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Stereoselective quenching of cedryl carbocation in epicedrol biosynthesis

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ABSTRACT

Epicedrol synthase catalyzes the cyclization of achiral diphosphate substrate, (*E*,*E*)-farnesyldiphosphate (FPP) into epicedrol. GC–MS analysis of assay extracts obtained by incubating FPP with epicedrol synthase in 21.6 at % $H_2{}^{18}O$ buffer showed the molecular ion of 224 for epicedrol. The labeled oxygen study presented here unambiguously demonstrates that the hydroxyl group of the epicedrol synthase enzymatic product, epicedrol, is derived from a water molecule, not from the phosphate moiety of the FPP.

Introduction

Over 70,000 isoprenoid compounds with diverse chemical structures are known to date and possess numerous biological activities such as antibiotics, toxins, and pheromones.[1,2] Usually, divalent metal dependent sesquiterpene synthases catalyze the cyclization of the linear substrate, farnesyl diphosphate (FPP) into complex hydrocarbon skeletons through a series of reactions involving electrophilic cyclizations, Wagner-Meerwein rearrangements, hydride/methyl shifts and oxidation then occur to synthesize the final terpenoid products.[3,4] Indeed many sesquiterpene synthases, such as *epi*-cubenol synthase from *Streptomycetes*, patchoulol synthase from *Pogostemon cablin*, epicedrol synthase from *Artemisia annua*, τ-cadinol synthase from *Lavandula angustifolia* are known to produce sesquiterpenoid alcohols *epi*-cubanol,[5] patchoulol,[6] epicedrol,[7] and τ-cadinol[8] respectively by quenching the respective final carbocation with an oxygen nucleophile in a stereoselective manner (Fig. 1).

Cane and co-workers[9] have demonstrated that the hydroxyl group of the borneol synthase product, borneol, is derived from the diphosphate moiety of the acyclic geranyl diphosphate substrate (GPP) (Fig 1).[10] The active site contour of terpene synthases usually is hydrophobic in nature to avoid improper quenching of the cationic intermediate with an external nucleophile such as water.[11] However, isotope labeling studies using *epi*-cubenol synthase,[12] patchoulol synthase,[13] and fenchol synthase[14] demonstrated that the oxygen atoms of the alcohol groups of these compounds are derived from water molecules present in the active site.

Epicedrol synthase[7,15] has been cloned and functionally characterized from *Artemisia annua*, a source of the potent anti-malarial drug, Artimisinin,[16] and is known to catalyze the electrophilic cyclization of achiral universal diphosphate substrate FPP to epicedrol as a major enzymatic product with traces of cedrol and corresponding hydrocarbons. Furthermore, studies have been carried out for the production of epicedrol in metabolically tractable heterologous systems such as *Saccharomyces cerevicea*.[17] Although, epicedrol synthase has been well studied, very little is known regarding the mechanism involved in epicedrol biosynthesis and the source of the oxygen atom.

Our continued interest to study the interface of chemical and biology with a particular emphasis on reaction mechanism[18] and protein function in biology.[19] Herein we studied the source of oxygen atom in epicedrol biosynthesis by carrying out the enzyme assay in $H_2{}^{18}O$.

Results and discussion

Epicedrol synthase of *A. annua* was expressed in *Escherichia coli* and purified as reported elsewhere.[7] GC and GCMS analysis of the assay containing purified epicedrol synthase and (*E*,*E*)-FPP indicated the formation of epicedrol as a major metabolite (~90%)

