diverse as their structures are, all isoprenoids are constructed from two precursors, isopentenyl diphosphate (IPP, **2**) and its isomer dimethylallyl diphosphate (DMAPP, **3**, Scheme 1). Recently, bioinformatic analysis of sequenced and partially sequenced genomes revealed that the two isoprenoid biosynthetic pathways, the mevalonic acid (MVA) pathway and the deoxyxylulose phosphate (DXP) pathway, have a well-defined distribution among different kingdoms.[3–5] The DXP pathway is found in green algae, the chloroplasts of higher plants, and most eubacteria; while the MVA pathway is present in animals, fungi, and archaebacteria.[3] In the MVA pathway, the end product is IPP, which is then isomerized to DMAPP by isopentenyl diphosphate isomerase.[6–9] In contrast, IspH enzyme in the DXP pathway catalyzes the formation of both IPP and DMAPP (Scheme 1).[10–17]

Several models have been proposed in the literature to account for the IspH-catalyzed reductive dehydration of (*E*)-4-hydroxy-3-methyl-2-butenyl diphosphate (HMBPP, **1**) to form **2** and **3**, and most of them share a common allylic radical intermediate (Scheme 1).[13,16,18] The presence of a [4Fe–4S] cluster and its interaction with the C_4-hydroxyl group of HMBPP have been suggested based on recent preliminary EPR characterization of the iron–sulfur cluster and on studies using substrate analogues.[16,18] The reductive dehydration needs an active site acid to protonate the hydroxyl group to facilitate its leaving as a water molecule (Scheme 1). Recently, the fluorinated analogue (*E*)-3-(fluoromethyl)-2-butenyl diphosphate (**4**) was reported to be an IspH substrate, and it can



Scheme 1. IspH mechanistic model.

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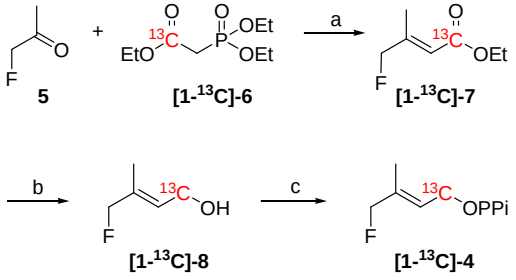
generate both IPP and DMAPP.[18] Different from the dehydration reactions, fluoride ion elimination does not need protonation. In addition, in aconitase-mediated dehydration, a prototype of iron–sulfur cluster-mediated dehydration, the dehydration process is reversible,[19] which complicates the studies on trapping and characterizing substrate-based reaction intermediates. In contrast, fluoride ion is a much weaker nucleophile. Thus, the fluorinated analogue might offer some advantage in the future for the detailed mechanistic studies on trapping and characterizing substrate- and enzyme-based intermediates in IspH-catalyzed reactions. In the present work, we report the syntheses of four C_1 position isotopically labeled substrate analogues, including [1-^{13}C]-**4**, [1,1-$^{2}H_2$]-**4**, (*R*)-[1-^{2}H]-**4**, and (*S*)-[1-^{2}H]-**4**.

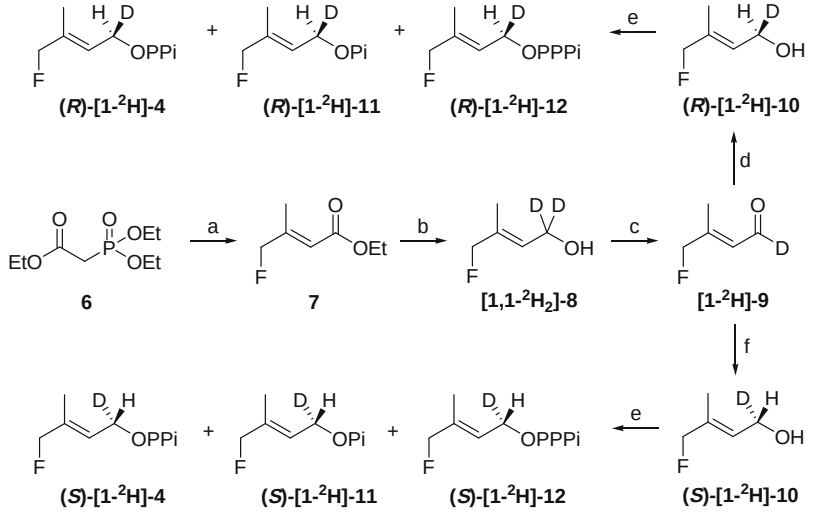
(*E*)-3-(Fluoromethyl)-2-[1-^{13}C]-butenyl pyrophosphate ([1-^{13}C]-**4**) was obtained via a three-step synthesis starting from the commercially available fluoroacetone (**5**) and [1-^{13}C]-triethyl phosphonoacetate ([1-^{13}C]-**6**) with an overall yield of 18% (Scheme 2). The α,β-unsaturated ester **7** was formed by Horner–Wadsworth–Emmons reaction using sodium hydride as a base to facilitate the coupling between the two starting materials in 60% yield.[20,21] Once the α,β-unsaturated ester **7** was obtained, it was subjected to reduction, halogenations, and pyrophosphorylation reactions following a reported procedure, and led to the formation of the desired [1-^{13}C]-**4** with a yield of 30% over the two steps (steps b and c in Scheme 2).[18,22,23] Compound [1-^{13}C]-**4** was purified by a combination of a cation-exchange chromatography (the ammonium form of DOWEX-50 WX8 resin) and a C18 reverse phase chromatography using a solvent system containing acetonitrile, 10% ammonium hydroxide, and water with an initial ratio of 10:2.5:0.5, then at 6:2.5:0.5.[24]



