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The Molecular Cloning of 8-Epicedrol Synthase[1] from *Artemisia annua*[2]

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A cDNA library was prepared from *Artemisia annua*, and a 129-bp fragment was amplified from this library using primers corresponding to sequences conserved in known dicot sesquiterpene synthases. A 1641-bp open reading frame that encoded a predicted protein 35–38% identical to dicot sesquiterpene synthases was cloned using this fragment as a hybridization probe. The gene product expressed in *Escherichia coli* cyclized farnesyl diphosphate to a 96:4 mixture of (−)8-epicedrol and cedrol. Neither cedrol epimer was detected by GC–MS in an *A. annua* extract prepared from the same specimen as the cDNA. © 1999 Academic Press

Artemisinin (Fig. 1, **1**) is an antimalarial sesquiterpene lactone produced by *Artemisia annua* (1). This compound and semisynthetic derivatives such as artemether are among the most potent chemotherapeutic agents against quinine-resistant forms of *Plasmodium falciparum* (2). The relatively low yield of artemisinin from plants results in prohibitive costs ($250/g) in the developing world where malaria is most prevalent [reviewed in (3)]. We are cloning sesquiterpene biosynthetic genes in an effort to identify those involved in artemisinin biosynthesis, with the eventual goal of making recombinant plants that overproduce these genes to increase artemisinin yields. As a sesquiterpene, artemisinin is presumably biosynthesized from farnesyl diphosphate (FDP,[4] **2**) (4). Although the initial cyclic hydrocarbon remains uncharacterized, the sesquiterpene carboxylic acid artemisinic acid (**3**) has been shown to be biosynthesized by *A. annua* (5) and converted to artemisinin (6), strongly suggesting the intermediacy of the sesquiterpene hydrocarbon 4,11-amorphadiene [**4,** which has been isolated from natural sources (7), but not from *A. annua*].

Several sesquiterpene synthase genes have been cloned from a variety of plants (reviewed in 4) that provide guidance for homology-based approaches, and we embarked on obtaining similar genes from *A. annua* with the expectation that an exhaustive search would provide the sesquiterpene synthase that forms the artemisinin skeleton. In the course of this work, we isolated and characterized a sesquiterpene synthase gene from *A. annua* that is apparently not involved in artemisinin biosynthesis, but that converts FDP to 8-epicedrol, which has not been previously reported as a natural product.

EXPERIMENTAL PROCEDURES

Plant materials, substrates, and reagents. *A. annua* plants were purchased from Teas Nursery (Houston, TX) and were grown in partial sun in Houston from April to June. Farnesyl diphosphate was synthesized according to literature procedures (8). mRNA isolation and library construction materials were from Gibco-BRL. Restriction enzymes were from New England Biolabs. Farnesol and reagents for FDP synthesis were from Aldrich, 8-epicedrol was from Fluka, and cedrol was from Lancaster. Other reagents were from Fisher Scientific.

A. annua mRNA preparation and cDNA library construction. The above-ground parts of budding *A. annua* were frozen in liquid nitrogen and ground in prechilled mortars to a fine power in liquid nitrogen. The powder was suspended in 10 vol of Trizol [guanidinium thiocyanate–phenol–chloroform (Gibco-BRL)] and total RNA was isolated using the Gibco-BRL MessageMaker kit according to the manufacturer's instructions. mRNA was obtained by two selections

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[4] Abbreviations used: FDP, farnesyl diphosphate; IPTG, isopropyl-β-D-thiogalactoside; DTT, dithiothreitol.

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over oligo(dT) columns (Gibco-BRL) according to the manufacturer's instructions. The Gibco-BRL Superscript Plasmid system was used to make the cDNA library according to the manufacturer's instructions. *Escherichia coli* host strain DH5α was transformed with the ligated library by electroporation. The resultant cDNA library had $\sim 10^6$ independent transformants, and the average size of 24 inserts was $\sim$1.3 kbp.