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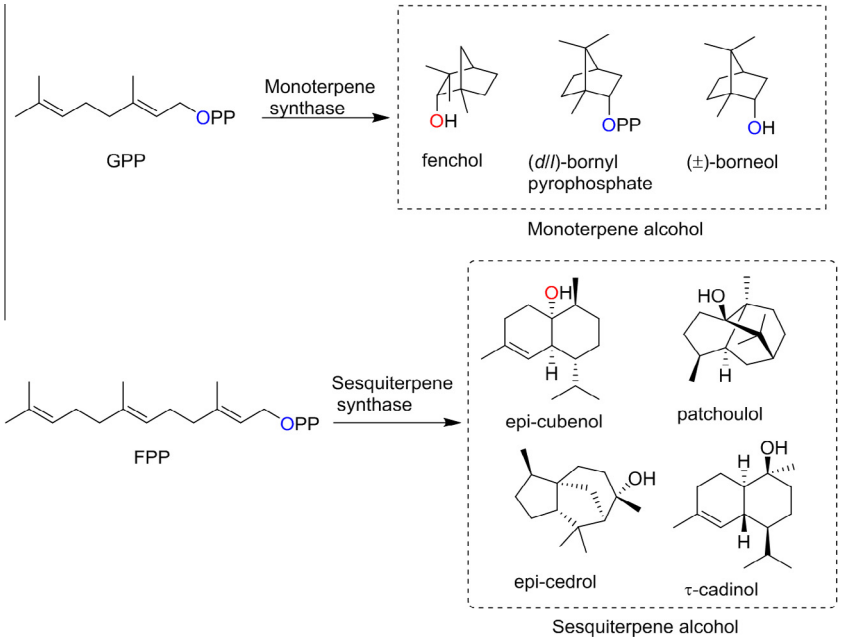
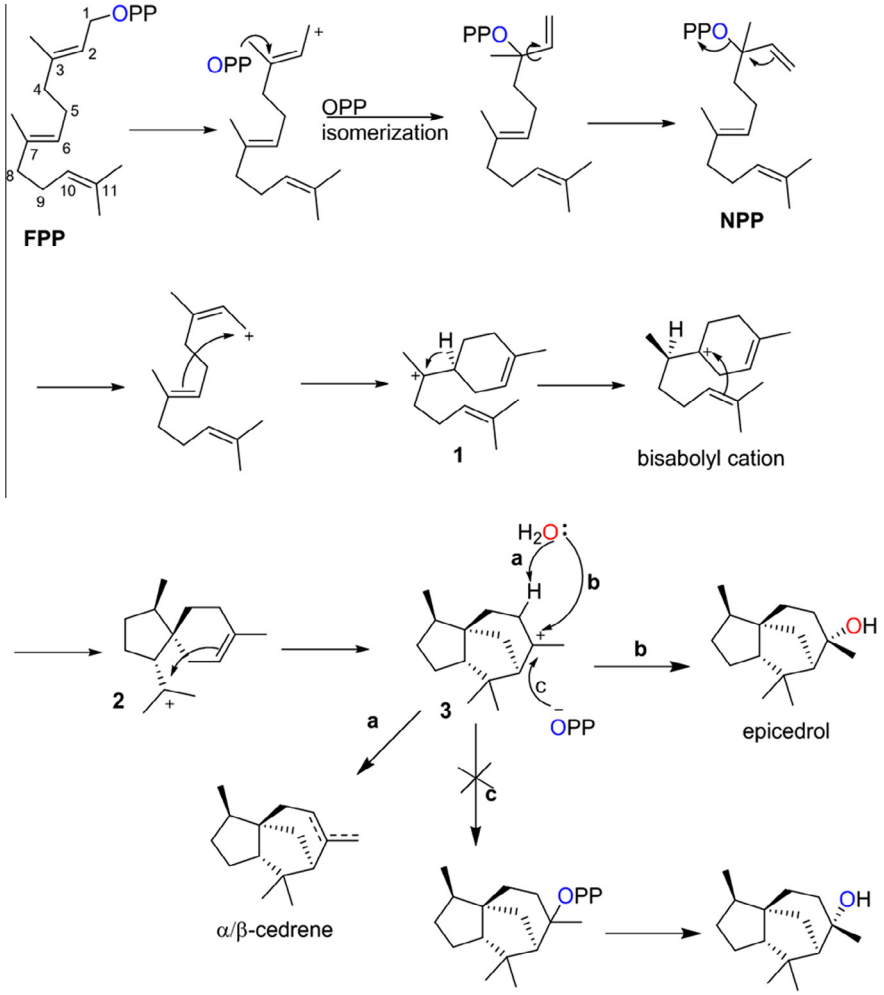


Figure 1. Monoterpene and sesquiterpene alcohols from corresponding linear prenyl pyrophosphates.



