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Cloning, Expression, and Characterization of *epi*-Cedrol Synthase, a Sesquiterpene Cyclase from *Artemisia annua* L[1,2]

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Sesquiterpene cyclases (synthases) catalyze the conversion of the isoprenoid intermediate farnesyl diphosphate to various sesquiterpene structural types. In plants, many sesquiterpenes are produced as defensive chemicals (phytoalexins) or mediators of chemical communication (i.e., pollinator attractants). A number of sesquiterpene synthases are present in *Artemisia annua* L. (annual wormwood). We have isolated a cDNA clone encoding one of these, *epi*-cedrol synthase. This clone contains a 1641-bp open reading frame coding for 547 amino acids (63.5 kDa), a 38-bp 5′-untranslated end, and a 272-bp 3′-untranslated sequence. The deduced amino acid sequence was 32 to 43% identical with the sequences of other known sesquiterpene cyclases from angiosperms. When expressed in *Escherichia coli*, the recombinant enzyme catalyzed the formation of both olefinic (3%) and oxygenated (97%) sesquiterpenes from farnesyl diphosphate. GC–MS analysis identified the olefins as α-cedrene (57% of the olefins), β-cedrene (13%), (*E*)-β-farnesene (5%), α-acoradiene (1%), (*E*)-α-bisabolene (8%), and three unknown olefins (16%) and the oxygenated sesquiterpenes (97% of total sesquiterpene generated, exclusive of farnesol and nerolidol) as cedrol (4%) and *epi*-cedrol (96%). *epi*-Cedrol synthase was not active with geranylgeranyl diphosphate as substrate, whereas geranyl diphosphate was converted to monoterpenes by the recombinant enzyme at a rate of about 15% of that observed with farnesyl diphosphate as substrate. The monoterpene olefin products are limonene (45%), terpinolene (42%), γ-terpinene (8%), myrcene (5%), and α-terpinene (2%); a small amount of the monoterpene alcohol terpinen-4-ol is also produced. The pH optimum for the recombinant enzyme is 8.5–9.0 (with farnesyl diphosphate as substrate) and the K_m values for farnesyl diphosphate are 0.4 and 1.3 μM at pH 7.0 and 9.0, respectively. The K_m for Mg^{2+} is 80 μM at pH 7.0 and 9.0.

***Key Words:* *epi*-cedrol synthase cDNA; sesquiterpene cyclase; monoterpene cyclase; bacterial expression; *Artemisia annua*.**

An important class of antimalarial compounds based on the sesquiterpenoid endoperoxide artemisinin (Fig. 1) from *Artemisia annua* L. (annual wormwood) has been developed from a traditional Chinese herbal remedy (Qinghaosu) (1). Total synthesis of artemisinin and its derivatives, dihydroartemisinin and artemether (Fig. 1), on a commercial scale has yet to be achieved and the yield of artemisinin from the plant is generally low (ca. 0.1% dry wt) (2). Attempts to obtain high-yielding strains by classical breeding and selection techniques have been of limited success (3) and cell culture methods do not appear to offer a viable production alternative (4). Of far more benefit would be the development of a new strain of *A. annua*, genetically engineered for increased production of artemisinin, which could be grown as a cropping plant in the area where the drug is actually required.

When sesquiterpene overproduction is induced in an intact plant (for example, by fungal elicitation of terpenoid C_{15}-phytoalexins in *Nicotiana tabacum*), the

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[2] The nucleotide sequence data reported in this article have been deposited with the GenBank/EMBL Database under Accession No. AJ001539.

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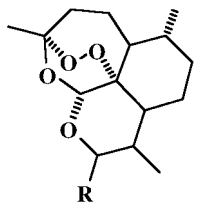


FIG. 1. Structures of artemisinin and its derivatives. R═O, artemisinin; R═OH, dihydroartemisinin; R═OCH_3, artemether; R═OCH_2CH_3, arteether.

mechanism of upregulation involves increases in the steady-state mRNA levels and the enzymatic activity for the respective sesquiterpene synthase and downregulation of squalene synthase (5, 6). Thus, a possible strategy for increasing the formation of artemisinin in *A. annua* would be upregulation of the synthase involved in the initial cyclization of farnesyl diphosphate (FDP),[4] the universal precursor of sesquiterpenoids, to the olefinic precursor of the drug. While a biosynthetic route from farnesyl diphosphate to artemisinin has been suggested (7), the exact identity of this key olefinic precursor remains unknown, although it is likely a cadinene.

A number of sesquiterpene synthases are expressed in *A. annua* since sesquiterpenes belonging to different structural classes have been isolated from the plant (8–10). Variable sesquiterpene compositions among different populations of *A. annua* have been observed, although some sesquiterpenes such as germacrene D, β-farnesene, β-caryophyllene, α-copaene, arteannuinic acid, and artemisinin appear to be present in most chemotypes of the plant (8–10). The synthases involved in the biosynthesis of these sesquiterpenes are most likely similar to other terpene synthases, particularly those from other angiosperms. A number of such enzymes have been isolated, characterized, and/or cloned, and the primary structures of these enzymes show a relatively high level of homology; it is clear that these enzymes have evolved from a common ancestor gene (11).

To investigate the biosynthesis of sesquiterpenes, and in particular artemisinin, by *A. annua,* we have begun to isolate sesquiterpene synthase clones from a cDNA library prepared from *A. annua* leaf tissue mRNA. Our aim is to identify the cyclase involved in artemisinin biosynthesis and to use the corresponding cDNA clone for metabolic engineering of *A. annua* plants. As a consequence of these efforts we have identified a new sesquiterpene cyclase, *epi*-cedrol synthase, in *A. annua* which is described in this communication. This is the first report on the cloning of a cDNA which encodes a sesquiterpene synthase that catalyzes the formation of a sesquiterpene alcohol (i.e., *epi*-cedrol).

[4] Abbreviations used: DTT, dithiothreitol; FDP, farnesyl diphosphate; GDP, geranyl diphosphate; GGDP, geranylgeranyl diphosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria–Bertani.

