Abietadiene Synthase from Grand Fir (*Abies grandis*): Characterization and Mechanism of Action of the "Pseudomature" Recombinant Enzyme[†]

Reuben J. Peters,[‡] Janice E. Flory,[‡] Reinhard Jetter,[‡] Matthew M. Ravn,[§] Hyung-Jae Lee,[§] Robert M. Coates,[§] and Rodney B. Croteau*,[‡]

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340, and Department of Chemistry, University of Illinois, 600 South Mathews Avenue, Urbana, Illinois 61801

Received August 22, 2000

ABSTRACT: The oleoresin secreted by grand fir (*Abies grandis*) is composed of resin acids derived largely from the abietane family of diterpene olefins as precursors which undergo subsequent oxidation of the C18-methyl group to a carboxyl function, for example, in the conversion of abieta-7,13-diene to abietic acid. A cDNA encoding abietadiene synthase has been isolated from grand fir and the heterologously expressed bifunctional enzyme shown to catalyze both the protonation-initiated cyclization of geranylgeranyl diphosphate to the intermediate (+)-copalyl diphosphate and the ionization-dependent cyclization of (+)-copalyl diphosphate, via a pimarenyl intermediate, to the olefin end products. Abietadiene synthase is translated as a preprotein bearing an N-terminal plastidial targeting sequence, and this form of the recombinant protein expressed in *Escherichia coli* proved to be unsuitable for detailed structure−function studies. Since the transit peptide−mature protein cleavage site could not be determined directly, a truncation series was constructed to delete the targeting sequence and prepare a "pseudomature" form of the enzyme that resembled the native abietadiene synthase in kinetic properties. Both the native synthase and the pseudomature synthase having 84 residues deleted from the preprotein converted geranylgeranyl diphosphate and the intermediate (+)-copalyl diphosphate to a nearly equal mixture of abietadiene, levopimaradiene, and neoabietadiene, as well as to three minor products, indicating that this single enzyme accounts for production of all of the resin acid precursors of grand fir. Kinetic evaluation of abietadiene synthase with geranylgeranyl diphosphate and (+)-copalyl diphosphate provided evidence for two functionally distinct active sites, the first for the cyclization of geranylgeranyl diphosphate to (+)-copalyl diphosphate and the second for the cyclization of (+)-copalyl diphosphate to diterpene end products, and demonstrated that the rate-limiting step of the coupled reaction sequence resides in the second cyclization process. The structural implications of these findings are discussed in the context of primary sequence elements considered to be responsible for binding the substrate and intermediate and for initiating the respective cyclization steps.

Conifer oleoresin (pitch) is a defensive secretion comprised of an approximately equal proportion of monoterpene olefins (turpentine) and diterpenoid resin acids (rosin), with lesser amounts of sesquiterpenes (*1*), that is produced by pines, spruces, and related species as a primary response to wounds caused by physical injuries, insects, large herbivores, and microbial diseases (*2*). Oleoresin is toxic toward foliage feeders and stem-boring insects and their pathogenic fungal symbionts, and this secreted material is also responsible for physically sealing wound sites by solidification of the resin acids following evaporation of the volatile turpentine (*3*). In true fir species (*Abies*), constitutive (primary) oleoresin is produced and stored in specialized anatomical structures termed resin blisters (*4*). Additionally, in the stems of *Abies* species, the synthesis of secondary oleoresin (which differs in composition from that of constitutive resin) can be induced in nonspecialized parenchyma cells by mechanical wounding that mimics attack by bark-boring insects (*5*).

Diterpenoid resin acids of the abietane family, characterized by the perhydrophenanthrene-type tricyclic ring structure of normal absolute configuration (C10 β-methyl) with an isopropyl group at C13 of the C ring, are the principal constituents of the rosin fraction of grand fir (*Abies grandis*) oleoresin (*1*). The biosynthesis of these resin acids involves formation of the abietane skeleton via cyclization of the universal diterpenoid precursor (*E*,*E*,*E*)-geranylgeranyl diphosphate (GGPP,[1] **1**), followed by stepwise oxidation of the C18 methyl group of the derived olefin as, for example, in the formation of abietic acid (**5**) from abieta-7(8),13(14)-diene

[†] This work was supported by National Institutes of Health Grants GM31354 to R.B.C. and GM13956 to R.M.C., by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund for Medical Research to R.J.P., and by a postdoctoral fellowship from the Deutsche Forschungs Gemeinschaft to R.J.

* To whom correspondence should be addressed. Phone: (509) 335-1790. Fax: (509) 335-7643. E-mail: croteau@mail.wsu.edu.

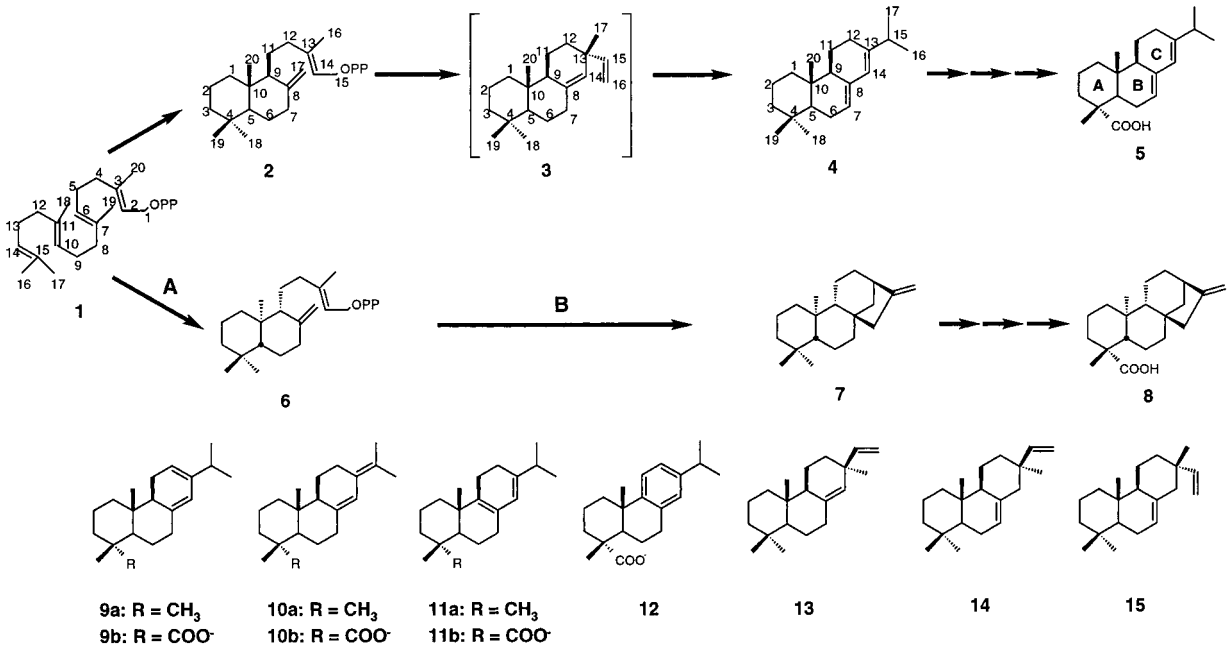
[‡] Washington State University.

[§] University of Illinois.

[1] Abbreviations: AS, abietadiene synthase (the prefix n or r denotes the native or recombinant enzyme, respectively); CPP, copalyl diphosphate; GC, gas chromatography; GGPP, (*E*,*E*,*E*)-geranylgeranyl diphosphate; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; MS, mass spectrometry; NMR, nuclear magnetic resonance (spectrometry); PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi001997l CCC: $19.00
Published on Web 11/18/2000

Scheme 1: Biosynthetic Pathways from Geranylgeranyl Diphosphate (**1**) either to Abietic Acid (**5**), via (+)-Copalyl Diphosphate (**2**), Sandaracopimaradiene (**3**), and Abietadiene (**4**), or to *ent*-Kaurenoic Acid (**8**), via (−)-Copalyl Diphosphate (**6**) and *ent*-Kaurene (**7**)[a]



[a] In plants, the synthesis of kaurene requires two enzymes, kaurene synthase A (for the conversion of **1** to **6**) and kaurene synthase B (for the conversion of **6** to **7**). In grand fir, the conversion of **1** to **4** is accomplished by a single enzyme. The structures of levopimaradiene (**9a**), levopimaric acid (**9b**), neoabietadiene (**10a**), neoabietic acid (**10b**), palustradiene (**11a**), palustric acid (**11b**), dehydroabietic acid (**12**), pimara-8(14),15-diene (**13**), pimara-7,15-diene (**14**), and isopimara-7,15-diene (**15**) are also illustrated.

