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Stereochemical Analysis of Isopentenyl Diphosphate Isomerase Type II from *Staphylococcus aureus* Using Chemically Synthesized (*S*)- and (*R*)-[2-^{2}H]Isopentenyl Diphosphates

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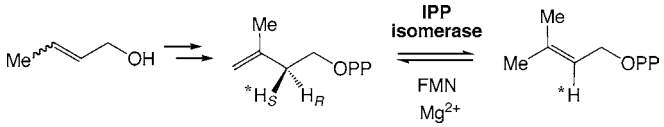
ABSTRACT

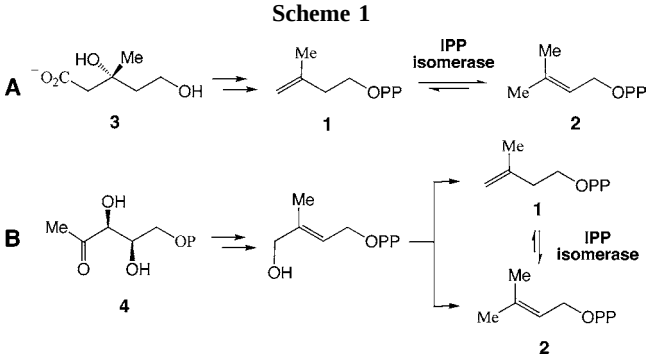


To study the catalysis of isopentenyl diphosphate (IPP) isomerase type II from *Staphylococcus aureus*, which is a flavoprotein catalyzing the interconversion of IPP and dimethylallyl diphosphate, we have chemically synthesized (*S*)- and (*R*)-[2-^{2}H]IPP and carried out stereochemical analysis of the reaction. Our results show that the C-2 deprotonation of IPP by this enzyme is pro-*R* stereospecific, suggesting a similar stereochemical course as the type I enzyme.

Isoprenoids, such as steroids, terpenoids, carotenoids, and ubiquinones, play important roles in all living organisms.[1] The basic building blocks for assembling these compounds are two 5-C precursors, isopentenyl diphosphate (IPP, **1**) and dimethylallyl diphosphate (DMAPP, **2**). In eukaryotic organisms IPP is the primary product of the mevaloante (**3**) pathway,[2] and DMAPP is derived from IPP by the action of IPP isomerase (Scheme 1, route A).[3] In contrast, both IPP and DMAPP are produced from 1-deoxy-D-xylulose (**4**) in most bacteria and green algae,[4] where IPP isomerase, if it is present, may play a role to fine-tune the ratio of **1** and **2** to meet the specific needs for the downstream processes (Scheme 1, route B).[5]

Two types of IPP isomerase are known. The type I enzyme,[6] which requires only divalent metal ions for activity, catalyzes isomerization via a carbocation intermediate and