MATERIALS AND METHODS

Reagents. The methods for preparation of [1-^{3}H]GDP (250 Ci/mol) (12), [1-^{3}H]FDP (125 Ci/mol) (13), and [1-^{3}H]GGDP (118 Ci/mol) (14) have been reported previously. [1-^{3}H]FDP (16 mCi/mmol) was also purchased from Amersham (Piscataway, NJ). Cedrol, α-cedrene, and β-cedrene were prepared by pentane extraction of wood from locally grown *Juniperus* sp.; α-acoradiene was a gift from Larry Cool (University of California, Berkeley, CA); other terpenoid standards were from our own collection. All other biochemicals and reagents were purchased from Sigma Chemical Co. or Aldrich Chemical Co. unless otherwise noted.

Plant growth and extraction. A. annua was grown in a greenhouse at 20°C with a 16-h photoperiod. Plants were vegetatively propagated from cuttings of mature plants.

Leaves or flowers of *A. annua* were extracted with excess pentane at room temperature for 2 h. The pentane was collected by filtration and passed through a 2-ml column of $MgSO_4$–silica gel (Mallinckrodt SilicAR-60) to provide the olefin fraction; the column was then washed with diethyl ether to provide the oxygenated terpene fraction. Both fractions were analyzed for monoterpene and sesquiterpene content by GC–MS (15). Since cedrol and *epi*-cedrol may exist in *A. annua* as conjugates (ester, glycoside, etc.), an aqueous extract of leaves was prepared, and then either HCl or NaOH was added to 0.25 M at room temperature to cleave the conjugate. The extract was then partitioned into diethyl ether and the separate ether phases from each preparation were backextracted with aqueous 0.1 M NaOH or 0.1 M HCl, respectively, and then passed over a $MgSO_4$–silica gel column to prepare the samples for GC–MS analysis.

Sesquiterpene cyclase enzyme assay. An aliquot of bacterial extract or partially purified recombinant enzyme was assayed in a total of 100 μl containing 25 mM Tris/HCl (pH 8.5), 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% glycerol, and 18 μM [1-H^3]FDP (100 mCi/mmol, Amersham). After a 10-min incubation at 25°C, the reactions were stopped by addition of an equal volume of 0.2 M KOH containing 0.1 M EDTA. Subsequently, the reaction mixtures were extracted with pentane (2 × 0.5 ml) and the pentane extracts were passed over a small column filled with 200–250 mg silica (size 0.2–0.5 μm) and the column was rinsed with additional pentane (1.0 ml). The pentane extract was examined for radioactivity by scintillation counting. Oxygenated compounds were eluted from the column into scintillation vials with 2 ml of diethyl ether for analysis. Two replications were run for each sample.

Construction and screening of the cDNA library. A λZAPII cDNA library was constructed from poly(A)$^+$ RNA isolated from young fresh leaves of mature *A. annua* plants according to the manufacturer's instructions (Stratagene). This library was screened for sesquiterpene cyclase cDNA clones using as a probe the full-length cDNA encoding *epi*-aristolochene synthase from tobacco (16) (a kind gift from J. Chappell, University of Kentucky) that had been ^{32}P-labeled by random oligonucleotide-primed synthesis (Pharmacia). Twelve positive clones were identified and sequenced. One clone of about 1100 bp with high homology to known terpenoid synthases was identified and used as a ^{32}P-labeled probe for a second round of screening. Four putative full-length synthase clones were thus isolated and one clone, pAC12, was selected for further characterization.

DNA sequencing. DNA sequencing of selected cDNAs was performed according to the dideoxy-chain termination method (17). Purification of single-stranded template DNA from pBluescript SK cDNA and sequencing were carried out as in the protocol from the

Sequenase 2.0 kit (U.S. Biochemical Corp.) using M-13 reverse as an initial sequencing primer. Oligonucleotides (15-mers) were synthesized according to sequence information obtained and used directly as primers for further sequencing.

Bacterial expression and partial purification of cloned sesquiterpene cyclase. *Escherichia coli* XL1-Blue harboring pAC12 was grown at 37°C in LB medium (500 ml) containing ampicillin (50 μg/ml) until OD_{600} = 0.5. IPTG (1 mM) was added and the incubation temperature was reduced to ≤25°C. After 3 h, the cells were harvested and centrifuged and then resuspended in 20 ml of 20 mM Hepes buffer, pH 8.0, containing 10% glycerol, 10 mM β-mercaptoethanol, and 5 mM EDTA. Sonication of the cells was carried out for 2 min in 5-s pulses with 5 s between pulses at 0°C (Vibracell power setting 12–15 W). Streptomycin sulfate (1% w/v) was added and the sonicate was centrifuged at 17,400*g* for 10 min at 4°C. The supernatant was filtered through a 0.2-μm Filtropur syringe filter and then loaded onto a MonoQ HR 10/10 column (Pharmacia). Elution was achieved in the previous sonication buffer with a gradient of NaCl (0–1.0 M in 30 min) and fractions (1.0 ml) were collected at a flow rate of 1.0 ml/min. Fractions containing sesquiterpene cyclase activity were pooled and desalted on a PD10 column equilibrated with 3 mM Hepes buffer, pH 7.5, containing 10% glycerol and 1 mM DTT. Aliquots of the partially purified cyclase were stored at −80°C.

