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Abstract
Terpenoid natural products are generally derived from isoprenyl diphosphate precursors with trans double-bond configuration, and no diterpenoid derived from the cisoid precursor (Z,Z,Z)-nerylneryl diphosphate (1) has yet been identified. Here further investigation of a terpenoid biosynthetic gene cluster from tomato is reported, which resulted in identification of a biosynthetic pathway from 1, in a pathway featuring a number of interesting transformations. Compound 1 is first cyclized to a tricyclene core ring structure analogous to that found in α-santalene, with the resulting diterpene termed here lycosantalene (2). Quantum chemical calculations indicate a role for the diphosphate anion coproduct in this cyclization reaction. Subsequently, the internal cis double bond of the neryl side chain in 2 is then further transformed to an α-hydroxy ketone moiety via an epoxide intermediate (3). Oxygen labeling studies indicate 3 undergoes oxidative conversion to lycosantalonol (4). Thus, in addition to elucidating the cisoid origins of 4, this work has further provided mechanistic insight into the interesting transformations required for its production.

Keywords
diphosphate, terpenoid, cisoid

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Comments

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Biosynthesis of Lycosantalonol, a cis-Prenyl Derived Diterpenoid

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Supporting Information

ABSTRACT: Terpenoid natural products are generally derived from isoprenyl diphosphate precursors with trans double-bond configuration, and no diterpenoid derived from the cisoid precursor (Z,Z,Z)-nerelyldiphosphate (1) has yet been identified. Here further investigation of a terpenoid biosynthetic gene cluster from tomato is reported, which resulted in identification of a biosynthetic pathway from 1, in a pathway featuring a number of interesting transformations. Compound 1 is first cyclized to a tricyclic core ring structure analogous to that found in α-santalene, with the resulting diterpene termed here lycosantalene (2). Quantum chemical calculations indicate a role for the diphosphate anion coproduct in this cyclization reaction. Subsequently, the internal cis double bond of the neryl side chain in 2 is then further transformed to an α-hydroxy ketone moiety via an epoxide intermediate (3). Oxygen labeling studies indicate 3 undergoes oxidative conversion to lycosantalonol (4). Thus, in addition to elucidating the cisoid origins of 4, this work has further provided mechanistic insight into the interesting transformations required for its production.

Until recently, terpenoid biosynthesis was thought to be exclusively derived from transoid linear precursors, with C10 monoterpenes derived from geranyl diphosphate, C15 sesquiterpenes from (E,E)-farnesyl diphosphate (6,6-FPP), and C20 diterpenes from (E,E)-geranylgeranyl diphosphate (GGPP). These precursors are formed by short-chain trans-isoprenyl diphosphate synthases,1 with the separate family of cis-prenyl transferases (CPTs) thought to be confined to the production of longer chain length isoprenoids.2 However, it has now been reported that CPT family members do participate in terpenoid biosynthesis, as tomato produces the linear cis-nerelyl diphosphate (NPP) as a monoterpenoid precursor3 and (Z,Z)-farnesyldiphosphate (z,z-FPP) as a sesquiterpene precursor,4 and lavender produces the irregular lavandulyl diphosphate as a monoterpenoid precursor.5

The CPTs producing NPP and z,z-FPP are orthologues found in domesticated and wild tomato plants; Solanum lycopersicum and Solanum habrochaites, respectively. This CPT1 gene is found in a region that also contains the separate, subsequently acting terpene synthases (TPSs).3,4 In addition, this region contains a gene encoding another CPT, CPT2, along with those encoding several additional TPSs as well as uncharacterized CYPs, alcohol acyltransferases, and an alcohol oxidase.6 Thus, it appears that tomatoes contain a terpenoid biosynthetic gene cluster, which is an unusual but not entirely uncommon occurrence in plant genomes.7

Previous characterization of the S. lycopersicum CPT2 (SlCPT2) suggested that this produces (Z,Z,Z)-nerelyldiphosphate (NNPP, 1),8 with subsequent production of an unidentified olefin by a nearby TPS (SlTPS21).9 In addition, there are two CYPs in the cluster, suggesting that these also might function in biosynthesis of the final terpenoid natural product, although one or the other of these appears to be nonfunctional in specific species of Solanum (i.e., encode a pseudogene). For example, in S. lycopersicum only one CYP appears to be functional, CYP71D51, which is present as the only gene in between SlCPT2 and SlTPS21. While the NPP derived monoterpenes are readily detected from S. lycopersicum, the unknown olefin derived from the activity of SlCPT2 and SlTPS21 is not,6 suggesting that this might be further transformed by at least the mono-oxygenase activity of CYP71D51.