Scheme 1



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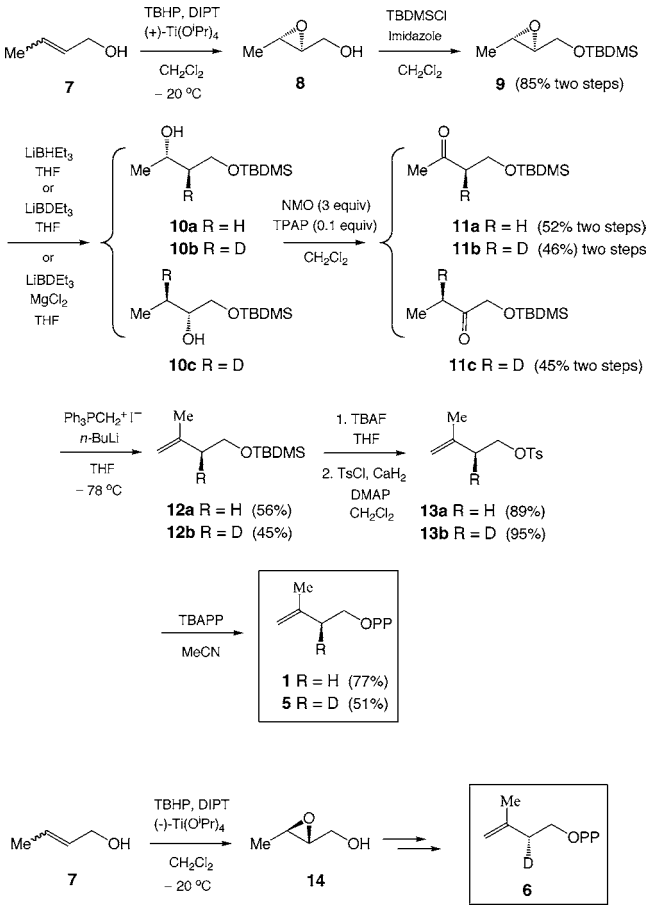
has been extensively characterized.[7] In contrast, the more recently discovered type II isomerase[5,8] is a flavoprotein whose activity requires divalent metal ion and NAD(P)H.[9] Since the cofactor requirements for these two types of IPP isomerase are so different, the type II isomerase may operate by a mechanism distinct from that of the type I enzyme.[10] Thus far, no mechanistic information about the type II isomerase has been reported,[11] presenting an exciting opportunity to initiate an investigation into this unusual enzyme. In addition, the fact that the type II isomerase is essential for some pathogens, including multidrug-resistant strains of *Staphylococcus aureus*, *Streptococci*, and *Enterococci*, whereas only type I isomerase is used in mammals, makes it an attractive target for therapeutic agents.[4b,5,8]

The reaction catalyzed by type I isomerase is a well-established reversible 1,3-antarafacial process involving the loss of the 2*R*-hydrogen of IPP and the addition of a solvent hydrogen to C-4 of DMAPP.[12] Although the mechanism of type II isomerase remains elusive, the stereochemical course of the deprotonation step for the type II enzyme from *Bacillus subtilis* has been determined to be similar to that of type I enzyme on the basis of an elegant, yet complex, labeling study.[13] In a separate report, prolonged incubation of IPP/DMAPP with the type II isomerase from *Synechocystis* PCC 6803 in ^2H_2O led to the incorporation of one deuterium at C-2 and two deuteria at C-4 of IPP.[14] Meanwhile, all three hydrogens of the (*E*)-methyl group of DMAPP were exchanged with deuterium. Interestingly, under similar conditions with the type I enzyme from yeast, only the protons at C-1 of IPP and DMAPP remained unexchanged. It was thus concluded that the reaction catalyzed by the *Synechocystis* type II enzyme is more stereoselective than the one catalyzed by the yeast type I isomerase.[14] However, the actual stereospecificity of the C-2 deprotonation step of the *Synechocystis* isomerase was not determined.

As part of our efforts to investigate the mechanism of type-II isomerase, we have recently expressed the gene for the type II isomerase from *Staphylococcus aureus* in *Escherchia coli* according to the reported protocols[8a] and carried out a stereochemical analysis of the reaction. Reported herein are the results, which clearly indicate that the C-2 deprotonation of IPP by the *Staphylococcus* isomerase is pro-*R* stereospecific, suggesting an analogous stereochemical course as the type I isomerase.

To facilitate the analysis, a new and convenient chemical synthesis for preparing the stereospecifically labeled (*S*)-[2-^{2}H]IPP (**5**) and (*R*)-[2-^{2}H]IPP (**6**) was developed.[15] As depicted in Scheme 2, the synthesis of (*S*)-[2-^{2}H]IPP (**5**) begins with (2*R*)-*trans*-3-methyloxiranemethanol (**8**), which was the product of Sharpless epoxidation of crotyl alcohol

Scheme 2



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(9) Under aerobic conditions, NADPH is required for enzyme activity. However, if purified and assayed under strictly anaerobic conditions, the addition of NADPH is no longer necessary.[5]

(10) The crystal structure of the type II isomerase from *Bacillus subtilis* was recently determined; however, the substrate (**1**/**2**) binding site could not be clearly defined (Steinbacher, S.; Kaiser, J.; Gerhardt, S.; Eisenreich, W.; Huber, R.; Bacher, A.; Rohdich, F. *J. Mol. Biol*. **2003**, *329*, 973−982).