Bacterial expression for product identification. *E. coli* XL1-Blue harboring pAC12 or empty pBluescript vector was grown overnight at 37°C in LB medium containing 100 μg ampicillin/ml. A 500-μl aliquot of the overnight culture was used to inoculate 50 ml of fresh medium supplemented with 100 μg/ml ampicillin. The culture was grown at 37°C with vigorous agitation to A_{600} = 0.5 before induction with 1 mM IPTG. The culture was immediately transferred to a 20°C incubator and allowed to grow overnight with agitation. The suspended cells were centrifuged (1000*g*, 15 min, 4°C), the medium was removed, and the pelleted cells were resuspended in 4 ml of cold assay buffer containing 25 mM Mopso (pH 7.0), 10 mM sodium ascorbate, 25 mM KCl, 10 mM DTT, 1 mM EDTA, and 10% glycerol. The cells were disrupted by sonication (Braun-Sonic 2000 microprobe at maximum power for 2 × 40-s bursts with a 1-min chilling period on ice between bursts) and then centrifuged at 4500*g* for 15 min. The supernatant was transferred to a 10-ml Teflon-capped, glass screw-top tube and adjusted to a final concentration of 10 mM $MgCl_2$ and 20 μM $MnCl_2$. [1-^{3}H]GDP, [1-^{3}H]FDP, or [1-^{3}H]GGDP (4.5, 7.3, or 10 μM, respectively) was added to the reaction mixture which was overlaid with 1 ml of pentane (except for the tube containing GGDP) and the sealed tube was incubated at 30°C for 2 h. The mixture was extracted a total of three times with 1 ml of pentane each and the organic phase passed over a $MgSO_4$–silica gel column to provide the olefin fraction. The remaining aqueous phase was subsequently extracted with diethyl ether and the ether passed over the corresponding silica column to provide the oxygenated terpene fraction. An aliquot from each fraction was taken for liquid scintillation counting and the remaining organic phase was then concentrated for GC–MS analysis as described previously (15).

Characterization of epi-cedrol synthase. For characterization of *epi*-cedrol synthase, the partially purified recombinant enzyme preparation was used (see above). The pH optimum was determined in buffers of 50 mM Bis–Tris, 50 mM Hepes, and 50 mM Tris containing 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 10% glycerol, in the pH range from 6.0 to 9.3. Samples were incubated at 25°C for 15 min. Kinetic constants for FDP and Mg^{2+} (at pH 7.0 and 9.0) were determined in the same buffer as was used for pH optimum determination.

RESULTS AND DISCUSSION

Essential oil analysis. The sesquiterpene olefin composition of the extracted oil of *A. annua* was highly variable between individual plants; the most common olefins were germacrene D, β-caryophyllene, α-copaene, and α-longipinene. A single plant contained α-cedrene (ca. 0.2% of total sesquiterpene olefins). The (*E*)-β-farnesene content varied greatly from nondetectable to comprising greater than 25% of the sesquiterpene olefin fraction. (*E*)-α-Bisabolene was not detected in any extract even though it is a minor product of *epi*-cedrol synthase (see below). Germacrene D, β-caryophyllene, α-copaene, and β-farnesene have previously been reported to be present in extracts of *A. annua* (9, 10).

Neither cedrol nor *epi*-cedrol could be detected in extracts of either leaf or flower tissue of *A. annua*. It is possible that these alcohols may be further metabolized to products not separable by GC or recognizable by MS. However, acid or base hydrolysis of aqueous extracts also gave no indication that hydrolyzable derivatives of cedrol or *epi*-cedrol were present. Neither cedrol nor *epi-cedrol* has been reported from *A. annua*, but cedrol has been reported from the related species *A. mongolica*, *A. feddei*, *A. vestita*, *A. dubia*, and *A. scoparia* (18). α-Cedrene has been reported from *A. herba-alba* (19).

cDNA isolation and sequence analysis. The cDNA encoding a full-length version of tobacco *epi*-aristolochene synthase (16) was employed as a heterologous probe to screen an *A. annua* leaf cDNA library. This approach yielded a high proportion of false positives, since 5′-terminal sequencing of 12 selected clones larger than 1.0 kb provided only one (of 1.1 kb) which showed significant sequence similarity to other terpene synthases. This 1.1-kb fragment was subsequently employed as a probe in a higher stringency screen of the library, from which 4 putative, full-length clones were ultimately isolated. Three of these clones were identical in size (1951 bp). The fourth clone (pAC12) lacked 21 nucleotides in the 5′-untranslated region but was otherwise identical in sequence thereafter. All 4 clones were identical to the 1.1-kb fragment except that this fragment was greatly truncated in the 5′ terminus.

A full-length clone contained an insert of 1951 bp with a putative plant translation starting sequence CAAGCATGT at positions 34–42 followed by an open reading frame of 547 amino acids. This sequence (at positions 34–42) matches the consensus for plant gene translation start sites (5′-G/CAANNATGG). Upstream of the putative starting codon is a stop codon in frame with the open reading frame indicating that there can be no prior start codon. The start codon and a typical poly(A) signal occurring at position 1932–1937 (AATAAT) establish that this clone is full length.

The deduced mass of the encoded protein is 63.5 kDa and the p*I* is calculated to be 4.94. The low p*I* and the molecular weight are similar to those reported for

plant sesquiterpene cyclases (15, 16, 20–22). The deduced sizes of mono- and diterpene synthases are usually somewhat larger, i.e., around 600 aa, because of the additional plastidial targeting signal sequence located at the N-terminus of these enzymes (23). To date, no plastidial targeting sequence has been found in any sesquiterpene cyclase from plants, and it is assumed that sesquiterpene cyclases are exclusively localized to the cytosolic compartment (23). The isolated clone does not encode a plastidial targeting sequence.

The deduced amino acid sequence of the *Artemisia* enzyme shows relatively high homology to other sesquiterpene synthase sequences and especially to those from angiosperms. An alignment of sesquiterpene synthases from angiosperms, including the *Artemisia* cyclase, has been carried out and a consensus sequence obtained (Fig. 2). One region contains the highly conserved aspartate-rich motif (DDXXD) which is found in all terpenoid synthases (23), including those of microbial origin, as well as in prenyltransferases which operate by a related mechanism on the common prenyl diphosphate substrates (24, 25). Based on directed mutagenesis and X-ray structural investigation, it has been concluded that this motif is responsible for binding the divalent metal ion(s) of the substrate diphosphate–metal ion(s) complex (26). Chen *et al.* (24) have identified an additional five conserved regions in terpenoid cyclases, as indicated in Fig. 2 (21). The seven sesquiterpene synthases aligned show some additional conserved regions which may be related to substrate/product specificity of this type of enzyme.

