The Primary Diterpene Synthase Products of *Picea abies*
Levopimaradiene/Abietadiene Synthase (PaLAS) Are Epimers of a Thermally Unstable Diterpenol

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The levopimaradiene/abietadiene synthase from Norway spruce (*Picea abies*; PaLAS) has previously been reported to produce a mixture of four diterpene hydrocarbons when incubated with geranylgeranyl diphosphate as the substrate: levopimaradiene, abietadiene, neoabietadiene, and palustradiene. However, variability in the assay products observed by GC-MS of this and orthologous conifer diterpene synthases over the past 15 years suggested that these diterpenes may not be the initial enzyme assay products but are rather the products of dehydration of an unstable alcohol. We have identified epimers of the thermally unstable allylic tertiary alcohol 13-hydroxy-8(14)-abietene as the products of PaLAS. The identification of these compounds, not previously described in conifers, as the initial products of PaLAS has considerable implications for our understanding of the complexity of the biosynthetic pathway of the structurally diverse diterpene resin acids of conifer defense.

Diterpene resin acids play an important role in conifer defense against herbivores and pathogens (1, 2). They are biosynthesized by the cyclization of geranylgeranyl diphosphate by single- or multi-product diterpene synthases (see Fig. 1) and the subsequent oxidation by cytochromes P450 at carbon-18 (3).

To date, only two types of diterpene synthases have been characterized in conifer resin acid biosynthesis: those that produce isopimaradiene and sandaracopimaradiene (4, 5) and those that produce a mixture of abietadiene, levopimaradiene, neoabietadiene, and palustradiene (4–7). So far, only one cytochrome P450 of diterpene resin acid biosynthesis, CYP720B1 from loblolly pine (*Pinus taeda*) (7), and Sitka spruce (*Picea sitchensis*) (4), as well as a paralogous diterpene synthase from two of these species, the levopimaradiene/abietadiene synthase; PaIso,2 which was originally reported to produce 98% abietadiene (9). Interestingly, the products of AgAS were subsequently demonstrated to be sensitive to the assay workup conditions; when passed through silica gel and MgSO4 prior to GC-MS analysis, abietadiene was the major product (9). However, without this treatment step, levopimaradiene, abietadiene, and neoabietadiene were seen in nearly equal amounts, along with minor amounts of palustradiene (6). This inconsistency was hypothesized to be the result of isomerization of levopimaradiene and neoabietadiene to abietadiene with the silica gel and MgSO4 treatment (6) but was not investigated further until now. Consequently, AgAS was described as a multi-product diterpene synthase (6).

Subsequent research has identified three orthologous diterpene synthases, the levopimaradiene/abietadiene synthases from Norway spruce (*Picea abies*; PaLAS) (5), loblolly pine (*P. taeda*) (7), and Sitka spruce (*P. sitchensis*) (4), as well as a paralogous diterpene synthase from two of these species, the isopimaradiene synthases from Norway spruce (Palso) (5) and Sitka spruce (4). All of these subsequent studies did not use the silica gel-MgSO4 assay workup, and all of the LAS enzymes produced similar multi-products: levopimaradiene, abietadiene, neoabietadiene, and palustradiene.

In the process of identifying the residues that determine product profile differences between the two paralogous diterpene synthases from Norway spruce (Palso and PaLAS) through site-directed mutagenesis (10), we were surprised to find that the relative ratios of the LAS-like products were completely unaffected by the various mutants we prepared even though some mutants also produced isopimaradiene. Our existing understanding of the mechanism of this and other terpene synthases would have predicted that mutations in the active site were very likely to change the proportion of products of multi-product enzymes (11–18). However, we did notice anecdotally that there was a significant change in the ratio of these PaLAS-like products that depended upon the conditions and temperature of the GC-MS injector and column. We also observed two products that had not previously been described (although seen in Ref. 10) from these or related diterpene synthases when analyzed on a brand new GC-MS system. These observations, and the prior observations with AgAS, led us to re-examine the product profile of PaLAS.