(**4**) (*6*, *7*) (Scheme 1). On the basis of mechanistic considerations and by analogy to the cyclization of GGPP (**1**) to *ent*-kaurene (**7**) [en route to *ent*-kaurenoic acid (**8**) and the gibberellin family of plant growth hormones (*8*−*10*) (Scheme 1)], a reaction scheme for the formation of abietadiene has been proposed that involves two distinct intermediates (*7*). In the first step of the sequence, a proton-initiated cyclization leads to closure of the A and B rings to yield (+)-copalyl diphosphate (CPP, **2**) in a reaction analogous to that mediated by kaurene synthase A of the gibberellin biosynthesis pathway (*11*, *12*) but of opposite enantiospecificity (Scheme 1). In the next step of the sequence, ionization of (+)-CPP leads to formation of the C ring of a pimarane-type intermediate in a reaction that corresponds to that mediated by kaurene synthase B (*11*, *12*) (Scheme 1). In the final step, a 1,2-methyl shift in the pimarane intermediate generates the C13 isopropyl group characteristic of the abietane skeleton, and a deprotonation completes the reaction cycle (*7*). Because the critical C13 stereochemistry and the double bond placement in the pimarenyl intermediate are eliminated in the final reaction steps, the precise structure of this intermediate cannot be predicted. However, the relative potency of 15-aza-15,16-dihydropimaradienes as cyclization inhibitors,[2] together with the stereospecific cyclization of 8α-hydroxy-17-nor-CPP to 17-normanoyl oxide (*13*), points to a 13*β*-methyl configuration and 8,14 double bond placement, i.e., isopimara-8(14),15-diene (sandaracopimaradiene, **3**) (Scheme 1). Additional experiments have revealed that the conversion of (+)-CPP to abietadiene occurs by initial anti-S_N' cyclization to form a sandaracopimar-15-en-8-yl carbocation (see Scheme 3, $\mathbf{3a}^+$), followed by a stereospecific intramolecular transfer of a proton from C14 to the *si* face of the vinyl group, syn-related methyl migration, and final elimination of a C7 proton (*13*, *14*).

The abietadiene synthase (AS) of grand fir (*7*) has been purified and characterized as an operationally soluble, monomeric 84 kDa protein with properties typical of other terpenoid synthases (*15*). Significantly, the complex, multistep transformation of GGPP to abietadiene was shown to be mediated by this single enzyme, a result that contrasts with formation of (−)-kaurene in higher plants which requires the two distinct gene products kaurene synthase A [(−)-copalyl diphosphate synthase] and kaurene synthase B (kaurene synthase) (*11*, *12*). Internal microsequencing of the purified, but N-blocked, AS provided a means for isolating the cDNA encoding this enzyme, which was confirmed by functional expression in *Escherichia coli* (*15*). Abietadiene synthase is translated as a preprotein bearing a substantial transit peptide for targeting the preprotein to the plastids where proteolytic processing occurs (*15*) and where the precursor GGPP also arises (*9*). Since the preprotein form of the enzyme is unsuitable for detailed mechanistic or structural investigation, particularly for defining the role(s) of the multiple aspartate-rich motifs of AS (Figure 1) which in most other terpenoid synthases are represented by a single version of this substrate binding element (*16*), a series of truncations was constructed and tested for catalytic capability. Such a "pseudomature" form of AS was employed to evaluate intermediates of the reaction and their stereochemistries, to demonstrate that the enzyme synthesizes multiple diterpene olefin products, and to indicate that two distinct active sites are responsible for mediating the two different cyclization steps of the catalytic cycle.

[2] M. M. Ravn, R. J. Peters, R. Croteau, and R. M. Coates, manuscript in preparation.

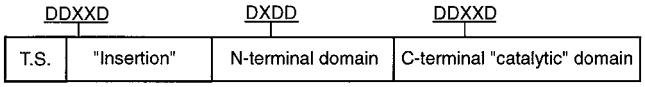


FIGURE 1: Schematic diagram of abietadiene synthase illustrating general structural features and the locations of the aspartate-rich elements. T.S. indicates the transit sequence. The unusual insertional element of AS and of a few other terpenoid synthases has been previously described (*16*). The overall structure is modeled on epi-aristolochene synthase (*49*).

EXPERIMENTAL PROCEDURES

Materials and General Procedures. Native abietadiene synthase (nAS) was prepared from wound-induced grand fir saplings as previously reported (*15*). Liquid scintillation counting, SDS−PAGE, and product analysis by GC−MS were carried out as previously described (*7*, *15*). The concentration of recombinant enzyme was determined by absorbance at 280 nm using the calculated extinction coefficient (138 350 M^{-1} cm^{-1}) (*16*). The preparations of [1-^{3}H]geranyl diphosphate (120 Ci/mol) (*17*), (*E*,*E*)-[1-^{3}H]farnesyl diphosphate (100 Ci/mol) (*18*), and (*E*,*E*,*E*)-[1-^{3}H]geranylgeranyl diphosphate (120 Ci/mol) (*7*) have been described, as have syntheses of the 13-cyclopropylidene analogue of GGPP (**16**) and the vinyl analogue (16-methylidene-GGPP, **17**) (*19*). The preparation of the abietane diterpenes, abietadiene (**4**), levopimaradiene (**9a**) neoabietadiene (**10a**), and palustradiene (**11a**), from the corresponding resin acids (**5**, **9b**, **10b**, and **11b**) has been described elsewhere (*20*). An authentic sample of sandaracopimaradiene (isopimara-8(14),15-diene, **3**) was prepared by reduction of the corresponding 18-phenylthio derivative (*21*) with lithium in ether/ethylamine at −78 °C as described previously for 18-(phenylthio)stemar-13-ene (*22*, *23*). Pimaric acid and isopimaric acid were converted to pimara-8(14),15-diene (**13**) and isopimara-7,15-diene (**15**), respectively, by procedures similar to those reported previously (*21*), except that the reductions of the 18-phenylthio derivatives with lithium in tetrahydrofuran/ammonia at −78 °C were stopped after 3 min by adding 3-hexyne and methanol to minimize over-reduction. A sample of pimara-7,15-diene (**14**) remained from previous work (*21*). All four pimaradiene isomers were purified by flash chromatography on $AgNO_3$-impregnated silica gel and/or silica gel, and the purities were established by GC at 95−98% except for pimara-7,15-diene which was 86%. Structures were confirmed by ^{1}H NMR spectra, and in some cases by ^{13}C NMR and MS data, which agreed with literature data (*21*).

For the preparation of (+)-[1-^{3}H]CPP, pure (+)-manool (98% by GC) was isolated from a heartwood extract of *Dacrydium biforme* (∼75% manool, provided by Dr. Peter Grant, Department of Chemistry, University of Otago, Dunedin, New Zealand) by flash chromatography on silica gel with 6:1 (v/v) hexane−ethyl acetate as eluant. Oxidation of manool (2.18 g) with pyridinium chlorochromate (4.86 g) in CH_2Cl_2 (65 mL) at room temperature for 12 h according to a literature procedure (*24*) afforded a 1:1.2 mixture of (*Z*)- and (*E*)-(+)-copalals (1.66 g, 77%). Purification by flash chromatography on silica gel with 9:1 (v/v) hexane−ether gave pure (*Z*)-copalal (0.8 g) and (*E*)-copalal (1.85 g). Reduction of the latter (28 mg) with NaB^3H_4 (25 mCi, 1000 Ci/mol) in absolute ethanol at room temperature for 1.5 h gave pure (+)-(*E*)-[1-^{3}H]copalol (250 Ci/mol) following preparative TLC purification on silica gel with 7:3 (v/v) hexane−ethyl acetate. The alcohol was diphosphorylated and purified as before (*7*). (−)-(*E*)-[1-^{3}H]Copalyl diphosphate (120 Ci/mol) was similarly prepared from (−)-(*E*)-copalal derived by pyridinium chlorochromate oxidation of (−)-(*E*)-copalol that was obtained by $LiAlH_4$ reduction of the methyl ester of (−)-copalic acid from copal resin (*25*).

