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Mechanism of monoterpene cyclization: stereochemistry of the transformation of noncyclizable substrate analogs by recombinant (−)-limonene synthase, (+)-bornyl diphosphate synthase, and (−)-pinene synthase

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Abstract

All monoterpene cyclases investigated to date are capable of overcoming the topological impediment to direct cyclization of the universal, acyclic C10 intermediate of isoprenoid biosynthesis geranyl diphosphate. Although strong suggestive evidence has been accumulated for the intermediary linalyl diphosphate in cyclase catalysis, all previous efforts to directly observe this product at the mandatory isomerization step have failed. (−)-4*S*-Limonene synthase from spearmint (*Mentha spicata*), (+)-bornyl diphosphate synthase from sage (*Salvia officinalis*), and (−)-pinene synthase from grand fir (*Abies grandis*) have been expressed in *Escherichia coli* and the recombinant enzymes have been isolated and purified. These enzymes were examined with the noncyclizable substrate analogs 6,7-dihydrogeranyl diphosphate and 2,3-methanogeranyl diphosphate to gain insight into the normally cryptic isomerization step of the reaction sequence. The analogs were catalytically active, affording acyclic olefins and alcohols as products. Chiral phase gas chromatography and mass spectrometry analysis provided evidence that the normal cyclization of geranyl diphosphate by (−)-4*S*-limonene synthase and by (−)-pinene synthase proceeds via preliminary isomerization to the bound tertiary intermediate 3*S*-linalyl diphosphate, whereas the cyclization catalyzed by (+)-bornyl diphosphate synthase proceeds via the intermediate 3*R*-linalyl diphosphate.

Keywords: (−)-4*S*-Limonene synthase; (−)-Pinene synthase; (+)-Bornyl diphosphate synthase; Linalyl diphosphate; Geranyl diphosphate

1. Introduction

The monoterpenoids are the simplest class of terpenoids in that they contain only 10-carbon atoms, and most members of this group are constructed by the monoterpene synthases (cyclases) that catalyze the conversion of geranyl diphosphate, the universal, acyclic C10 intermediate of isoprenoid biosynthesis, to the cyclic parents of the various monoterpene skeletal types. All monoterpene cyclases investigated to date are capable of overcoming the topological impediment to direct cyclization of geranyl diphosphate, imposed by the *trans*-geometry of the C2=C3 double bond, by way of a preliminary isomerization step that occurs without the formation of detectable free intermediates (Fig. 1). Although strong evidence

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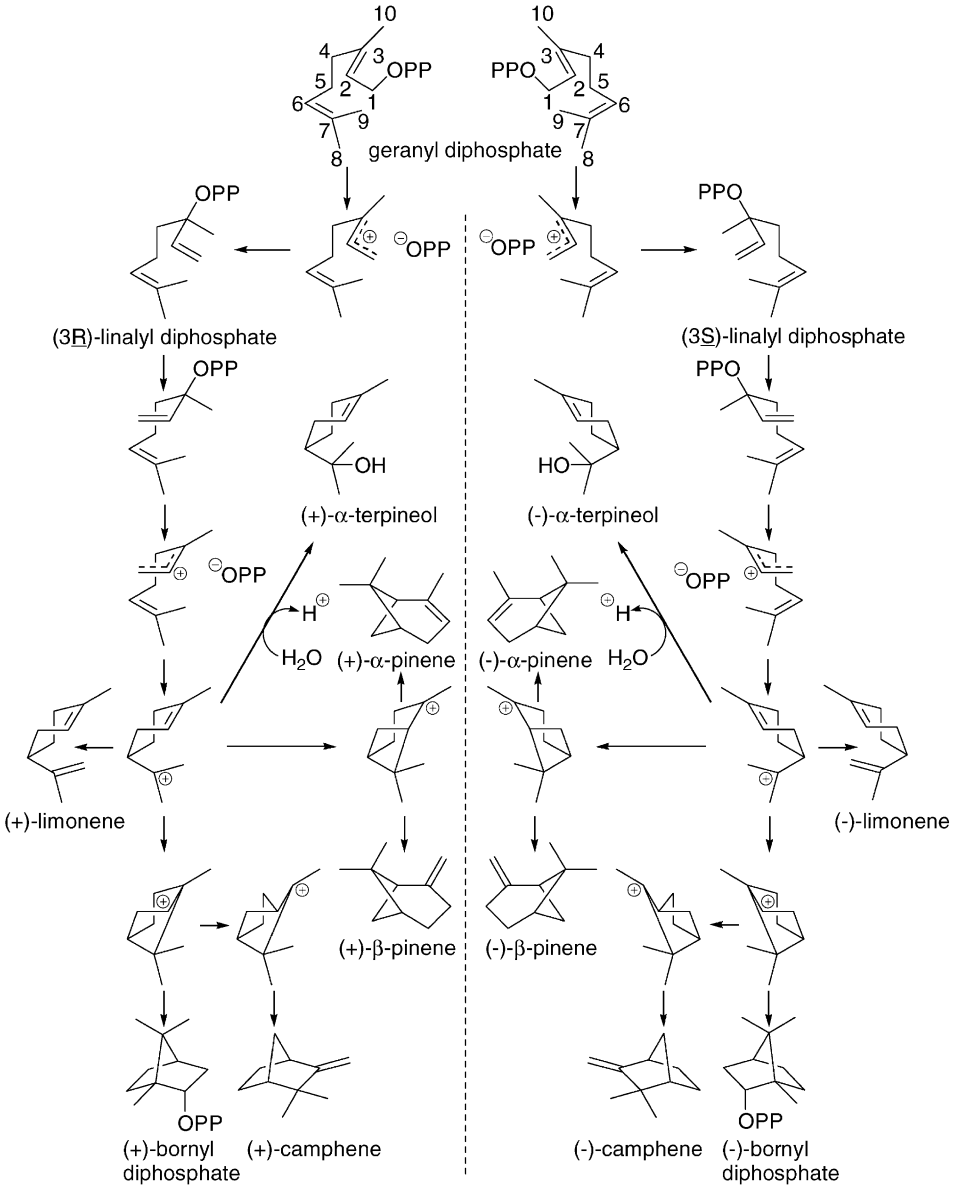

