JOC Note

Synthesis and Evaluation of Substrate Analogues as Mechanism-Based Inhibitors of Type II Isopentenyl Diphosphate Isomerase

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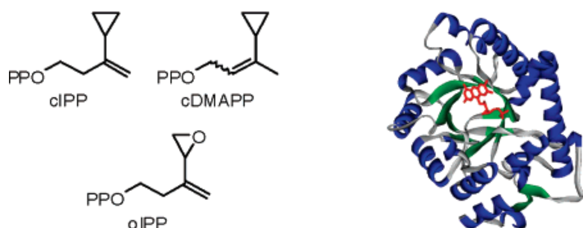

Type 2 isopentenyl diphosphate isomerase (IDI-2), which catalyzes the interconversion of isopentenyl diphosphate and dimethylallyl diphosphate, contains a tightly bound molecule of FMN. To probe the mechanism of the reaction, cyclopropyl and epoxy substrate analogues, designed to be mechanism-based irreversible inhibitors, were synthesized and evaluated with IDI-2 from *Thermus thermophilus*. The cyclopropyl analogues were alternative substrates. The epoxy analogue was an irreversible inhibitor, with $k_I = 0.37 \pm 0.07$ min^{-1} and $K_I = 1.4 \pm 0.3$ μM. LC−MS studies revealed formation of an epoxide-FMN adduct.

Isopentenyl diphosphate isomerase (IDI) catalyzes the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the two fundamental building blocks in the isoprenoid biosynthetic pathway. IPP is synthesized from acetyl CoA in eukarya, archaea, and some bacteria by the mevalonate pathway (MVA),[1] and isomerization of IPP to DMAPP is an essential step required for survival of the organisms. In plant chloroplasts and most bacteria, IPP and DMAPP are synthesized from pyruvate and D-glyceraldehyde phosphate by the methylerythritol phosphate (MEP) pathway.[2] IDI activity, while typically found, is not required for survival of these organisms.

IDI was discovered in the 1950s during studies of cholesterol metabolism in yeast and rats.[3−6] The enzyme is a zinc metalloprotein and requires a second divalent metal, typcially Mg^{2+}, for activity.[7] Isomerization occurs by a protonation/deprotonation mechanism that proceeds through a tertiary carbocation as shown in Scheme 1.[8−10] A second isomerase, IDI-2, was discovered in 2001.[11] It is clear from comparisons of amino acid sequences[12,13] and X-ray structures[14,15] that IDI-1 and IDI-2 evolved independently. IDI-2, like IDI-1, requires Mg^{2+} for activity. In addition, IDI-2 contains a tightly bound molecule of FMN and requires a reductant, typically NADPH, for activity.[16] Two mechanisms have been proposed for IDI-2: a protonation/deprotonation sequence similar to the mechanism for IDI-1 and a hydrogen atom addition/abstraction (Scheme 1).[16−20] While flavins normally facilitate oxidation/reduction reactions, there are examples of the cofactors participating in isomerizations with concomitant transient changes in oxidation state.[17]

Most bacteria synthesize isoprenoid compounds by the MEP pathway, and although not required for their survival, most have either IDI-1 or IDI-2 activity.[21] However, a few pathogenic bacteria, including *Streptococcus pneumoniae* and *Staphylococcus aureus*, utilize the MVA pathway for isoprenoid biosynthesis and rely on IDI-2 for isomerization of IPP to DMAPP. For these bacteria, IDI-2 presents an attractive target for the development of antibacterial drugs.[22]

We were drawn to cyclopropyl or epoxy analogues of IPP, where the methyl group has been replaced by a substituent, as potential substrate analogues or mechanism-based inhibitors that might be useful for distinguishing between the proton and hydrogen atom mechanisms. Protonation of the double bond in a cyclopropyl analogue, cIPP, would generate a cyclopropylcarbinyl cation, which could isomerize to cDMAPP or irreversibly inhibit the enzyme by reacting with an active site nucleophile.[23] Protonation of the oxirane ring in epoxy analogue,

