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Type II Isopentenyl Diphosphate Isomerase: Probing the Mechanism with Alkyne/Allene Diphosphate Substrate Analogues[†]

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ABSTRACT: Isopentenyl diphosphate isomerase (IDI) catalyzes the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the basic five-carbon building blocks of isoprenoid molecules. Two structurally unrelated classes of IDIs are known. Type I IPP isomerase (IDI-1) utilizes a divalent metal in a protonation−deprotonation reaction. In contrast, the type II enzyme (IDI-2) requires reduced flavin, raising the possibility that the reaction catalyzed by IDI-2 involves the net addition or abstraction of a hydrogen atom. As part of our studies of the mechanism of isomerization for IDI-2, we synthesized allene and alkyne substrate analogues for the enzyme. These molecules are predicted to be substantially less reactive toward proton addition than IPP and DMAPP but have similar reactivities toward hydrogen atom addition. This prediction was verified by calculations of gas-phase heats of reaction for addition of a proton and of a hydrogen atom to 1-butyne (**3**) and 1,2-butadiene (**4**) to form the 1-buten-2-yl carbocation and radical, respectively, and related affinities for 2-methyl-1-butene (**5**) and 2-methyl-2-butene (**6**) using G3MP2B3 and CBS-QB3 protocols. Alkyne **1-OPP** and allene **2-OPP** were not substrates for *Thermus thermophilus* IDI-2 or *Escherichia coli* IDI-1 but instead were competitive inhibitors. The experimental and computational results are consistent with a protonation−deprotonation mechanism for the enzyme-catalyzed isomerization of IPP and DMAPP.

The conversion of isopentenyl diphosphate (IPP)[1] to dimethylallyl diphosphate (DMAPP), catalyzed by IPP isomerase (IDI), is an important step in the early stages of isoprenoid metabolism. DMAPP is the initial electrophilic substrate for the chain elongation reactions that lead to most of the isoprenoid compounds found in nature, including mono-, sesqui-, and diterpenes, carotenoids, sterols, ubiquinones, and dolichols (*1*). In those organisms that synthesize isoprenoid units by the mevalonate (MVA) pathway, IDI is an essential enzyme (*2*). However, IDI is also found in most organisms that synthesize IPP and DMAPP by the methylerythritol phosphate (MEP) pathway, where a mixture of both is produced from hydroxydimethylallyl diphosphate in the final step. In this case, IDI activity is presumably important for balancing the pools of IPP and DMAPP to match the stoichiometry of the two substrates required for subsequent chain elongation reactions (*3*).

Two structurally unrelated forms of IDI have been identified. The type I enzyme (IDI-1) was discovered in the late 1950s (*4*−*9*). IDI-1 is a zinc metalloprotein that also requires Mg^{2+} for activity (*10*−*12*). A second form IDI was reported in 2001 (*13*). The structure of the type II enzyme (IDI-2) is unrelated to IDI-1. In contrast to IDI-1, IDI-2 is a flavoprotein that requires the reduced form of flavin mononucleotide (FMN) and Mg^{2+} for activity (*14*−*16*). There is no strict correlation between the two forms of IDI found in an organism and the pathway (MVA or MEP) for synthesis of IPP (*17*). For example, organisms that synthesize IPP and DMAPP from MVA have IDI-1 (Eukaryota) or IDI-2 (Archaea and a few Bacteria), while organisms that utilize the MEP pathway also have IDI-1 (plant chloroplasts and Bacteria) or IDI-2 (Bacteria).