The highest sequence homology is observed between the *Artemisia* cyclase and δ-cadinene cyclases from *Gossypium* (21, 27) with similarities between 65 and 67% and identities between 41 and 43%. Germacrene C synthase from tomato (*Lycopersicon esculentum*) (20), vetispiradiene synthase from *Hyoscyamus muticus* (22), and *epi*-aristolochene synthase from pepper (*Capsicum annuum*) (28) and tobacco (16) all show similarities of 61–64% and identities of 40.5–42.5%. A somewhat lower homology is observed for (*E*)-β-farnesene synthase from peppermint (63.6% similarity, 32.5% identity) (15). Sesquiterpene cyclases (γ-humulene, δ-selinene, and *E*-α-bisabolene synthase) from the gymnosperm *Abies grandis* exhibit 56.0–58.5% similarity and 29.0–33.9% identity to the *Artemisia* clone (29, 30). The homology to monoterpene cyclases, such as pinene synthase from *A. grandis* (31) and *S*-linalool synthase from *Clarkia breweri* (32), varies but is in general somewhat lower (47–57% similarity, 20–32% identity). The diterpene synthase from *Ricinus communis* L, casbene synthase (33), shows the highest homology among the diterpene cyclases (63.4% similarity, 39.0% identity) to the *Artemisia* enzyme.

Alignments of the deduced amino acid sequences of mono-, sesqui-, and diterpene cyclases show a number of conserved regions, suggesting that the plant terpene synthases have evolved from one ancestral synthase gene. However, analysis of exon/intron organization of some terpenoid synthase genes indicates that these plant enzymes may have evolved from two distinct ancestral genes (34). The 3′-termini exhibit the same exon/intron organization while the organization of the 5′-terminus differs considerably. It is interesting to note that the level of homology between deduced amino acid sequences of synthases showing identical substrate/product specificity from different species can be lower than the homology between two synthases catalyzing the formation of completely different terpene structural types (11). At present, only the general class of terpene synthase (mono-, sesqui-, and di-) can be predicted by sequence analysis. Prediction of the exact terpene structural type is not currently possible by sequence analysis alone and awaits further understanding of synthase active-site structure.

Bacterial expression. Crude extracts of *E. coli* harboring pAC12 displayed no activity with [1-^{3}H]GGDP as substrate, but GC–MS analysis of the enzymatic products derived from [1-^{3}H]GDP and [1-^{3}H]FDP revealed complex mixtures. Eight sesquiterpene olefins, α-cedrene (57% of the olefins), β-cedrene (13%), (*E*)-β-farnesene (5%), α-acoradiene (1%), (*E*)-α-bisabolene (8%), unknown 1 (4%), unknown 2 (8%), and unknown 3 (4%), together comprised 3% of the total products generated in the assay from FDP (exclusive of farnesol and nerolidol) (Fig. 3). The oxygenated sesquiterpenes (97% of total sesquiterpenes generated exclusive of farnesol and nerolidol) consisted of cedrol (4%) and *epi*-cedrol (96%), the major product for which the synthase is named (Fig. 3). Farnesol and nerolidol were detected in amounts similar to those found in extracts of *E. coli* harboring empty plasmid, and these products are likely derived by solvolysis and/or from the action of nonspecific phosphatases. The cedrol epimer is likely the C-3 epimer (farnesol numbering system; Fig. 4) and not di-*epi*-cedrol (C1 methylene bridge epimer bearing the funebrane skeleton) as this stereochemistry is consistent with the absolute configuration of α-cedrene, β-cedrene, and cedrol generated by the recombinant enzyme. Neither di-*epi*-α-cedrene (α-funebrene) nor di-*epi*-β-cedrene (β-funebrene) was detected as an enzymatic product. The retention time by GC analysis and the mass spectrum of the biosynthetic *epi*-cedrol also matched those of a minor component tentatively identified as *epi*(C3)-cedrol in extracts of *Juniperus.* Although (*E*)-β-farnesene occurs in the essential oil of *A. annua, epi*-cedrol synthase cannot be the sole source of this olefin since it accumulates in large amounts even when other coproducts of *epi*-cedrol synthase are not present. Therefore, (*E*)-β-farnesene must also be produced by other synthase(s).