Fig. 1. Stereochemical scheme for the enzymatic conversion of geranyl diphosphate to monoterpene enantiomers. Formation of cyclic products requires preliminary isomerization of geranyl diphosphate to either 3*R*- or 3*S*-linalyl diphosphate. OPP denotes the diphosphate moiety.

has been accumulated for the intermediacy of linalyl diphosphate in the synthase catalysis [1], all previous efforts to observe this product directly have failed. In an attempt to dissect the cryptic isomerization step of the normally coupled reaction sequence, several strategies were devised for employing substrate analogs (**1** and **2**) (Figs. 2 and 3) that were competent to undergo the normal cyclase-catalyzed ionization–isomerization step but that would generate a corresponding tertiary diphosphate that could

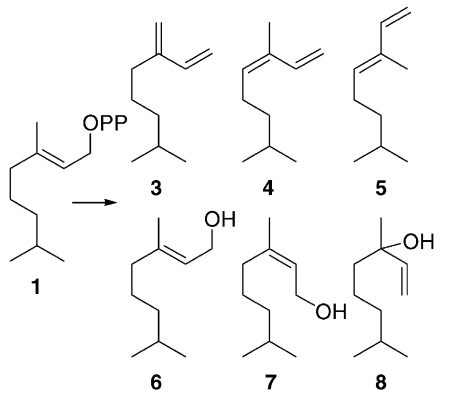

Fig. 2. Products formed by the monoterpene synthases from 6,7-dihydrogeranyl diphosphate **1**.

not cyclize or that was sufficiently unreactive in the subsequent ionization-cyclization step to allow escape from the active site [2,3]. Among other products 6,7-dihydrolinalol **8** (Fig. 2) and homolinalol **15** (Fig. 3) were formed by (+)-bornyl diphosphate synthase from **1** and **2**, respectively. However, the stereochemistry of **8** and **15** remained unknown due to the very small amounts of materials available from the native enzymes and the lack of suitable means for chiral resolution at the small scale. With the molecular cloning and functional heterologous expression of a range of monterpene cyclases, and the availability of chiral phase capillary columns to permit the GC-based resolution of monoterpene enantiomers, it has become possible to informatively revisit these earlier experiments with a more refined approach focused on stereochemical considerations. Recently, the monoterpene synthases (−)-4*S*-limonene synthase from spearmint (*Mentha spicata*), (+)-bornyl diphosphate synthase from sage (*Salvia officinalis*), and (−)-pinene synthase from grand fir (*Abies grandis*) have been expressed in *Escherichia coli* and the recombinant enzymes have been isolated and purified [4–6]. According to a mechanistic paradigm proposed by Croteau [1], (−)-4*S*-limonene synthase and (−)-pinene synthase form the cryptic intermediate 3*S*-linalyl diphosphate whereas (+)-bornyl diphosphate synthase generates the intermediate 3*R*-linalyl diphosphate (Fig. 1). To gain insight into the sereochemistry of the normally cryptic isomerization step of the reaction sequence, these enzymes were examined with the noncyclizable substrate analogs **1** and **2**.

