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Cis or *Trans* with class II diterpene cyclases†

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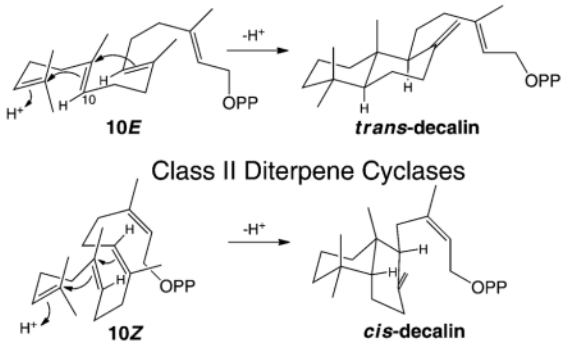
Abstract

Isoprenoid precursors readily undergo (poly)cyclization in electrophilic reaction cascades, presumably as internal addition of the carbon-carbon double-bonds from neighboring isoprenyl repeats readily forms relatively stable cyclohexyl tertiary carbocation intermediates. This hypothesis is agnostic regarding alkene configuration (i.e., *Z* or *E*). Consistent with this, here it is shown that certain class II diterpene cyclases, which normally convert (*E,E,E*)-geranylgeranyl diphosphate to 13*E*-*trans*-decalin bicycles, will also act upon (*Z,Z,Z*)-nerylneryl diphosphate, producing novel 13*Z*-*cis*-decalin bicycles instead.

Graphical Abstract



Keywords

(*Z,Z,Z*)-nerylneryl diphosphate; class II diterpene cyclases; labdane-related diterpenoids; metabolic engineering

Terpenoids form the largest class of natural products, with almost 55,000 compounds already known.[1] Underlying this chemical diversity is the array of hydrocarbon backbone structures that are largely produced by cyclization and/or rearrangement of acyclic isoprenoid precursors in electrophilic reactions that proceed via carbocationic intermediates.[2] The precursors are composed of repeating isoprenyl units that contain methyl branched alkenes. Accordingly, internal addition from these alkenes to carbocations

†Electronic supplementary information available: Experimental methods, structural data, supplemental tables and figures.

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in the adjoining isoprenyl unit to form cyclohexyl intermediates can proceed via relatively stable tertiary carbocations.

This intrinsic reactivity can be readily seen in the bicyclization reactions catalyzed by class II diterpene cyclases (DTCs), which act upon the general diterpene precursor (*E,E,E*)-geranylgeranyl diphosphate (GGPP, **1**),[3] in highly exothermic reactions (i.e., these are energetically favorable by > 30 kcal mol^{-1}).[4] Initial bicyclization forms a labda-13*E*-en-8-yl diphosphate (PP) carbocation intermediate, characterized by a decalin ring structure wherein the bridgehead carbons have *trans* substituents, as dictated by the *E* configuration of the relevant alkene (i.e., 10*E*). This initial bicycle can be generated with distinct stereochemistries resulting from different conformations of **1** imposed by the relevant DTC active site prior to bicyclization, and which are designated by comparison to the analogous A/B ring structures in cholesterol – e.g., those with the same configuration are termed "normal". Formation of labda-13*E*-en-8-yl^{+} PP can be followed by a series of alternating 1,2-hydride and methyl shifts that similarly proceed through tertiary carbocation intermediates. While these carbocationic cascade reactions are terminated by deprotonation, this can occur following the addition of water to form hydroxylated bicycles (Scheme 1).[5]

The above suggests the hypothesis that terpenoid diversity stems, in part, from the intrinsic reactivity of the oligo-isoprenyl precursors. Notably, this does not *a priori* depend on the configuration of the internal alkenes. It has been recently discovered that diterpenes can be produced from the alternative precursor (*Z,Z,Z*)-nerylneryl diphosphate (NNPP, **2**), but **2** has only been shown to be utilized by a single class I diterpene synthase, which catalyzes a distinct type of reaction initiated by ionization of the allylic diphosphate ester bond.[6] Even very promiscuous class I diterpene synthases preferentially react with substrates in which this allylic alkene is in the *E* rather than *Z* configuration.[7] On the other hand, DTCs initiate their reactions by protonation of the terminal alkene, which does not differ between **1** and **2**.[5] Accordingly, it seemed possible that DTCs might be able to react with **2**, enabling further examination of the intrinsic reactivity of isoprenoid precursors, although the distinct alkene configuration of **2** obviously will impact how it is bound and (bi)cyclized.