8-Epicedrol synthase gene cloning. Conserved segments of *Hyoscyamus muticus* vetispiradiene synthase (9), *Nicotiana tabacum* epiaristolochene synthase (10), and two *Gossypium arboreum* cadinene synthase genes (11, 12) were identified, and 11 forward and 11 reverse PCR primers were designed. All possible combinations of these primers were tested for their ability to amplify DNA from the *A. annua* cDNA library. Each PCR included 200 ng cDNA, 5.0 μl 10$\times$ buffer (500 mM Tris–HCl, pH 9.1, 160 mM ammonium sulfate, 35 mM $MgCl_2$), 4.0 μl dNTPs (each at 2.5 mM), 5.0 μl of each degenerate primer (20 pmol/μl), and H_2O to 50 μl. The PCR program included a 4-min 95°C hot start during which 0.5 μl *Taq* polymerase (Fisher, 5.0 U/μl) was added and 40 cycles with 1 min annealing using a temperature gradient from 68 to 48°C ($-$0.5°C/cycle), 3 min extension at 72°C, and denaturation for 45 s at 95°C. The program was terminated with a 5-min extension at 72°C. Each PCR (5 μl) was analyzed on a 1% agarose gel. Of all these combinations, only the pair of forward primer GAYMGNGYNGTNGARKGNTAYTTYTGG [corresponding to DR(A/V)VE(C/G)YFW] and reverse primer SCRTANGMRTCRWANGTRTCRTC [corresponding to the reverse complement of DDT(F/Y)D(A/S)Y(G/A)] gave a clear band at the expected size (129 bp). The PCR fragment was subcloned into T-tailed (13) pBluescript II KS(+) to make plasmid LH4.0. The DNA sequence was consistent with a fragment of a sesquiterpene synthase, and the insert was used as a probe to hybridize to the original cDNA library.

The 129-bp insert of plasmid LH4.0 was excised with *Eco*RI and *Hin*dIII, labeled with [α-^{32}P]dCTP by oligonucleotide priming (14) using the primer LH4F1 (GACAGGGCGGTGGAAGG), and used to probe 1.2×10^6 colonies from the cDNA library by hybridization (14). DNA from three of the four hybridizing colonies gave a PCR-amplified band of the appropriate size using primers designed to specifically amplify the target gene (LH4F2, TGGGCATTAGCAGTGTAC; and LH4R1, CATAGGAGTCGTACGTGTC). Inserts from these three colonies ranged in size from 1.2 to 1.9 kbp, and restriction analysis indicated that they all derived from the same gene. The sequence of the longest insert was determined on both strands, but was not full length based on homology to sesquiterpene synthases.

The 5′ end of the cDNA was amplified from the library using anchored nested PCR with the T7 primer and the primer LH4.2R1 (CGTCCCTCTTTGTCTTTG) in the first round and the T7 primer and the primer LH4.2R2 (TGTCGCATGAGTCGAAAC) in the second round. The 450-bp fragment obtained from this PCR was subcloned into pBluescript KS(+) and sequenced. The sequence was used to design a primer (LH4.6F1, CCGTACTAATTCGCATCT) upstream of the presumed initiator codon. LH4.6F1 and LH4.2R2 were used to amplify the cDNA library using the high-fidelity polymerase *Pfu* (Stratagene). This fragment was cloned into the longest cDNA obtained (using the *Nde*I site at nucleotide 277 of the coding sequence to join the two segments) to give LH4.8, which has a 1.7-kbp insert that encodes the complete 8-epicedrol synthase cDNA.