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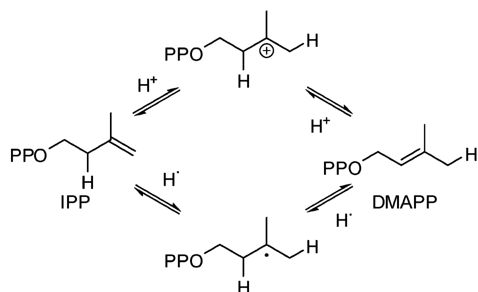
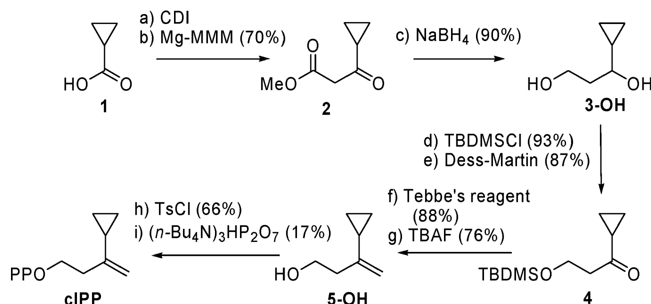
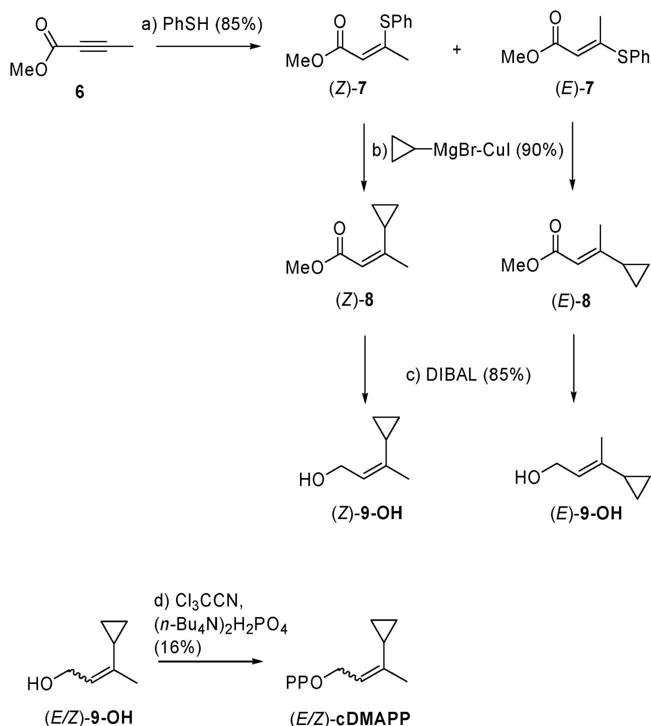
SCHEME 1. Protonation/Deprotonation and Hydrogen Atom Addition/Abstraction Mechanisms



SCHEME 2. Synthesis of cIPP



SCHEME 3. Synthesis of (*E*)-9-OH, (*Z*)-9-OH, and (*E*,*Z*)-cDMAPP



oIPP, could also result in inactivation of IDI by activating the epoxide to nucleophilic capture.[24] Addition of a hydrogen atom to cIPP or oIPP would generate cyclopropylcarbinyl[25] or epoxy carbinyl[26] radicals, which could then isomerize to cDMAPP or oDMAPP upon abstraction or isomerize to ring-opened homoallylic radicals that could inactivate the enzyme by hydrogen atom abstraction or recombination reactions. We now report the synthesis of cIPP, cDMAPP, and oIPP and an evaluation of these compounds as alternative substrates and inhibitors of *Thermus thermophilus* IDI-2.[16]

Synthesis of cIPP. The synthesis of cIPP is outlined in Scheme 2. Cyclopropane carboxylic acid (**1**) was treated with carbonyl diimidazole (CDI), and the resulting imidazolide was condensed with the magnesium salt of monomethyl malonate (MMM)[27] to give a good yield of the Claisen product (**2**).[28]

Reduction of ketoester **2** with sodium borohydride to diol **3-OH**, followed by protection of the primary hydroxyl group and Dess−Martin oxidation[29] of the secondary hydroxyl group, gave cyclopropyl ketone **4**. Treatment of **4** with Tebbe's reagent[30] and removal of the silyl-protecting group gave vinylcyclopropane alcohol **5-OH**. Conversion of **5-OH** to the corresponding tosylate, followed by treatment with tetrabutyl ammonium pyrophosphate, gave cyclopropyl analogue cIPP.

Synthesis of cDMAPP. We originally planned to prepare the *E* and *Z* isomers of cDMAPP from the corresponding alcohols (*E*)- and (Z)-**9-OH**. As shown in Scheme 3, Micheal addition of thiophenol to ethyl 2-butynoate (**6**) gave a mixture of (*E*)- and (*Z*)-**7**,[31,32] which were then separated by column chromatography. Each double bond isomer was treated with cyclopropyl cuprate to give stereoisomeric cyclopropyl esters (*E*)- and (*Z*)-**8**, and the esters were reduced with DIBAL to yield cyclopropyl allylic alcohols (*E*)- and (*Z*)-**9-OH**.

