Terpene Biosynthesis

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The Stereochemical Course and Mechanism of the IspH Reaction**

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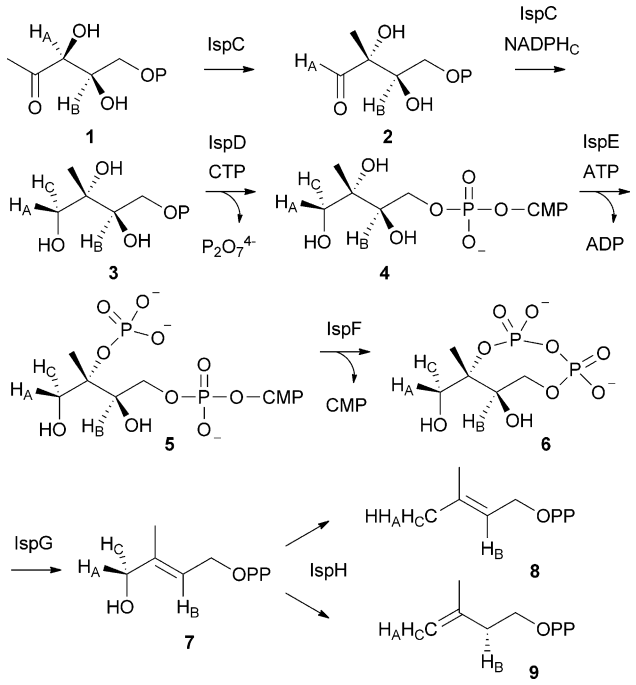
Two pathways exist for the biosynthesis of the terpene monomers dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP): the mevalonate pathway[1] and the deoxyxylulose phosphate (DOX) pathway.[2,3] Many pathogenic bacteria and the malaria parasite *Plasmodium falciparum* exclusively use the DOX pathway, which is essential in these organisms.[4,5] Since this pathway is not present in humans, its enzymes represent attractive targets for new antimicrobial drugs.[6] Therefore, a detailed understanding of the enzyme mechanisms is of high interest.

The intermediates and enzymes of the DOX pathway have all been identified (Scheme 1).[7] In particular, the unique mechanism of IspH that catalyzes the conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate (HMBPP, **7**) into an approximate 1:5 mixture of DMAPP (**8**) and IPP (**9**) has attracted considerable interest and is still under discussion. The crystal structure of IspH showed a central Fe_3S_4 cluster,[8] while other data were in favor of an Fe_4S_4 cluster.[9] Recently, the crystal structure of IspH in the presence of its substrate **7** was refined to a structure with an Fe_4S_4 cluster.[10] The fourth labile iron center binds the substrate **7**,[9d,10] as well as potent inhibitors such as pyridine diphosphates and alkynes.[11] Mössbauer parameters of the substrate-free enzyme suggested that this Fe center is coordinated by three S and two or three other ligands (O or N) that dissociate upon substrate binding.[9b,d]



Scheme 1. Stereochemistry of the DOX pathway. CTP = cytidine triphosphate, ATP = adenosine triphosphate, ADP = adenosine diphosphate, CMP = cytidine monophosphate.

Three mechanisms have been proposed for the IspH reaction (Scheme 2). Mechanism A, suggested by Rohdich et al. (Scheme 2 A), resembles a Birch reduction of complex **10** by a one-electron transfer, protonation, and elimination of water to the allyl radical **11**, and a second electron transfer to give the allyl anion **12**. Its protonation at C-2 or C-4 results in IPP and DMAPP, respectively.[12] This mechanism is supported by isotopic labeling experiments with substrate analogues.[13] Mechanism B, proposed by Wang et al. (Scheme 2 B), is based on the ENDOR spectroscopic detection of a paramagnetic intermediate trapped in the unreactive E126A mutant that was interpreted as a metallacyclopropane species.[14] Alternatively, this intermediate may also be described as an η^2-alkenyl π complex. The delineated mechanism proceeds from **10** by one-electron reduction to form the η^2-alkenyl/metallacycle **13** followed by a protonation/dehydration to give the η^1-allyl complex **14**. A second electron transfer yields **12** as the precursor for IPP and DMAPP. Mechanism C was proposed by Altincicek et al. and includes the initial protonation/dehydration of **10** assisted by the Lewis acidity of the metal center to generate the allyl cation **15**, which undergoes two reduction steps via **11** to **12** and a subsequent protonation to afford IPP and DMAPP.[15] This mechanism is contradicted by the minor effect of electron-withdrawing substituents in substrate analogues.[16]