8-Epicedrol synthase expression, incubation with farnesyl diphosphate, and product structural characterization. PCR was used to introduce a *Xho*I site immediately upstream of the 8-epicedrol synthase initiator codon to facilitate subcloning into pET15b. The reassembled 8-epicedrol synthase (plasmid LH4.8) was PCR-amplified using *Pfu* and the primers ATCTCGAGATGTCTCTTATA (the introduced *Xho*I site is underlined) and LH4.2R2 (see 8-epicedrol synthase gene cloning). The PCR product was digested with *Xho*I and *Nde*I and ligated into LH4.8 cut with the same two enzymes. The insert was excised from the resultant construct with *Xho*I and *Not*I and subcloned into a pET15b (Novagen) derivative in which a *Not*I site had been inserted in the polylinker (30). The resultant plasmid encodes a protein tagged with six histidine residues at its amino terminus and was expressed in the *E. coli* strain BL21(DE3)pLysS (Novagen). To express protein, 1-ml overnight cultures were diluted into 14 ml LB supplemented with 100 μg/ml carbenicillin and 25 μg/ml chloramphenicol. These cultures were shaken 2 h at 30°C (OD_{600} = 0.7), induced with 100 μM IPTG, and harvested by centrifugation after an additional 3 h at 30°C. The cell pellet was resuspended in 750 μl of lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, and 100 mM NaCl) and 50 μl of 10 mg/ml lysozyme and incubated at room temperature for 1 h.

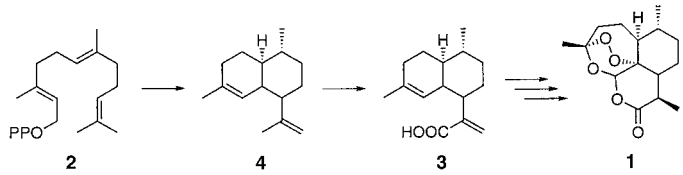

FIG. 1. The artemisinin biosynthetic pathway. FDP (**2**) is cyclized to 4,11-amorphadiene (**4**) and oxidized to artemisinic acid (**3**). Further oxidation generates artemisinin (**1**).

The recombinant enzyme was tested for activity by adding 30 μl lysate and 10 μl 1 μg/μl FDP to a 500-μl aliquot of assay buffer as described for assaying casbene synthase (15). The enzymatic reactions were incubated at room temperature for 12 h. The reaction was extracted with pentane and the organic layer was dried with sodium sulfate, concentrated, and analyzed by GC–MS. The reaction was monitored by GC (Restek Rtx-5 30-m, 0.25-mm-i.d., 0.10-μm df crossbond, 5% diphenyl–95% dimethyl polysiloxane column, 70°C, 1 min, 15°C/min to 250°C). The effect of glycerol, reductants, and divalent cations was examined by assaying in 50 mM Tris, pH 8.0, with varying glycerol (0 or 15%), β-mercaptoethanol (5 or 10 mM), DTT (0.5–2.0 mM), Mn^{2+} (1–4 mM), or Mg^{2+} (1–4 mM) content.

A large-scale incubation was conducted by inoculating 80 ml of overnight culture into a 4-liter flask containing 1.2 liters LB with 100 μg/ml carbenicillin and 25 μg/ml chloramphenicol. The flask was shaken at 30°C until the OD_{600} reached 0.8. IPTG was added to 100 μM and the flask was shaken another 3 h. The cells were harvested by centrifugation at 4°C for 20 min in a Sorvall GS-3 rotor at 5000 rpm. The 5.8-g cell pellet was washed once with lysis buffer, resuspended in lysis buffer at 100 mg/ml, and passed three times through a French press at 7000 psi.

The crude lysate was incubated with 150 μmol FDP in 400 ml 50 mM Tris, pH 8.0, 15% glycerol, 2.5 mM DTT, 1 mM $MgCl_2$ for 14 h. The reaction was quenched by adding 400 ml ethanol, and the precipitate was removed by centrifugation. The supernatant was extracted three times with 1 vol of hexane, which was then dried with sodium sulfate and concentrated to dryness. The crude product was chromatographed and eluted in hexane:CH_2Cl_2:EtOAc [50:45:5 (v/v)].