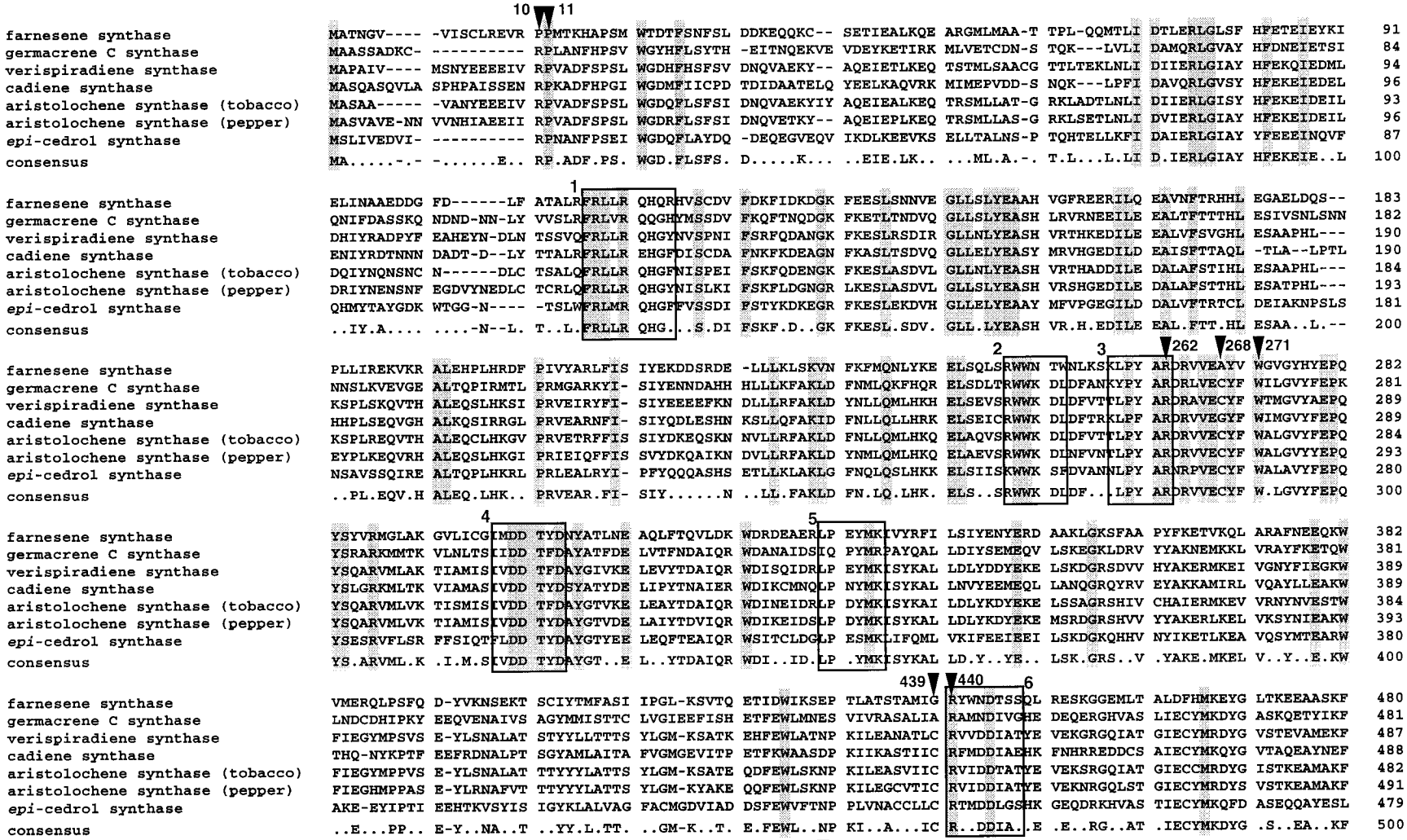
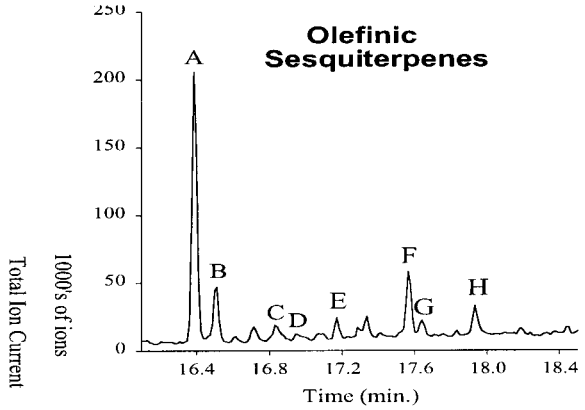
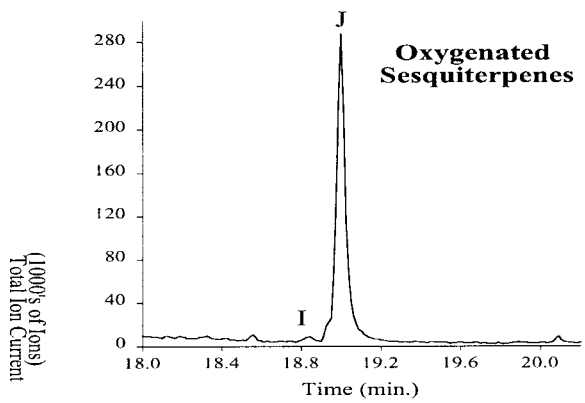
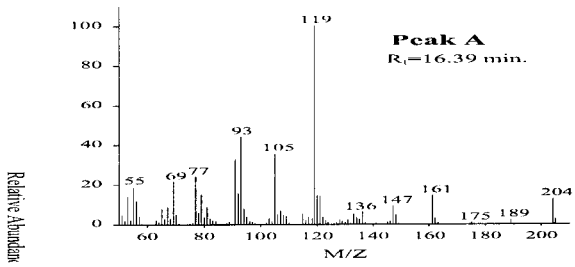


FIG. 2. Alignment of the deduced amino acid sequences of seven sesquiterpene synthases from angiosperms. The six regions of high similarity found among terpene synthases [after Chen *et al.* (21)] are boxed and numbered. Amino acid residues discussed in the text are labeled with an arrowhead and the residue number in *epi*-cedrol synthase. Amino acids identical in all seven enzymes are in shaded boxes. The consensus sequence (four of the seven synthases containing the same residue) is indicated.


Olefinic Sesquiterpenes
A
B
C
D
E
F
G
H
1000's of ions
Total Ion Current
Time (min.)


Oxygenated Sesquiterpenes
I
J
(1000's of Ions)
Total Ion Current
Time (min.)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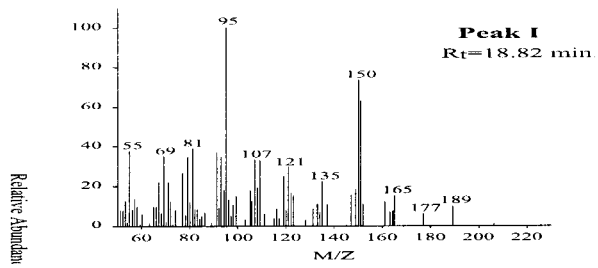
Rt=16.39 min.
Relative Abundance
M/Z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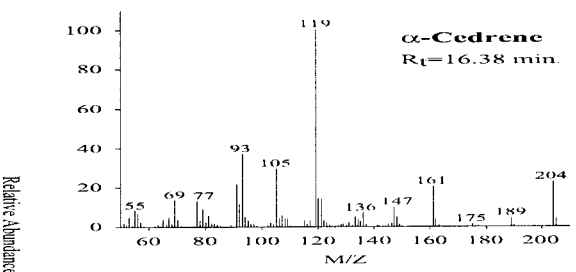
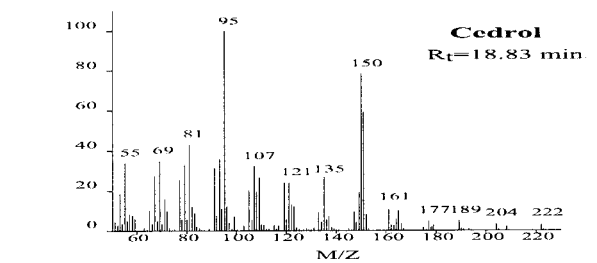
Relative Abundance
M/Z