Here the ability of DTCs to react with **2** was examined using a panel of 12 functionally distinct DTCs (Table S1). In particular, these were examined in the context of a previously developed modular metabolic engineering system,[8] including increased flux to isoprenoid metabolism.[9] As previously reported,[7] when expressed in *E. coli* also engineered to produce **1** all but one of these DTCs efficiently converts **1** almost completely to the expected decalin diphosphate product (these are observed here by GC-MS as the derived primary alcohol derived from hydrolysis of the diphosphate ester by endogenous phosphatases). However, when expressed in *E. coli* also engineered to produce **2**, only four of these DTCs appeared to be able to react with this alternative precursor – i.e., AgAS:D621A,[10] SmCPS/KSL1:D501A/D505A,[11] NgCLS[12] and MtHPS[13] (note that the first two of these are bifunctional and the mutations knock-out the associated class I diterpene synthase activity). In all four cases, each DTC appears to produce only a single product, and the mass spectra of the derived dephosphorylated compound closely resembles that for the analogous dephosphorylated derivative of the product formed by reaction with **1** by the same DTC (Figure 1). This suggests structural similarity between the products from **1** and **2**, although

their distinct retention times demonstrate that they do differ. Intriguingly, these four DTCs all catalyze reactions that proceed via initial formation of a labda-13*E*-en-8-yl$^+$ PP intermediate in the "normal" conformation. Accordingly, the active sites of these four DTCs, which presumably bind **1** in a similar fashion to produce the normal labda-13*E*-en-8-yl$^+$ PP intermediate they share in common, also are able to bind **2** in a reactive orientation.

To determine how **2** might be bound, it was necessary to elucidate the structure of at least one of the resulting products. The DTC that most efficiently reacts with **2** is AgAS:D621A, which reacts with **1** to produce normal copalyl diphosphate (CPP, **3**) stemming from direct deprotonation of the methyl substituent of the C8 carbocation in labda-13*E*-en-8-yl$^+$ PP (i.e., C17), and is the only DTC that appears to convert more than half of **2** to product (~70%, while all the others exhibited yields of < 30%). To characterize the structure of the product (**4**), the relevant culture was grown at a larger scale, and the resulting dephosphorylated derivative (**4′**) was purified. This was then structurally characterized by comprehensive NMR analysis (Figures S1–8 and Table S2). The structure was assigned as copalol on the basis of the proton and carbon chemical shifts (Table S2) from 1D spectra (Figures S2 and S3, respectively), and the correlations observed in DQF-COSY, HMQC-COSY, HSQC and HMBC spectra (Figures S4–S7, respectively). The alkene in the isoprenyl tail was found to retain the *Z* configuration (i.e., contain a 13*Z*-ene group), as verified by the strong NOE correlation between H_3-16/H-14 (Figure S8). NOE correlations between H-5 and H_3-20 were consistent with the *cis* configuration of the decalin ring structure expected from the configuration of the relevant alkene in **2** (i.e., 10*Z*). Moreover, NOE correlations between H-9 and H-5, as well as between H-9 and H_3-20, further indicated the relative *syn* configuration of the hydride and methyl substituents at C9 and C10, respectively. Hence, **4′** appears to be 13*Z*-*cis*-*syn*-copalol, derived from dephosphorylation of the 13*Z*-*cis*-*syn*-CPP (**4**), both of which appear to be novel (i.e., these are not found in SciFinder). Although the absolute stereochemical configuration of **4** remains unknown, it exhibits optical rotation of $[\alpha]_D^{25}=+4$ (c = 3.30, $CHCl_3$). Here it is simply depicted as derived from folding of **2** into the normal conformation for the 'A' ring in common with the known (bi)cyclization of **1** to form **3** (Scheme 2).