GC–MS analysis of A. annua tissue. A portion of the *A. annua* specimen from which the cDNA library was prepared was extracted with ether in a Soxhlet extractor. The resultant oil was examined by GC–MS as described above for the product of the recombinant protein.

RESULTS AND DISCUSSION

Cloning of a sesquiterpene cyclase gene from A. annua. Significant progress has been made toward elucidating the artemisinin biosynthetic pathway (Fig. 1) (5, 6, 16–18). Although the initial steps in the pathway (cyclization of FDP to 4,11-amorphadiene and subse-

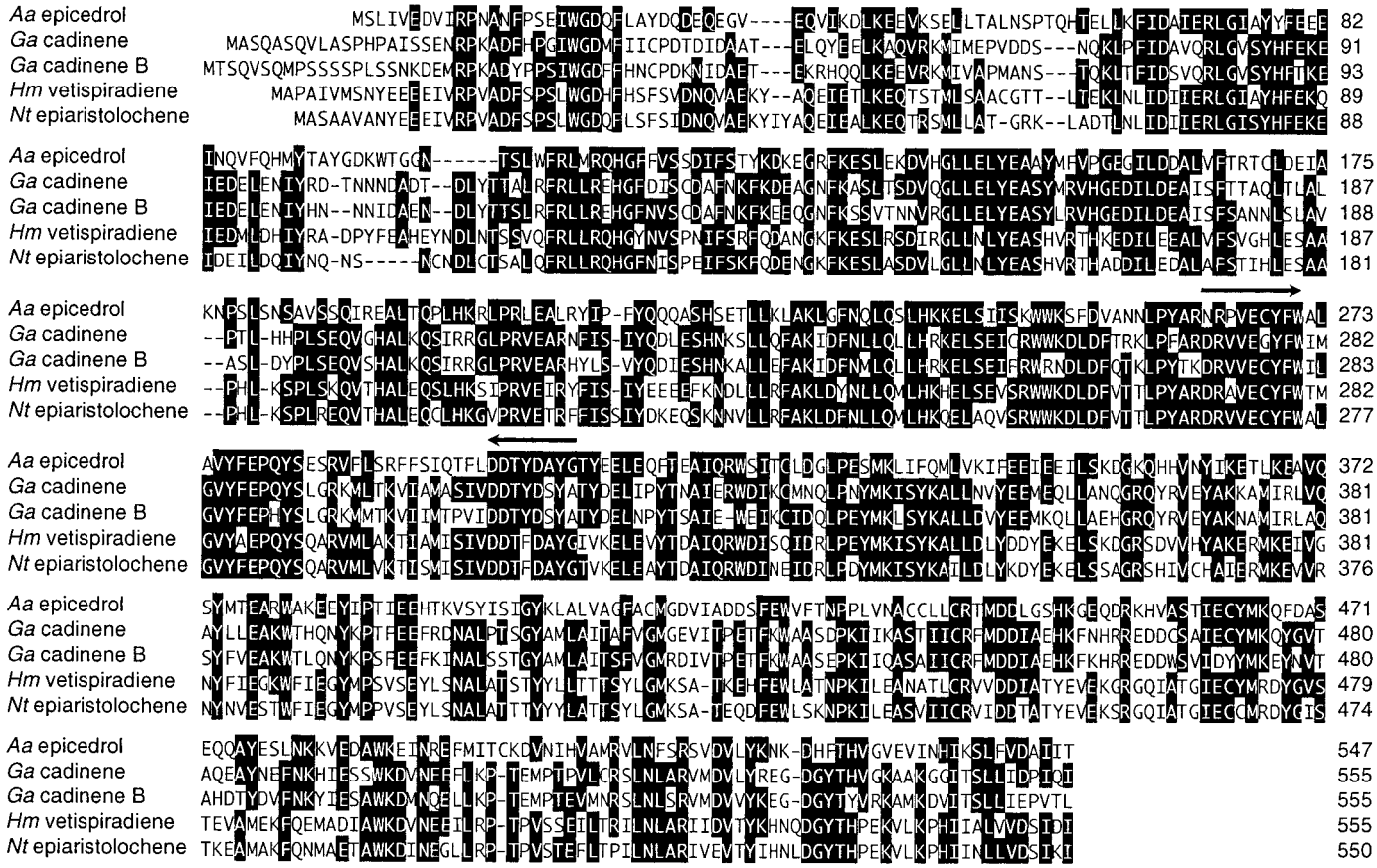

FIG. 2. Alignment of plant sesquiterpene cyclases. Sequences were aligned with the Megalign program (DNAStar, Madison, WI) by using the Clustal method. Shown are *A. annua* epicedrol synthase, *G. arboreum* cadinene synthase (11), *G. arboreum* cadinene synthase B (12), *H. muticus* vetispiradiene synthase (9), and *N. tabacum* epiaristolochene synthase (10). Amino acid residues identical in at least three of the five sequences are shaded, and hyphens indicate gaps introduced to maximize alignment. Arrows mark the regions used to design degenerate PCR primers (see Experimental Procedures).

quent oxidation to artemisinic acid) have not yet been experimentally verified, no other route seems plausible. It is generally accepted that sesquiterpenes are derived from FDP (4), and artemisinic acid has been shown to be an artemisinin precursor (5, 6). A reasonable mechanism can be drawn for the cyclization to 4,11-amorphadiene, and enzymatic allylic oxidations such as that from 4,11-amorphadiene to artemisinic acid are common. Sesquiterpene synthases cyclize FDP to structurally related compounds, and these enzymes have similar sequences.