α-Cedrene
Rt=16.38 min.
Relative Abundance
M/Z


Cedrol
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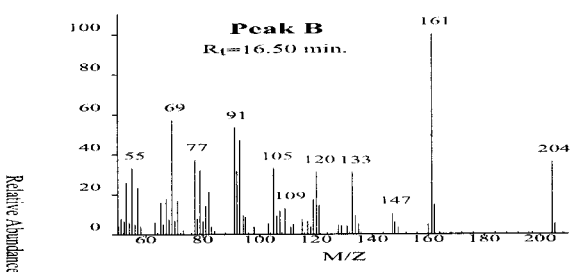
Relative Abundance
M/Z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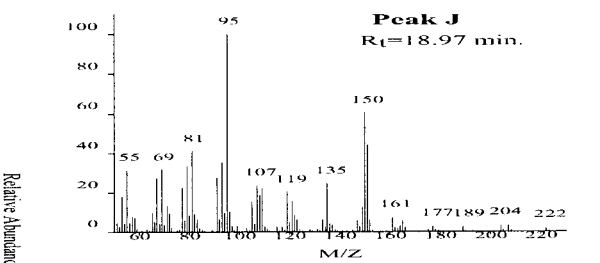
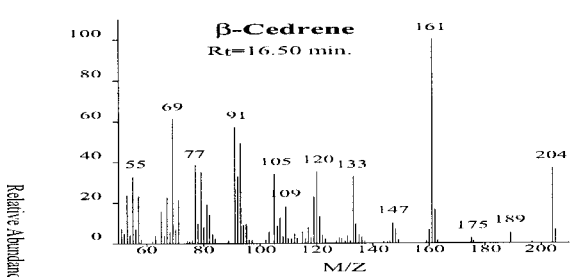
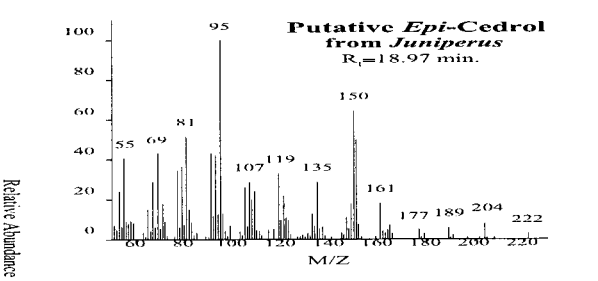
Relative Abundance
M/Z