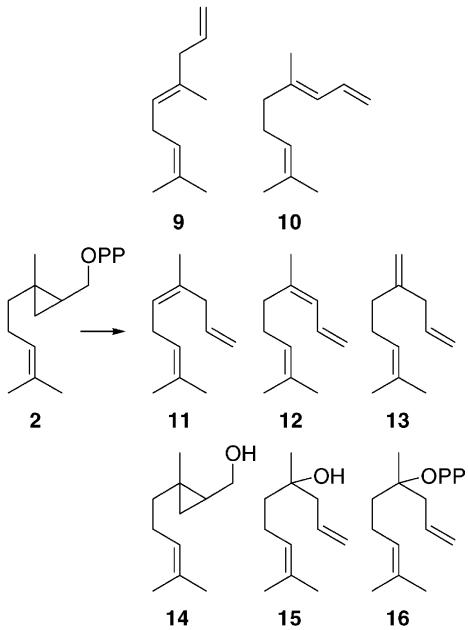

Fig. 3. Products formed by the monoterpene synthases from 2,3-methanogeranyl diphosphate **2**.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Fluka (Buchs, Switzerland). 6,7-Dihydrogeraniol and 6,7-dihydronerol were separated from a mixture (BASF AG, Ludwigshafen, Germany) by silica gel argentation chromatography with diethyl ether–pentane mixtures of increasing polarities. Identities of the products were confirmed by GC–MS analysis. Geranyl diphosphate, **1** and **2** were synthesized according to published procedures [2,3,7].

2.2. 6,7-Dihydrolinalol

Racemic 6,7-dihydrolinalol was synthesized by acid (1N HCl) catalyzed rearrangement of 6,7-dihyrogeraniol. *R*-6,7-Dihydrolinalol and *S*-6,7-dihydrolinalol were prepared from *R*-linalol and *S*-linalol (isolated

from coriander oil), respectively [8–10]. Racemization did not occur as products showed the same enantiomeric excess (ee) as the starting material *R*- and *S*-linalol with 80 and 40% ee, respectively.

2.3. Homolinalol

Racemic homolinalol was synthesized by the Grignard reaction [11]. Enantiomerically enriched homolinalol was prepared by the procedure of Boldrini et al. [12]. Assignment of the stereoisomers was achieved after degradation to 4-methylhexane-1,4,7-triol by ozonolysis [13] and synthesis of 2,2-dimethyl-4-(3′-hydroxypropyl)-4-methyl-1,3-dioxane by acetonide formation [14]. *S*-2,2-dimethyl-4-(3′-hydroxypropyl)-4-methyl-1,3-dioxane was synthesized from *S*-3-hydroxy-citronellol by ozonolysis [13] followed by acetonide formation [14]. *S*-3-Hydroxy-citronellol was obtained from *S*-linalol (isolated from coriander oil) according to the procedure of Ohloff et al. [8].

2.4. 2,3-Methanogeraniol

Racemic 2,3-methanogeraniol was prepared by modified Simmons–Smith reaction [11,15,16]. (+)-(2*S*,3*R*)- and (−)-(2*R*,3*S*)-methanogeraniol was synthesized according to the method of Charette et al. [17–19].

2.5. Isolation of enzymes, enzyme activity assay and protein determination

(−)-4*S*-Limonene synthase from spearmint (*M. spicata*), (+)-bornyl diphosphate synthase from sage (*S. officinalis*), and (−)-pinene synthase from grand fir (*A. grandis*) were expressed in *E. coli* and were isolated as previously described [4–6].

2.6. GC–MS analysis

Analysis was performed by using a Fisons MD 800 Quadrupol mass spectrometer coupled to a Fisons GC. A J & W DB-Wax 20 M fused silica capillary column (25 m × 0.25 mm i.d.; df = 0.25 μm), which was maintained at 50 °C for 3 min, then programmed to 240 °C at 4 °C/min, was used with helium gas at a flow rate of 3 ml/min.