Enzyme Assays and Inhibition Studies. Abietadiene synthase was assayed essentially as described previously for the native enzyme [50 mM HEPES, pH 7.2, 100 mM KCl, 7.5 mM $MgCl_2$, 20 μM $MnCl_2$, 5% (v/v) glycerol, 5 mM fresh DTT, and 5 μM GGPP] (*7*) by a method involving pentane extraction of the diterpene products from the reaction mixture and passage of the extract through a short column of $MgSO_4$ and silica gel to dry the sample and remove oxygenated diterpenoids (i.e., geranylgeraniol liberated from the substrate by contaminating phosphohydrolases). The latter precaution was unnecessary for assay of the recombinant synthase expressed in *E. coli*, and so the chromatography step was eliminated and the initial pentane extract was simply back-washed with water before aliquot counting and product analysis by capillary GC−electron impact MS [Hewlett-Packard 6890 GC−MSD, cool on-column injection; 70 eV full spectra were recorded (*7*)]. For determination of steady-state kinetic parameters, enzyme concentration was reduced to 3 nM, α-casein (0.1 mg/mL) was added to stabilize the enzyme at this low concentration, the assay times were reduced to 5 min (with GGPP) or 1 min (with CPP), and the reactions were terminated by the addition of KOH (to 0.2 M) and EDTA (to 15 mM) as described (*26*). Control reactions without enzyme were performed at substrate concentrations throughout the range used in the kinetic assays to monitor the low rates of solvolysis of the allylic diphosphate substrates under assay conditions. Duplicate sets of enzyme assays were performed at each substrate concentration, and the data, corrected for the corresponding background rates (<0.01% conversion), were fitted to the described equations using Kaleidagraph (Synergy Software). For determination of product profiles, reactions were run for 3 h with 50 nM AS at saturating concentrations of GGPP or (+)-CPP, and the reaction products were extracted three times with 1 mL of pentane. The pooled organic phase was washed with distilled H_2O and concentrated under N_2 for GC−MS analysis as above.

For studies with the four pimaradiene isomers (Scheme 1, **3**, **13**, **14**, and **15**), 5 nmol of each olefin was dissolved in pentane and added to a dry test tube. The solvent was evaporated at room temperature under a stream of argon and the olefin then suspended in 1 mL of assay buffer by sonication [the pimaradienes were added in slight excess of their solubility limit (∼5 μM) to ensure saturation]. Conversions were monitored with or without the addition of 100 μM inorganic diphosphate, or AS activity was assayed directly as described above for the steady-state kinetic experiments.

The conversions of [1-^{3}H]geranyl diphosphate and [1-^{3}H]-farnesyl diphosphate were determined at concentrations of 10 μM using the standard assay, with the addition of a pentane overlay (this did not adversely influence the reaction) to trap volatile monoterpenes in the case of geranyl diphosphate as substrate. To assess inhibition of AS with the cyclopropyl and vinyl analogues of GGPP (see Scheme 2),

the potential inhibitors (50 μM in assay buffer) were individually preincubated with the enzyme overnight at 30 °C, and either the preparations were assayed directly with GGPP or residual inhibitor was removed by repeated dilution and ultrafiltration (30 kDa cutoff, Amicon) before the assay.

Expression of Truncated Abietadiene Synthase from pGEX. The cDNA insert encoding the AS preprotein was excised from the original pBluescript clone pAC22.1 (*15*) by sequential digestion with *Eco*RI and *Xho*I. Since there is an *Eco*RI recognition site at nucleotide (nt) position 639 of the insert, conditions were first optimized for partial digestion with this endonuclease. In a second digest, the insert was released by cleavage with *Xho*I (other fragments were further digested with *Bgl*I to render the cleavage products easily separable by agarose gel electrophoresis). The resulting gel-purified insert fragment was dephosphorylated with alkaline phosphatase (New England Biolabs) and ligated into *Eco*RI- and *Xho*I-digested vector pGEX-4T-3 (Pharmacia) to yield construct pG22.

Truncated versions of pG22 were generated by one of two methods. The Pharmacia Unique Site Elimination kit was used to make deletions from amino acids (aa) 25 through 93 by oligonucleotide-directed mutagenesis of pG22. In each case, a new in-frame *Bam*HI recognition site was introduced at the corresponding N-terminus of the synthase coding sequence. The mutagenesis reactions were performed in the presence of two additional mutagenic primers in order to eliminate two unique recognition sites (*Pst*I to *Sac*II, *Nar*I to *Nhe*I) from the vector DNA, so that restriction with *Pst*I and *Nar*I could be used as a selection against unmutagenized plasmid. The resulting truncated synthase sequences were subcloned as *Bam*HI/*Xho*I fragments into pGEX4T-3 that was similarly digested.

Truncations at aa positions 109 through 115 were constructed by PCR using undigested pAC22.1 as template and *Pfu* polymerase (Stratagene). The primer pairs used employed the T7 primer in combination with a mutagenic primer designed to introduce a new *Bam*HI cleavage site at the truncated position. After restriction with *Bam*HI and *Xho*I, the gel-purified products were ligated into the vector pGEX-4T-3 to yield the new plasmids designated pG22Δ1-108 through pG22Δ1-114 (recombinant proteins designated M109 through M115). These pG22-derived constructs were transformed into *E. coli* strain XL1-Blue. For each new plasmid, one clone with maximum expressed activity was selected by screening 10 mL liquid cultures grown, harvested, and assayed as described previously (*15*). The selected clones were first subjected to PCR evaluation and restriction analysis to verify insert size, and the inserts of the purified plasmids were then fully sequenced to confirm that no unwanted alterations had occurred in the coding region during the mutagenesis procedure. The confirmed plasmids were then transformed into *E. coli* XL1-Blue for expression studies.

Transformed *E. coli* cells harboring the full-length pG22 sequence or the corresponding truncated versions were grown in 1 L of Luria−Bertani medium at 37 °C with shaking at 350 rpm to an A_{550} of approximately 1.0. After induction with 200 μM IPTG, the cultures were immediately cooled to 20 °C and following temperature adjustment were incubated for 6 h. Bacteria were harvested by centrifugation (45000*g*, 15 min, 4 °C), resuspended in 50 mL of assay buffer containing 1 mg of lysozyme, incubated for 1 h at 0 °C, and then disrupted by mild sonication. The lysates were cleared by centrifugation (4000*g*, 30 min, 4 °C), and this crude extract was added to 1 mL of glutathione−Sepharose (Pharmacia) followed by gentle shaking of the matrix for 2 h at 4 °C. The slurried matrix was then poured into a column and washed with 50 mL of assay buffer. Thrombin (10 units, ICN) was then added in the assay buffer to cleave the fusion protein while still bound to the affinity column. The recombinant synthase thus eluted was free of contaminating proteins and was assayed directly.

Expression of Truncated Abietadiene Synthase from pSBET. The construct designated pJF-M84 was prepared by oligonucleotide-directed mutagenesis of template pG22. Silent mutations were introduced to eliminate two internal *Nde*I recognition sites. In each case (aa 139−141 and aa 184−186), the coding sequence was converted from gCATATGac to gCTTATGac. Next, an in-frame methionine codon nested in an *Nde*I site was inserted immediately upstream of codon Val85 (CATTGG changed to CATATG), and a *Bam*HI site was added downstream of the stop codon (nt 2776−2781 changed from GGAGTC to GGATCC). The coding region was then resequenced to ensure that no undesired mutations had been introduced. This plasmid containing the truncated version was then restricted with *Nde*I and *Bam*HI, and the insert was subcloned into the unique *Nde*I/*Bam*HI sites of pSBETa (*27*) to create pJF-M84, which was then transformed into *E. coli* BL21 (DE3) (*28*) for expression studies.

Transformed bacterial cells harboring pJF-M84 were grown in 1 L of Luria−Bertani medium at 37 °C with shaking to an A_{600} of about 0.6 transferred to 20 °C for 1 h, then induced with 200 μM IPTG, and incubated with shaking for 16−20 h. Bacteria were harvested by centrifugation as before and suspended in 100 mM Bis-Tris buffer (pH 6.8). After sonication, the lysate was cleared by centrifugation (15000*g*, 30 min, 4 °C).