Several lines of evidence were used to establish the mechanism for the isomerization reaction catalyzed by IDI-1. In particular, studies with IPP analogues provide strong support for protonation of the double bond in IPP to generate a transient carbocationic intermediate, which upon elimination of a proton, gives DMAPP. Epoxide and diene analogues of IPP and DMAPP irreversibly inhibit the enzyme by formation of covalent adducts with an active site cysteine residue (*18*, *19*). In both cases, protonation activates the analogue for alkylation of the active site nucleophile. *N*,*N*-Dimethyl-2-amino-1-ethyl diphosphate, a reactive intermediate analogue with a positively charged ammonium group at physiological pH, binds to IDI-1 with subnanomolar affinity (*20*). In addition, IPP and DMAPP analogues substituted with powerful electron-withdrawing fluorine groups that destabilize the carbocationic intermediate (*17*, *21*, *22*) are poor substrates for isomerization (*20*, *23*). All of these studies support a protonation−deprotonation mechanism for IDI-1.

While the reactions catalyzed by IDI-1 and IDI-2 are identical, the enzymes have different protein scaffolds and use different cofactors. In other enzymes, the flavin cofactor required by IDI-2 is used to facilitate redox reactions, to mediate covalent

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[1]Abbreviations: DMAPP, dimethylallyl diphosphate; IDI, isopentenyl diphosphate isomerase; IPP, isopentenyl diphosphate; MEP, methylerythritol phosphate; MVA, mevalonate.

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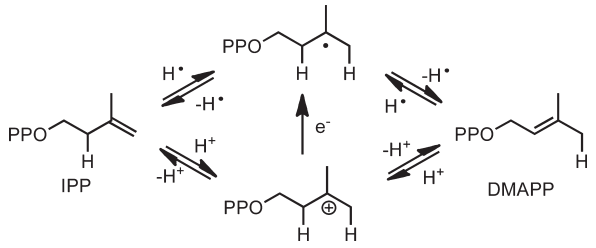
Scheme 1: Radical and Carbocation Mechanisms for IDI-2



catalysis, or to fulfill a structural role (*24*). Several mechanisms have been suggested for IDI-2, and the associated role of reduced flavin. A variety of studies with substrate and flavin analogues have been interpreted as evidence of protonation−deprotonation or radical-based mechanisms (Scheme 1) (*14*, *16*, *24*−*31*).

We now report a study with 3-butyn-1-yl diphosphate (**1-OPP**) and 2,3-butadien-1-yl diphosphate (**2-OPP**), acetylenic and allenic analogues of IPP and DMAPP, respectively, designed to differentiate between mechanisms initiated by proton and hydrogen atom addition. Calculated heats of reaction for addition of a hydrogen atom or a proton to the alkyne and allene moieties indicate that protonation of a monosubstituted triple bond is ∼15 kcal/mol more endothermic than protonation of unsymmetrically disubstituted and trisubstituted double bonds, while the corresponding hydrogen atom additions are ∼2 kcal/mol more endothermic. Analogues **1-OPP** and **2-OPP** were not substrates for IDI-2 but instead were competitive inhibitors. These results support the protonation−deprotonation mechanism for isomerization.