2.7. Multidimensional gas chromatography (MDGC)

MDGC analyses were performed with two Fisons GCs (8160 and 8130). The first GC was fitted with a split injector (1:10, temperature 230 °C) and both GCs were equipped with FIDs at 250 °C. A J & W DB-Wax 20 M fused silica capillary column (25 m × 0.25 mm i.d., df = 0.25 μm) was used in the first GC for the pre-separation of volatiles. Separation of enantiomers was achieved in the second GC using a fused silica capillary column coated with 2,3-di-*O*-ethyl-6-*O*-*tert*-butyl-dimethylsilyl- β - cyclodextrin/PS086 (25 m × 0.25 mm i.d., df = 0.15 μm). The column in GC 1 was connected by a multicolumn switching system (MCSS, Fisons) to the column in GC 2.

2.8. NMR analysis

^{1}H and ^{13}C NMR spectra were recorded on a Bruker WM 400 (400 MHz) spectrometer with $CDCl_3$ (Merck, Darmstadt, Germany) as solvent.

3. Results and discussion

3.1. Incubation with geranyl diphosphate

(−)-4*S*-Limonene synthase from spearmint (*M. spicata*), (+)-bornyl diphosphate synthase from sage (*S. officinalis*), and (−)-pinene synthase from grand fir (*A. grandis*) were expressed in *E. coli* and isolated as previously described [4–6]. The purified enzymes were incubated with geranyl diphosphate and the stereochemistry of the isolated products were determined by gas chromatographic separation of their enantiomers and comparison with reference compounds. (−)-4*S*-Limonene synthase formed exclusively (−)-4*S*-limonene (ee > 90%) and (−)-pinene synthase produced (−)-alpha-pinene (ee > 90%) and (−)-beta-pinene (ee > 90%) as expected [4,6]. In contrast, (+)-bornyl diphosphate synthase formed (+)-alpha-pinene (ee > 90%), (+)-camphene (ee > 90%), (−)-4*S*-limonene (ee = 82%), *R*-(−)-linalol (ee = 6%), alpha-terpineol, borneol and geraniol. The formation of bornyl diphosphate by (+)-bornyl diphosphate synthase was confirmed by the detection of borneol after enzymatic hydrolysis of the residual

polar products. Except for (−)-4*S*-limonene, the product pattern was in accordance with recently published results for the heterologously expressed synthase [5].

3.2. Incubation with 6,7-dihydrogeranyl diphosphate *1*

The product pattern generated from **1** with the recombinant (+)-bornyl diphosphate synthase was identical to that previously published for the native enzyme counterpart [2]. In contrast, recombinant (−)-4*S*-limonene synthase from spearmint produced less terpenols and in the assay with recombinant (−)-pinene-synthase only trace amounts of 6,7-dihydrogeraniol were detected (Fig. 4). Enzymatically formed 6,7-dihydrolinalol **8** was only detected in the assays with (+)-bornyl diphosphate synthase. Regarding the olefins (−)-pinene-synthase was highly selective forming only 6,7-dihydromyrcene **3**. MDGC analysis of **8** formed by (+)-bornyl diphosphate synthase revealed an enantiomeric excess of 60% for the

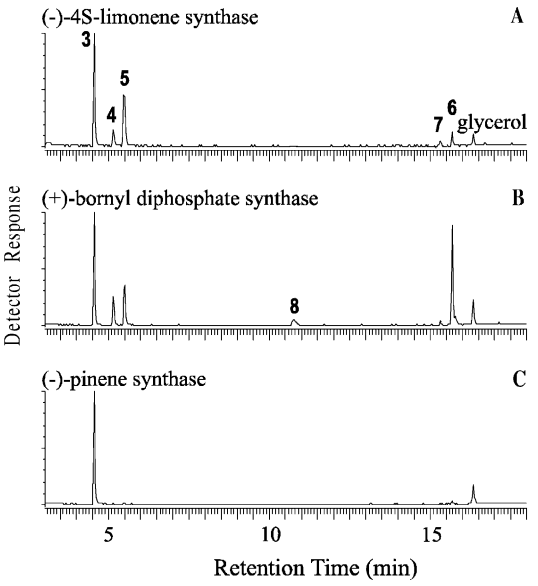

Fig. 4. Chiral phase capillary GC–MS analysis of products formed by recombinant (−)-4*S*-limonene synthase (A), (+)-bornyl diphosphate synthase (B), and (−)-pinene synthase (C) from 6,7-dihydrogeranyl diphosphate. See Fig. 2 for structure of the compounds corresponding to peaks numbered **3**–**8**. Glycerol is a constituent of the assay buffer that is extracted and carried through the analysis.