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2 The abbreviations used are: AgAS, *A. grandis* abietadiene synthase; PaLAS, *P. abies* levopimaradiene/abietadiene synthase; Palso, *P. abies* isopimaradiene synthase; APCI, atmospheric pressure chemical ionization; TMS, trimethylsilyl.
**Norway Spruce Diterpene Synthase Products**

**EXPERIMENTAL PROCEDURES**

Preparation of Recombinant Enzyme Assay Products—Recombinant pseudomature enzymes (PaIso and PaLAS) were produced from 1-liter *Escherichia coli* cultures and nickel affinity-purified as described previously (10). Single-vial enzyme assays with geranylgeranyl diposphate (Sigma) as substrate were completed in 50 mM HEPES, 100 mM KCl, 7.5 mM MgCl₂, 0.02 mM MnCl₂, and 5% glycerol (pH 7.2) as described previously (10). Unlike some prior methods for terpene synthase assays, this method does not pass the products through a silica column with MgSO₄ and does not require concentrating the pentane extract prior to GC-MS analysis. Standards—Abietic acid was obtained from Orchard Cellmark (New Westminster, British Columbia, Canada). Abietadiene and abietadienol were prepared by Best West Laboratories Inc. (Salt Lake City, UT) from abietic acid as described previously (3). The synthesis of (+)-13-hydroxy-8(14)-abietane from (+)-α-cyclocitral (Toronto Research Chemicals, North York, Ontario, Canada) was attempted by Best West Laboratories Inc. as described previously (19) with the exception of the final isopropylation step, for which the method of Gu et al. (20) was used.

GC-MS Analyses—GC-MS analyses were conducted on Agilent HP-5 (5% phenylmethyl silicone, 30 m, 250-μm inner diameter, 0.25-μm film) and DB-WAX (polyethylene glycol, 30 m, 250-μm inner diameter, 0.25-μm film) columns at 1 ml/min helium on an Agilent 6890N GC 7683B series autosampler (vertical syringe position of 8) and an Agilent 5975 inert XL mass spectrometer with a high structural similarity of PaLAS and PaIso with a root mean square deviation of 0.7 Å between all Cα atoms. Molegro Virtual Docker 2010.4.0.0 (27) was applied to perform the molecular docking of (+)-copalyl diphosphate and 13-hydroxy-8(14)-abietene in the presence of the cleaved diphosphate group into the predicted class I cavity of PaLAS and PaIso. Protein Data Bank coordinates of ligands were generated using the Dundee PRODRG2 server (28).

RESULTS

From the series of experiments detailed below, we obtained multiple lines of evidence that the initial diterpene products formed and released by PaLAS in the *in vitro* assays with geranylgeranyl diphosphate were not hydrocarbons but were instead epimeric thermally unstable allotypic tertiary alcohols that subsequently dehydrated under the conditions of analysis to the multiple diterpene hydrocarbons previously described as the reaction products of LAS enzymes. On the basis of these results, we propose that the reaction mechanism of PaLAS proceeds via water quenching of the abieta-8(14)-en-13-yl carbocation, resulting in 13-hydroxy-8(14)-abietene (Fig. 1).

"GC-MS Analyses—We found that PaLAS, which is thought to utilize a previously described rearrangement route for product formation (Fig. 1, route a), produced two new compounds in an approximate 1:20 ratio that have not previously been reported as products in these or related enzymes, in addition to levopimaradiene, abietadiene, neoabietadiene, and palustra-"
The proposed mechanisms of both PaLAS and PaIso begin with the formation of an isopimar-15-en-8-yl carbocation from geranylgeranyl diphosphate. The mechanisms diverge at this intermediate. For PaIso, loss of a proton results in the formation of isopimara-diene (route b). For PaLAS, intramolecular proton abstraction and the subsequent Wagner-Meerwein 1,2-methyl shift result in formation of an abieta-8(14)-en-13-yl carbocation (route a). Previously, loss of a proton from this intermediate was thought to immediately give rise to the hydrocarbons abietadiene, levopimaradiene, neoabietadiene, and palustradiene. However, the data presented in this work suggest that the abieta-8(14)-en-13-yl carbocation is quenched by water to form 13-hydroxy-8(14)-abietene, a tertiary allylic alcohol. The apparent products abietadiene, levopimaradiene, neoabietadiene, and palustradiene previously reported are consequently the result of thermally induced dehydration of 13-hydroxy-8(14)-abietene.
diene (Fig. 2A, peaks 6 and 7). Their retention indices were 2216/2820 and 2230/2894 on the HP-5ms and DB-WAX columns, respectively, compared with 2060/2354, 2067/2369, 2131/2495, and 2199/2596 for palustradiene, levopimaradiene, abietadiene, and neoabietadiene, respectively. The retention indices of these additional products suggested that they were much more polar than the other diterpenes. The mass spectra of these new peaks (Fig. 2B) were identical to each other and nearly identical to that of abietadiene (Fig. 2C), except for an additional strong peak at m/z 247. As loss of a fragment at m/z 25 from a molecular ion of 272 to get to 247 was not plausible, we postulated that this ion was indicative of loss of an isopropyl group, [M − 43]⁺, from a trimethylsilyl (TMS) diterpenol (with a molecular weight of 362), paralleling a similar loss of an isopropyl group from the underivatized compound. Also, we observed a weak fragment at m/z 347, [M − 15]⁺, suggesting a loss of a methyl group from the TMS group. We postulated that these newly described peaks in the GC-MS product profile of PaLAS were the epimers of 13-hydroxy-8(14)-abietene due to water quenching of the abieta-8(14)-en-13-yl carbocation (Fig. 1, route a).

