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A diterpene synthase from the clary sage *Salvia sclarea* catalyzes the cyclization of geranylgeranyl diphosphate to (8*R*)-hydroxy-copalyl diphosphate ☆

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This manuscript is dedicated to memory of late Professor Dr. MeinhartZenk (Zenk Memorial issue of Phytochemistry).

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ABSTRACT

The bicyclic diterpene (−)-sclareol is accumulated in glandular trichomes in *Salvia sclarea* (Schmiderer et al., 2008), and is a major terpenoid component of this plant species. It is used as the starting material for Ambrox synthesis, a synthetic ambergris analog used in the flavor and fragrance industry. In order to investigate the formation of sclareol, cDNA prepared from secretory cells of glandular trichomes from *S. sclarea* inflorescence were randomly sequenced. A putative copalyl diphosphate synthase encoding EST, *SscTPS1*, was functionally expressed in *Escherichia coli*. Whereas reaction of geranylgeranyl diphosphate with the putative copalyl diphosphate synthase followed by hydrolysis with alkaline phosphatase yielded a diastereomeric mixture of (13*R*)- and (13*S*)-manoyl oxide, HCl hydrolysis yielded (−)-sclareol (**1**) and 13-*epi*-sclareol as products. The product of the reaction of SscTPS1 with geranylgeranyl diphosphate was subjected to analysis by LC-negative ion ESI-MS/MS without prior hydrolysis. EPI scans were consistent with copalyl diphosphate to which 18 mass units had added (*m/z* 467 $[M+H]^-$). The enzymatic reaction was also carried out in the presence of 60% $H_2{}^{18}O$. LC-negative ion ESI-MS/MS analysis established an additional reaction product consistent with the incorporation of ^{18}O. Incubation in the presence of 60% ^2H_2O resulted in the incorporation of one deuterium atom. These results suggest water capture of the carbocation intermediate, which is known to occur in reactions catalyzed by monoterpene synthases, but has been described only several times for diterpene synthases.

1. Introduction

Salvia sclarea L. (clary sage) is a commercially important aromatic plant of the mint family (Lamiaceae) that produces the bicyclic diterpene (−)-sclareol (**1**) (Volmar and Jermstad, 1928), and which is used as starting material for synthesis of Ambrox (**2**) (Fig. 1), one of the principle active constituents of ambergris used in the flavor and fragrance industry (Ohloff, 1990). Early studies on the biosynthesis of (−)-sclareol (**1**) in *S. sclarea* indicated that although $^{14}CO_2$ administered to flowering stalks was efficiently incorporated into (−)-sclareol (**1**), [2-^{14}C]-acetic acid and [2-^{14}C]-mevalonic acid did not efficiently label the diterpene (Nicholas, 1961a,b, 1964). It is well established now that diterpenes in plants are synthesized in plastids and that the five-carbon units that are required for their formation are produced *via* the methylerythritol phosphate pathway (Eisenreich et al., 1998). Cell-free extracts of *Nicotiana tabacum* leaf midvein epidermal peels were shown to contain diterpene synthase activity that catalyzed the cyclization of cis-abienol from geranylgeranyl diphosphate (GGPP) (**3**) (Fig. 2) (Guo et al., 1994), and equivalent experiments with *Nicotiana glutinosa* resulted in the formation of labdenediol and (−)-sclareol (**1**) (Guo and Wagner, 1995). Guo and Wagner proposed in 1995 that after cyclization of GGPP (**3**), the carbocation at C-8 could be neutralized by hydroxyl anion capture to form the 8-hydroxy moiety.

Based on the absolute configuration of the diterpene (−)-sclareol (**1**), it was originally reasoned that the biosynthesis likely begins with GGPP (**3**) that is cyclized to a (+)-copalyl diphosphate (**4**) analog. Abietadiene synthase has been studied in detail due to its relevance in the biosynthesis of (−)-abietadiene, the precursor to (−)-abietic acid, the principal diterpenoid resin acid of wound-induced oleoresin secreted by grand fir (*Abies grandis*) (Vogel et al., 1996). This enzyme has been characterized in recombinant form to function as a bifunctional diterpene cyclase. The amino-terminal sequence of abietadiene synthase from grand fir is similar

☆ The nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI Data Bank ID: HQ641451.

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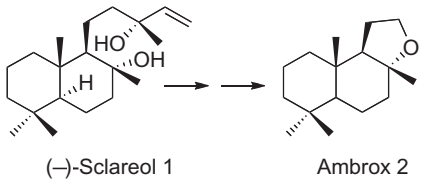

Fig. 1. Structures of (−)-sclareol (**1**) and Ambrox (**2**).

in amino acid sequence to class II terpene cyclases that use substrate double bond protonation that initiates a carbocationic reaction cascade and leaves intact the diphosphate ester linkage, such as *ent*-copalyl diphosphate synthase (kaurene synthase A) involved in gibberellin biosynthesis (Yamaguchi, 2008), whereas the carboxy-terminal sequence is more similar to class I cyclases that ionize the allylic diphosphate ester of the substrate to initiate cyclization such as *epi*-aristolochene synthase (Back and Chappell, 1995; Starks et al., 1997) and *ent*-kaurene synthase (kaurene synthase B, gibberellin biosynthesis) (Yamaguchi, 2008). A detailed review of labdane-forming class I and II terpene synthases has recently been published (Peters, 2010) and will not be further elaborated here. The grand fir abietadiene synthase is bifunctional and catalyzes both the protonation initiated and the ionization initiated cyclization steps to form (−)-abietadiene from GGPP (**3**) (Vogel et al., 1996). Although modification of the diterpene carbon skeleton by the insertion of oxygen was thought to be catalyzed almost invariably by cytochrome P450 monooxygenases (Peters, 2010), recent mechanistic studies on the functionally equivalent diterpene synthase from Norway spruce suggest that an intermediary isopimar-15-en-8-yl carbocation initially forms from GGPP. Loss of a proton from this intermediate could give rise to abietadiene (Vogel et al., 1996); however, it is now suggested that the abieta-8(14)-en-13yl carbocation is quenched by the addition of water to 13-hydroxy-8(14)-abietadiene which is subsequently dehydrated to abietadiene (Keeling et al., 2011). Water capture of the carbocation intermediate is known to occur in reactions catalyzed by monoterpene synthases, but it has been described only several times for diterpene synthases. One example from an early land plant is the bifunctional *ent*-kaurene synthase from the moss *Physcomitrella patens* which catalyzes the cyclization of GGPP to *ent*-kaurene and *ent*-16α-hydroxykaurene (Hayashi et al., 2006).