Characterization of the hypothesized cis-prenyl derived diterpenoid product of the SlTPS21-CYP71D51-SlCPT2 subcluster from S. lycopersicum was undertaken using a previously described modular metabolic engineering system.9 To verify the production of 1 by SlCPT2, we expressed SlCPT2 in Escherichia coli along with a previously described plasmid that overexpresses key enzymes from the endogenous isoprenoid precursor metabolic pathway, increasing flux into terpenoid production.10 This enabled isolation of the dephosphorylated derivative (Z,Z,Z)-nerelylnerol in amounts (ca. 2 mg) sufficient for comprehensive NMR analysis, with comparison of the resulting series of NOE correlations between H17/H14, H14/H18, H18/H10, H10/H19, H19/H6, H6/H20, and H20/H2, to those observed with the dephosphorylated derivative of GGPP, (E,E,E)-geranylgeranial (Figures S1–S11 and Table S1, Supporting Information (SI)), confirming the cis configuration of the internal double-bonds and hence the production of 1 by SlCPT2 (Scheme S1, SI).

Coexpression of SlTPS21 along with SlCPT2 led to production of the previously reported unknown olefin,9 which also was isolated in amounts (ca. 3 mg) sufficient for comprehensive NMR analysis (Figures S2 and S12–S17 and Table S2, SI). This compound was found to contain a

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tricyclo[2.2.1.0^2,6]heptane ring structure analogous to that found in tricyclic, albeit here with a neryl side-chain. On the basis of the match between this core ring structure and that found in the sesquiterpene α-santalene, the term lycosantalen e (2) is proposed for the diterpene characterized here (Scheme S2, SI). Given the production of small amounts of 7R-(+)-α-santalene by SI TP S21 from z.z-FPP, it seems likely that lycosantalen e also exhibits the corresponding absolute stereochemistry. Consistent with this hypothesis, 2 also exhibits positive optical rotation: [α]^{25}_D + 7.9 (c 0.1, CHCl₃).

Interestingly, (+)-α-santalene is the major product of the z.z-FPP specific TPSs from S. habrocat es, which might suggest some role for the cisoid configuration in formation of the observed ring structure. Use of a cis-isoprenyl diphosphate precursor does enable direct formation of the cyclohexanyl (terpinyl-type) carbocation intermediate, which otherwise requires isomerization of the allylic diphosphate from C1 to C3 to enable rotation around the C2–C3 bond (Scheme S3, SI). However, it should be noted that α-santalene also is the major product of e.e-FPP specific TPSs from other plants.¹¹

Formation of the strained tricyclo[2.2.1.0^2,6]-heptane ring structure is of some mechanistic interest and was explored here by quantum chemical calculations (QCC; see SI for details)¹²–¹⁸ the application of which to terpene cyclization has recently been experimentally validated.¹⁹ The reaction proceeds through a series of intermediates, with the initial allylic carbocation formed by diphosphate ionization (A) undergoing C1–C6 cyclization to a terpinyl-type carbocation (B), which undergoes C2–C7 cyclization to a bicyclic pinyl-type carbocation (C) that rearranges to a bornyl-type secondary carbocation (D), a transition state rather than intermediate, and is further rearranged to a camphyl-type carbocation (E) that is predicted to undergo concerted C3–C4 ring closure/C4 deprotonation to form the tricyclic core ring structure (Scheme 1). Notably, some support for the suggested stereoselective loss of the pro-R-hydrogen in tricyclic formation has been previously reported.²⁰

Scheme 1. Carboxylation Series for Tricyclic Ring Forming Reaction Indicated by QCC (R = H, Prenyl, or Neryl for Mono-, Sesqui-, or Diterpene Cyclization, Respectively)

