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Unraveling the Mechanisms of Isoprenoid Biosynthetic Enzymes: Mechanistic Studies of the Early Stage Enzymes

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Abstract: Isoprenoids (or terpenoids) are a large and structurally diverse class of biomolecules that are essential for the survival of all forms of life. Despite the vast differences in their final structures and functions, the early steps of isoprenoid biosynthesis in all organisms follow one of only two known biosynthetic pathways: the mevalonate pathway or the methyl erythritol phosphate (MEP) pathway. Interestingly, while humans utilize the mevalonate pathway, many human pathogens rely exclusively on the MEP pathway for the biosynthesis of their isoprenoid compounds. This has led to a number of mechanistic studies of the MEP-specific pathway enzymes, with the ultimate goal of developing small molecule inhibitors as potential drugs. In addition to their therapeutic value, many of the MEP pathway enzymes also catalyze unusual chemical transformations that are not well understood. In this review, we will highlight the recent work by us and others towards the elucidation of the catalytic mechanisms of several key enzymes involved in the early stages of isoprenoid biosynthesis. These include 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) and 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase (IspH) of the MEP pathway, and the type II isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI-2) from *Staphylococcus aureus*. The functions of these enzymes are validated or identified as potential drug targets.

Keywords: Biosynthesis · Catalysis · Enzyme mechanism · Isoprenoids

Introduction

With over 55,000 known representatives, isoprenoids (or terpenoids) comprise one of the largest and most structurally diverse class of biomolecules found in Nature. These compounds are involved in numerous biological processes including electron transport, bacterial cell wall biosynthesis, membrane stabilization, plant defense, and photosynthesis. They also function as hormones, precursors to vitamins, and as antibiotics. While the final structures of these compounds differ markedly, all isoprenoids are derived from two isoprene units (Scheme 1), isopentenyl pyrophosphate (IPP, **1**) and dimethylallyl pyrophosphate (DMAPP, **2**). There are two biosynthetic pathways for the formation of IPP and DMAPP: the mevalonate pathway[1–3] which is present in higher eukaryotes and archaea, and the non-mevalonate (or methyl erythritol phosphate, MEP) pathway[4,5] which is present in most eubacteria, algae, and in the chloroplasts of plants. In the mevalonate pathway, three molecules of acetyl-CoA (**3**) are condensed to yield 3-hydroxy-3-methyl-glutaryl-CoA (**4**), which is then converted to mevalonate (**5**) by HMG-CoA reductase. Following two successive phosphorylations, diphosphomevalonate (**6**) is converted to IPP by ATP-dependent decarboxylation. Organisms that utilize the mevalonate pathway for isoprene biosynthesis require an isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI) activity to generate DMAPP from IPP. IPP and DMAPP are then condensed to initiate long-chain isoprenoid biosynthesis (reviewed in ref. [6]).

In the MEP pathway, isoprenoids are derived from the glycolytic intermediate/product, pyruvate (**7**) and glyceraldehyde-3-phosphate (**8**). In the first step of the MEP pathway, **7** and **8** are condensed into 1-deoxy-D-xylulose-5-phosphate (**9**), which is then converted to MEP (**10**) in the committed step to the MEP pathway. Subsequent cytidylylation, phosphorylation, and cyclization yields compound **11**. In the last two steps of the MEP pathway, **11** is converted to IPP and DMAPP *via* intermediate **12** by two separate [Fe-S]-containing proteins. Even though an isomerase activity (to convert IPP to DMAPP) is not essential in organisms that utilize the MEP pathway, some bacteria that use the MEP pathway also have an isomerase, presumably to balance the cellular pools of IPP and DMAPP.[7]

Human cells employ the mevalonate pathway for isoprenoid biosynthesis, whereas most bacteria rely on the MEP pathway. Because all of the enzyme in the MEP pathway are foreign to human tissues, but are required for the survival of these bacteria, all of the MEP pathway enzymes are potential drug targets.[8,9] This review will focus on recent mechanistic studies of two MEP pathway enzymes involved in bacterial isoprenoid biosynthesis: 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), which catalyzes the conversion of **9** to **10** in the committed step to the MEP pathway, and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (IspH) which catalyzes the final step of the MEP pathway (**12** → **1** + **2**). We will also discuss recent mechanistic studies of the type II isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI-2), which differs structurally and mechanistically from the human isomerase and which is required for isoprenoid biosynthesis in several human Gram-positive pathogens that utilize the mevalonate pathway.