Anticipating that the *A. annua* sesquiterpene synthases would resemble other dicot sesquiterpene synthases, we sought representatives of this class using a homology-based PCR approach. Conserved segments were identified in *H. muticus* vetispiradiene synthase (9), *N. tabacum* epiaristolochene synthase (10), and two *G. arboreum* cadinene synthase genes (11, 12), and 11 forward and 11 reverse PCR primers were designed. All possible pairs of these primers were examined for their ability to amplify an *A. annua* cDNA library, but only one combination gave an appropriately sized product. The sequence of this 129-bp fragment was consistent with being a sesquiterpene synthase. The fragment was used to probe the *A. annua* cDNA library, and the three clones that were isolated represented the same gene, but even the longest of these was only a partial cDNA compared to other dicot sesquiterpene synthases. PCR with an internal primer and a sequence from the vector provided the 5′ end of the gene, and the full-length gene was reconstructed from the two fragments. The 1641-bp open reading frame encodes a 64-kDa predicted protein with 35–38% identity to sesquiterpene synthases (Fig. 2) and significant similarity to monoterpene and diterpene synthases (data not shown).

Protein expression and optimizing the in vitro enzymatic reaction. The protein was expressed as a His-Tag fusion to facilitate future purification. The gene was subcloned into a pET15b derivative, and the expressed protein was examined for sesquiterpene syn-

thase activity using conditions described for trichodiene synthase (19). The recombinant protein converted FDP into a new compound evidenced by GC analysis. Since a parallel cell-free extract of *E. coli* carrying empty vector did not produce this compound, the protein product of the expressed gene catalyzed the reaction. The reaction was optimized by varying divalent metal cofactors, glycerol, and reductant (see Experimental Procedures). As with other angiosperm terpene synthases (4), epicedrol synthase is activated by Mg^{2+} (1 mM $MgCl_2$ is optimal) but not by Mn^{2+}. Either DTT or β-mercaptoethanol can serve as reductant, but 2.5 mM DTT gave slightly better epicedrol yield. The optimal buffer used for the large-scale reaction was 50 mM Tris, pH 8.0, 15% glycerol, 2.5 mM DTT, 1 mM $MgCl_2$.