Scheme 2. Synthesis of the [1-^{13}C]-**4**. Reagents and conditions: (a) NaH, THF, room temperature, 60%; (b) DIBAL-H, CH_2Cl_2, −78 °C, 72%; (c) (i) PBr_3, pentane, 0 °C; (ii) TBAPP, MeCN, room temperature, 41% (two steps). TBAPP = tris(tetra-*n*-butylammonium) hydrogen pyrophosphate.

Following the same scheme (Scheme 2), (*E*)-3-(fluoromethyl)-2-[1,1-$^{2}H_2$]-butenyl diphosphate ([1,1-$^{2}H_2$]-**4**) was also synthesized. To synthesize the [1,1-$^{2}H_2$]-**4**, the same procedure as that in Scheme 2 was followed except $LiAlD_4$ instead of DIBAL-H was utilized for the reduction of **7** to [1,1-$^{2}H_2$]-**8** in 72% yield. The overall yield of [1,1-$^{2}H_2$]-**4** was 13% over 3 steps.[25]

Enzymatic reactions are stereo-selective in general. Also, if the allylic radical is indeed one of the substrate-based intermediates in the IspH-catalyzed reductive dehydration, the interaction between the two hydrogen atoms at C_1 position and the allylic radical can be different depending on their relative orientations. Instead of synthesizing the racemic mixture of the two mono-deuterated stereoisomers, two stereo-specifically deuterium-labeled (*R*)- and (*S*)-[1-^{2}H]-**4** were also synthesized (Scheme 3). The double deuterium-labeled alcohol [1,1-$^{2}H_2$]-**8,** an intermediate in the synthesis of [1,1-^{2}H]-**4**, was oxidized by pyridinium chlorochromate (PCC) in 82% yield to produce the deuterium-labeled aldehyde [1-^{2}H]-**9**. Using BITIP catalysts prepared from titanium tetraisopropoxide ($Ti(O^iPr)_4$) with (*S*)- and (*R*)-BINOL, respectively, and using Bu_3SnH as the reductant, stereo-specifically labeled (*R*)-[1-^{2}H]-**10** and (*S*)-[1-^{2}H]-**10** can be generated in ~70% yield after a stereo-specific reduction.[26] The optical purity of (*R*)-[1-^{2}H]-**10** and (*S*)-[1-^{2}H]-**10** was determined by converting them to the corresponding Mosher ester.[27] The C_1 protons in the ^{1}H NMR spectra of the diastereomeric esters from (*R*)-[1-^{2}H]-**10** and (*S*)-[1-^{2}H]-**10** with (*R*)-(−)-Mosher's chloride were cleanly resolved, giving peaks at 4.827 and 4.852 ppm, respectively. The enantiomeric ratios for the synthesized (*R*)-[1-^{2}H]-**10** and (*S*)-[1-^{2}H]-**10** were 97:3 and 4:96, respectively, based on the integration of the corresponding peak intensities. To retain the absolute stereochemistry of C_1, pyrophosphorylation of the two chiral alcohols was achieved by the treatment with a mixture of trichloroacetonitrile and inorganic phosphate TEAP (bis-triethylammonium phosphate) following a reported procedure,[28] which was reported to be able to retain the absolute stereochemistry of the corresponding alcohols during phosphorylation processes. However, the product generated in the phosphorylation reaction was a mixture of mono-, di-, and triphos-



Scheme 3. Syntheses of (*R*)-[1-^{2}H]-**4** and (*S*)-[1-^{2}H]-**4**. Reagents and conditions: (a) Fluoroacetone (**5**), NaH, THF, room temperature, 56%; (b) $LiAlD_4$, Et_2O, room temperature, 72%; (c) PCC, CH_2Cl_2, room temperature, 82%; (d) *S*-BITIP, Bu_3SnH, Et_2O, −20 °C, 24 h, 70%; (e) TEAP, CCl_3CN, MeCN, 15 min, 21% for (*R*)-[1-^{2}H]-**4**, 25% for (*S*)-[1-^{2}H]-**4**, 18% for (*R*)-[1-^{2}H]-**11**, 21% for (*S*)-[1-^{2}H]-**11**, 6.4% for (*S*) and (*R*)-[1-^{2}H]-**12**; (f) *R*-BITIP, Bu_3SnH, Et_2O, −20 °C, 24 h, 68%. BITIP: catalyst prepared from BINOL and titanium tetraisopropoxide; TEAP = bis-triethylammonium phosphate.