The resin of *Cistus creticus* contains labdane-type diterpenes that contain oxygen (Falara et al., 2010). A recombinant diterpene synthase isolated from *C. creticus* has been shown to cyclize GGPP (**3**) into copal-8-ol diphosphate, which was considered a likely intermediate in the formation of oxygen-containing diterpenes in resin. It has been postulated that the reaction is initiated by protonation of the terminal double bond of GGPP. After cyclization to a bicyclic carbocation, it is hypothesized that a hydroxyl anion is captured (Guo and Wagner, 1995). Incorporation of oxygen into the diterpene structure is, thereby, a consequence of the cyclization process, rather than the result of an independent action of a monooxygenase. To test this hypothesis in the formation of (−)-sclareol (**1**) in *S. sclarea*, a cDNA library was prepared from RNA of secretory cells from glandular trichomes Schmiderer et al. (2008) isolated from *S. sclarea* inflorescence. ESTs were randomly sequenced and the predicted amino acid sequences were compared with protein sequences available in public databases to identify a putative copalyl diphosphate synthase that was functionally expressed and characterized.

2. Results

2.1. Isolation of a diterpene synthase cDNA from S. sclarea

A cDNA library was prepared from RNA of secretory cells from glandular trichomes isolated from *S. sclarea* inflorescence. The size of the cDNA inserts in the λ-library was monitored by PCR and only those clones that contained an insert >500 bp were chosen for sequencing. A total of 1500 single-pass sequenced expressed sequence tags (ESTs) were generated. A homology search using the BLASTX algorithm identified 49 ESTs (4%) potentially involved in terpenoid metabolism. Twenty-one ESTs encoded monoterpene synthases, 3 were sesquiterpene synthases and 6 were diterpene synthases (3 copalyl diphosphate synthase, 3 kaurene synthase). Eight ESTS were putatively involved in the terpenoid anabolism as cytochromes P-450 and 11 encoded enzymes involved in either the methylerythritol phosphate or mevalonic acid pathways. The three ESTs with overlapping identity to each other and similarity to copalyl diphosphate synthase from *Solanum lycopersicum* were

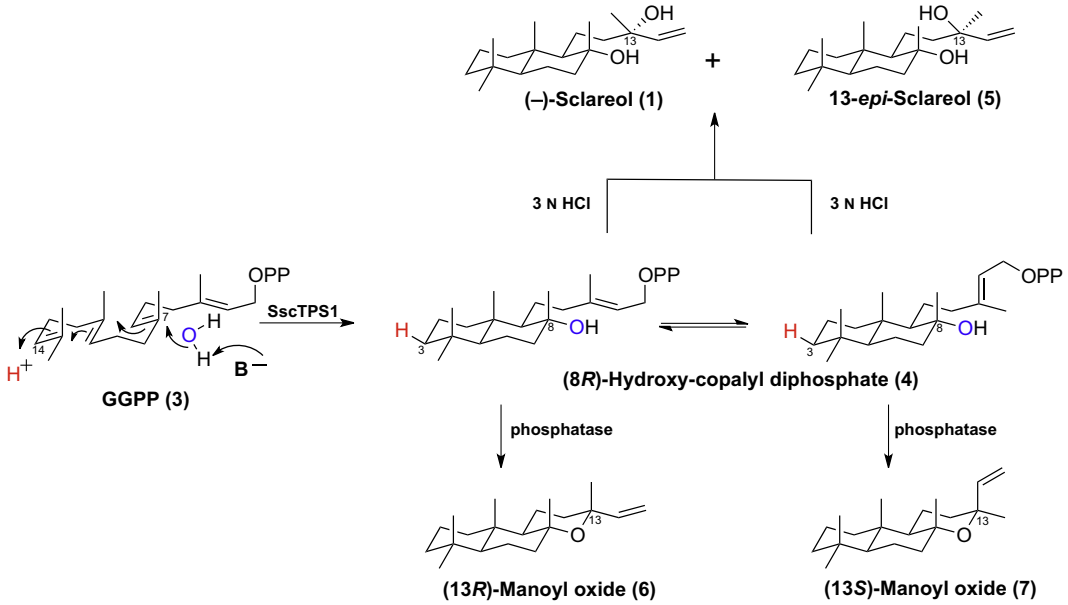

Fig. 2. Mechanism of conversion of GGPP by SscTPS1. Conversion of GGPP into (8*R*)-hydroxy-copalyl diphosphate by SscTPS1 from *S. sclarea,* followed by derivatization to (13*R*)- and (13*S*)-manoyloxide (**6**) and (**7**), (−)-sclareol (**1**) and 13-*epi*-sclareol (**5**). Blue, ^{18}O; red, ^{2}H. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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