EXPERIMENTAL PROCEDURES

3-Butyn-1-yl Diphosphate (***1-OPP***). Tris(tetrabutylammonium) hydrogen pyrophosphate trihydrate (9.14 g, 9.3 mmol) was dissolved in 50 mL of anhydrous acetonitrile followed by addition of 3-butyn-1-yl tosylate (**1-OTs**, 0.500 g, 2.2 mmol). The reaction mixture was allowed to stir at room temperature (rt) for 4 h before solvent was removed under vacuum. The pale yellow residue was dissolved in 3 mL of ion-exchange buffer [2 g of NH_4HCO_3 in 1 L of a 1:49 (v/v) isopropyl alcohol/water mixture], and the resulting clear solution (25 mM NH_4HCO_3) was loaded onto a column of Dowex AG 50W-X8 (100−200-mesh) cation-exchange resins (ammonium form). Fractions containing product were collected and lyophilized. The material was dissolved in 5 mL of 0.05 M ammonium bicarbonate; 20 mL of a 1:1 (v/v) acetonitrile/isopropyl alcohol mixture was added, and the contents were mixed thoroughly on a vortex mixer, during which time a white precipitate formed. The suspension was cleared by centrifugation for 5 min at 2000 rpm. The supernatant was removed, and the residue was suspended in 5 mL of 0.05 M ammonium bicarbonate and 8 mL of a 1:1 (v/v) acetonitrile/isopropyl alcohol mixture. The mixture was concentrated to ca. 5 mL under reduced pressure at 40 °C. Half of the concentrated extract was dissolved in an equal volume of chromatography buffer (50 mM NH_4HCO_3 in a 1:2:1 CH_3CN/*i*PrOH/H_2O solvent) and loaded onto a cellulose flash column. The column was eluted with chromatography buffer; fractions were analyzed by thin layer chromatography, and those containing diphosphate were pooled, concentrated at reduced pressure, and lyophilized. The chromatography was repeated to give 0.364 g (56%) of a white powder: ^{1}H NMR (D_2O) δ 3.85 (q, 2H, J = 6.8 Hz), 2.46 (m, 2H), 2.20 (t, 1H, J = 2.7 Hz); ^{13}C NMR (D_2O) δ 82.3, 70.7, 64.1, 20.3; ^{31}P NMR (D_2O) δ −7.6 (d, J = 21 Hz), −9.9 (d, J = 21 Hz); negative ion ESMS (M − 1) 228.9682, calcd 228.9673.

2,3-Butadien-1-yl Diphosphate (***2-OPP***). Following the procedure described for **1-OPP**, 250 mg (1.1 mmol) of **2-OTs** gave 170 mg (54.5%) of a white powder: ^{1}H NMR (D_2O) δ 5.22 (m, 1H, J = 6.8 Hz), 4.76 (m, 2H), 4.25 (t, 2H, J = 2.4 Hz); ^{13}C NMR (D_2O) δ 209.0, 87.8, 76.6, 64.0; ^{31}P NMR (D_2O) δ −7.7 (d, J = 21 Hz), −9.9 (d, J = 21 Hz); mass (m/z) 229.97; negative ion ESMS (M − 1) 228.9677, calcd 228.9673.

Computational Methods. Calculations were conducted using Gaussian 09 (*32*). The gas-phase proton affinity (PA), hydrogen atom affinity (HA), and heats of formation were calculated with modern composite chemical models, G3MP2B3 (*33*) and CBS-QB3 (*34*, *35*). Both calculations involve predefined electronic structure calculations combined with empirical corrections to produce molecular energies typically within ∼1 kcal/mol of experimental values (*33*, *36*). PA and HA are defined as follows:

$$M + H^+ \rightarrow MH^+ \quad \Delta H_{rxn} = -PA$$

$$M + H^\bullet \rightarrow MH^\bullet \quad \Delta H_{rxn} = -HA$$

These values were obtained directly from the calculated enthalpy differences between the enthalpies of products and reactants at 298.15 K. Standard heats of formation for 1-butyne and 1,2-butadiene were also calculated and compared to experimentally determined values (*37*) to give an independent check of the accuracy of the calculated results. The standard heat of formation was derived using the method described by Nicolaides et al. (*38*), which uses the atomization energy of a molecule in conjunction with the experimental heats of formation of its constituent atoms. A working example can be found in the article titled "Thermochemistry in *Gaussian*" on the Gaussian company website (www.gaussian.com) under the white papers and technical notes section. All optimized structures were confirmed to be true minima with zero imaginary frequencies within the frequency calculation jobs. Because the harmonic vibrational frequencies calculated were consistently larger than experimental values, a scaling factor of 0.96 was employed to scale the calculated vibrational frequencies and zero-point energies (ZPE) (*39*).