Attempts to phosphorylate the alcohols using a two-step chlorination/phosphorylation procedure we developed for allylic diphosphates[33] were unsuccessful. Introduction of the cyclopropane substituent was apparently too activating, and the intermediate allylic chlorides decomposed. We then turned to the direct phosphorylation of the alcohols using the Danilov modification of the Cramer procedure.[34,35] Because of the low yields typically associated with Cramer phosphorylations, we decided to use a 75:25 mixture of (*E*)- and (*Z*)-**9-OH** from reduction of (*E*)- and (*Z*)-**8** obtained by a Wittig condensation of methylcyclopropyl ketone and triethyl phosphonoacetate[36] to optimize the phosphorylation step. We discovered that both isomers of cDMAPP were unstable at room temperature when dissolved in water, and the diphosphates decomposed with a half-life of ~5 h at pH 8 and ~10 h at pH 11. As a result, a substantial amount of material was lost during purification by ion exchange and column chromatography, and we did not pursue synthesis of the individual double bond isomers of cDMAPP.

Inorganic pyrophosphate is an excellent leaving group whose reactivity depends on its total negative charge.[37] The pH dependence of the half-life of cDMAPP in water suggested that

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SCHEME 4. Synthesis of oIPP

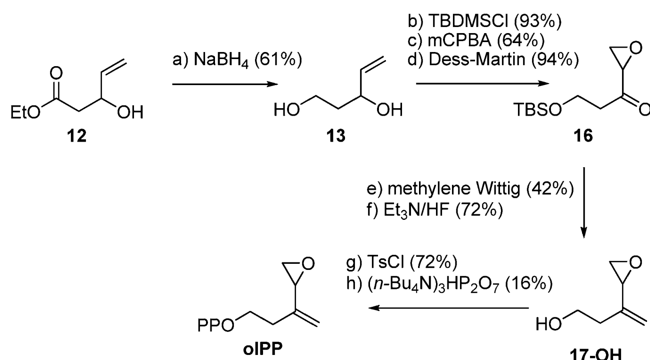
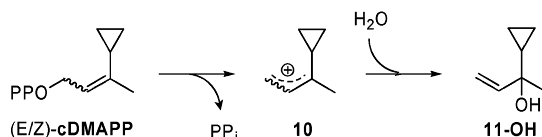


TABLE 1. Inhibition of IDI-2 with Substrate Analogues[a]

compound	IC_{50} (μM)
cIPP	100 ± 15 at 25 °C, pH 8.6
(*E*/*Z*)-cDMAPP	278 ± 185 at 25 °C, pH 8.6
oIPP	1.14 ± 0.27 at 37 °C, pH 7.0

[a] Incubations were for 10 min in 50 μL of 200 mM Tris buffer containing 8 nM IDI-2, 5 μM IPP (59 μCi/μmol), 20 μM FMN, 5 mM NADPH, and 10 mM $MgCl_2$. The higher pH and lower temperature for (*E*/*Z*)-cDMAPP were used to minimize decomposition and for cIPP to permit a direct comparison between the allylic isomers.

the diphosphate had solvolyzed. ^{1}H NMR analysis of the reaction showed a first-order disappearance of cDMAPP to give vinylcyclopropyl alcohol **11-OH**[38] as the sole product, suggesting that vinyl-substituted cyclopropylcarbinyl cation **10** is an intermediate in the reaction.



Synthesis of oIPP. The synthesis of oIPP is shown in Scheme 4. Ethyl ester **12**, obtained by condensation of ethyl acetate with acrolein,[39] was reduced with sodium borohydride to give allylic diol **13**. The primary hydroxyl group was selectively protected with TBSCl, followed by treatment with *m*-chloroperbenzoic acid. The epoxide was then subjected to a Dess−Martin oxidation to give epoxy ketone **16**. Attempts to convert the ketone to an olefin with Tebbe's reagent as described for conversion of **4** to **5-OH** were unsuccessful. After exploring a variety of Wittig conditions, we were able to obtain **17-OH** in a modest yield using methyltriphenylphosphonium bromide and sodium hexamethyl disilazide. The alcohol was converted to oIPP using the phosphorylation procedure described for cIPP.