Scheme 1. Proposed mechanisms of epicedrol synthases, path (a) elimination of H. path (b) addition of OH from water and path (c) by OPP addition then hydrolysis.

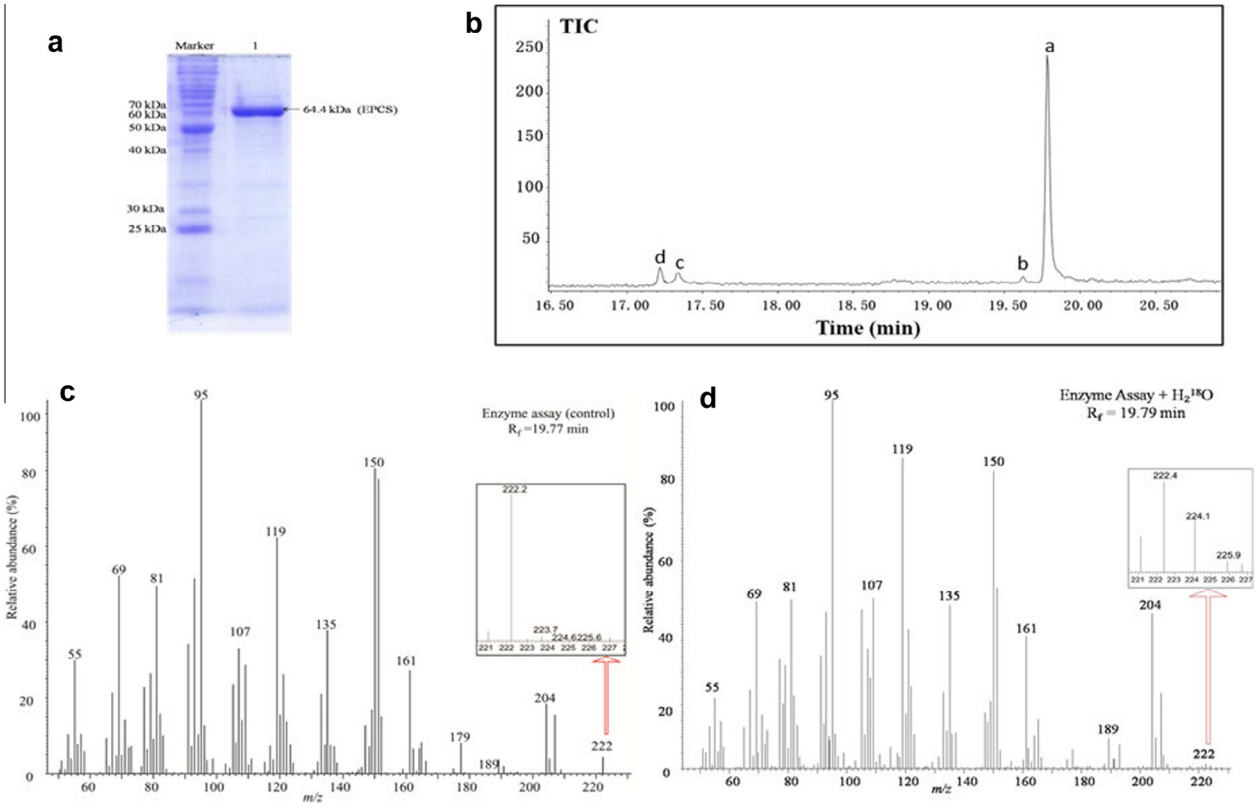


Figure 2. (a) Marker and purified epicedrol synthase (lane 1). (b) GC–MS analysis (TIC) of epicedrol synthase assay of FPP and $H_2{}^{18}O/H_2O$. (c) MS of product from FPP and H_2O assay. (d) MS of product from FPP and $H_2{}^{18}O$ assay.