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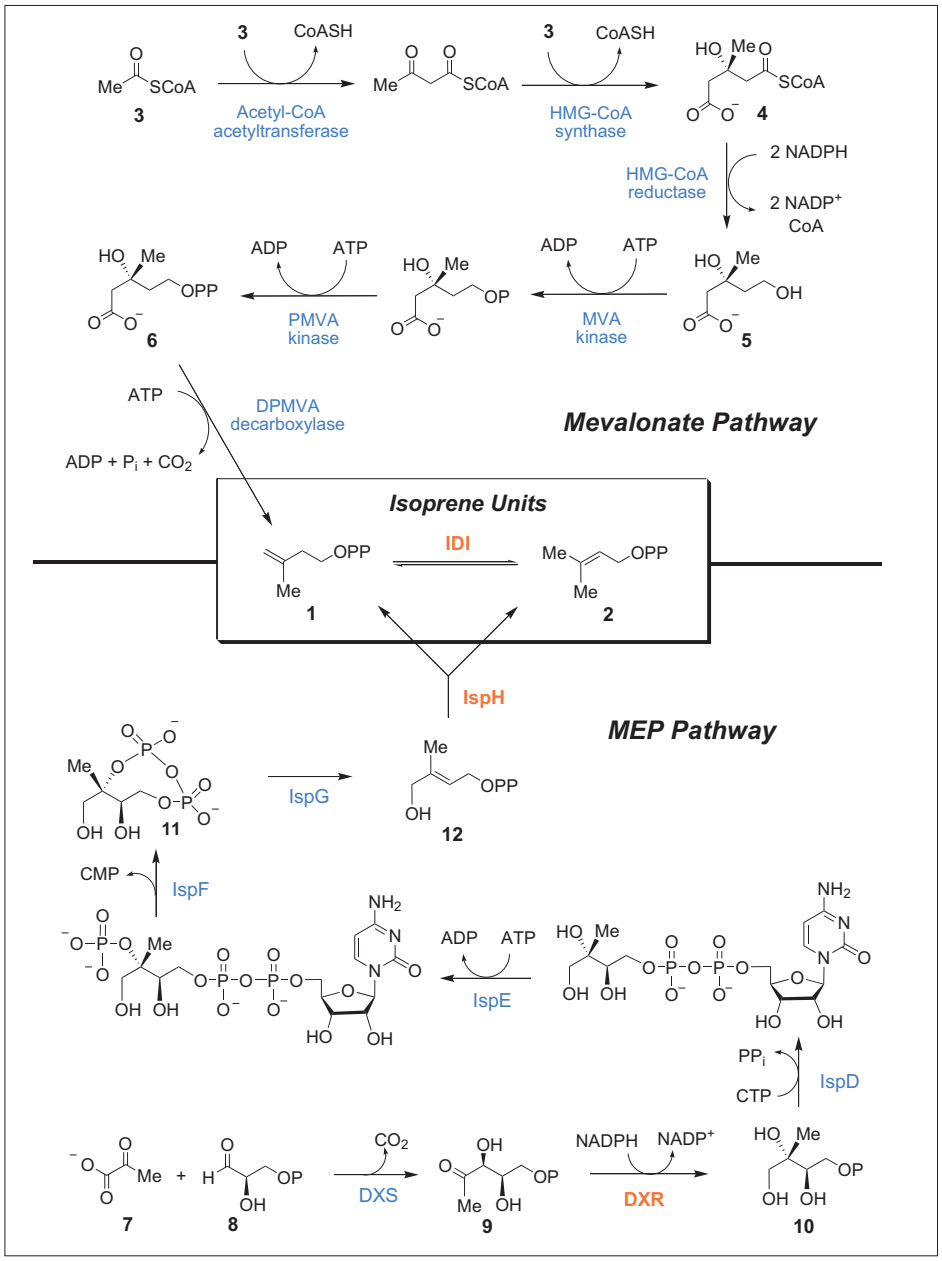
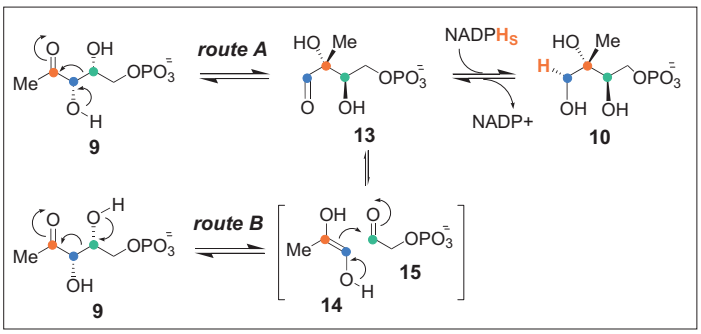


Scheme 1.