Studies with IDI-2. cIPP, *E*/*Z*-cDMAPP, and oIPP were examined as inhibitors of recombinant *T. thermophilus* IDI-2.[16] Preliminary experiments to determine IC_{50} values gave substantial errors (Table 1) due to a combination of chemical instability of cDMAPP and irreversible inhibition by **oIPP** (see the following paragraph). The isomerization of **cIPP** to **cDMAPP** was followed by ^{1}H NMR spectroscopy. Initially, signals for **cIPP** decrease with a concomitant increase in the intensity of the signals for **cDMAPP**. This was followed by a decrease in the peaks for **cDMAPP** as the intermediate solvolyzed to give tertiary alcohol **11-OH**.[19] A similar experiment starting with **cDMAPP** resulted in the transient production of **cIPP** along with the competing solvolysis reaction. These diphosphates did not appear to irreversibly inhibit IDI-2.

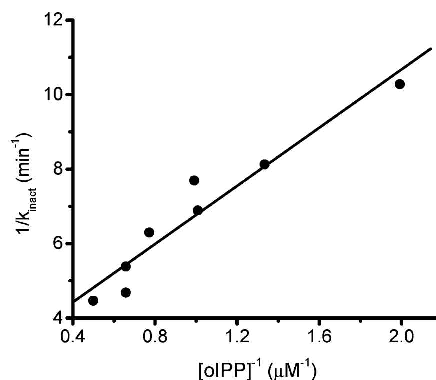


FIGURE 1. Double reciprocal plot of k_{inact} versus [oIPP].

Incubation of IDI-2, which had been reduced with NADPH, with **oIPP** resulted in a rapid first-order irreversible loss of activity of the enzyme. Kinetic parameters for the time-dependent inactivation were calculated from a double reciprocal plot of the first-order rate constants (k_{inact}) at different inhibitor concentrations (Figure 1); $k_I = 0.37 \pm 0.07$ min^{-1} and $K_I = 1.4 \pm 0.3$ μM. oIPP was not an irreversible inhibitor in the absence of NADPH.

In a preparative-scale experiment, reduced IDI-2 was incubated with oIPP. Negative ion LC−MS analysis of the enzyme·inhibitor complex gave a peak at *m/z* 731 for a FMN·oIPP adduct and fragment ions at *m/z* 456 and 274 for the flavin and oIPP components of the adduct.[19] A UV spectrum of the inhibited enzyme was characteristic of an alkylated flavin. A transient absorption seen at 600 nm when the enzyme·inhibitor complex was denatured with guanidinium hydrochloride was similar to spectra reported for N5 flavin adducts.[16,40−43] The FMN·oIPP adduct from inactivation of IDI-2 in D_2O followed by workup in H_2O did not contain deuterium.[19] This result shows that the epoxide ring was activated for inactivation of IDI-2 by protonation at oxygen rather than by hydrogen atom addition to the double bond of oIPP, followed by trapping of a radical intermediate by flavin semiquinone. This mechanism for irreversible inhibition by oIPP is similar to that for inhibition of IDI-1 by eIPP, the epoxy derivative of IPP.[9] Recently, Hoshino and co-workers[20] reported that eIPP also inhibits IDI-2 with concomitant formation of a covalent adduct with the reduced flavin cofactor. These observations are consistent with the recent report by Kittleman and co-workers[44] that IDI-2·1-deazaFMN is active when incubated with IPP and NADPH, while the 5-deazaFMN complex is not, suggesting that the proton at N5 is an integral part of the catalytic machinery.

In summary, cyclopropyl analogues of IPP and DMAPP and an epoxy analogue of IPP were synthesized. cIPP and cDMAPP

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were substrates for IDI-2, and no evidence was found for irreversible inhibition by either compound. In contrast, oIPP was a potent irreversible inhibitor that formed a covalent adduct with the reduced FMN cofactor in the enzyme. At this point, a protonation/deprotonation mechanism provides the simplest explanation for our observations.