IC_{50} and K_i Measurements. The half-maximal inhibitory concentrations (IC_{50}) were determined using the acid lability assay (*19*, *20*). Reactions were performed in duplicate and initiated by addition of 5 μL of IDI (100 nM) to 40 μL of buffer [200 mM HEPES (pH 7.0) containing 2 mM $MgCl_2$, 40 μM FMN, 2 mM NADPH, and 50 μM [^{14}C]IPP for IDI-2; 50 mM HEPES (pH 7.2) containing 10 mM $MgCl_2$, 200 mM KCl, 1 mg/mL BSA, 0.5 mM DTT, and 50 μM [^{14}C]IPP for IDI-1] and varying amounts of inhibitor. The reaction mixtures were incubated at 37 °C, quenched, and extracted as described for the acid lability assay. Values of IC_{50} and K_i were determined from plots of activity versus inhibitor concentration using Grafit.

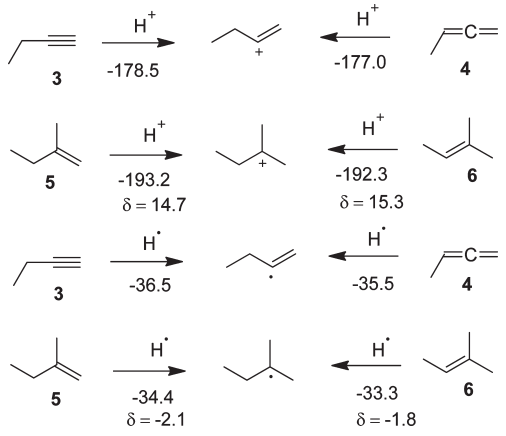
Product Studies. Following the procedure of Walker et al. (*40*), an NMR sample was prepared in D_2O containing 12 μM IDI-2, 10 mM **1-OPP** or **2-OPP**, 20 μM FMN, 2 mM NADPH, and 10 mM $MgCl_2$ in 50 mM HEPES buffer (pD 7.3), using components that were exchanged with D_2O before being mixed. The concentration of the stock solution of enzyme was detemined

Table 1: Heats of Reaction for Protonation and Hydrogen Atom Addition and Heats of Formation for 1-Butyne (**3**), 1,2-Butadiene (**4**), 2-Methyl-1-butene (**5**), and 2-Methyl-2-butene (**6**)[a]

	$\Delta_R H°(H^+$ addition) (kcal/mol)		$\Delta_R H°(H^•$ addition) (kcal/mol)		$\Delta_f H°$ (kcal/mol)		
	G3MP2B3	CBS-QB3	G3MP2B3	CBS-QB3	G3MP2B3	CBS-QB3	exptl
3	−178.74	−178.25	−36.26	−36.76	38.57	39.40	39.50
4	−177.17	−176.91	−34.69	−35.42	37.00	38.06	38.70
Δ[b]	−1.57	−1.34	−1.57	−1.34	1.57	1.34	0.80
5	−193.51	−193.27	−33.79	−34.97	−9.54	−10.00	−8.39
6	−192.49	−192.04	−32.77	−33.74	−10.56	−11.23	−9.92
Δ[c]	−1.02	−1.23	−1.02	−1.23	1.02	1.23	1.53

[a]See Experimental Procedures. [b]Δ is the difference in $\Delta_R H°$ and $\Delta_f H°$ between the values of **3** and **4**. [c]Δ is the difference in $\Delta_R H°$ and $\Delta_f H°$ between the values of **5** and **6**.

Scheme 2: Comparison of Heats of Reaction for Proton and Hydrogen Atom Addition for Alkyne **3**, Allene **4**, and Isomeric Alkenes **5** and **6**

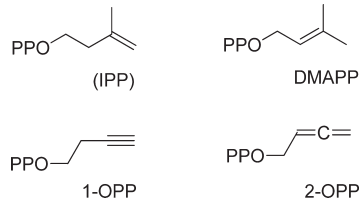


by UV spectrometry (*16*, *41*), and its activity was determined by the acid lability assay. Samples were incubated at 37 °C, and NMR spectra were recorded at 500 MHz. Each spectrum was the average 32 scans taken with 10 s intervals between each pulse sequence.