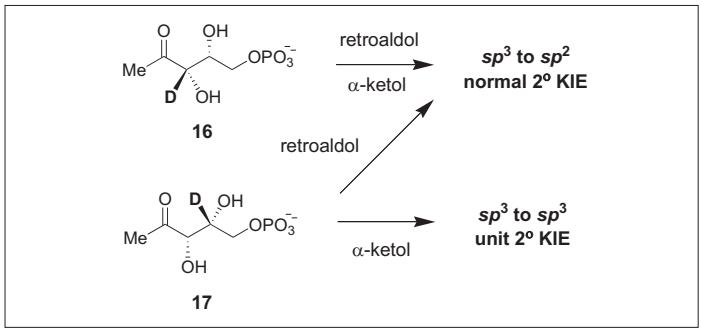
Scheme 2.

1-Deoxy-D-xylulose-5-phosphate Reductoisomerase (DXR)

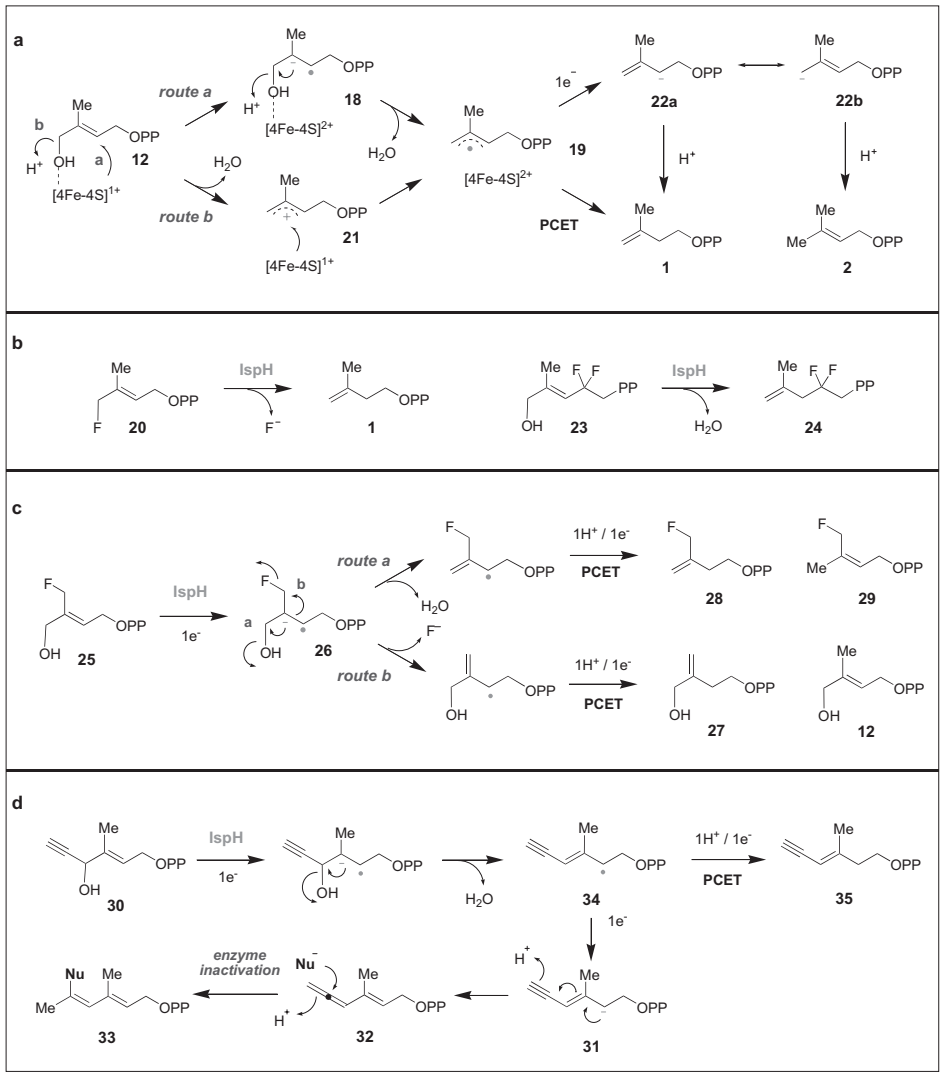
DXR catalyzes the NADPH-dependent reductive rearrangement of 1-deoxy-D-xylulose-5-phosphate (DXP, **9**) into methyl-D-erythritol-4-phosphate (MEP, **10**, Scheme 2) in a reaction that also requires a divalent metal ion for catalysis.[10] DXR catalyzes the committed step to the MEP biosynthetic pathway and is an established antimalarial drug target.[9] Two mechanisms have been proposed for the DXR-catalyzed reaction.[10–13] In the α-ketol rearrangement mechanism (route A), deprotonation of the C(3)-hydroxyl group, followed by C–C bond migration gives methylerythrose phosphate (**13**), which is then reduced to MEP (**10**) by NADPH. In the retroaldol/aldol rearrangement mechanism (route B), the C(3)–C(4) bond of **9** is cleaved to generate two fragments (**14** and **15**), which subsequently recombine in an aldol reaction to form a new C–C bond – yielding the same aldehyde intermediate (**13**) formed in the α-ketol mechanism. Subsequent reduction of **13** by NADPH affords MEP (**10**). Stereochemical studies have shown that the pro *S* hydride of NADPH is transferred to the pro *R* position at C(1) of MEP and that the pro *S* hydrogen at C(1) of MEP is derived from C(3)-H of **9**.[13–15] Several different divalent metal ions support catalysis[10,11,13] and crystallographic studies[16–22] suggest that the divalent metal ion likely coordinates to the C(2)=O and C(3)-OH functional groups of **9** as well as to several acidic active site residues.[23] The exact function of the metal ion is unknown, but it likely aids substrate binding, and could also be involved in substrate deprotonation and/or electrophilic catalysis.[13]