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(15) Compounds **5** and **6** were traditionally prepared enzymatically from labeled mevalonic acid (see: Cornforth, J. W.; Cornforth, R. H.; Donninger, C. Popjak, G. *Proc. R. Soc. London, Ser. B* **1965**, *163*, 492−514. Sagami, H.; Ogura, K.; Seto, S. *Biochemistry* **1977**, *16*, 4616−4622). They could also be chemically synthesized from dimethylally alcohol (Suga, T.; Ohta, S.; Ohmoto, T. *Chem. Soc., Perkin Trans. 1* **1987**, 2845−2848). A synthesis of **6** starting from D-mannitol 1,2:5,6-bis-acetonide in high enantiomeric excess was recently reported (Leyes, A. E.; Poulter, C. D. *Org. Lett*. **1999**, *1*, 1067−1070).

(**7**) using (+)-diisopropyltartarate as a catalyst.[16] The enantiomeric purity of **8** was estimated to be greater than 95% by the comparison of its optical rotation data with the reported value.[16] Silyl protection of the hydroxyl group followed by reduction of **9** by lithium triethylborohydride (super hydride) led to the formation of **10a** as the sole product. To our surprise, an opposite regiospecific ring opening was observed when the reduction of **9** was carried out using lithium triethylborodeuterride (super deuteride), in which compound **10c** emerged as the only identifiable product (58% yield). Interestingly, the regioselectivity was sensitive to the presence of Lewis acid in the reaction mixture. An extensive survey of different conditions led to the finding that an 11:1 product ratio of **10b** and **10c** could be achieved when the reaction was carried out with a stoichiometric amount of anhydrous $MgCl_2$.

Although the byproduct **10c** could be removed by silica gel chromatography, the isolated yield of **10b** was low as a result of its high volatility. Thus, to avoid the unnecessary loss of **10b**, the mixture of **10b** and **10c** was treated with oxidizing agents without purification to make the corresponding keto products. Among the various oxidants tested, including Dess−Martin periodant, *O*-iodoxybenzoic acid (IBX), RuO_4, and *N*-methylmorpholine *N*-oxide (NMO)-tetrapropylammonium perruthenate (TPAP), NMO-TPAP, was found to be most effective. However, the pretreatment of the reaction mixture with excess NMO was crucial for consistent results. In addition to its co-oxidant role, this reagent may serve as a scavenger to neutralize the residual reducing species remaining from the previous step. The resulting 3-keto and 2-keto products (**11b** and **11c**, respectively) were separated, and compound **11b** was subjected to olefination under Wittig conditions to give *tert*-butyldimethyl-((2*S*)-3-methyl-[2-^{2}H]-but-3-enyloxy)-silane (**12b**) in 45% yield.

Deprotection of **12b** by anhydrous tetrabutylammonium fluoride (TBAF) followed by tosylation of the isopentenyl alcohol product afforded **13b** in 95% yield. The remaining moisture from the deprotection reaction was found to complicate the subsequent tosylation, so that the inclusion of anhydrous CaH_2 in the tosylation step was important to ensure a complete transformation. The final pyrophosphorylation step to generate **5** was accomplished based on a literature procedure[17] in 51% yield. The (*R*)-[2-^{2}H]IPP (**6**) was prepared from the corresponding 2*S*-isomer of **8** (i.e., **14**) in an analogous manner.[18]

With these labeled IPPs in hand, we proceeded to determine the stereospecificity of the C-2 deprotonation step catalyzed by the *Staphylococcus* enzyme, which was purified and established to be a flavoprotein as previously reported.[8a] Analogous to other members of type II isomerases, NAD(P)H as well as a divalent metal ion are required for its activity. Accordingly, the reaction mixture containing 10 mM of labeled IPP, 1 mM NADPH, 10 μM FMN, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 10 μM of enzyme in 1 mL of 100 mM potassium phosphate buffer (pH 7.0) was prepared. The reaction was incubated at 37 °C for 20 h and stopped by rapid freezing in liquid nitrogen. The samples were lyophilized, redissolved in D_2O, and lyophilized again. The dry residue was then dissolved in D_2O and subjected to NMR analysis. The sample derived from (*R*)-[2-^{2}H]IPP (**6**) shows two methyl singlets at δ 1.56 (3H, s) and 1.61 (3H, s), an olefinic proton resonance at δ 5.30 (1H, m), and a methylene signal at δ 4.31 (2H, t, J = 7 Hz) (Figure 1, top),