Peak J
Rt=18.97 min.
Relative Abundance
M/Z


β-Cedrene
Rt=16.50 min.
Relative Abundance
M/Z


Putative Epi-Cedrol
from Juniperus
Rt=18.97 min.
Relative Abundance
M/Z

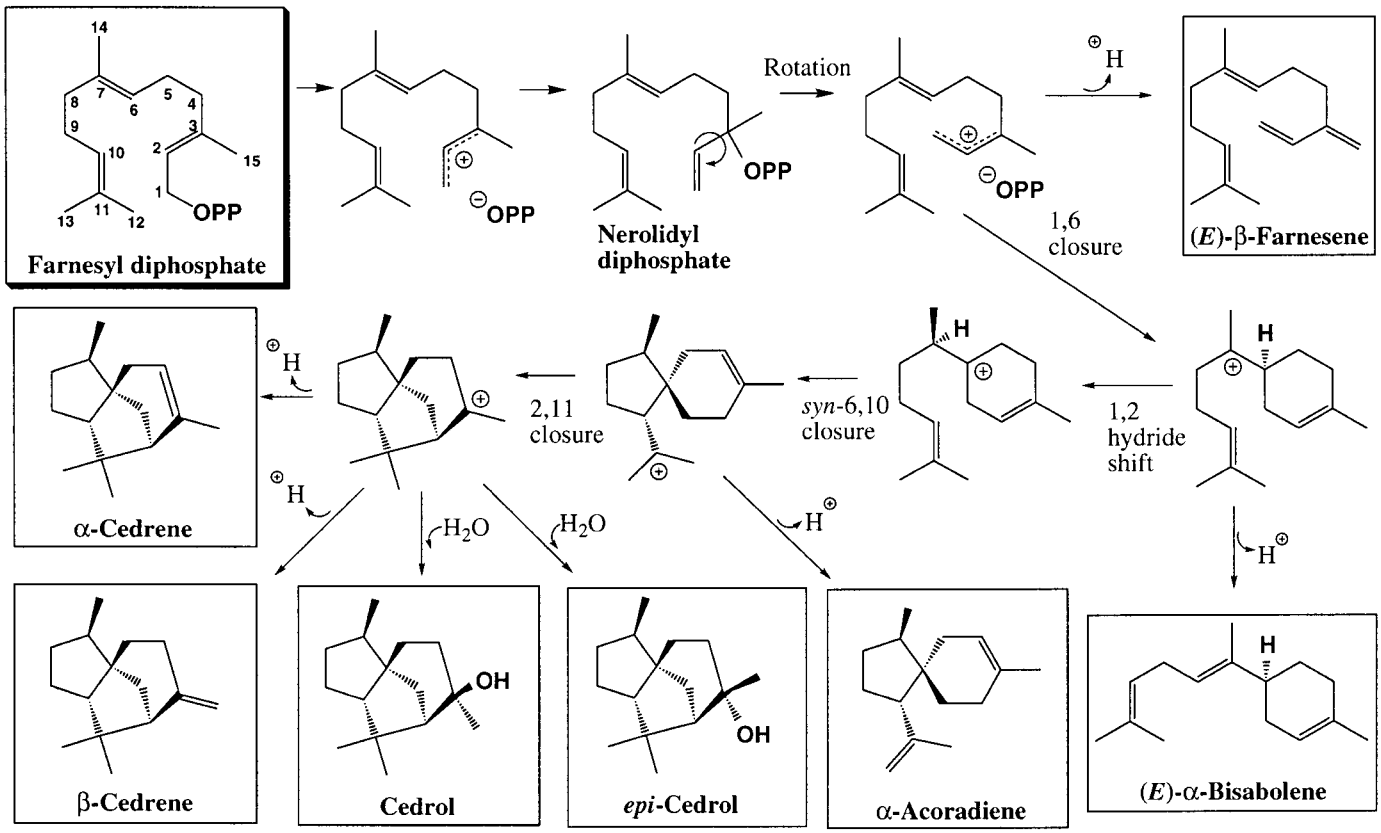


FIG. 4. Proposed mechanism for the formation of sesquiterpenes from FDP by *epi*-cedrol synthase. Structures enclosed in boxes have been identified by GC–MS and only relative stereochemistry is implied. (*E*)-*β*-Farnesene could also be derived by ionization of FDP and deprotonation of the resulting cation. OPP denotes diphosphate moiety.

epi-Cedrol synthase can also catalyze the divalent metal ion-dependent conversion of [1-^{3}H]GDP to a mixture of monoterpene olefins (major products) and a single monoterpene alcohol. Under saturating substrate concentration, the rate of monoterpene synthesis by the recombinant enzyme is about 15% of the rate of sesquiterpene synthesis from FDP. Limonene (45%), terpinolene (42%), γ-terpinene (8%), myrcene (5%), and α-terpinene (2%) comprise the olefinic products of *epi*-cedrol synthase when incubated with the C10 substrate (Fig. 5). The only significant oxygenated product detected with GDP as substrate was terpinen-4-ol, at 0.3% of the total oxygenated terpene fraction (Fig. 5) from the assay. Linalool, α-terpineol, nerol, and geraniol were also produced, but in amounts consistent with their formation by solvolysis (or phosphatase action) on the substrate. The adventitious use of GDP as a substrate by sesquiterpene synthases has been observed previously (15, 30, 31); however, with the previously reported sesquiterpene olefin synthases, only monoterpene olefins are generated, presumably because of the exclusion of reactive water from the active site. Since the formation of cedrols requires water as a nucleophile to quench the terminal carbocation of the reaction sequence, water would be expected to be available for quenching of the terpinen-4-yl cation in the monoterpene series as well. As with all reported sesquiterpene synthases which can utilize GDP as a substrate (15, 30, 31), limonene is the most abundant product, regardless of the sesquiterpene structural type produced. This simple olefin is apparently the "default" monoterpene, perhaps generated as a consequence of the most favorable folding of the geranyl substrate at an active site that is too large to enforce

FIG. 3. Total ion chromatograms of the sesquiterpene products derived from FDP by recombinant *epi*-cedrol synthase. In the column below each chromatogram are mass spectra and retention times for selected (lettered) peaks. The mass spectrum for peak I is compromised due to its low abundance. Other lettered peaks in the chromatograms denote the sesquiterpenes (*E*)-*β*-farnesene (C), α-acoradiene (D), unknown 1 (E), unknown 2 and a coeluting alkane (F) (*E*)-α-bisabolene (G), and unknown 3 (H) (spectral data not shown).

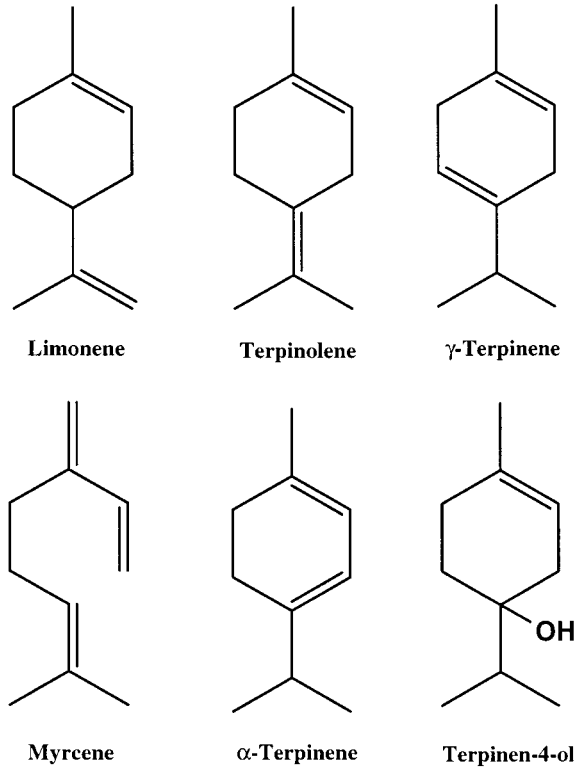


FIG. 5. Monoterpenes generated from the adventitious substrate GDP by recombinant *epi*-cedrol synthase.

additional conformational restraint. Extended substrate conformers which would yield acyclic products like myrcene (only 5% of the olefinic monoterpene products) are clearly disfavored.

Characterization of epi-cedrol synthase. The recombinant, partially purified *epi*-cedrol synthase exhibited maximum activity at a relatively high pH (8.5–9.0; Fig. 6). This optimum is the same when enzyme activity is measured as olefins or as alcohols produced. The ratio of olefin to alcohol formation remains essentially constant (0.24–0.28) throughout the pH range investigated. For other sesquiterpene synthases, pH optima in the range of 6.5–8.0 have been reported (15, 21).