Steady-state kinetic and inhibition studies of the *E. coli* enzyme indicate that the DXR-catalyzed reaction likely follows an ordered, sequential kinetic mechanism with NADPH binding before DXP, though a random sequential mechanism has been suggested for the *Mycobacterium tuberculosis* enzyme.[11,13] The overall reaction is freely reversible[11,13] and the rate-limiting step in the *E. coli* enzyme occurs prior to NADPH-mediated reduction and product release,[24] implying that the rearrangement step limits catalysis. To distinguish among the α-ketol and retroaldol/aldol mechanisms, a number of mechanistic probes and substrate analogues have been synthesized and evaluated,[11–13,25–29] but none of these studies provided direct, conclusive evidence for either mechanism. Furthermore, while compound **13** has been synthesized and shown to be converted by DXR into MEP or DXP in the presence of NADPH or $NADP^+$, respectively, the putative intermediates **13**, **14**, and/or **15** have never been detected in reaction mixtures.[11,12,30]

Because kinetic studies suggested a rate-limiting rearrangement step for the *E. coli* enzyme, we probed the reaction in a kinetic isotope effect (KIE) study using DXP stereospecifically labeled with deuterium at C(3) or C(4) (**16** and **17**, respectively, Scheme 3).[31] If the reaction proceeds *via* the α-ketol rearrangement mechanism, incubation with [3-^{2}H]-DXP (**16**) is expected to produce a normal 2° KIE whereas [4-^{2}H]-DXP (**17**) should give a 2° KIE of unity.[32] This is because C(3) undergoes sp^3 to sp^2 rehybridization during the α-ketol rearrangement, but the hybrid-



Scheme 3.



Scheme 4.

ization of C(4) is unchanged. In contrast, in the retroaldol mechanism, the hybridization of both C(3) and C(4) change from sp^3 to sp^2 in the C–C bond scission step, which should result in a normal 2° KIE for both compounds.

