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Type-2 Isopentenyl Diphosphate Isomerase: Evidence for a Stepwise Mechanism

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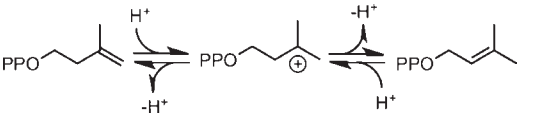
Supporting Information

ABSTRACT: Isopentenyl diphosphate isomerase (IDI) catalyzes the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These two molecules are the building blocks for construction of isoprenoid carbon skeletons in nature. Two structurally unrelated forms of IDI are known. A variety of studies support a proton addition/proton elimination mechanism for both enzymes. During studies with *Thermus thermophilus* IDI-2, we discovered that the olefinic hydrogens of a vinyl thiomethyl analogue of isopentenyl diphosphate exchanged with solvent when the enzyme was incubated with D_2O without concomitant isomerization of the double bond. These results suggest that the enzyme-catalyzed isomerization reaction is not concerted.

Isopentenyl diphosphate isomerase (IDI) catalyzes the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). This is a required reaction in the mevalonate pathway for isoprenoid biosynthesis in Eukaryotes and Archaea and, while not required, can balance the relative concentrations of IPP and DMAPP in Bacteria and plant chloroplasts, which synthesize isoprenoid compounds by the methylerythritol phosphate pathway. Two independently evolved forms of IDI are known. IDI-1, discovered in the 1950s, is a zinc metalloprotein found in Eukaryotes and some Bacteria.[1,2] IDI-2, discovered in 2001, is a flavoprotein found in Archaea and other Bacteria.[3] A few bacterial species contain both forms of IDI and others apparently lack both.

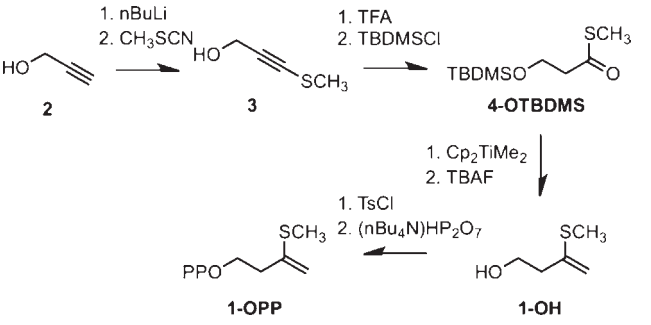
Studies with IDI-1 and IDI-2 provide strong evidence for a protonation−deprotonation mechanism for the isomerization of IPP to DMAPP, where a proton from solvent is added to C4 of IPP and the pro-R hydrogen is removed from C2.[4−7] Epoxide and diene analogues of IPP and DMAPP are irreversible inhibitors of IDI-1 and IDI-2, which are activated by protonation and form covalent adducts with nucleophiles in the active site.[6,8−12] Furthermore, IPP and DMAPP analogues that do not support formation of a carbocationic intermediate are poor substrates for the enzymes.[6,13] The data are consistent with a stepwise reaction that proceeds through a tertiary carbocationic intermediate (Scheme 1) or a concerted process that proceeds through a late transition state with substantial carbocationic character. During studies with IPP analogues where the methyl group was replaced by a variety of functional groups with different electronic properties, we discovered that *Thermus thermophilus* IDI-2 catalyzed exchange of the vinyl hydrogens in a vinyl thioether analogue (**1-OPP**) without concomitant isomerization. These experiments and their implications with regard to the mechanism of the isomerization reaction are now discussed.

Scheme 1. Stepwise Mechanism for Isomerization of IPP to DMAPP



1-OPP was prepared as shown in Scheme 2. Propargyl alcohol[2] was deprotonated with *n*-butyl lithium, followed by addition of methylthiocyanate to give thiomethyl alkyne **3**.[14] Treatment with trifluoroacetic acid gave thioester **4-OH**.[15] The hydroxyl group was protected as a *t*-butyldimethylsilyl ether, and **4-OTBDMS** was converted to vinyl thioether **1-OTBDMS** upon treatment with Petasis reagent.[16] The protecting group was removed with tetra-*n*-butylammonium fluoride and alcohol **1-OH** was phosphorylated by treatment with tosyl chloride to give **1-OTs** followed by tris-(tetra-*n*-butylammonium) hydrogen pyrophosphate.[17] **1-OTs** was unstable and was converted to **1-OPP** immediately after purification by chromatography.