The K_m value for FDP with the recombinant synthase was calculated to be 0.4 μM at pH 7.0, a value typical of other sesquiterpene synthases of plant origin (15, 27). At pH 9.0, the K_m for FDP is somewhat higher at 1.3 μM. These K_m values were, as expected, the same when the activity was measured as olefins or alcohols produced. A K_m value of 80 μM was determined for Mg^{2+} at both pH 7.0 and 9.0. The enzyme also exhibits activity in the presence of Mn^{2+} and demonstrates a roughly threefold increase in activity with 60 μM Mn^{2+} compared to saturating (2 mM) levels of Mg^{2+}. At higher levels of Mn^{2+} (120 μM), a significant reduction in activity was monitored. This inhibition of activity by Mn^{2+} has been reported for (*E*)-β-farnesene synthase and partially purified β-selinene synthase (15, 35).

Enzyme mechanism. As with many other plant terpenoid synthases, *epi*-cedrol synthase catalyzes the formation of multiple products from a single substrate, probably as a consequence of the highly reactive series of carbocationic intermediates formed sequentially during the reaction cycle (25). With tobacco *epi*-aristolochene synthase, binding of FDP results in closure of an α-helical loop over the active site that shields the developing carbocation from solvent water (26). This loop is held in place by hydrophobic interactions between Trp^{273} and Tyr^{527}; these residues are conserved as Trp^{271} and Phe^{526} in *epi*-cedrol synthase. Loop closure brings two basic residues (Arg^{264} and Arg^{441}) in *epi*-aristolochene synthase, and presumably in *epi*-cedrol synthase (Arg^{262} and Arg^{440}), near to each other. This concentration of charge assists in ionization of the substrate diphosphate ester and helps to move the diphosphate anion away from the developing carbocation, with the assistance of the divalent metal cation(s) coordinated by the DDXXD motif present in all known terpene synthases (11). Following ionization of FDP (Fig. 4), capture of the paired diphosphate anion at C3 to form nerolidyl diphosphate allows rotation about C2–C3 to the *cisoid* conformer necessary for C1,C6 closure. Ionization of nerolidyl diphosphate and subsequent ring closure generate the bisabolyl cation, while deprotonation of either the farnesyl or nerolidyl cation allows the formation of (*E*)-β-farnesene (see Fig. 2). Grand fir (*E*)-α-bisabolene synthase (30) contains tan-

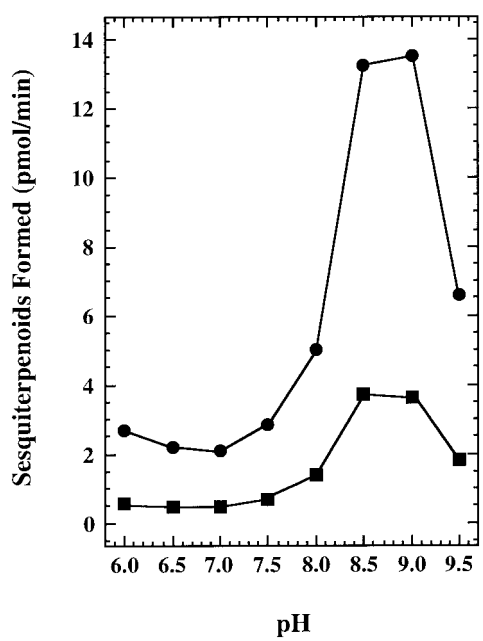


FIG. 6. *epi*-Cedrol synthase activity as a function of pH. (●) Oxygenated products; (■) olefinic products.

dem arginine residues near the N-terminus which presumably function to aid ionization and isomerization to the tertiary allylic isomer nerolidyl diphosphate, in a manner analogous to linalyl diphosphate formation by the monoterpene cyclase limonene synthase, in order to orchestrate C1,C6 closure (36). Substitution of the second arginine in limonene synthase results in an enzyme incapable of catalyzing this obligatory rearrangement of the geranyl diphosphate ester (36). Although the formation of *epi*-cedrol from FDP (and indeed all the monoterpenes except myrcene that are generated from GDP by *epi*-cedrol synthase) proceeds by isomerization to the tertiary allylic form with rotation about C2–C3 of this intermediate, *epi*-cedrol synthase lacks the second arginine of the N-terminal arginine pair and contains instead an Arg^{10}–Pro^{11} pair. The Arg–Pro pair motif is conserved among all cloned angiosperm sesquiterpene synthases (Fig. 2) (15, 16, 20–22), regardless of whether an intervening isomerization step to nerolidyl diphosphate is required.

The bisabolyl cation generated by *epi*-cedrol synthase (Fig. 4) may deprotonate directly, releasing (*E*)-α-bisabolene as a minor product, or undergo a 1,2-hydride shift with subsequent attack on C10, *syn* to C14. The acorane skeleton is thus generated and may suffer deprotonation at either C12 or C13 to afford α-acoradiene which is detectable as a minor product. Interestingly, cedrane sesquiterpenes have not been reported from *A. scoparia;* instead, this species accumulates α-acoradiene, the synthesis of which may be catalyzed by a cedrol synthase that possesses an altered active site which blocks further progression to the cedrane skeleton (20). Because closure to the acorane skeleton places the C11 cationic center in close proximity to C2, flux in the *epi*-cedrol reaction cycle proceeds through a third closure to assemble the tricyclic cedrane skeleton. Two alternatives for deprotonation of the cedryl cation, at C4 or C15, yield either α- or β-cedrene, whereas capture by water from either face gives rise to the cedrols. The 96:4 ratio of *epi*-cedrol to cedrol produced by this enzyme may result from preferential facial attack by the placement of coordinated water at the active site or from steric hindrance from the C12,C13 methyl groups.

epi-Cedrol synthase represents the first sesquiterpene cyclase to be described that produces the mechanistically complex cedrane structural family and the first that predominately quenches the cationic reaction sequence with water. While of considerable interest in the context of sesquiterpene synthase structure–function relationships, the rationale for the presence of such an enzyme in *A. annua* is uncertain since the cedranes do not appear to be significant metabolites of this species. Efforts are underway to better define the functional roles of this enzyme *in planta*.

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