The KIEs were measured using the equilibrium perturbation method developed by Cleland and coworkers.[33] Accordingly, DXR was added to a mixture of deuterated DXP (either **16** or **17**), MEP, NADPH, and $NADP^+$ at chemical equilibrium. After enzyme addition, labeled DXP is converted into labeled MEP in the forward direction, with the concomitant oxidation of NADPH to $NADP^+$. In the reverse direction, unlabeled MEP is converted to unlabeled DXP and $NADP^+$ is reduced to NADPH. Thus, if there is a normal KIE on the forward reaction, there will be a temporary increase in the concentration of NADPH (monitored by its absorbance at 340 nm) because the net flux in the reverse direction (**10** → **9**) will be greater. Conversely, if there is an inverse isotope effect, a temporary decrease in the concentration of NADPH would be expected because the net flux in the forward direction (**9** → **10**) will be greater.

For both [3-^{2}H]-DXP and [4-^{2}H]-DXP, a transient increase in the concentration of NADPH was observed before the NADPH/$NADP^+$ levels returned to equilibrium, corresponding to a normal 2° KIE for both compounds.[31] The 2° KIE values were determined by kinetic simulation of the experimental progress curves to be 1.04 ± 0.02 and 1.11 ± 0.02 for [3-^{2}H]-DXP and [4-^{2}H]-DXP, respectively. The observed perturbation patterns strongly favor the retroaldol/aldol rearrangement mechanism. Interestingly, the simulated KIE values for DXR are smaller than the 2° KIE values determined for muscle aldolase,[34] which catalyzes a similar reaction. This indicates that either the retroaldol reaction in DXR is only partially rate-limiting, or that the C(3)–C(4) bond cleavage step has an early transition state where little rehybridization at C(3) and C(4) has occurred. The size of the KIE for [4-^{2}H]-DXP is slightly larger than that of [3-^{2}H]-DXP, suggesting that C(3) may have less *s*-character than C(4) in the transition state. This could be due to the incomplete delocalization of the incipient negative charge on C(3) into the C(2)-carbonyl.

1-Hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate Reductase (IspH)

IspH (or LytB) catalyzes the final reaction of the MEP pathway, where 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (**12**) can be converted to either IPP (**1**) or DMAPP (**2**) by reductive elimination (Scheme 4a).[35,36] Amino acid sequence alignments of IspH enzymes revealed a relationship to [Fe-S] cluster-containing proteins as well as the presence of three absolutely conserved cysteine residues,[35] which were shown to be essential for activity and iron binding.[37,38] Subsequent metal analysis, and absorbance and EPR spectroscopic studies demonstrated that the purified enzyme indeed contains a [4Fe-4S] cluster, and X-ray crystallographic studies of the *Aquifex aeolicus* IspH revealed the presence of a [3Fe-4S] cluster coordinated by the three conserved cysteine residues.[36–39] Following [Fe-S] cluster reconstitution under anaerobic conditions, the enzyme is active in the presence of several reducing systems including dithionite and a flavodoxin/flavodoxin reductase system in the presence of NADPH, suggesting that the reduced $[4Fe\text{-}4S]^{1+}$ cluster is required for catalysis.[35,36,39,40] The fourth iron atom is likely lost from the enzyme during crystallization. Interestingly, IspH appears to form a stable complex with flavodoxin, suggesting that this may be the physiological enzyme system that is used to deliver reducing equivalents to IspH during turnover.[41]

The chemical mechanism for IspH catalysis has been suggested to resemble the Birch reduction of allylic alcohols (Scheme 4a, route a).[40] Namely, electron transfer to **12** by the reduced $[4Fe\text{-}4S]^{1+}$ cluster could generate the key radical anion intermediate (**18**) that then eliminates the C(1)-OH group to form the allylic radical (**19**). Following electron transfer (**12** → **18**), coordination of the C(1)-OH to the oxidized $[4Fe\text{-}4S]^{2+}$ cluster may favor OH elimination over diphosphate elimination, which would otherwise be thermodynamically favored. While no spectroscopic evidence has been provided for this putative coordination event, kinetic studies of a C(1)-fluorinated analogue (**20**, Scheme 4b) revealed a larger K_m for the fluorinated compound, suggesting that coordination to the [4Fe-4S] cluster may be important for substrate binding.[42] In addition, docking simulations using the X-ray crystal structure of IspH suggest that two absolutely conserved histidine residues bind to the pyrophosphate moiety of **12** and position the C(1)-OH group 1.9 Å from the [Fe-S] cluster.[38]