Product characterization reveals 8-epicedrol. A reaction with 150 μmol FDP was done to obtain material for structural characterization. GC of the crude product with flame-ionization detection showed that the sesquiterpene product of the *A. annua* enzyme had two distinct isomers with retention times of 9.84 and 9.96 min in a 4:96 ratio, respectively. GC–MS analysis showed that these two compounds are $C_{15}H_{26}O$ sesquiterpene alcohols (M_r 222) and not $C_{15}H_{24}$ sesquiterpene hydrocarbon products (M_r 204). Subsequent GC–MS analysis uncovered no sesquiterpene hydrocarbons. The minor product is cedrol, as its EI fragmentation matched that of cedrol, and it coeluted with cedrol in GC coinjection experiments. Although the EI mass spectrum of the major product also resembled that of cedrol, these compounds had different GC mobilities. Silica gel chromatography did not separate the two sesquiterpene alcohols, but afforded 7.0 mg of the mixture (21% yield).

^{1}H NMR, ^{13}C NMR, and optical rotation showed that the major product is (−)8-epicedrol (numbered according to the tricyclo[5.3.1.0(1,5)]undecane skeleton). The ^{13}C NMR spectrum is within experimental error of 8-epicedrol literature values (20, 21), and the ^{1}H NMR and ^{13}C NMR spectra and optical rotations were indistinguishable from authentic (−)8-epicedrol. ^{1}H NMR (500 MHz, Bruker AMX500 spectrometer, 25°C, 5 mM in $CDCl_3$, referenced to tetramethylsilane) δ: 0.851 (d, 7.1 Hz, 3H), 1.014 (d, 0.6 Hz, 3H), 1.139 (br d, 0.5 Hz, 3H), 1.322 (s, 3H), 1.275 (m, 1H), 1.339 (m, 1H), 1.397 (m, 1H), ~1.519 (m, 1H), 1.527 (m, 1H), 1.56 (m, 1H), 1.57 (m, 1H), 1.59 (m, 1H), ~1.6 (m, 1H), ~1.678 (m, 1H), 1.733 (m, 1H), 1.883 (td, 12, 6 Hz, 1H), 1.902 (dd, 11.7, 1.6 Hz, 1H). ^{13}C NMR ($CDCl_3$, 62.5 MHz, Bruker AC250 spectrometer, 25°C, 15 mM in $CDCl_3$, referenced to tetramethylsilane) δ: 15.4 (C-12), 25.3 (C-4), 28.1 (C-14), 29.0 (C-13), 30.5 (C-10), 30.6 (C-15), 34.3 (C-9), 36.9 (C-3), 39.9 (C-11), 41.8 (C-2), 41.9 (C-6), 53.4 (C-1), 56.2 (C-5), 61.5 (C-7), 73.2 (C-8) ppm. The optical rotation is −8°, 577 nm, $CHCl_3$.