Beyond elucidation of the AgAS:D621A product, particularly given the similarly close mass spectral data, it seems likely that the other DTC products from **2** observed here also share an analogous relationship to their products with **1**. Accordingly, SmCPS/KSL1:D501A/D505A, which produces *endo*-CPP (**5**) from **1**,[7] presumably produces 13*Z*-*cis*-*syn*-*endo*-CPP (**6**) from **2**; NgCLS, which produces 8α-hydroxy-CPP (**7**) from **1**,[14] presumably produces 8α-hydroxy 13*Z*-*cis*-*syn*-CPP (**8**) from **2**; and MtHPS, which produces halima-5,13*E*-dienyl diphosphate (HPP, **9**) from **1**,[13] presumably produces 13*Z*-*cis*-*syn*-HPP (**10**) from **2** (Chart S1). Regardless of exact outcome, it is striking that in all four cases, each DTC only yields a single product, indicating that both **1** and **2** can only assume a single reactive conformation in their active sites. Although this pre-catalytic conformation is not directly investigated here, the characterized production of **4** does provide some insight into how **2** may be folded in the AgAS DTC active site. In particular, modeling suggests that **2** can be folded such that key features occupy similar positions as in the pre-catalytic conformation of **1** (Scheme 2). Specifically, the carbons that will be protonated (C14) and deprotonated (C17 in the relevant

labda-13*E*-en-8-yl$^+$ PP intermediate), allowing use of the same enzymatic general acid and base (i.e., the conserved DxDD motif that characterizes DTCs,[15] and a hydrogen-bonded His-Tyr pair in AgAS,[14] respectively).

Conclusions

The results reported here provide evidence supporting the intrinsic suitability of oligo-isoprenyl precursors for (poly)cyclization, which may have further driven the evolutionary elaboration of terpenoid natural products. More specifically, given the ability of DTCs to react with **2** in addition to their usual substrate **1**, it can be speculated that this reactivity may be utilized in as yet undiscovered diterpenoid biosynthetic processes. Regardless, the DTC promiscuity found here provides novel biosynthetic access to stereochemically distinct *cis*-decalin bicycles. Hence, it will be of interest to both explore the ability of a wider range of DTCs to react with **2**, along with the ability of the subsequently acting class I diterpene synthases to catalyze further elaboration of *cis*-decalin bicycles such as **4**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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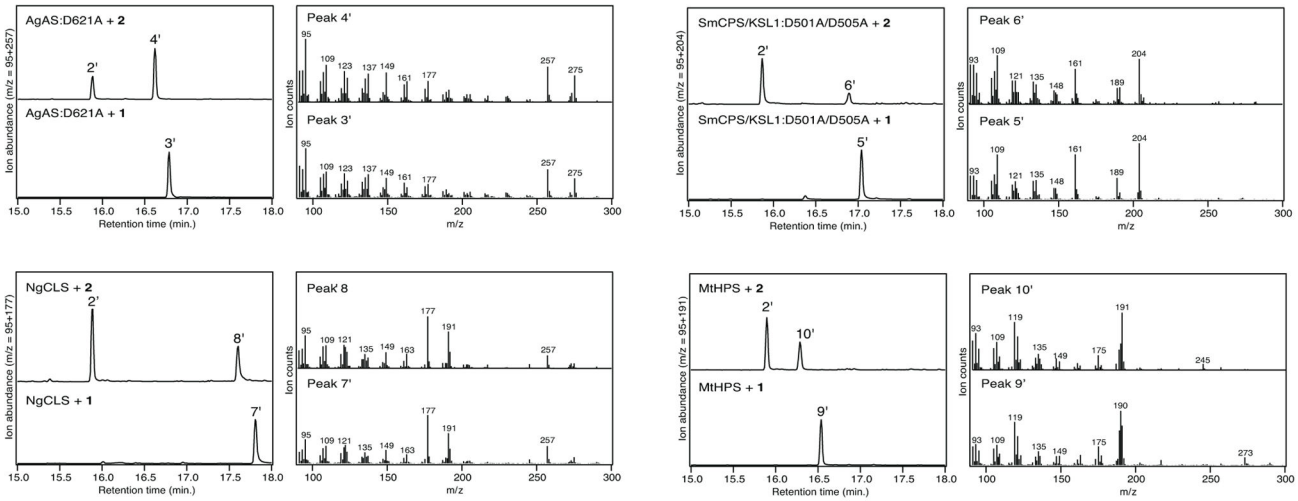
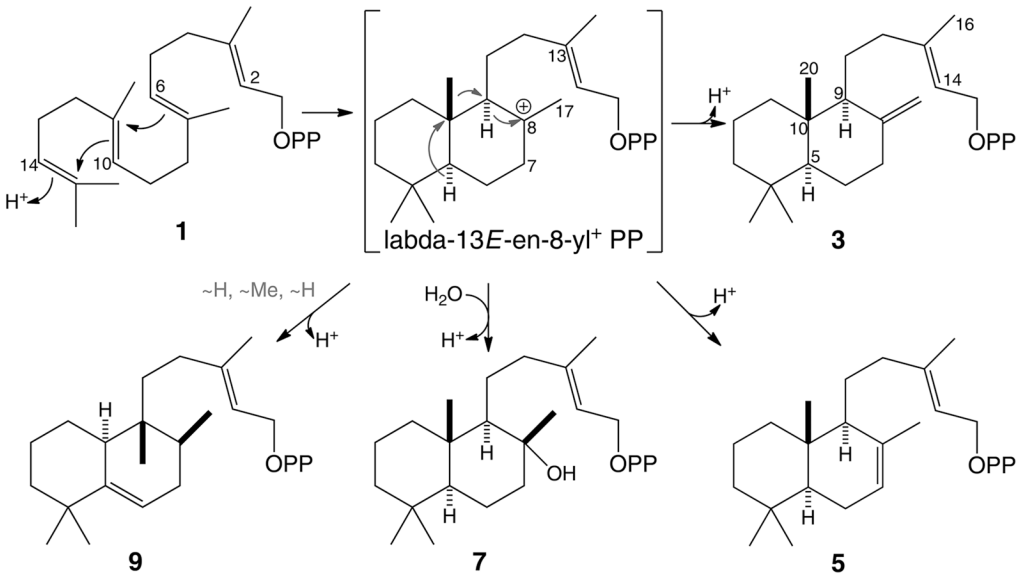