Common intermediates of all three mechanisms are the initial complex **10** and the allyl anion **12**. The product ratio of IPP and DMAPP was suggested to be controlled by the proton transfer from the terminal phosphate group of HMBPP, where the two oxygen atoms are at a distance of 3.4–3.5 Å to C-1 and C-3 (Scheme 2 A).[10,12b] This hypothesis is in full agreement with the observed stereospecific C-3 protonation of HMBPP from the *Si* face.[17] The stereochemical course of almost all the steps of the DOX pathway have been studied.[18] Along this pathway, the C-3 hydrogen atom of **1** (H_A, Scheme 1) becomes the aldehyde hydrogen atom in **2** and ends up as the *pro-S* hydrogen atom at C-1 in **3**, whereas the *pro-R* hydrogen atom (H_C) is introduced by NADPH.[19] All subsequent transformations from **3** to **6** proceed with an

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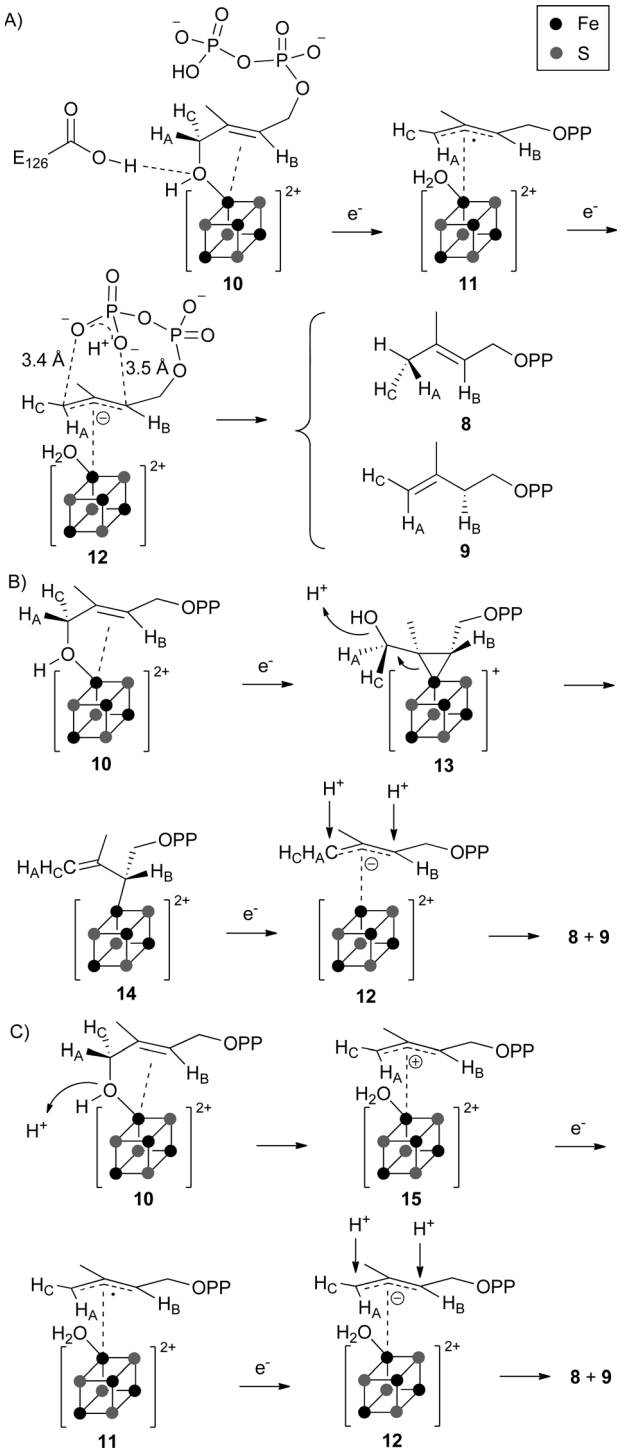
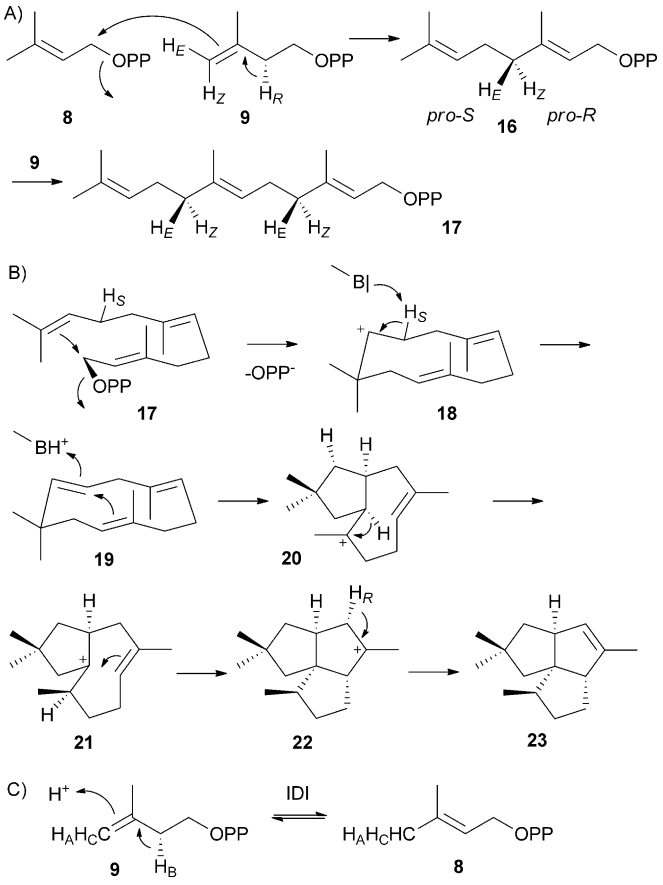