Purification of Pseudomature Abietadiene Synthase. The supernatant from above containing the truncated, but otherwise unmodified, AS was initially separated on a 30 mL column of type II ceramic hydroxyapatite (Bio-Rad) using a peristaltic pump. The column was washed with 3 column volumes of 50 mM Bis-Tris buffer (pH 6.8) containing 150 mM KCl and then eluted with 200 mM sodium phosphate buffer (pH 6.8), and the elutant was further clarified by 0.2 μm filtration. By using a perfusion chromatography system (BioCAD Sprint), AS was further purified over a 16 × 100 mm POROS HQ/M column at 20 mL/min. The column was washed with 3 column volumes of 50 mM Bis-Tris buffer (pH 6.8), and elution was performed in the same buffer with a 0−1 M NaCl gradient over 10 column volumes. Abietadiene synthase eluted at approximately 300 mM NaCl, and the appropriate fractions were pooled, $MgCl_2$ was added to 10 mM, and the solution was then loaded directly onto a 4.6 × 100 mm column of type II ceramic hydroxyapatite (Bio-Rad) that was previously equilibrated with 50 mM Bis-Tris buffer (pH 6.8) at 5 mL/min. The column was washed with 3 column volumes of 50 mM Bis-Tris buffer (pH 6.8) and eluted with a 0−200 mM sodium phosphate buffer (pH 6.8) gradient over 15 column volumes. Abietadiene synthase eluted at approximately 120 mM phosphate. Pooled fractions were diluted 1:1 with distilled water, and the material was loaded onto a 16 × 100 mm POROS PI/M column at 10

mL/min. The column was washed with 3 column volumes of 50 mM Bis-Tris buffer (pH 6.8). AS was eluted with a 0−1 M NaCl gradient over 5 column volumes of the Bis-Tris buffer. Fractions (AS elutes at about 0.5 M NaCl) were pooled and then assayed for activity (using GGPP as substrate) and protein content before combining. This protocol results in an overall yield of about 70%, and the rAS obtained by this protocol is >98% pure, as judged by SDS−PAGE followed by Coomassie (*29*) and silver staining (*30*).

RESULTS

Truncation of Abietadiene Synthase. Diterpene biosynthesis is compartmentalized in plastids of higher plants (*9*, *31*); thus, all diterpene synthases cloned to date (*16*) are encoded as preproteins bearing an amino-terminal transit peptide for import of these nuclear gene products into plastids (leucoplasts in the present instance) where they are proteolytically processed to the mature forms (*32*, *33*). The AS cDNA from grand fir (*A. grandis*) was functionally expressed in *E. coli* as a means of confirming the clone (*15*); however, this recombinant preprotein form of the synthase proved to be difficult to purify (because of self-association and tight binding to host chaperones) and was catalytically impaired compared to the native enzyme (*7*). Similar difficulties have been encountered previously with recombinant monoterpene synthases which are also translated as preproteins (*34*). Since the native AS is amino-terminally blocked, direct determination (by N-terminal sequencing) of the transit peptide−mature protein cleavage junction was not possible. Thus, the construction and testing of a series of N-terminally truncated versions of the synthase were necessary in order to prepare a pseudomature form of the enzyme suitable for detailed mechanistic and structural investigations.

Although plastidial targeting amino acid sequences are characterized by a low degree of similarity (*35*), they all share common features in being rich in serine, threonine, and small hydrophobic residues but with few acidic residues, such that predictive algorithms to locate the transit peptide cleavage site have been developed (*36*, *37*). Therefore, initial efforts to produce a pseudomature AS were directed to truncation near Ser111, which was considered to define the approximate cleavage site based upon the predictive methods of von Heijne and associates (*36*, *37*). A series of truncations starting at Ala109 and sequentially shortened by deletion of successive residues to Arg115 was constructed. Each truncated version of the synthase was expressed as a glutathione *S*-transferase (GST) fusion protein (from pGEX in *E. coli* XL1-Blue) to facilitate purification by affinity chromatography and thus (following thrombin cleavage of the affinity tag) simplify kinetic comparison of the various forms. Unfortunately, this entire set of truncated synthase constructs was substantially less active than was the preprotein (Figure 2), which was itself less active than the native enzyme (*7*, *15*). Subsequent analysis of this group of truncated enzymes by SDS−PAGE showed that they were too short relative to the native form from grand fir stems (*15*). It is notable that the Ala109 through Arg115 truncations were detectably active (by comparison of V_{max} at saturating substrate; Figure 1), although one of the aspartate-rich, potential substrate-binding motifs (DDXXD) had been entirely deleted (Figure 1).

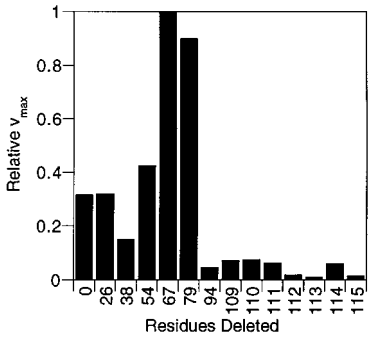


FIGURE 2: Activity of N-terminal truncated forms of abietadiene synthase. The numbers correspond to the total residues deleted from the N-terminus. Truncations 94−115 also exhibited K_m values 2 to 4 times higher than the preprotein (denoted 0), whereas K_m values for truncations 26−79 were similar. The rate of the native enzyme corresponds to 1.1 on this scale.

To define a suitable pseudomature form of the synthase, a second series of N-terminal deletions was constructed by truncation at spaced intervals within the first 100 residues. The activities of these constructs were evaluated as before by expression, purification of the corresponding protein, and assay with GGPP. These results (Figure 2) indicated that the transit peptide cleavage site was near Leu79, since truncations up to this position showed a general trend toward increasing V_{max}, whereas truncation at residue Trp93 exhibited greatly decreased activity.

The AS cDNA specifies a relatively high number of arginine residues by AGG or AGA (*15*), which studies with other terpenoid synthases of plant origin (*19*, *34*) have indicated can compromise expression in the *E. coli* host [in which these codons are rarely utilized (*27*)]. Therefore, truncated constructs were transferred into pSBETa, a pACYC-derived, T7 expression vector that carries a supplemental copy of the *E. coli argU* tRNA gene for rare arginine codon usage (*27*). For this purpose, and in order that the pseudomature form of the enzyme most closely resemble the native synthase, the glutathione *S*-transferase N-terminal tag was not employed, and a new starting methione was installed immediately upstream of the truncation by the addition of an *Nde*I restriction site. Expression and testing as before led to selection of the construct made by truncation of 84 N-terminal residues as the optimum pseudomature form (designated M84). The M84 enzyme was the shortest protein with activity comparable to the longer versions (e.g., M78) and to the native synthase, and the expression levels were good (~20% of soluble protein) and provided a stable form (soluble to at least 10 mg/mL and stable for months at −20 °C) that was readily purified (>98%) by combination of anion-exchange and hydroxyapatite chromatography. SDS−PAGE analysis of the recombinant, M84 pseudomature synthase also showed this protein to be about the same size as the native AS from grand fir stem (data not shown).

Product Profile of Abietadiene Synthase. Previous GC−MS analyses of the product profile of AS indicated that this diterpene cyclase produces almost exclusively abietadiene (**4**) with small amounts of sandaracopimaradiene (**3**) (*7*). However, these earlier studies utilized the standard practice of purifying the pentane-extractable olefinic products by chromatography over a small column of silica gel−$MgSO_4$ (*38*) to remove geranylgeraniol released from the substrate by contaminating phosphohydrolases. In the course of

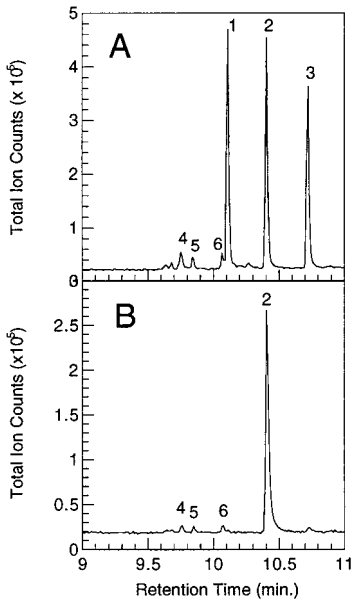


FIGURE 3: Total ion chromatograms of the products of abietadiene synthase [with GGPP or (+)-CPP as substrate] before silica gel−$MgSO_4$ chromatography (A) and after the chromatography step (B). The diterpene olefins identified are levopimaradiene (1), abietadiene (2), neoabietadiene (3), pimara-8(14),15-diene (4), sandaracopimaradiene (5), and palustradiene (6).

working with the recombinant, pseudomature AS, it was shown that the chromatographic separation of the product extract was unnecessary since contaminating phosphohydrolase activity from the *E. coli* host was negligible. Surprisingly, GC−MS analysis of the products derived from GGPP and contained in the untreated pentane extract revealed the presence of two major new diterpene olefins as well as two additional minor olefins (Figure 3). These products of the rAS were identified, by comparison of retention time and mass spectrum to those of authentic standards (*20*), as levopimaradiene (**9a**, 34%; note that levopimaradiene is structurally an abietane diterpene but retains the pimarane designation for historical reasons), neoabietadiene (**10a**, 28%), the previously observed abietadiene (**4**, 31%), the minor products pimara-8(14),15-diene (**13**, 3%), palustradiene (**11a**, 2%), and the previously noted sandaracopimaradiene (**3**, 2%).