Experimental Section

Epoxy Ketone 16. Epoxy alcohol **16** (520 mg, 2.25 mmol) in 5 mL of CH_2Cl_2 was added via cannula to 9.5 g (15 wt %, 3.38 mmol) of Dess−Martin periodinane in 100 mL of CH_2Cl_2. After being stirred for 18 h at rt, a saturated solution of $NaHCO_3$ and solid $Na_2S_2O_3$ were added. After 30 min, the mixture was diluted with ether and washed with water and brine. The aqueous layers were extracted with ether. The combined organic layers were dried ($MgSO_4$) and concentrated at reduced pressure. The residue was chromatographed on silica (8:2 hexanes/ethyl acetate) to give 486 mg (94%) of an oil. ^{1}H NMR ($CDCl_3$): δ 0.03 (s, 6H), 0.86 (s, 9H), 2.42−2.65 (m, 2H), 2.92−3.00 (m, 2H), 3.43−3.45 (m, 1H), 3.80−3.97 (m, 2H); ^{13}C NMR ($CDCl_3$): δ −5.6, 18.1, 25.7, 39.2, 45.3, 53.6, 58.3, 206.7; HRMS (CI) calcd for $C_{11}H_{22}O_3Si$ (M + H) 231.1416, found 231.1421.

Silyl-Protected Alcohol 17-OTBS. A suspension of methyltriphenylphosphonium bromide (232 mg, 0.65 mmol) in 20 mL of THF was cooled to 0 °C before sodium bis(trimethylsilyl)amide (1.0 M, 0.52 mL, 0.52 mmol) was added by syringe. The reaction was stirred for 30 min and then cooled to −78 °C before a solution of 100 mg (0.43 mmol) of epoxy ketone **16** in 5 mL of THF was added by cannula. The reaction was stirred for 1 h while warming to rt and was then quenched with a saturated solution of NH_4Cl. Ether was added, and the resulting solution was washed with water and brine. The aqueous layers were extracted with ether, and the combined organic layers were dried ($MgSO_4$) and concentrated at reduced pressure. The residue was chromatographed on silica (95:5 hexanes/ethyl acetate) to give 42 mg (42%) of an oil. ^{1}H NMR ($CDCl_3$): δ 0.05 (s, 6H), 0.88 (s, 9H), 2.17 (td, 2H, J = 1.2, 6.9 Hz), 2.66 (dd, 1H, J = 2.7, 5.4 Hz), 2.87 (dd, 1H, J = 4.2, 5.4 Hz), 3.35−3.37 (m, 1H), 3.66−3.74 (m, 2H), 5.01−5.02 (m, 1H), 5.21 (s, 1H); ^{13}C NMR ($CDCl_3$): δ −5.3, 18.3, 25.9, 34.1, 47.8, 53.8, 62.6, 114.3, 142.8; HRMS (CI) calcd for $C_{12}H_{24}O_2Si$ (M + H) 229.1624, found 229.1617.

oIPP. To a solution of *p*-toluenesulfonyl chloride (175 mg, 0.92 mmol) and DMAP (150 mg, 1.23 mmol) in methylene chloride was added by cannula a solution of 70 mg (0.61 mmol) of alcohol **17-OH** in 2 mL of methylene chloride. The mixture was stirred at rt for 16 h. Solvent was removed at reduced pressure, and the residue was chromatographed on silica (7:3 hexanes/ethyl acetate) to give 118 mg (72%) of an oil. The tosylate was immediately used in the next step without further characterization.

A solution of **17-OTs** (118 mg, 0.44 mmol) in 2 mL of acetonitrile was added dropwise to 595 mg (0.66 mmol) of tris-(*tetra*-butylammonium) pyrophosphate in 20 mL of acetonitrile. The mixture was allowed to stir for 2 h at rt and was concentrated at reduced pressure. The residue was loaded onto an ion exchange column (DOW-EX 50W NH_4^+ form) in 25 mM NH_4HCO_3/2% (v) IPA. The resin was washed with two column volumes of 25 mM NH_4HCO_3/2% (v) IPA. The eluent was concentrated by lyophilization, and the residue was chromatographed on cellulose (8:2 IPA/25 mM NH_4HCO_3) to give 22 mg (16%) of a white powder. ^{1}H NMR (DMSO-d_6): δ 2.18 (br s, 2H), 2.65−2.67 (m, 1H), 2.83−2.86 (m, 1H), 3.39 (br s, 1H), 3.84 (br s, 2H), 5.06 (s, 1H), 5.18 (s, 1H); ^{13}C NMR (DMSO-d_6): δ 31.2, 47.2, 53.2, 62.0, 114.1, 142.6; ^{31}P NMR (DMSO-d_6) δ −8.45 (br s), −9.90 (br s); HRMS (MALDI) calcd for $C_6H_{12}O_8P_2$ (M−H) 272.9929, found 272.9943.

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Supporting Information Available: General methods, procedures for synthesis of all other compounds, enzymatic assays, and ^{1}H and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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