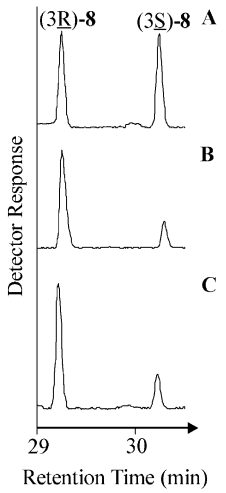

Fig. 5. Chiral phase capillary MDGC analysis of racemic 6,7-dihydrolinalol **8** (A) and **8** formed by recombinant (+)-bornyl diphosphate synthase after 1 h (B) and 24 h (C) of incubation with 6,7-dihydrogeranyl diphosphate **1**.

R-configurated alcohol by comparison with synthesized reference compounds (Fig. 5).

The stereochemistry of the linalol derivative is in accordance with the mechanistic paradigm proposed by Croteau [1] (Fig. 1). Thus, **1** is initially ionized and isomerized to form a 3*R*-linalyl intermediate (i.e. 3*R*-linalyl diphosphate). In the case of (+)-bornyl diphosphate synthase, the enzyme pocket for the substrate might be larger compared with the other enzymes under investigation as the diphosphate moiety is preserved in the product (+)-bornyl diphosphate. The diphosphate probably remains close to the active site, so that water has access to serve as the terminating nucleophile and to capture the cation forming *R*-6,7-dihydrolinalol (and *R*-linalol after the incubation with geranyl diphosphate) [1]. We assume that the intermediate of the (−)-4*S*-limonene synthase and (−)-pinene synthase is tightly bound in the smaller and nonpolar active site of these enzyme preventing the invasion of water.

3.3. Incubation with 2,3-methanogeranyl diphosphate *2*

2,3-Methanogeranyl diphosphate **2** served as a second noncyclizable substrate analog. The product pattern obtained with the recombinant (+)-bornyl

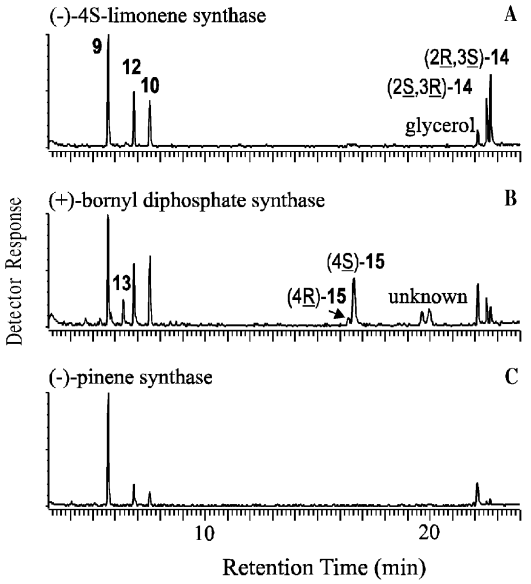

Fig. 6. Chiral phase capillary GC–MS analysis of products formed by recombinant (−)-4*S*-limonene synthase (A), (+)-bornyl diphosphate synthase (B), and (−)-pinene synthase (C) from racemic 2,3-methanogeranyl diphosphate. See Fig. 3 for structures of the compounds corresponding to peaks numbered **9**–**15**. Glycerol is a constituent of the assay buffer that is extracted and carried through the analysis.

diphosphate synthase confirmed the results achieved with the native enzyme counterpart [3]. (−)-Pinene synthase was again the most selective enzyme whereas only (+)-bornyl diphosphate synthase produced homolinalol **15** (Fig. 6). Chiral phase capillary GC–MS and MDGC analysis revealed the formation of *S*-homolinalol (ee = 64%) by (+)-bornyl diphosphate synthase. The enantiomeric excess of *S*-homolinalol remained stable (ee = 64%) during a time course experiment (0–24 h) with (+)-bornyl diphosphate synthase (Fig. 7). In addition, we were able to separate and assign the enantiomers of 2,3-methanogeraniol **14** by chiral phase capillary GC analysis (Fig. 6) by comparison with synthesized reference compounds [17–19]. We observed an enrichment of (2*S*,3*R*)-methanogeraniol in the case of (+)-bornyl diphosphate while the opposite enantiomer (2*R*,3*S*) was enriched in the case of (−)-4*S*-limonene synthase and (−)-pinene synthase. After phosphatase treatment of the residual **2**, the enantiomeric excess of the released alcohols was even more obvious (Fig. 8). 2,3-Methanogeraniol **14**,