This unexpected difference from the previously described product profile (*7*, *15*) also was observed with the full-length recombinant preprotein form of AS, as well as with the partially purified native enzyme from grand fir stem extracts, both of which produced from GGPP the same olefins, in similar proportions, as produced by the M84 pseudomature enzyme (data not shown). Also, the same multiple product profile was generated from either GGPP or (+)-CPP (see below) with all versions of AS, confirming that the ability to synthesize these diterpene olefin isomers was an inherent feature of the enzyme and not an artifact generated by the recombinant form.

Since equivalent amounts of radioactive enzymatic products (generated from [1-^{3}H]GGPP) could be recovered by extraction of the reaction mixture with pentane, diethyl ether, or ethyl acetate, either with or without subsequent passage over silica gel, neutral alumina, or $MgSO_4$ alone or in combination with gel matrix, it was clear that the alteration in product composition was not caused by differential losses during processing. Rather, product analysis before and after the chromatography step (Figure 3) demonstrated that interaction on the activated matrix (especially $MgSO_4$) in aprotic solvent led to rearrangement of levopimaradiene (**9a**) and neoabietadiene (**10a**) to the thermodynamically more stable abieta-7(8),13(14)-diene (*39*). Although palustradiene (**11a**) is also known to be thermodynamically less stable than abietadiene, it does not rearrange to abietadiene under these conditions (see Figure 3), presumably because proton transfer to the sterically hindered C9 position is relatively slow.

It is important to note that the two new products, levopimaradiene and neoabietadiene, correspond in structure to resin acids previously identified (*1*) in grand fir rosin [10% levopimarate (**9b**) and 10% neoabietate (**10b**), respectively, along with 42% abietate (**5**) and 38% dehydroabietate (**12**)], which are almost certainly derived from these olefins by sequential oxidation of the appended C18-methyl group, as with abietic acid (**5**) itself (*6*, *7*). The observed ease of double bond rearrangement in the abietane diterpenes suggests that the precise composition of rosin likely reflects the summation of chemical (and thermal) isomerization processes (*40*) that occur at the level of the olefin precursors, intermediates, and final resin acid products prior to, and during, the analysis.

Substrates, Intermediates, and Inhibitors of Abietadiene Synthase. Mechanistic considerations suggest that the multistep reaction catalyzed by AS (see Scheme 3) involves the initial protonation-dependent cyclization of GGPP (**1**) to (+)-CPP (**2**), followed by ionization-dependent cyclization of the latter to a pimarenyl intermediate (*7*) which recent studies (*13*, *14*) indicate should correspond in stereochemistry to sandaracopimaradiene (**3**). To test these potential intermediates leading up to the final methyl migration and terminating deprotonation steps of the reaction cycle, both (+)- and (−)-[1-^{3}H]CPP were prepared, as were all four possible isomers of pimaradiene differing in the placement of the endocyclic double bond at the 7(8) or 8(14) position and in the stereochemistry of the methyl and vinyl substituents of C13 (i.e., **3**, **13**, **14**, **15**).

(+)-[1-^{3}H]CPP and (−)-[1-^{3}H]CPP were tested as substrates at a concentration corresponding to saturation for GGPP (~3 μM). (+)-CPP was converted to the previously noted mixture of diterpene olefins at a rate comparable to that of GGPP, whereas (−)-CPP was inactive as a substrate, thereby confirming the proposed stereochemistry of the copalyl intermediate. At the same substrate concentration, the recombinant M84 version of AS was unable to convert sandaracopimaradiene [**3**, the predicted C17-β-methyl epimer of pimara-8(14),15-diene] or any other of the possible pimaradiene intermediates (**13**, **14**, **15**) to detectable levels of abietane products, even in the presence of excess (100 μM) inorganic diphosphate added to provide the counterion for presumptive carbocation intermediates formed upon protonation of the vinyl group (see Scheme 3, route a) to initiate methyl migration. Moreover, none of the pimaradiene isomers inhibited AS (3 μM GGPP as substrate) at their solubility limit (~5 μM) (*41*), in either the absence or presence of 100 μM inorganic diphosphate, indicating that the synthase did not recognize these diterpene olefins. Similar attempts to intercept multistep terpenoid cyclizations using presumptive olefinic intermediates of the reaction have also failed (*19*, *41*). However, negative results regarding the intervention and conversion of potential intermediates of the

reaction do not eliminate the possible involvement of the corresponding, tightly bound, transient intermediate that is not accessible to, or exchangeable with, exogenous material due to temporal considerations or conformational alteration in the protein during the catalytic cycle. In the present instance, the pimarenyl intermediate arises deep in the cyclization cascade, and recent evidence has indicated that deprotonation at C14 of the intermediate C8-pimarenyl cation (**3a**$^+$) involves the stereospecific intramolecular transfer of this hydrogen atom from the C14 position to the *si* face of the vinyl group to initiate the subsequent methyl migration to yield the abietane skeleton (*13*, *14*). Thus, two considerations are likely to apply. On binding the CPP intermediate, AS could undergo conformational modification as a means of shielding the subsequently formed pimarenyl cation from premature capture by water. As a consequence, such conformational change would also prevent egress of this intermediate from the active site and disallow binding of the exogenous intermediate from solution. Second, the neutral pimaradiene intermediate (i.e., sandaracopimaradiene) would appear to arise only transiently at the midpoint of the presumably rapid intramolecular hydrogen-transfer step and thus would be effectively inaccessible for productive substitution by the exogenous olefin.

The steady-state kinetics of the two cyclization steps catalyzed by AS were investigated with [1-^{3}H]GGPP and (+)-[1-^{3}H]CPP as substrates for diterpene olefin formation. Interestingly, the enzyme exhibited marked substrate inhibition with GGPP but not with CPP (Figure 4). Fitting the kinetic data for GGPP to the standard substrate inhibition equation (*42*)

$$v = \frac{k_{cat}[rAS][GGPP]}{K_m + [GGPP]\left(1 + \frac{[GGPP]}{K_i}\right)}$$

yielded $K_m = 3 \pm 0.8\ \mu M$, $K_i = 5 \pm 2\ \mu M$, and $k_{cat} = 0.75 \pm 0.15\ s^{-1}$. In contrast, the conversion of (+)-CPP to the diterpene olefin product mixture clearly followed standard Michaelis−Menten kinetics, and the fitted data ($R > 0.99$) yielded a K_m value of $0.35 \pm 0.10\ \mu M$ with a k_{cat} of $0.75 \pm 0.15\ s^{-1}$. Even at very high concentrations of CPP (to 50 μM), the reaction showed no sign of substrate inhibition. Since k_{cat} for the two substrates was nearly identical, the catalytic efficiency for CPP ($k_{cat}/K_m = 2.0 \times 10^6\ s^{-1}\ M^{-1}$) is higher than that of GGPP ($k_{cat}/K_m = 2.5 \times 10^5\ s^{-1}\ M^{-1}$), reflecting the substantially lower K_m for CPP which contributes to the inability to observe the latter as a free intermediate of the two-step cyclization reaction (*7*, *15*).