QCC analysis in the absence of the pyrophosphate (PPI) coproduct indicated that the conversion of cation B to cation E is concerted (but asynchronous), while inclusion of PPI led to a stepwise mechanism (in which cation D is still not a minimum), due to stabilization of particular carbocations by the anionic PPI. However, such stabilization required substantial shifts in the relative orientation of the PPI and various carbocationic intermediates (particularly B and C relative to the initial A and terminal E; see SI for details). Given the tight binding of the diphosphate/PPI observed in TPS cocystal structures,²¹ such reorientation seems unlikely to occur in the restricted context of the active site. Intriguingly, inclusion of PPI in the QCC analysis does seem to bias the reaction toward cyclopropane formation as the C3–C2–C4 angle in E is significantly more acute (79° versus 95°), shortening the distance between C3–C4 from 2.28 to 2.01 Å (Figure 1 and Schemes S4 and S5, SI). Thus, beyond orienting the substrate to enable primary and secondary cyclization, simply restricting the conformational freedom of the olefinic carbocation intermediates relative to the PPI coproduct may help explain the ability of certain TPSs to form tricyclic structures. Such restriction may be enabled by increased hydrocarbon chain length (i.e., size of the C7 substituent, due to mass effects and/or increased interaction with the TPS), as SITPS21 quite specifically produces 2 (C_{20}, R = neryl), as any other product being formed must be present as less than 3% of 2. By contrast, the previously characterized α-santalene (C_{15}, R = prenyl) synthases are less specific (i.e., produce significant amount of other sesquiterpenes), and the only molecularly characterized monoterpene synthase that produces tricyclic (C_{10}, R = H) only yields this as a minor product.²²

To characterize the ability of CYP71D51 to react with 2 we obtained a synthetic gene, codon optimized and N-terminally modified as previously shown to enable functional expression of plant microsomal CYP in E. coli,²³–³⁰ and coexpressed this (sCYP71D51) along with SICPT2 and SITPS21, as well as a plant CYP reductase to provide the requisite high-energy electrons. In addition to 2, two new products were observed in these cultures, with masses suggestive of successive oxygenation reactions, i.e., product 3 had an apparent molecular ion of m/z = 288 versus 272 for 2, while that for 4 was 304 (Scheme 2 and Figures S18 and S24, SI), consistent with the calculated molecular masses for a diterpene olefin (272 Da), epoxy derivative (288 Da), and dioxy derivative (304 Da). Compounds 3 and 4 were isolated in amounts (ca. 0.7 and 1.5 mg, respectively)²³ for NMR analysis, which indicated that 3 is the Δ^{12,13} epoxide derivative of 2 (Figures S2 and S19–23 and Table S3, SI), while 4 contains a C13,12 α-hydroxy ketone (Figures S25–30 and Table S4, SI) and also exhibits positive optical rotation; [α]^{25}_D + 13.8 (c 0.2, CHCl₃). Purified 3 fed to CYP71D51 in vitro led to the production of 4.
Production of the α-hydroxy ketone moiety in 4 by CYP71D51 was probed by labeling experiments using $^{18}$O$_2$. In vitro reactions run under $^{18}$O$_2$ with 2 as substrate led to production of doubly labeled 4 (Figure S32, SI), demonstrating that both oxygens are inserted by CYP71D51. Formation of the α-hydroxy ketone in 4 from the epoxide found in 3 can be envisioned as proceeding via the addition of oxygen to either C12 or C13. In vitro reactions run under $^{18}$O$_2$ with 3 as substrate led to production of singly labeled 4, MS fragmentation (Figure S33, SI) indicates 4 arises from the addition of oxygen to C12 of 3, suggesting formation of either a epoxide-hemiketal or 12-hydroperoxy-13-ol intermediate that goes on to form the observed α-hydroxy ketone.

The studies reported here illuminate the biosynthesis of (+)-lycosantalol (4), whose production is encoded by a SlTPS21-CYP71D51-SlCPT2 subcluster of a larger terpenoid SlTPS21-CYP71D51-SlCPT2 subcluster of a larger terpenoid (Figure S31, SI), indicating the intermediacy of 3 (3) Schilmiller, A. L.; Schauvinhold, I.; Larson, M.; Xu, R.; Charbonneau, A. L.; Schmidt, A.; Wilkerson, C.; Last, R. L.; Pichersky, E. Proc. Natl. Acad. Sci. U.S.A. 2009, 106, 10865.

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