phates. The desired pyrophosphate products, (*R*)-[1-^{2}H]-**4** and (*S*)-[1-^{2}H]-**4**, were purified using semi-preparative HPLC method developed in our laboratory (Waters Atlantis® Prep T3 column (10 × 250 mm), 3 mL/min, 50 mM ammonium bicarbonate) with 21% and 25% yields, respectively.[29] At the same time, the monophosphate, (*R*)-[1-^{2}H]-**11** and (*S*)-[1-^{2}H]-**11**, and triphosphates, (*R*)-[1-^{2}H]-**12** and (*S*)-[1-^{2}H]-**12**, could also be purified with 6–21% yields.[30]

In conclusion, we have developed efficient methods to synthesize four C_1 position isotopically labeled IspH substrate analogues, [1-^{13}C]-**4**, [1,1-$^2H_2$]-**4**, (*R*)-[1-^{2}H]-**4**, and (*S*)-[1-^{2}H]-**4**. They were all synthesized following practical routes using commercially available isotopic reagents. With these isotopically labeled probes, a systematic characterization of the proposed substrate-based IspH intermediates is now under investigation.

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24. Pyrophosphate [1-^{13}C]-**4:** ^{1}H NMR (D_2O, 400 MHz): δ 5.60 (t, J = 28.0 Hz, 1H), 4.67 (d, J = 61.2 Hz, 2H), 4.18 (dd, J = 10.8, 6.4 Hz, 1H), 1.57 (s, 3H); ^{13}C NMR of 1-^{13}C (100 MHz, D_2O) δ 62.56 (d, J = 5.2 Hz); ^{31}P NMR (162 MHz, D_2O) δ −10.92 (d, J = 15.39 Hz); −13.75 (dd, J = 17.8, 3.9 Hz); ^{19}F NMR (376 MHz, $CDCl_3$): −192.39 (t, J = 37.6 Hz).
25. Pyrophosphate [1,1-$^2H_2$]-**4:** ^{1}H NMR (D_2O, 400 MHz): δ 5.61 (s, 1H), 4.68 (dd, J = 47.2, 0.8 Hz, 2H), 1.59 (s, 3H); ^{31}P NMR (162 MHz, D_2O) δ −12.39 (d, J = 16.4 Hz), −13.80 (d, J = 17.2 Hz); ^{19}F NMR (376 MHz, D_2O): −192.57 (dt, J = 37.6, 3.0 Hz); HRMS (ESI, negative ion detection mode) for $C_5H_8D_2FO_7P_2$ gives a major signal at m/z 264.9915 (calculated $[M-H]^-$ for [1,1-$^2H_2$]-**4** is m/z 265.0011).
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30. Monophosphate (*R*)-[1-^{2}H]-**11**: ^{1}H NMR (400 MHz, D_2O): δ 5.59 (s, 1H), 4.68 (d, J = 46.8 Hz, 2H), 4.19 (d, J = 3.6 Hz, 1H), 1.58 (s, 3H); ^{31}P NMR (162 MHz, D_2O) δ −2.24 (s); ^{19}F NMR (376 MHz, D_2O): δ −191.76 (t, J = 38.7 Hz). Triphosphate (*R*)-[1-^{2}H]-**12**: ^{1}H NMR (400 MHz, D_2O): δ 5.63 (s, 1H), 4.70 (d, J = 46.8 Hz, 2H), 4.42 (s, 1H), 1.61 (s, 3H); ^{31}P NMR (162 MHz, D_2O) δ −10.37 (d, J = 16.4 Hz), −13.78 (d, J = 15.6 Hz), −23.05 (t, J = 16.0 Hz); ^{19}F NMR (376 MHz, D_2O): δ −192.64 (t, J = 37.6 Hz). Monophosphate (*S*)-[1-^{2}H]-**11**: ^{1}H NMR (400 MHz, D_2O): δ 5.56 (s, 1H), 4.66 (d, J = 47.2 Hz, 2H), 4.16 (d, J = 4.8 Hz, 1H), 1.56 (s, 3H); ^{31}P NMR (162 MHz, D_2O) δ −2.18 (s); ^{19}F NMR (376 MHz, D_2O): δ −191.81 (t, J = 37.6 Hz). Triphosphate (*S*)-[1-^{2}H]-**12**: ^{1}H NMR (400 MHz, D_2O): δ 5.61 (s, 1H), 4.68 (d, J = 46.8 Hz, 2H), 4.40 (d, J = 2.8 Hz, 1H), 1.59 (s, 3H); ^{31}P NMR (162 MHz, D_2O) δ −10.42 (d, J = 16.4 Hz), −13.80 (d, J = 15.4 Hz), −23.07 (t, J = 15.9 Hz); ^{19}F NMR (376 MHz, D_2O): δ −192.68 (tt, J = 38.0, 2.6 Hz).