Two critical implications derive from the steady-state kinetic analysis. First, it is clear from the comparable k_{cat} values with the two substrates that the rate-limiting step for the overall reaction must reside downstream of CPP in the second cyclization step. Second, substrate inhibition with GGPP implicates two active sites, one for the conversion of GGPP to (+)-CPP and the second for the conversion of CPP to the olefinic products, with the second accessible to nonproductive binding by GGPP to block the overall reaction. The multiple aspartate-rich patches of AS (*15*) likely demarcate these multiple active sites (Figure 1), which in most other terpenoid synthases are represented by a single

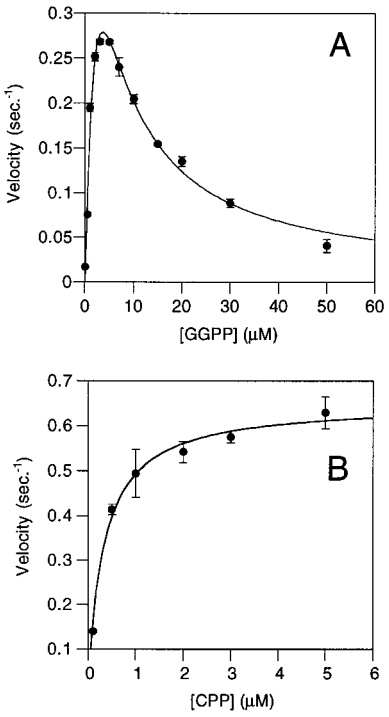


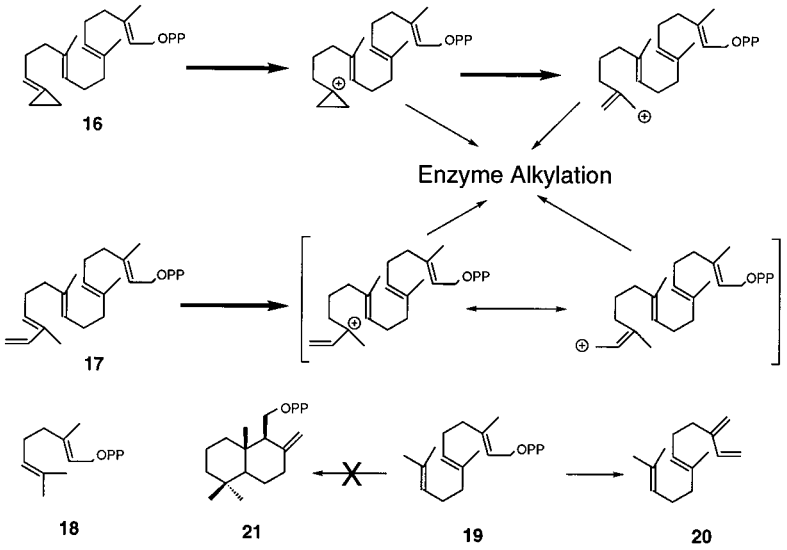
FIGURE 4: Steady-state kinetic plots for recombinant abietadiene synthase. Data are corrected for background and averaged from duplicate assays; the error bars represent the standard deviation. The fit to the substrate inhibition equation (see Results) is illustrated with GGPP as substrate (A), and the fit to the Michaelis−Menten equation is illustrated with (+)-CPP as substrate (B).

DDXXD motif for binding and ionizing the prenyl diphosphate substrate (*16*).

In an attempt to distinguish these two sites of AS, we turned to the use of two previously prepared (*19*) potential, mechanism-based inhibitors, the 13-cyclopropylidene analogue of GGPP (**16**, Scheme 2) and the vinyl analogue of GGPP [16-methylidenegeranylgeranyl diphosphate (**17**), Scheme 2]. These substrate analogues are designed to reposition and/or stabilize positive charge in the initial protonated intermediate generated in the course of the normal cyclization reaction (Scheme 2), with the expectation that alteration in charge configuration of the carbocation could result in alkylation of a proximate basic residue of the synthase at the first active site. This general approach has been utilized to inactivate (and specifically label) certain monoterpene synthases (*43*, *44*) and has been widely employed to probe the structure and mechanism of triterpene synthases (*45*).

In the present instance, neither the cyclopropylidene analogue (**16**) nor the vinyl homologue (**17**) at a concentration of 50 μM exhibited significant time-dependent inactivation of AS when incubated under standard conditions (at 7.5 mM Mg^{2+}) with the M84 version of the enzyme. Even after overnight incubation and removal of the inhibitors by ultrafiltration, the vinyl analogue inactivated only 60% of the enzyme, and the cyclopropyl analogue only 20%, relative to control incubations. Both inhibitors were shown to bind to the synthase, exhibiting apparent competitive inhibition with I_{50} values in the 40−50 μM range. When tested as substrates under standard assay conditions, the vinyl analogue does not react to give product. However, the cyclopropyl analogue was very slowly ($<10^{-3}\ s^{-1}$) converted to a

Scheme 2: Possible Mechanisms for the Utilization of Substrate Analogues **16** and **17** by Abietadiene Synthase[a]



[a] Geranyl diphosphate (**18**) and the conversion of farnesyl diphosphate (**19**) to (*E*)-β-farnesene (**20**) are also illustrated.

pentane-extractable product. This product appears by GC−MS analysis to be an acyclic olefin resulting from ionization and direct deprotonation of **16**, since it has a shorter retention time than the cyclopropylidene analogue of geranylgeraniol but yields a very similar mass spectrum. These results suggest that neither substrate analogue is readily recognized by AS, which exhibits a relatively low K_m value of 3 μM for the normal GGPP substrate, and that any analogue that does productively bind is turned over, albeit inefficiently, to olefinic product, thereby escaping the possibility of alkylation.

A further attempt to distinguish the two presumptive active sites was made with the shorter isoprenologues of GGPP, geranyl diphosphate (**18**) and farnesyl diphosphate (**19**), which might preferentially bind and undergo reaction at only one of the two sites to give a diagnostic product. Farnesyl diphosphate was converted by AS at a rate of about 0.02 s^{-1} to the acyclic sesquiterpene olefin (*E*)-β-farnesene [**20**, identified by GC−MS comparison to the authentic standard (*46*)]; no product corresponding to the sesquiterpene homologue (**21**) of CPP was detected. These results indicate that the acyclic prenyl diphosphate farnesyl diphosphate (and thus by implication GGPP) can bind and undergo ionization and deprotonation at the second (CPP reactive) site, although catalysis is quite inefficient. The C10 analogue geranyl diphosphate was such a poor substrate for AS ($k_{cat} < 10^{-4}$ s^{-1}) that it was not possible to obtain sufficient product for identification. Finally, unlike the monoterpene synthases (*47*), neither the native AS (*7*) nor the recombinant enzyme was inhibited by inorganic diphosphate at concentrations up to 100 μM. The recombinant enzyme was also not measurably inhibited by *all-trans*-geranylgeraniol or (+)-copalol, either alone at 100 μM or in the presence of 100 μM inorganic diphosphate, indicating that the free prenol plus inorganic diphosphate does not effectively mimic the natural substrate or intermediate of the reaction.

DISCUSSION

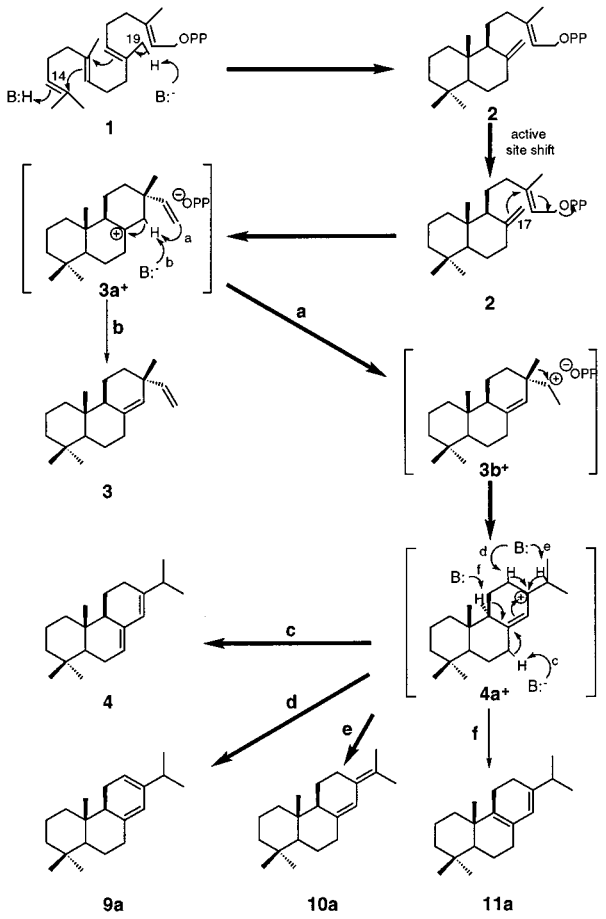
Abietadiene synthase is translated as an operationally compromised preprotein bearing a substantial N-terminal plastidial targeting peptide, the proteolytic processing site for which was not possible to determine directly because the native enzyme is N-blocked (*15*). A truncation series was therefore constructed to delete the transit sequence and provide a pseudomature form of the synthase more suitable for mechanistic and structural investigation. As judged by relative activity of the corresponding expressed proteins (Figure 2) and their approximate size by SDS−PAGE, it appeared that the appropriate cleavage site resides near Leu79. Considerations of expression yield, ease of purification, and other operational properties led to the selection of the construct with an initiation codon followed by Val85 and the remaining sequence that provided a pseudomature synthase with kinetic constants and product profile characteristic of the native enzyme. Similar results have been obtained recently with a truncation series of the taxadiene synthase preprotein, responsible for the first step of Taxol biosynthesis in yew species and the only other diterpene synthase with which deletion of the transit peptide has been attempted (*19*). The proximity of the apparent cleavage site of these two diterpene synthases from conifers may allow more straightforward design of pseudomature constructs in the future. It is notable that the initial truncation series to residue Ser111 had deleted an N-terminal DDXXD motif (aa 96−100) (Figure 1) that is an unusual characteristic of AS, since in most other terpene synthases such a typical substrate binding element (*48*, *49*) resides toward the C-terminus (*16*). These truncated proteins [even that devoid of the first 114 residues (Figure 1)] were capable of making olefinic products, although at a significantly diminished rate, indicating that this aspartate-rich element may not be absolutely required for cyclization. Consistent with this result, Kamiya and associates (*50*) recently have demonstrated, by directed mutagenesis, that a similarly placed element of an unusual bifunctional *ent*-kaurene synthase of fungal origin (in which the two related cyclization activities reside in a single protein, much like AS) is not essential for activity, although the production of the corresponding intermediate (−)-CPP is very greatly impaired by an alanine for aspartate substitution.