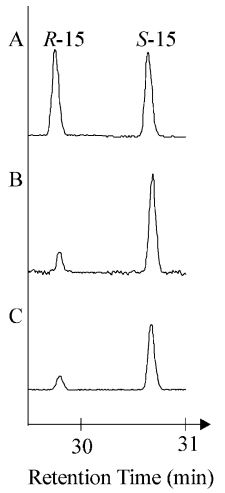

Fig. 7. Chiral phase capillary MDGC analysis of racemic homolinalol **15** (A) and **15** formed by recombinant (+)-bornyl diphosphate synthase after 1 h (B) and 24 h (C) of incubation with 2,3-methanogeranyl diphosphate **2**.

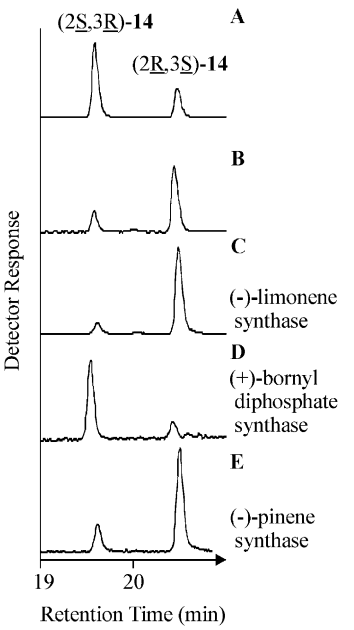

Fig. 8. Chiral phase capillary MDGC analysis of (2*S*,3*R*)- (A) and (2*R*,3*S*)- (B) enriched 2,3-methanogeraniol **14** obtained by chemical synthesis and of **14** released by phosphatase treatment of the residual **2** remaining after incubation with recombinant (−)-4*S*-limonene synthase (C), (+)-bornyl diphosphate synthase (D) and (−)-pinene synthase (E).

released by phosphatase treatment in the control experiments (heat-inactivated enzymes) was racemic. This result implies that (+)-bornyl diphosphate synthase prefers (2*R*,3*S*)-methanogeranyl diphosphate as substrate while (2*S*,3*R*)-methanogeranyl diphosphate accumulates during the enzymatic transformation. In the case of (−)-4*S*-limonene synthase and (−)-pinene synthase, the (2*S*,3*R*)-enantiomer is transformed and the (2*R*,3*S*)-enantiomer accumulates.

The generation of the 4*S*-homolinalol antipode from racemic **2** is the opposite absolute stereochemistry of nucleophile capture from that observed in the preferential conversion of 6,7-dihydrogeranyl diphosphate **1** to 3*R*-dihydrolinalol by this enzyme. We postulate that the opposite stereochemistry in the former case appears to be a direct consequence of the stereoselective utilization of (2*R*,3*S*)-methanogeranyl diphosphate by (+)-bornyl diphosphate synthase. Thus, upon binding, (2*R*,3*S*)-methanogeranyl diphosphate can be assumed to adopt a left-handed helical folding appropriate for the stereochemically consistent *syn*-isomerization, anti,endo-cyclization conducted by this enzyme. In this conformation, the cyclopropyl methylene group of (2*R*,3*S*)-methanogeranyl diphosphate faces downward from the C1–C2–C3 plane. Ionization and ring opening, while removing the steric constraint imposed by the downward facing cyclopropyl methylene, exposes the pro*S*-face of the tertiary carbocation to which water capture effectively competes with protective repositioning of the terminal isopropylidene group to shield the cation from 'backside' access. The formation of 3*R*-dihydrolinalol and 4*S*-homolinalol by (+)-bornyl diphosphate synthase therefore can be seen to result from the spatial orientation of the respective substrate analog at the active site and mimicry of the isomerization step of the normal reaction (in the absence of any possibility for cyclization to move positive charge to a more distal location) [20].

4. Conclusions

Although the work described here has failed to provide direct evidence for the formation of linalyl diphosphate as an intermediate in monoterpene synthase catalysis, these studies have provided further stereochemical details about the coupled isomerization–cyclization sequence, and have unexpectedly revealed a fundamental difference between (+)-bornyl diphosphate synthase and the two cyclic olefin synthases with respect to water access to the active sites and participation of water as terminating nucleophile in these electrophilic reactions.

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