RESULTS

Calculation of Heats of Reaction for Protonation and Hydrogen Atom Addition. Gas-phase heats of reaction ($\Delta_r H°$) for protonation and hydrogen atom addition were calculated for 1-butyne (**3**), 1,2-butadiene (**4**), 2-methyl-1-butene (**5**), and 2-methyl-2-butene (**6**) using composite chemical models, G3MP-2B3 (*33*) and CBS-QB3 (*34*, *35*), which incorporate predefined electronic structure calculations combined with empirical corrections to produce accurate molecular energies, typically within ∼1 kcal/mol of experimental values (see Table 1) (*33*, *36*). Values for the standard heats of formation ($\Delta_f H°$) of the four hydrocarbons were also calculated and compared with experimental values. In each case, the differences between the calculated and experimental values for $\Delta_f H°$ for the pairs of isomeric hydrocarbons (Δ) were <1 kcal/mol. Furthermore, our calculated value for the proton affinity of alkene **5** is similar to the experimental value reported for isobutene (*42*). The heats of reaction for the individual proton and hydrogen atom transfers are illustrated in Scheme 2. The data indicate that addition of a proton to either member of the alkyne/allene pair is approximately 15 kcal/mol less exothermic (δ) than protonation of the isomeric alkenes, reflecting the substantial difference in the stabilities of the vinyl and tertiary carbocations produced in the reactions. Interestingly, the data suggest that addition of a hydrogen atom to alkyne **3** and allene **4** is ∼2 kcal/mol more exothermic (δ) than addition of a hydrogen atom to alkenes **5** and **6**. Thus, the alkyne/allene pair should be substantially less reactive than the isomeric alkenes for isomerization by a protonation−deprotonation mechanism and of comparable reactivity for a hydrogen atom addition−abstraction mechanism.

Enzymatic Studies. 3-Butyn-1-yl diphosphate (**1-OPP**) and 3,4-butadien-1-yl diphosphate (**2-OPP**) were synthesized by conversion of the alcohols (*43*−*45*) to the corresponding tosylates, followed by treatment with tris(tetrabutylammonium) hydrogen pyrophosphate by the procedure reported for synthesis of IPP (*20*). The diphosphates were purified by chromatography on cellulose and stored at −80 °C until they were needed. Recombinant *Escherichia coli* IDI-1 and *Thermus thermophilus* IDI-2 were purified from overproducing strains of *E. coli* as previously described (*16*) and stored at −80 °C in buffer containing glycerol.



Inhibition and turnover experiments were conducted for IDI-1 and IDI-2 with alkyne analogue **1-OPP** and allene analogue **2-OPP**. The stability of the enzymes in the presence of **1-OPP** and **2-OPP** was measured by incubation with either analogue at 37 °C. Samples were removed at 10 min intervals; [^{14}C]IPP was added, and activity was measured by the acid lability assay (*19*, *20*). The small decreases in activity seen over a period of 48 min were characteristic of slow nonspecific loss of enzyme activity rather than irreversible inactivation by the analogues. In preliminary reversible inhibition studies of the isomerization of IPP to DMAPP catalyzed by IDI-1, the alkyne and allene analogues gave an IC_{50} of ∼200 μM, while similar measurements with IDI-2 gave an IC_{50} of ∼50 μM. In a more extensive set of kinetic studies, **1-OPP** and **2-OPP** were found to be competitive inhibitors of the isomerization of IPP to DMAPP catalyzed by IDI-1 and by IDI-2 with K_I values that were 4−8-fold higher than the K_M values of the two enzymes (see Table 2).