In the course of these studies it was demonstrated that AS is a multiple product enzyme that generates three principal olefins with the abietane skeleton [abietadiene (**4**), levopimaradiene (**9a**), and neoabietadiene (**10a**)], as well as lesser amounts of pimaradiene (**13**), sandaracopimaradiene (**3**), and palustradiene (**11a**). Abietadiene synthase is the first diterpene synthase demonstrated to be quite so promiscuous in product formation (*31*); however, multiple product synthases are well-known in the monoterpene (*47*, *51*), sesquiterpene (*52*), and triterpene (*45*, *53*) series. It is important to note that AS provides all of the necessary precursors for subsequent oxidation at C18 to the component resin acids of grand fir rosin (*6*, *7*). This is of significance since only one diterpene synthase enzyme (and cDNA) was observed in grand fir and so the origin of the complex mixture of resin acids was not obvious (*7*). It can now be seen that this single enzyme can account for the entire resin acid complex of this species. Because the same product set is generated from GGPP and (+)-CPP, it is clear that multiple product formation derives from the second cyclization step (of CPP) by variations on the same theme. Thus, after methyl migration generates the intermediate C13 abietanyl cation (**4a**$^+$), four alternatives for deprotonation would give rise to abietadiene (**4**), levopimaradiene (**9a**), neoabietadiene (**10a**), and palustradiene (**11a**) (Scheme 3). The trace products 8(14),15-pimaradiene (**13**) and 8(14),15-isopimaradiene (**3**, sandaracopimaradiene) can be accounted for by the two alternative stereochemistries of the S_{N}' cyclization of CPP followed by deprotonation at C14 of the resulting C8-pimarenyl (or isopimarenyl) cation (Scheme 3). Present evidence[2] (*13*, *14*) suggests that the bulk of the C8-isopimerenyl cation (**3a**$^+$) formed in the course of the reaction undergoes intramolecular proton migration to the C15-pimerenyl cation (**3b**$^+$) and thence methyl migration to form the abietenyl cation (**4a**$^+$) followed by deprotonation to products (Scheme 3). It seems highly unlikely that the epimeric C8-pimerenyl cation could proceed by a similar route, since stereoelectronically favorable intramolecular proton transfer in this case would require either conformational inversion of the C ring or a long migration of the C14α-H to the β-oriented vinyl group.

Abietadiene synthase efficiently utilizes (+)-CPP, but not (−)-CPP, as a substrate with k_{cat} comparable to that of the normal substrate GGPP. This result confirms the predicted stereochemistry of the copalyl intermediate, consistent with that of the ring junctions of the olefin products, and it also indicates that the rate-limiting step of the coupled reaction sequence resides in the second (ionization-dependent) cyclization process. Too little information is presently available to address this point in detail. In most mechanistically related monoterpene cyclizations, it is the prenyl diphosphate ionization step that is slow (*47*, *51*); however, in certain sesquiterpene cyclizations it is product release that is rate limiting (*26*, *52*). It is worth noting that the cyclization catalyzed by AS appears to represent the overall rate-determining step of resin acid biosynthesis (*6*, *54*).

Abietadiene synthase exhibits notable substrate inhibition with GGPP but not with (+)-CPP. The most reasonable explanation for this unusual behavior is the presence of two functionally distinct active sites, one for binding GGPP and conversion to the intermediate (+)-CPP and a second for binding (+)-CPP and conversion to product olefins but that is accessible to, and capable of being blocked by, GGPP to prevent the intermediate transfer and completion of the reaction cycle. While more detailed studies with the protein will be required to define these sites, it would appear that the N-terminal aspartate-rich DXDD motif (aa 402−405; Figure 1) delineates the site involved in the proton-initiated cyclization of GGPP to CPP since in both sequence and placement it resembles the corresponding site in kaurene synthase A (*55*) and triterpene synthases thought to be responsible for protonation of the terminal double bond (or corresponding epoxide) of squalene to initiate the cyclization reaction (*45*, *53*). On the other hand, the C-terminal aspartate-rich element (DDXXD comprising aa 621−625; Figure 1) is identical in sequence and consistent in placement with this motif found in kaurene synthase B (*56*) and in most monoterpene and sesquiterpene synthases where it is involved in binding and ionizing the prenyl diphosphate substrate of these typical cyclization reactions (*16*, *51*, *52*). This motif almost certainly delineates the site for the conversion of CPP to diterpene olefins by AS. Consistent with this hypothesis

Scheme 3: Proposed Reaction Mechanism for Abietadiene Synthase[a]



[a] GGPP (**1**) is bound at the N-terminal active site and protonated at C14 to initiate A/B ring closure followed by deprotonation at C19 to terminate the reaction to (+)-CPP (**2**). (+)-CPP is then transferred to the C-terminal active site where ionization of the diphosphate ester initiates anti-S_{N}' cyclization to the C8-isopimarenyl cation (**3a**$^+$), which may be deprotonated at C14 to yield sandaracopimaradiene (**3**) (pathway b) or undergo direct intramolecular proton transfer from C14 to C16 (pathway a) to afford the C15-pimarenyl cation (**3b**$^+$). 1,2-Methyl migration in **3b**$^+$ generates the abietenyl cation (**4a**$^+$), from which deprotonation at various positions yields the observed abietane products (pathways c−f).

is a recent analysis of a bifunctional *ent*-kaurene synthase from the fungus *Phaeosphaeria* sp. L487, the enzyme which most closely resembles AS in function (*50*). Directed mutagenesis clearly indicated the presence of two distinct active sites corresponding to those proposed for AS, and related evidence was provided for substrate inhibition of the bifunctional kaurene synthase by GGPP but not (−)-CPP. In the two active sites, at which the corresponding substrate or intermediate must be properly aligned to conduct the cyclization with appropriate regio- and stereochemistry, there must also be present specifically positioned base(s) to terminate the respective cyclizations. In the first site, a base must be positioned to deprotonate at C19 and terminate the cyclization to CPP. In the second site, one or more bases must be present to effect the various regiospecific deprotonations leading to the different abietadiene isomers. A base may also be deployed to mediate the intramolecular proton transfer from C14 of the pimarenyl intermediate (**3a**$^+$) to initiate the methyl migration step (Scheme 3); however, it is possible that this transfer is unassisted as appears to be the case in a related intramolecular hydrogen migration in the cyclization to taxadiene catalyzed by taxadiene synthase from yew (*57*). Efforts to define the two active sites of AS and their associated bases more precisely, and to determine the rate-limiting step of the overall coupled reaction, are underway.