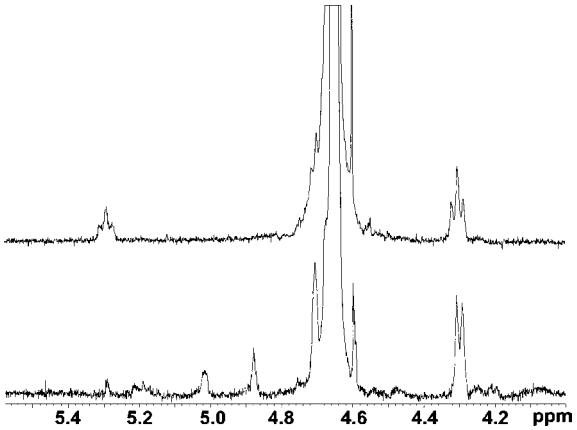

Figure 1. ^{1}H NMR spectra showing the C-1 methylene and C-2 olefinic region of DMAPP generated in the incubation of (*R*)-[2-^{2}H]IPP (**6**) (top) or (*S*)-[2-^{2}H]IPP (**5**) (bottom) with the *Staphylococcus* type II IPP isomerase.

which are identical to an authenic DMAPP spectrum.

In contrast, the spectrum of the sample derived from (*S*)-isomer (**5**) exhibits signals at δ 1.59 (3H, s), 1.63 (3H, s), and 4.30 (2H, d, J = 5 Hz) (Figure 1, bottom). The disappearance of the δ 5.30 signal and the corresponding change of the δ 4.30 signal to a doublet clearly demonstrated retention of the deuterium label at C-2 in this sample. On the basis of these observations, it can be concluded that the enzyme removes only the hydrogen resided at the H_{re} position of C-2 of IPP during catalysis. A complementary experiment was also performed with the type I IPP isomerase isolated from *E. coli*.[19] As expected, the same pro-*R* stereochemical preference was noted for this enzyme. This observation also confirms the stereochemical assignment of the isotope labeling in **5** and **6**.[20]

This work is significant for two reasons. First, a convenient chemical method for the preparation of stereospecifically

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(18) See Supporting Information for details.

(19) The *Escherichia coli* type I isomerase gene was amplified from the genomic DNA, cloned into pET24b(+) vector and expressed in *E. coli* BL21 under the induction by 0.5 mM isopropyl β-D-thiogalactoside (IPTG). The *C*-terminal His_6-tagged enzyme was purified to greater than 90% purity using Ni-NTA chromatography. The assay mixture contained 10 mM labeled IPP, 200 mM KCl, 20 mM $MgCl_2$, 1 mM DTT, 5 μM isomerase in 1 mL of 100 mM potassium phosphate buffer, pH 7. The workup procedure and subsequent NMR analysis were the same as those used for type II enzyme.

(20) Attempt to determine the chiral purity of **5** and **6** in poly γ-benylglutamate methylene chloride solution based on a published method[14] was unsuccessful.

labeled IPP, which is the biosynthetic precursor for isoprenoids in all living organisms, was developed. The ready availability of these labeled IPPs should find wide application in stereochemical studies of enzymes involved in the biosynthesis of a great variety of secondary metabolites. Second, the stereoselectivity of C-2 deprotonation catalyzed by the *Staphylococcus* IPP isomerase has been unambiguously determined to be pro-*R* specific, a preference identical to its counterpart from *Bacillus subtilis*. The pro-*R* stereospecificity is conserved among all type I and type II IPP isomerases studied thus far. Clearly, despite the different cofactor requirements for these two classes of IPP isomerases, the active sites of type II isomerases likely share features with those of the type I enzymes in controlling the orientation of the enzyme−substrate complex. Experiments are in progress to explore the implications of these results on the mechanism of this unusual enzyme.

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Supporting Information Available: Experimental procedures for the preparation of stereospecifically labeled **5** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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