Scheme 2. Proposed mechanisms for the IspH reaction.

unchanged carbon backbone. Different mechanisms have been suggested for the transformation of **6** to HMBPP,[20] but it is well-established that C-2 and C-3 are deoxygenated and H_B is retained at C-3.[21] Only the stereochemical course of the IspH reaction has not been fully established: It is not known whether H_A or H_C in HMBPP ends up as the terminal olefinic (*E*)- or (*Z*)-hydrogen atom in IPP, a fate that is predetermined in intermediate **12** (Scheme 2). However, a prediction of the stereochemcial course can be made for mechanisms A and C: The OH group of HMBPP remains bound to Fe up to the step that determines the stereochemical outcome of the reaction. This should result in H_A being directed to the *Z* position and of H_C to the *E* position. In mechanism B, this OH group is released in **13**, thereby allowing rotation of the CH_2OH group. Consequently, no prediction of the stereochemical outcomes of H_A and H_C is possible. Protonation at C-1 delivers a potentially chiral methyl group in DMAPP with unknown configuration, but the absolute configuration shown in Scheme 2 A should result from mechanisms A and C. We report here on the stereochemical course of the IspH reaction and its implications for the mechanism of the enzyme.

Deuterated isotopologues of 1-deoxy-D-xylulose, phosphorylated intracellularly to yield **1**, were used in feeding experiments to investigate the stereochemical course of the IspH reaction. Deuterium labeling of H_A can, in particular, give important insights. A suitable system to follow the deuterium labeling is pentalenene biosynthesis in streptomycetes, since the detailed stereochemical course of all the transformations to this sesquiterpene are known.

The reaction of DMAPP or any advanced polyisoprenoid diphosphate such as geranyl diphosphate (GPP, **16**) proceeds with attack of IPP at C-4 from its *Si* face[22] and abstraction of the *pro-R* hydrogen atom (Scheme 3 A).[23] Thus, the terminal olefinic H_E of IPP ends up in the 4-*pro-S* position and H_Z is