Scheme 2. Synthesis of IPP Thiomethyl Analogue 1-OPP



A 0.7 mL solution of 25 μM *T. thermophilus* IDI-2 in D_2O, phosphate buffer, pH 7.0, was prepared in an NMR tube. Incubations at 37 °C were initiated by addition of **1-OPP** (12 mM final concentration), and ^{1}H spectra were collected at 5 min intervals for up to 18 h. Samples were assayed for IDI activity before and after the incubation to verify that the enzyme was active at the end of the experiments. The results are shown in Figure 1. At $t = 0$, peaks for **1-OPP** are seen at 2.21 (methyl group), 2.57 (C2 methylene), 4.05 (C1 methylene), 4.8 (C4 H), and 5.20 (C4 H). Additional peaks are seen at 3.45, 3.55, and 3.68 ppm for

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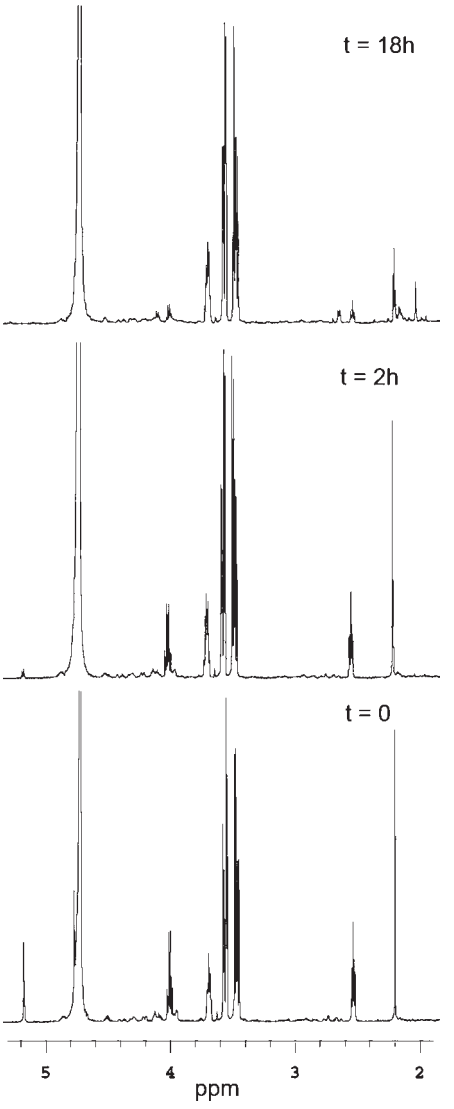


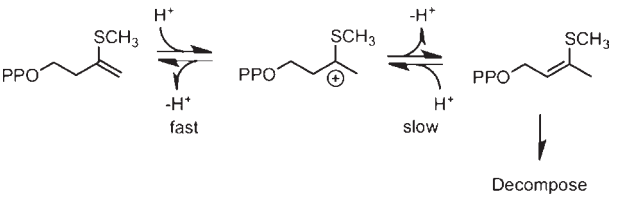
Figure 1. NMR spectra for incubations of **1-OPP** and IDI-2 in D_2O.

glycerol from the enzyme storage buffer. After 2 h, the intensities of peaks at 2.21, 2.37, and 4.05 have decreased by ~20%, while the intensities for those at 4.8 and 5.20 have decreased by >90% of their original values. No peaks were seen that could be attributed to the allylic isomer of **1-OPP**. After 18 h, the peaks at 2.21, 2.37, and 4.05 had decreased by ~85%, while the peaks at 4.8 and 5.20 disappeared. A portion of the 18 h sample was analyzed by HPLC/MS on a C_{18} reversed-phase column. A peak eluting at 3.8 min (1:20 acetonitrile/water), with the same retention time as **1-OPP**, gave a negative ion electrospray mass spectrum with a peak at m/z 278.9, while a control run with **1-OPP** gave a peak at m/z 276.9, confirming the NMR results that the vinyl hydrogens exchanged with D_2O. In a control experiment **1-OPP** was incubated in the reaction buffer without IDI-2 for 24 h. During this time, there was no discernible change in the NMR spectrum. The slow disappearance of **1-OPP** in the presence of IDI-2 is consistent with isomerization of the homoallylic diphosphate to its allylic isomer at a rate that is substantially slower than the rate for proton exchange, followed by solvolysis of the more reactive allylic isomer. Thus, IDI-2 catalyzes the facile exchange of the vinyl hydrogens at C4 of **1-OPP** without formation of detectable amounts of its allylic isomer.