Alternatively, with the aid of a suitably positioned catalytic acid group (perhaps a conserved glutamate residue),[38] the [4Fe-4S] cluster may act as a Lewis acid to facilitate elimination of the C(1)-OH of **12** and to generate the allylic cation (**21**) in a mechanism reminiscent of the one used in the aconitase-catalyzed reaction (Scheme 4a, route b).[43] However, the requirement of reducing equivalents for IspH activity suggests that the catalytic cycle likely begins with the $[4Fe\text{-}4S]^{1+}$ cluster, and it is unclear whether the reduced cluster could facilitate an aconitase-like elimination reaction (aconitase employs an oxidized $[4Fe\text{-}4S]^{2+}$ cluster to catalyze hydroxide elimination). If formed, the allylic cation (**21**) would likely be reduced rapidly to **19** by the $[4Fe\text{-}4S]^{1+}$ cluster. Regardless of the mechanism of C(1)-OH bond scission, the fact that the C(1)-fluorinated analogue (**20**) is turned over to IPP by IspH suggests a heterolytic C(1)-OH bond cleavage event, which would be consistent with either mechanism.[42]

Following hydroxide elimination, the second electron required for reduction of the substrate radical (**19**) to the allylic anion (**22a/b**) may be supplied by the external reducing agent through the transiently oxidized $[4Fe\text{-}4S]^{2+}$ cluster of IspH. Protonation of **22** at either C(3) or C(1) would then generate IPP (**1**) or DMAPP (**2**), respectively. Alternatively, protonation could occur concomitantly with electron transfer to either C(3) or C(1) in a concerted proton-coupled electron transfer (PCET) pathway. Interestingly, a C(4)-difluorinated analogue (**23**) was found to be converted into the C(4)-difluoro-IPP product (**24**, Scheme 4b).[44] This observation is consistent with a mechanism involving a concerted proton-coupled electron transfer (**19** → **1** + **2**), because the C(3)-centered anion of the C(4)-difluoro phosphonate analogue (similar to **22a**) would likely eliminate a fluoride from C(4). However, no fluoride was detected in reaction mixtures and only the difluoro product (**24**) was obtained. The aforementioned conserved glutamate residue is the most likely candidate for the active site proton donor as its carboxylate group is located 4 Å from C(3) of **12** in the *A. aeolicus* IspH/**12** docking model.[38]