Table 1
Mass spectrometric analysis of molecular ion peaks of Epicedrol

EI mass (*m*/*z*)	222	224
Peak% ($H_2{}^{16}O$)	96.58	3.42
Peak% ($H_2{}^{18}O$)	80.13	19.86

along with small traces cedrene and cedrol (Supporting information, SI) which is in line with the earlier observation. This divalent metal dependent sesquiterpene synthase could initiate the reaction by the ionization of FPP to delocalized farnesyl/nerolidol carbocation followed by series of electrophilic cyclization including a hydride shift from C-6 to C-7 to the generation of bisabolyl cation (Scheme 1). Then *si*-face attack of the C-10 olefin initiated two subsequent cyclizations to form exo-cyclic acoradane skeleton and tricyclic cedrane skeleton by C-10 to C-6 and C-2 to C-11 ring closure respectively. Generated tricyclic intermediate carbocation (**3**, Scheme 1) may be quenched by following three pathways: (a) deprotonation of either 15-H or 4-H which produces two sesquiterpene hydrocarbons α-cedrene and β-cedrene, respectively or (b) stereo selective addition of water to form cedrol/epicedrol, or (c) capture pyrophosphate anion present in the active site followed by hydrolysis to generate cedrol/epicedrol.

To address the ambiguous source of the oxygen atom in epicedrol biosynthesis, epicedrol synthase was incubated with FPP in both isotopically labeled and unlabeled conditions, then the obtained extracted products were characterized by GC and GC-MS analysis (SI). GC and GC-MS analysis of the control assay extract obtained by incubating FPP with epicedrol synthase in normal buffer system (ESI) indicated the formation of epicedrol as a major product along with traces of cedrol (1.2%), α-cedrene (2.1%), and β-cedrene (3.2%) (SI, Table S1). The formation of these compounds was confirmed by comparing retention time and GC co-injection studies. EI MS of sesquiterpene alcohols, epicedrol, and cedrol gave detectable molecular ion (M^+) at *m*/*z* 222 along with other peaks including *m*/*z* 204 formed from loss of water molecule, respectively (Fig. 2). Notably, when epicedrol synthase was incubated with FPP in Tris–HCl buffer, pH 8.5, containing $MgCl_2$ in $H_2{}^{18}O$ (21.6 at %), the product ratios were slightly changed (Table 1). Interestingly, the enrichment of molecular ion at *m*/*z* 224 for both epicedrol and cedrol was observed. This shift in the molecular ion clearly indicates the insertion of ^{18}O to epicedrol and cedrol molecules. Furthermore, there was no shift observed for the peak at *m*/*z* 204 due to the loss of water molecule (Fig. 2d). These results demonstrate that epicedrol and cedrol are formed through the quenching of cedryl carbocation **3** by water molecule present in the active site either from *Si*- or *Re*-face, respectively. However, *Si*-face seems to be more favorable as the epicedrol is the major enzymatic product. Furthermore, insertion of ^{18}O derived from $H_2{}^{18}O$ implies that both epicedrol and cedrol might not have formed through recapture of pyrophosphate anion present in the active site followed by the hydrolysis of corresponding diphosphate. The formation of corresponding sesquiterpene hydrocarbons α-cedrene and β-cedrene might be formed through deprotonation at 4-H and 15-H of cedryl carbocation (**3**), respectively.

Conclusions

In conclusion, we have demonstrated that the oxygen atom of the hydroxyl functionality of epicedrol is derived from a water molecule, not from the oxygen of diphosphate anion of farnesyl diphosphate. The attack of water to obtain the isotopically labeled epicedrol is preferred due to the steric blocking effects of the proximal geminal dimethyl groups in cedryl carbocation. The detection of traces of isotopically labeled cedrol implies that attack on the unbound carbocation is at least partially responsible for the observed product mixture.

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Supplementary data

Supplementary data (GC–MS analysis data of enzyme products) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.01.109.

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