Product studies were conducted by incubation of **1-OPP** or **2-OPP** with IDI-2 at 37 °C in an NMR tube. Spectra were recorded at 1 h intervals over a period of 12 h. Incubations with **1-OPP** were analyzed for the appearance of a signal at ∼5.3 ppm for

Table 2: Inhibition and Michaelis Constants for IDI-1 and IDI-2[a]

		IPP	DMAPP	**2-OPP**	**3-OPP**
K_M (μM)	IDI-1	7.9[b]	14[b]	–	–
	IDI-2	5.6[c]	–	–	–
K_I (μM)	IDI-1	–	–	49 ± 5	31 ± 3
	IDI-2	–	–	48 ± 6	36 ± 5

[a]Acid lability assay (*19*, *20*). [b]From refs *12* and *15*. [c]From refs *12*, *15*, and *22*.

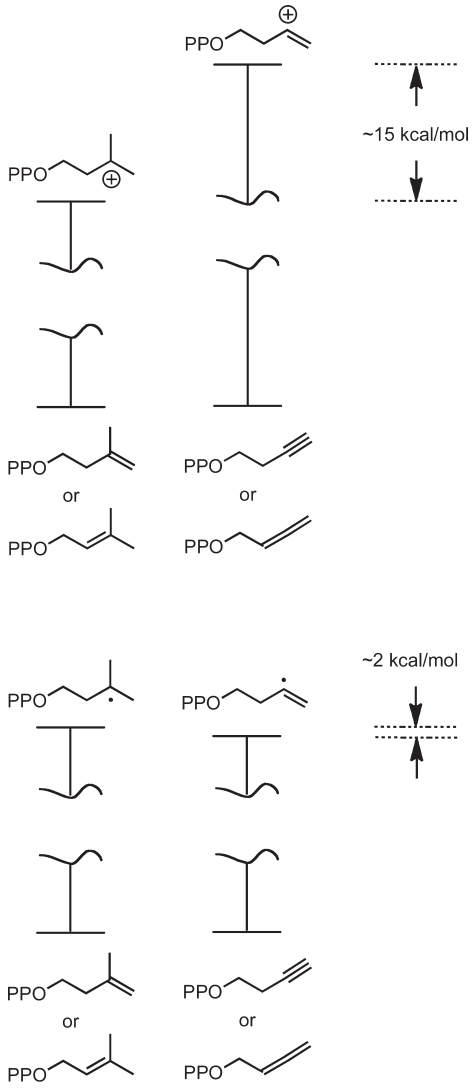


FIGURE 1: Energy differences between carbocations formed by addition of a proton and radicals formed by addition of a hydrogen atom to **1-OPP** or **2-OPP** and IPP or DMAPP.

the proton at C2 as evidence for the formation of **2-OPP**, while those for incubation with **2-OPP** were analyzed for the appearance of signals at 2.2 and 2.5 ppm for the protons at C4 and C2, respectively. In both cases, no evidence of isomerization was detected. On the basis of our previous NMR studies of the isomerization of IPP to DMAPP with IDI-1 (*46*), we estimate that the alkyne and allene analogues are at least 1000 times less reactive that the natural substrates. Similar results were obtained when **1-OPP** and **2-OPP** were incubated with IDI-1.

DISCUSSION

IDI-2 catalyzes the FMN-dependent isomerization of IPP to DMAPP. The cofactor is tightly bound in the *T. thermophilus* enzyme and is in the fully oxidized state (FMN) when the protein is purified under aerobic conditions (*15*, *16*, *47*). The oxidized flavin must be reduced to observe turnover. Upon incubation with NADPH, the IDI-2·FMN form is rapidly reduced to the IDI-2·$FMNH^-$ form, and upon addition of substrate, the cofactor is protonated to give the IDI-2·IPP·$FMNH_2$ form (*14*, *29*). In the presence of excess NADPH or in the absence of oxygen, the enzyme continues to turn over until equilibrium is established between IPP and DMAPP (*29*, *46*).