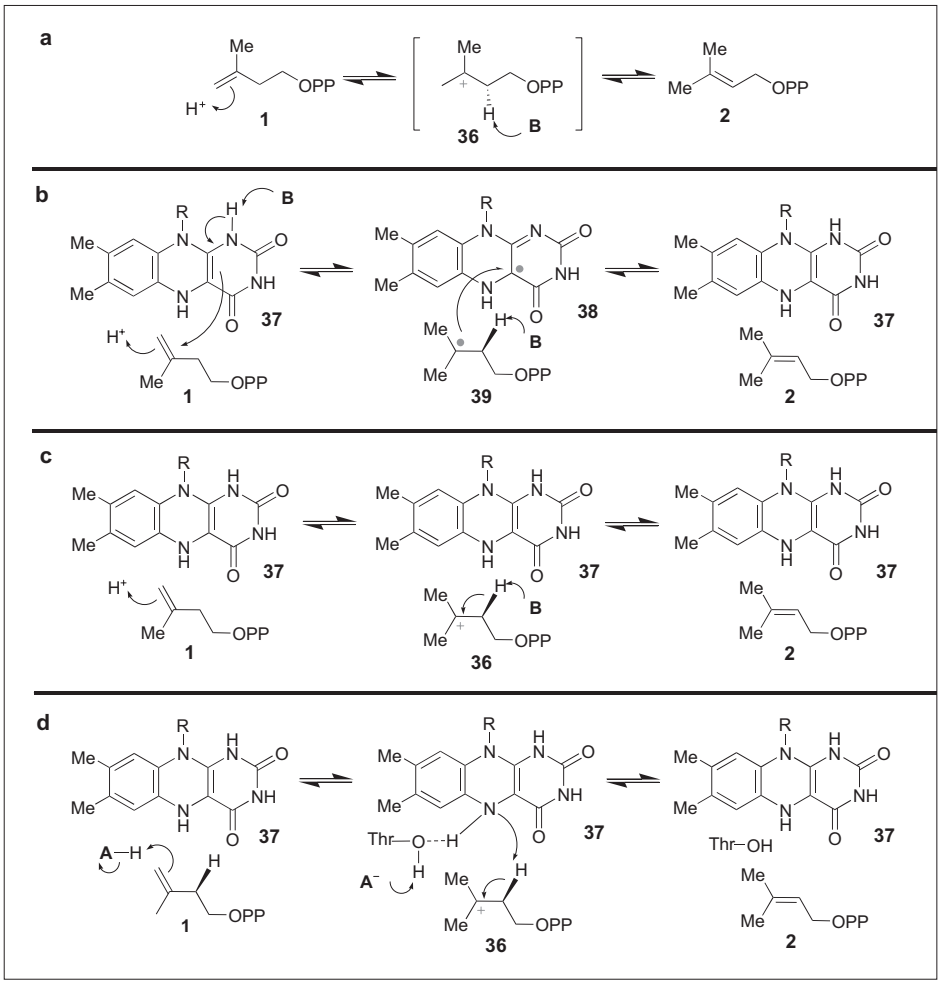
Our studies on the IspH-catalyzed reaction involve the synthesis and analysis of mechanistic probes designed to test for the presence of putative reactive intermediates during turnover. For example, compound **25** (Scheme 4c) can be used to determine if a radical anion intermediate, proposed to form in the first step of IspH catalysis, exists. If anion **26** indeed forms, this intermediate is expected to partition between hydroxide elimination (route a) and fluoride elimination (route b). Protonation and electron transfer could then give hydroxy compounds **27** and **12** and/or fluoro compounds **28** and **29**, depending on whether fluoride or hydroxide, respectively, is eliminated. In addition, increasing the number of fluorine atoms at C(1) of **25** may alter the partition ratio in favor of hydroxylated compounds by better facilitating fluoride elimination. The alkyne analogue **30** may be an irreversible inhibitor of IspH if the allylic anion forms the allene (**31** → **32**, Scheme 4d), which could then be trapped by an active site nucleophile to generate **33**. Alternatively, if both a proton and an electron are delivered simultaneously to the allylic radical **34** in a PCET mechanism, **30** may be turned over to **35**. These studies may provide the foundation for developing drugs targeting this unusual enzyme.

Type II Isopentenyl Diphosphate:dimethylallyl Diphosphate Isomerase (IDI-2)

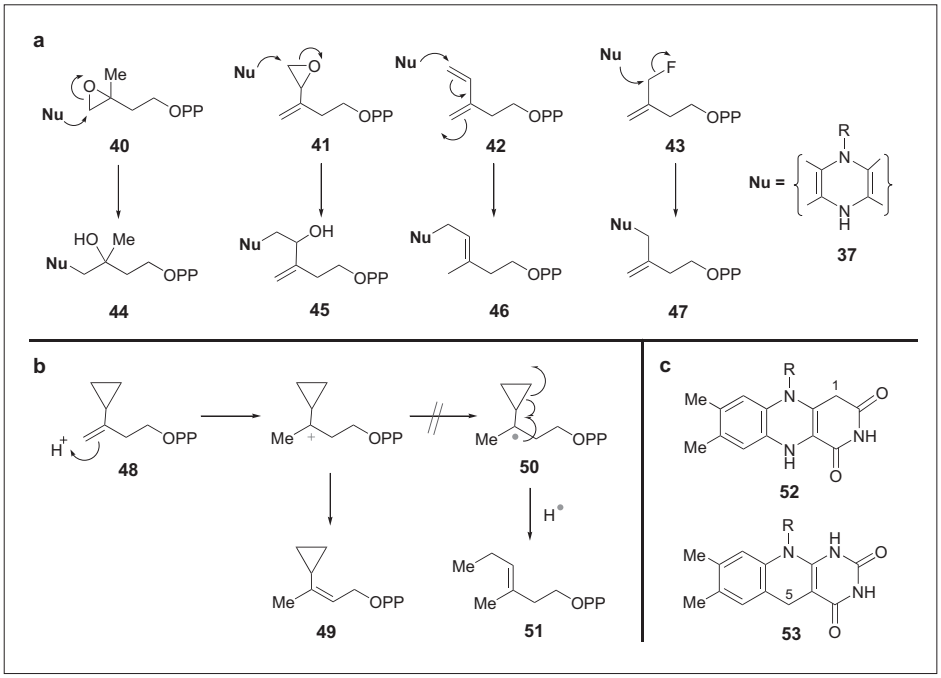
Isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI) catalyzes the reversible interconversion of the two ubiquitous isoprene units, IPP and DMAPP (**1** and **2**, respectively, Scheme 5). Two structurally and mechanistically distinct classes of IDI exist. The type I enzymes (IDI-1) employ divalent metal ions and active site acid/base chemistry to effect an overall proton addition/elimination reaction *via* a carbocation intermediate (**36**, Scheme 5a). In addition to a divalent metal ion, the type II enzymes (IDI-2) require a reduced flavin mononucleotide (FMN) coenzyme (**37**) for catalysis.[45] Because the interconversion between IPP and DMAPP involves no net change in redox state, the exact role of the flavin in IDI-2 catalysis is not obvious. While not unprecedented, flavoenzymes that catalyze reactions with no net redox change are rare.[46,47] Importantly, organisms that utilize the mevalonate pathway (including humans and bacterial pathogens in the *Streptococci*, *Staphylococci*, and *Enterococci* genera) require an IDI enzyme for survival. While humans have a type I enzyme, the aforementioned pathogens exclusively rely on a type II enzyme.