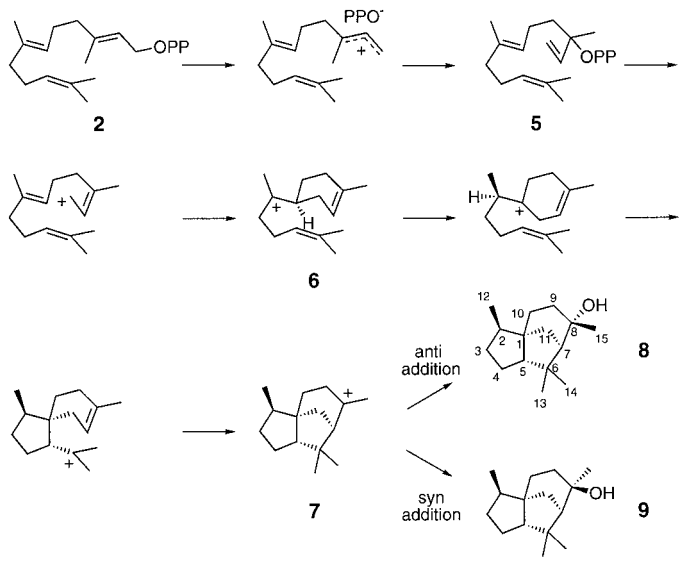

FIG. 3. Proposed mechanism of epicedrol cyclization. FDP (**2**) isomerizes to nerolidyl diphosphate (**5**), which ionizes and cyclizes to the bisabolyl cation (**6**). A hydride shift followed by two cyclizations yields the cedryl cation (**7**). Epicedrol (**8**) results from anti addition of water, whereas syn addition generates cedrol (**9**).

Reaction mechanism and relevance of 8-epicedrol synthase to artemisinin biosynthesis. Sesquiterpene synthases terminate cationic reactions either by proton elimination to make $C_{15}H_{24}$ hydrocarbons or by the equivalent of hydroxide addition to make $C_{15}H_{26}O$ sesquiterpene alcohols. Numerous sesquiterpene synthases that make hydrocarbons have now been identified (4), as have enzymes that make the acyclic sesquiterpene alcohol linalool (22, 23). The 8-epicedrol synthase gene is the first representative that encodes a cyclic sesquiterpene alcohol synthase. 8-Epicedrol synthase is more closely related to dicot sesquiterpene hydrocarbon synthases (35–38% identical, Fig. 2) than to the sesquiterpene alcohol linalool synthase (20% identical), indicating that the mechanism of quenching the carbocation has evolved more than once.

A mechanism for the 8-epicedrol synthase-mediated cyclization is proposed in Fig. 3. Allylic carbocation formation, isomerization to nerolidyl diphosphate (**5**), and cyclization generate the monocyclic bisabolyl cation (**6**). A hydride shift is followed by two cyclizations to yield the cedryl cation (**7**), and the reaction is terminated by quenching the tricyclic cation with a hydroxide equivalent (presumably by addition of water followed by proton loss) to form 8-epicedrol (**8**). The third cyclization is a syn addition and therefore cannot be concerted with the second cyclization. The formation of cedrol (**9**) as a minor product also suggests that the final carbocation has a significant lifetime. If water

addition were concerted with the third ring closure, antiperiplanar addition would mandate quantitative 8-epicedrol formation.

The best-studied sesquiterpene synthases terminate the cyclization reaction by abstracting a proton from an intermediate cation (4), but termination by quenching the intermediate has been demonstrated previously. Patchouli alcohol (24) and epicubenol (25) have been shown to be formed directly from FDP in this way. The 8-epicedrol synthase gene described here is the first example of this type to be cloned.

An *A. annua* extract prepared from the same specimen as the cDNA was examined by GC–MS (see Experimental Procedures). Neither cedrol epimer was detected. This is in agreement with decades of research isolating and identifying the natural products found in this medicinal herb (26–29). In fact, epicedrol has not been reported as a natural product from any organism. The isolation of a gene encoding an epicedrol synthase implies that this product is made in plants, even though this compound is undetectable in mature *A. annua* plants. Epicedrol is apparently rapidly metabolized to a different product *in vivo*, but is probably not an artemisinin precursor. Converting epicedrol to artemisinic acid would require rearrangements that do not seem plausible.

We have shown that a homology-based gene-cloning approach can be fruitful for isolating new sesquiterpene synthase genes from *A. annua* and for identifying new natural products. The epicedrol cloning experiments illustrate the power of molecular biology to obtain catalysts that make natural products so rare that they cannot be detected by chromatographic methods as sensitive as GC–MS. This is currently an underutilized approach in natural products chemistry and might be particularly useful for finding biosynthetic intermediates that do not accumulate because they are rapidly metabolized. We are continuing to seek the 4,11-amorphadiene synthase gene, and we are designing new PCR primers using the *A. annua* epicedrol synthase sequence as a guide.

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