In separate experiments, **1-OPP** was incubated with geranyl diphosphate (GPP) or DMAPP and avian farnesyl diphosphate synthase at 4 or 30 °C, under conditions normally used for the chain elongation reaction. Analysis of the mixture from **1-OPP** and GPP by negative ion electrospray HPLC/MS did not show peaks with retention times in the neighborhood of farnesyl diphosphate with a mass expected for the thiomethyl farnesyl analogue from the coupling of **1-OPP** and GPP. However, analysis of the incubation mixture from **1-OPP** and DMAPP by negative ion electrospray HPLC/MS gave a peak at m/z 445 characteristic of a dithiomethyl farnesyl diphosphate analogue. Repeated attempts to detect the **1-OPP**-GPP adduct were unsuccessful. In a separate experiment, the mixture from a 4 °C incubation of **1-OPP** and GPP was immediately treated with alkaline phosphatase to convert the putative diphosphate product to the corresponding alcohol. The mixture was extracted with methyl *t*-butyl ether, and analysis of the extract by HPLC/MS (positive ion electrospray) gave a peak at m/z 277.0 with a mass corresponding to the Na^+ adduct of the alcohol. These results indicate that **1-OPP** is a substrate for chain elongation when incubated with DMAPP or GPP, and gives the expected farnesyl analogues. However, it is apparent that allylic 3-alkyl-3-thiothiomethyl diphosphates are unstable to our standard incubation conditions and the amount of products isolated from the reactions was low.

The reactivity of IPP analogues in the isomerization and chain elongation reactions depends on the ability of the substituent at C3 to stabilize positive charge. If the methyl group in IPP is replaced by CF_3, the reactivity of the substrate decreases by more than 10^6-fold and by more than 10^2-fold when replaced by chlorine.[13,18,19] These trends for IDI-2 are consistent with the σ_p^+ values for CF_3 (0.612) and chlorine (0.114) relative to methyl (−0.311) and trends seen for IDI-1.[20] The thiomethyl group in **1-OPP** ($\sigma_p^+ = -0.604$) stabilizes positive charge at C3 more effectively than a methyl group. On the basis of the rate of disappearance of the vinyl peak during the first 20 min of the incubation, we estimate that $k_{cat}^{exchange} \sim 0.2\ s^{-1}$. Since the deprotonation step should selectively remove protium rather than deuterium from C4 in the early stages of the exchange reaction because of a primary deuterium isotope effect, $k_{cat}^{exchange}$ should reflect the rate of protonation of the double bond in IPP. However, $k_{cat}^{exchange}$ for **1-OPP** does not include a correction for a solvent deuterium isotope effect or a statistical correction to account for the requirement that two protonations are required to fully exchange the vinyl protons. In addition, **1-OPP** appears to bind to IDI-2 in a conformation that slows the deprotonation at C2 relative to protonation and deprotonation at C4. The result is a rapid exchange of the vinyl protons with solvent and a much slower isomerization of **1-OPP** to its allylic isomer (Scheme 3). Thus, **1-OPP** is more reactive than IPP, although less so than predicted by the relative σ_p^+ values for methyl and thiomethyl groups.

Scheme 3. Proton Exchange and Isomerization of 1-OPP



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The thiomethyl group also increases the reactivity of the allylic isomer toward solvolysis, which gives a thiomethyl hemiketal followed by elimination of methanethiol. We anticipate that methyl vinyl ketone produced from the thiomethyl DMAPP analogue would be unstable during the incubation. A related solvolysis reaction was seen following isomerization of 3-cyclopropyl-3-butenyl diphosphate to its allylic isomer, where σ_p^+ for the cyclopropane ring (−0.601) is similar to the thiomethyl value.[8,20] In this case, the solvolysis product from (*Z*)-3-cyclopropyl-2-butenyl diphosphate was sufficiently stable to be characterized.

A considerable body of evidence with analogues of IPP indicates that substantial positive charge develops at C3 of IPP during the isomerization to DMAPP. However, the previous data do not distinguish between a stepwise mechanism that proceeds through a carbocationic intermediate and a concerted process with a late transition state for their isomerization reactions. The rapid exchange of the vinyl protons in **1-OPP** with solvent without concomitant rearrangement to its allylic isomer shows that the analogue is protonated by IDI-2 to give an intermediate carbocation, which loses a proton to return to **1-OPP** without a mandatory isomerization. **1-OPP** is the first example of a substrate for IDI in which protonation and isomerization are decoupled. The trends in reactivity for IPP and other C3 analogues and the exchange results for **1-OPP** are consistent with a common protonation−deprotonation mechanism for their isomerization reactions.

■ ASSOCIATED CONTENT

Supporting Information. Procedures for synthesis of 1-OPP and NMR spectra; descriptions of product studies and mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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