To date, no convincing evidence for a cryptic redox cycle in IDI-2 catalysis has been reported. No flavin semiquinone (such as **38**, Scheme 5b) could be detected in single-turnover stopped-flow assays, and EPR studies failed to detect the presence of a coupled FMN_{sq}/substrate radical pair (**38/39**) bound in the IDI-2 active site.[48–50] Instead, the neutral reduced FMN (**37**) forms rapidly in the pre-steady state upon IPP or DMAPP binding, suggesting that **37** is the flavin intermediate that accumulates prior to the rate limiting step.[48,50] Rapid-mix chemical-quench experiments and kinetic isotope effect studies demonstrated that the rate-limiting step(s) in the pathway likely involve deprotonation of IPP at C(2) and may also involve protonation of the IPP double bond by a solvent exchangeable proton.[50] Based on these findings, mechanisms involving the neutral reduced flavin as an electrostatic (Scheme 5c) or acid/base catalyst (Scheme 5d) have been proposed.[47,48,50] While an acid/base role for the reduced flavin would be unusual, the available X-ray crystal structures of IDI-2 solved in the presence of oxidized FMN with and without pyrophosphate, suggest a relative dearth of amino acid residues in the putative IPP binding pocket that could mediate acid/base chemistry.[51–53]

Interestingly, several irreversible inhibitors (**40**–**43**) form stable N(5)-alkyl adducts (**44**–**47**, respectively) with reduced FMN when incubated with IDI-2 (Scheme 6a).[54–56] These observations suggest that not only do these inhibitors bind in close proximity to the flavin, but also that the N(5) atom of reduced $FMNH_2$ (**37**) accumulates sufficient electron density to attack the electrophilic moieties of **40**–**42** or to displace the fluoride leaving group in **43**. Importantly, the double bond of the cyclopropyl analogue (**48**) was isomerized to **49**, arguing against a mechanism involving the formation of a 3º substrate radical (**50**), which would likely rearrange rapidly to **51** (Scheme 6b). While the irreversible inhibitors are artificially activated towards nucleophilic attack relative to the normal substrates, the crystal structures revealed that a conserved threonine residue (Thr67 in the *S. aureus* enzyme) is positioned such that it can hydrogen bond with the flavin N(5) and, thus, may be able to activate



Scheme 5.



Scheme 6.

the flavin for a catalytic role. Indeed, preliminary studies on a T67A IDI-2 mutant have shown that k_{cat} for the native reaction is reduced about 20-fold relative to the wild-type enzyme. The putative hydrogen bond between Thr67 and N(5) may serve to modulate the pK_a of the N(5) atom for acid/base catalysis (as in Scheme 5d) or to alter the local electron density at N(5) for an electrostatic stabilization role. These proposals involving the N(5) atom of FMNH$_2$ in catalysis are consistent with the observation that reduced 1-deaza-FMNH$_2$ (**52**) supports catalysis, while 5-deaza-FMNH$_2$ (**53**) does not (Scheme 6c).[49,57] Studies designed to more clearly delineate the function of the flavin in catalysis and to better determine the nature of the chemical mechanism are currently in progress.

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