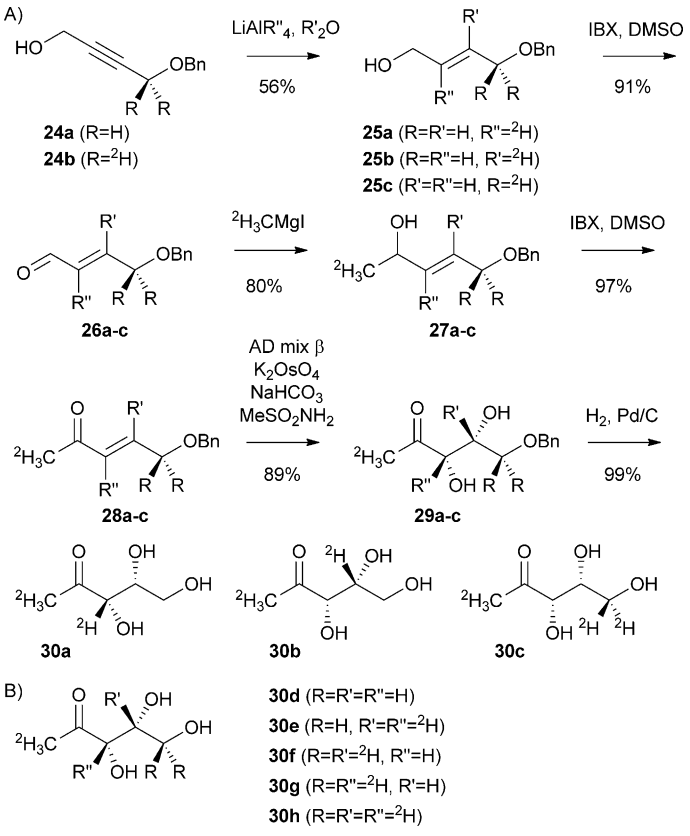


Scheme 3. Stereochemical course of A) polyisoprenoid biosynthesis, B) pentalenene biosynthesis, and C) the IDI reaction.

found as the 4-*pro-R* proton. The conversion of farnesyl diphosphate (FPP, **17**) into pentalenene (**23**, Scheme 3B) is initiated by the cyclization to the humulyl cation (**18**). Specific removal of the 9-*pro-S* hydrogen atom yields α-humulene (**19**). The abstracted proton is reintroduced at C-10 without significant exchange with the medium.[24] Concomittant cyclization to **20**, a 1,2-H^- shift to **21**, cyclization to **22**, and deprotonation yields **23**. The final deprotonation proceeds with specific loss of the *pro-R* hydrogen atom,[25] which can be traced back to H_E in IPP. Therefore, the conversion of H_A in **1** into either H_E or H_Z in IPP in the IspH reaction can be followed by its fate in this step.

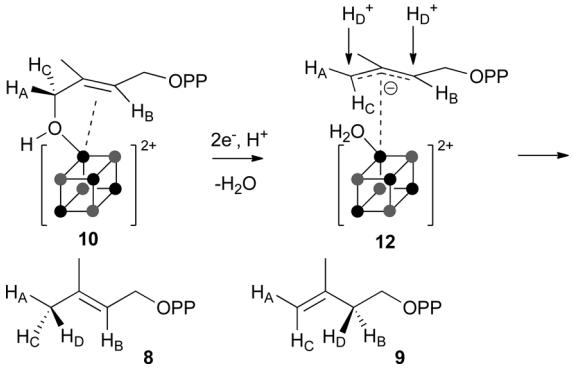
A series of deuterated isotopologues of 1-deoxy-D-xylulose (**30**) were synthesized for the feeding experiments (Scheme 4). All of these compounds were fed to *Streptomyces avermitilis* and the incorporation of deuterium into **23** was analyzed by GC-MS. An advantage of this analytical method is the possibility to separate the deuterated from unlabeled isotopologues gaschromatographically (see Figure S1 in the Supporting Information).[26] Since this effect becomes more significant as the deuterium content of the analyte increases, the methyl groups in all of the synthesized isotopologues of **30** were deuterated.

A flexible route for the introduction of deuterium into **30** was developed based on a procedure by Giner (Scheme 4A).[27] But-2-yn-1,4-diol monobenzyl ether (**24a**) was treated with $LiAl^2H_4$ and then quenched with water to obtain **25a**. Oxidation with IBX gave **26a** without any detectable loss of deuterium. The reaction with $^2H_3CMgI$ resulted in **27a**, which afforded **28a** after oxidation with IBX. Sharpless dihydroxylation with AD-mix β and catalytic hydrogenation furnished **30a**. For the synthesis of **30b**, alkyne **24a** was reduced with $LiAlH_4$ and then quenched with ^2H_2O, while **30c** was obtained from deuterated **24b**, which is available from *tert*-butyldimethylsilyl-protected propargylic alcohol (see the Supporting Information). The other isotopologues **30d–h** (Scheme 4B) were all obtained by the same route by using the appropriate combination of unlabeled and deuterated reagents.



Scheme 4. Synthesis of deuterated 1-deoxy-D-xylulose. IBX = 1-hydroxy-1,2-benziodoxol-3(1*H*)-one 1-oxide, DMSO = dimethyl sulfoxide.