Flavin cofactors are known to perform a variety of roles in catalysis, including mediating one- and two-electron oxidations and reductions (*14*, *24*), serving as a covalent catalyst (*17*, *26*), and serving as a structural unit in the active site (*30*, *47*). Mechanisms for isomerization of the unactivated carbon−carbon double bond in IPP by hydride transfer to give a tertiary carbanion or by covalent catalysis are unlikely. Several groups have proposed mechanisms involving radical or carbocationic intermediates (see Scheme 1) (*14*, *29*, *48*, *49*), although there is no precedent in which a reduced flavin serves as a sufficiently strong acid to protonate the unactivated double bond in IPP.

Analogues **1-OPP** and **2-OPP** were synthesized to distinguish between the mechanisms initiated by proton transfer and hydrogen atom transfer to the double bond in IPP. As seen in Figure 1, the calculated difference in energies for formation of a vinyl cation from **1-OPP** or **2-OPP** is ~15 kcal/mol greater than for formation of a tertiary carbocation from IPP or DMAPP, reflecting the substantial differences in the stability of tertiary and vinyl carbocations. While this difference would be smaller if the proton addition and elimination steps were concerted, studies with IPP analogues and IDI-1 indicate that the reaction involves a tertiary carbocation or a transition state with highly developed positive charge at C3 (*20*). In contrast, the corresponding difference for formation of a vinyl radical from **1-OPP** or **2-OPP** is ~2 kcal/mol less than for formation of a tertiary radical from IPP or DMAPP. Thus, one would predict that **1-OPP** and **2-OPP** would not be alternate substrates if IDI-2 catalyzed isomerization by a protonation−deprotonation mechanism but would be substrates, perhaps with k_{cat} values similar to those of the natural substrates, for a hydrogen atom addition−abstraction mechanism. As expected, neither analogue was a substrate for *E. coli* IDI-1, which catalyzes isomerization by a protonation−deprotonation mechanism, although both compounds were competitive inhibitors with K_I values that were only slightly above those for K_m^{IPP} and K_m^{DMAPP}. Although the lack of reactivity for the tightly bound analogues could result from unfavorable conformations within the active site, IDIs typically have a rather broad selectivity for alternate substrates and active site irreversible inhibitors (*19*, *20*, *23*, *27*). Similar results were obtained for *T. thermophilus* IDI-2. Thus, we conclude that isomerization does not proceed by addition of a hydrogen atom to the double bonds of IPP or DMAPP.

An alternative mechanism for flavin-dependent isomerization consistent with our observations involves protonation of the double bond with concomitant formation of $FMNH^-$, followed by the transfer of an electron from $FMNH^-$ to generate a tertiary radical-flavin semiquinone radical pair (*14*, *24*, *27*). Redox potentials for $FMNH^\bullet$ indicate that the semiquinone is stabilized relative to $FMNH^-$ when the flavins are bound to IDI-2 (*25*, *29*, *31*). However, no evidence of formation of radical intermediates using cyclopropylcarbinyl and epoxycarbinyl radical clock analogues of IPP that rearrange to homoallylic radicals with rate constants of ~10^7 and ~10^{10} s^{-1}, respectively, was found (*16*, *27*, *31*).

The cyclopropylcarbinyl analogue of IPP isomerized to the corresponding DMAPP derivative, while the epoxycarbinyl analogue was an irreversible inhibitor activated by protonation of the epoxide. If the tertiary carbocation were converted to the corresponding radical during the isomerization of IPP to DMAPP, we estimate that the ensuing abstraction reaction must have a rate constant of at least $\sim 10^8$ s^{-1}. At this point, application of Occam's razor favors the protonation−deprotonation mechanism.

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SUPPORTING INFORMATION AVAILABLE

General methods; experimental protocols for synthesis of **1-OTs** and **2-OTs**; ^{1}H, ^{13}C, and ^{31}P NMR and HRMS spectra for compounds **1-OPP** and **2-OPP**; and ^{1}H NMR spectra for incubations of **1-OPP** and **2-OPP** with IDI-2. This material is available free of charge via the Internet at http://pubs.acs.org.

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