ACKNOWLEDGMENT

We thank M. Phillips, D. Williams, and R. Long for technical assistance, Ram Mohan for preparing sandaracopimaradiene, Zhanqi Ho for purifying pimara-7,15-diene, Min Chu for preparing (+)-(*E*)-copalal and (+)-(*E*)-copalol, and Duane Zinkel and the USDA Forest Products Laboratory, Madison, WI, for samples of pimaric and isopimaric acids.

REFERENCES

1. Lewinsohn, E., Savage, T. J., Gijzen, M., and Croteau, R. (1993) *Phytochem. Anal. 4*, 220−225.
2. Phillips, M. A., and Croteau, R. (1999) *Trends Plant Sci. 4*, 184−190.
3. Gijzen, M., Lewinsohn, E., Savage, T. J., and Croteau, R. B. (1993) *ACS Symp. Ser. 525*, 8−22.
4. Lewinsohn, E., Gijzen, M., Savage, T. J., and Croteau, R. (1991) *Plant Physiol. 96*, 38−43.
5. Steele, C. L., Lewinsohn, E., and Croteau, R. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4164−4168.
6. Funk, C., and Croteau, R. (1994) *Arch. Biochem. Biophys. 308*, 258−266.
7. LaFever, R. E., Stofer Vogel, B., and Croteau, R. (1994) *Arch. Biochem. Biophys. 313*, 139−149.
8. Dennis, D. T., and West, C. A. (1967) *J. Biol. Chem. 212*, 3293−3300.
9. West, C. A., Dudley, M. W., and Dueber, M. T. (1979) *Recent Adv. Phytochem. 13*, 163−198.
10. Graebe, J. E. (1987) *Annu. Rev. Plant Physiol. 38*, 419−469.
11. Duncan, J. D., and West, C. A. (1981) *Plant Physiol. 68*, 1128−1134.
12. Saito, T., Abe, H., Yamane, H., Sakurai, A., Murofushi, N., Takio, K., Takahashi, N., and Kamiya, Y. (1995) *Plant Physiol. 109*, 1239−1245.
13. Ravn, M. W., Coates, R. M., Flory, J. E., Peters, R. J., and Croteau, R. (2000) *Org. Lett. 2*, 573−576.
14. Ravn, M. M., Coates, R. M., Jetter, R., and Croteau, R. (1998) *J. Chem. Soc., Chem. Commun. 1998*, 21−22.
15. Stofer Vogel, B., Wildung, M., Vogel, G., and Croteau, R. (1996) *J. Biol. Chem. 271*, 23262−23268.
16. Bohlmann, J., Meyer-Gauen, G., and Croteau, R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 4126−4133.
17. Croteau, R., Alonso, W. R., Koepp, A. E., and Johnson, M. A. (1994) *Arch. Biochem. Biophys. 309*, 184−192.
18. Dehal, S. S., and Croteau, R. (1988) *Arch. Biochem. Biophys. 261*, 346−356.
19. Williams, D. C., Wildung, M. R., Jin, A. Q., Dalal, D., Oliver, J. S., Coates, R. M., and Croteau, R. (2000) *Arch. Biochem. Biophys. 379*, 137−146.
20. Lee, H.-J., Ravn, M. W., and Coates, R. M. (2000) *J. Nat. Prod.* (submitted for publication).
21. Chu, M., and Coates, R. M. (1992) *J. Org. Chem. 57*, 4590−4597.
22. Mohan, R. S., Yee, N. K. N., Coates, R. M., Ren, Y.-Y., Stemenkovic, P., Mendez, I., and West, C. A. (1996) *Arch. Biochem. Biophys. 330*, 33−47.
23. Yee, N. K. N., and Coates, R. M. (1992) *J. Org. Chem. 57*, 4598−4608.
24. Sundararaman, P., and Herz, W. (1977) *J. Org. Chem. 42*, 806−813.
25. Coates, R. M., and Cavender, P. L. (1980) *J. Am. Chem. Soc. 102*, 6358−6359.
26. Mathis, J. R., Back, K., Starks, C. M., Noel, J., Poulter, C. D., and Chappell, J. (1997) *Biochemistry 36*, 8340−8348.
27. Schenk, P. M., Baumann, S., Mattes, R., and Steinbiss, H.-H. (1995) *BioTechniques 19*, 196−200.
28. Studier, F. W., Rosenburg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
29. Laemmli, U. K. (1970) *Nature 227*, 680−685.
30. Blum, H., Beier, H., and Gross, H. J. (1987) *Electrophoresis 8*, 93−99.
31. MacMillan, J., and Beale, M. H. (1999) in *Comprehensive Natural Products Chemistry: Isoprenoids Including Steroids and Carotenoids* (Cane, D. E., Ed.) pp 217−244, Elsevier Science, Oxford, U.K.
32. Keegstra, K., Olsen, J. J., and Theg, S. M. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 40*, 471−501.
33. Schnell, D. J. (1998) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 49*, 97−126.
34. Williams, D. C., McGarvey, D. J., Katahira, E. J., and Croteau, R. (1998) *Biochemistry 37*, 12213−12220.
35. Kouranov, A., and Schnell, D. J. (1996) *J. Biol. Chem. 271*, 31009−31012.
36. von Heijne, G., Steppuhn, J., and Herrmann, R. G. (1989) *Eur. J. Biochem. 180*, 535−545.
37. Gavel, Y., and von Heijne, G. (1990) *FEBS Lett. 261*, 455−458.
38. Croteau, R., and Cane, D. E. (1985) *Methods Enzymol. 110*, 383−405.
39. Takeda, H., Schuller, W. H., and Lawrence, R. V. (1983) *J. Org. Chem. 33*, 1683−1684.
40. Soltes, E. J., and Zinkel, D. F. (1989) in *Naval Stores. Production, Chemistry, Utilization* (Zinkel, D. F., and Russell, J., Eds.) pp 289−291, Pulp Chemical Association, New York.
41. Lin, X., Hezari, M., Koepp, A. E., Floss, H. G., and Croteau, R. (1996) *Biochemistry 35*, 2968−2977.
42. Schulz, A. R. (1994) in *Enzyme Kinetics from Diastase to Multi-enzyme Systems*, pp 38−41, Cambridge University Press, Cambridge, U.K.
43. Croteau, R., Alonso, W. R., Koepp, A. E., Shim, J.-H., and Cane, D. E. (1993) *Arch. Biochem. Biophys. 307*, 397−404.
44. McGeady, P., and Croteau, R. (1995) *Arch. Biochem. Biophys. 317*, 149−155.
45. Abe, I., and Prestwich, G. D. (1999) in *Comprehensive Natural Products Chemistry: Isoprenoids Including Steroids and Carotenoids* (Cane, D. E., Ed.) pp 267−298, Elsevier Science, Oxford, U.K.
46. Crock, J. E., Wildung, M., and Croteau, R. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12833−12838.
47. Wise, M. L., and Croteau, R. (1999) in *Comprehensive Natural Products Chemistry: Isoprenoids Including Steroids and*

Carotenoids (Cane, D. E., Ed.) pp 97−153, Elsevier Science, Oxford, U.K.
48. Joly, A., and Edwards, P. A. (1993) *J. Biol. Chem. 268*, 26983−26989.
49. Starks, C. M., Back, K., Chappell, J., and Noel, J. P. (1997) *Science 277*, 1815−1820.
50. Kawaide, H., Sassa, T., and Kamiya, Y. (2000) *J. Biol. Chem. 275*, 2276−2280.
51. Davis, E. M., and Croteau, R. (2000) *Top. Curr. Chem. 209*, 53−95.
52. Cane, D. E. (1999) *Comprehensive Natural Products Chemistry: Isoprenoids Including Steroids and Carotenoids* (Cane, D. E., Ed.) pp 155−200, Elsevier Science, Oxford, U.K.
53. Wendt, K. U., Poralla, K., and Schulz, G. E. (1997) *Science 277*, 1811−1815.
54. Funk, C., Lewinsohn, E., Stofer Vogel, B., Steele, C. L., and Croteau, R. (1994) *Plant Physiol. 106*, 999−1005.
55. Sun, T.-P., and Kamiya, Y. (1994) *Plant Cell 6*, 1509−1518.
56. Yamaguchi, S., Saito, T., Abe, H., Yamane, H., Murofushi, N., and Kamiya, Y. (1996) *Plant J. 10*, 101−111.
57. Williams, D. C., Carroll, B. J., Jin, Q., Rithner, C., Lenger, S. R., Floss, H. G., Coates, R. M., Williams, R. M., and Croteau, R. (2000) *Chem. Biol.* (in press).

BI001997L