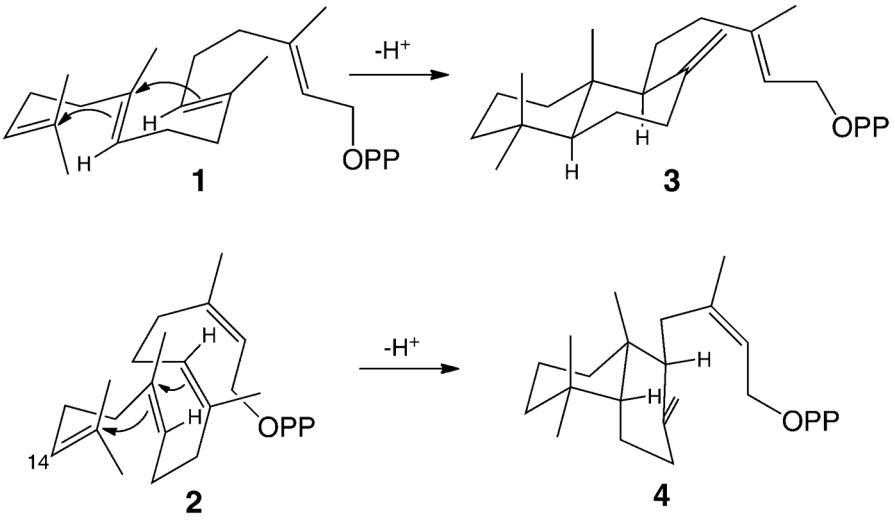
Figure 1.

Certain DTCs react with **2**. GC-MS chromatograms extracts from cultures wherein the indicated DTC was expressed in *E. coli* also engineered to produce either **1** or **2**. These contain dephosphorylated derivatives of the resulting products, as indicated by prime notation of the corresponding peak/compound numbers. Also shown are the corresponding mass spectra for the indicated derived product compounds.



Scheme 1.

Bicyclization of **1** to normal labda-13*E*-en-8-yl$^+$ diphosphate catalyzed by DTCs, in reactions that are terminated by deprotonation, either immediately (e.g., from C17 to form **3** or from C7 to form **5**, as catalyzed by AgAS:D621A or SmCPS/KSL1:D501A/D505A, respectively), or after the addition of water with subsequent deprotonation (e.g., to form **7**, as catalyzed by NgCLS), or a series of 1,2-hydride and methyl shifts (e.g., to form **9**, as catalyzed by MtHPS).



Scheme 2.

Three dimensional rendering of reaction catalyzed by AgAS:D621A with **1** or **2** (as indicated).