Feeding of **30a** afforded $[^2H_{11}]$-**23** (see Figure S2A,B in the Supporting Information). The biosynthesis of [4,5,5,5-$^2H_4$]DMAPP and [4,5,5,5-$^2H_4$]IPP can be expected from **30a** ($H_A = {}^2H$). Since only 11 of the 12 deuterium atoms in the three isoprene units of **23** are retained and one is lost, this experiment clearly demonstrates that the stereochemical course of the IspH reaction directs H_A in **10** into the *E* position and H_C into the *Z* position of IPP (Scheme 5). Although no direct evidence is obtained, the potentially chiral methyl group $-CH_AH_BH_D$ in DMAPP may have the absolute configuration shown.



Scheme 5. Stereochemical course of the IspH reaction.

Feeding of **30b** resulted in $[^2H_{10}]$-**23** (Figure S2C in the Supporting Information). This is fully consistent with a conversion of **30b** into [2,5,5,5-$^2H_4$]DMAPP and (2*R*)-[2,5,5,5-$^2H_4$]IPP ($H_B = {}^2H$) as well as the formation of $[^2H_{10}]$FPP by a loss of two deuterium atoms (H_B) from the IPP elongation units during the biosynthesis of polyisoprenoid. These data reinforce the previously observed stereospecific C-3 protonation of HMBPP from the *Si* face. The reason for some loss of an additional deuterium, as indicated by the ion at *m*/*z* 213, is the partial isomerization of (2*R*)-[2,5,5,5-$^2H_4$]IPP to [5,5,5-$^2H_4$]DMAPP by isopentenyl diphosphate isomerase (IDI, Scheme 3C), a reaction that specifically removes the *pro-R* hydrogen atom (H_B) for both type I and type II IDI,[17,23a,28] and its incorporation into **23**. A type I IDI, but no type II enzyme, is encoded in the genome of *S. avermitilis*.

Feeding of **30c** resulted in $[^2H_{15}]$-**23** (Figure S2D in the Supporting Information), with retention of all 15 deuterium atoms. This result corroborates the previous finding that the conversion of cation **18** via **19** into **20** (Scheme 3B) proceeds without proton exchange with the medium, in full agreement

with the known mechanism of the pentalenene synthase.[24,25] Feeding experiments with the isotopologues **30 d–h** confirmed the results obtained with **30 a–c** (see the Supporting Information).

The delineated stereochemical course of the IspH reaction (Scheme 5) has some important implications on the enzyme mechanism (Scheme 2). As outlined above, it is difficult to understand the stereochemical course of $H_A \rightarrow H_E$ and $H_C \rightarrow H_Z$ through mechanisms A and C, whereas in mechanism B the OH group of HMBPP is released from the Fe center in **13**. This allows rotation of the CH_2OH group into a conformation that readily explains the observations. Interestingly, such a conformation was recently described by Glide docking calculations and molecular mechanics optimization (Figure 1),[14] and was also recently observed in the crystal structures of several IspH mutants.[29] In summary, the stereochemical course of the IspH reaction is consistent with mechanism B.

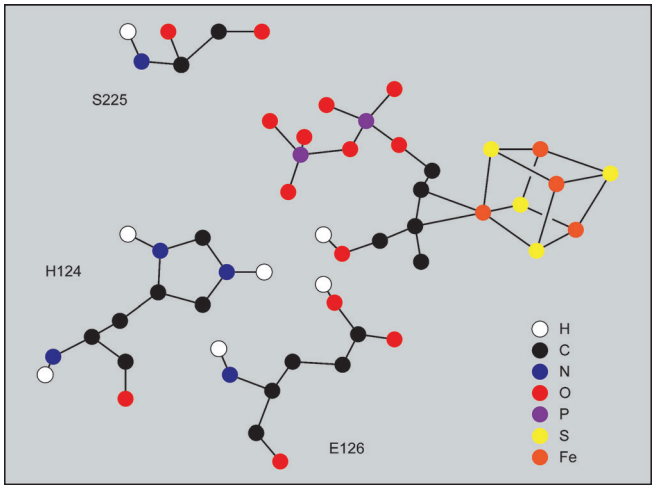


Figure 1. Calculated docking of HMBPP to the active site of IspH.[14]

In conclusion, we have presented the stereochemical course of the IspH reaction giving important insights in the enzyme mechanism. This will allow for the precise localization of deuterium in feeding experiments concerning the biosynthesis of terpenes made through the DOX pathway. The consequences of the mechanism of IspH may assist in the rational design of new IspH inhibitors as potential drugs.

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