From these observations, we suspected that the high temperature of the GC-MS injector was sufficient to dehydrate the allylic alcohol products into the diterpenes previously assigned as the products of PaLAS and other LAS-type diterpene synthases. Using a cold on-column injector but a similar GC oven temperature program, we observed that the diterpene peaks significantly decreased and the diterpenol peaks increased in intensity, confirming that the diterpenes were not the initial products of the in vitro enzyme activity (Fig. 4A). Interestingly, a hump in the base line was observed as the temperature of the

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**FIGURE 2.** GC-MS chromatogram and mass spectra of assay products from PaLAS. A, GC-MS chromatogram of PaLAS assay products on a clean GC-MS system with an HP-5ms column. TIC, total ion chromatogram. Peak 1, palustradiene; peak 2, levopimaradiene; peak 3, dehydroabietadiene; peak 4, abietadiene; peak 5, neoabietadiene; peaks 6 and 7, diterpenol products. B, mass spectrum of the most abundant diterpenol (peak 7). The mass spectrum of peak 6 was identical. The distinctive fragment at m/z 247 could not have arisen from a molecular ion at 272. C, mass spectrum of abietadiene (peak 4).
GC oven increased, which abruptly stopped when the second diterpenol (peak 7) eluted (Fig. 4A). The mass spectrum of this hump was very similar to that of abietadiene. This observation suggested that the diterpenols were also dynamically dehydrating to the less-retained hydrocarbons within the GC column as the oven temperature increased.

When combined with cold on-column injection and a lower final oven temperature (150 °C), the proportion of the diterpenol products increased further (Fig. 4B, peaks 6 and 7). With cold on-column injection and chemical ionization (positive mode and CH4 as the reagent gas), we observed a weak but apparent m/z 291, consistent with a diterpenol, [M/H]+. The significant intensity of the fragments at m/z 272 and 257 for the diterpenol peaks under any of the GC and MS conditions tested, including cold on-column injection and chemical ionization, suggested that dehydration of these compounds to diterpenes (m/z 272, [M – 18]+) and subsequent loss of a methyl group (m/z 257, [M – 18 – 33]+) were also occurring within the heated transfer line and the heated and high energy mass spectrometer.

Silica Chromatography—To confirm the polar nature of the products of the PaLAS in vitro enzyme activity, which are unlike the non-polar assay product isopimaradiene from Palso (Fig. 1, route b), and to avoid the confound of heating during the chromatographic step, we combined the assay products of these two enzymes and separated the individual compounds by silica chromatography at room temperature. The products of Palso and PaLAS in pentane were combined, loaded onto a silica-filled Pasteur pipette column, eluted with pentane (four 1-ml fractions) and diethyl ether (two 1-ml fractions), and analyzed by GC-MS. We observed that isopimaradiene eluted very early, as would be expected, in the first and second pentane fractions, but only very small amounts of LAS-like products were observed in any of these and subsequent pentane fractions (Fig. 5). However, the LAS-like products were observed in fractions that eluted from the silica column with diethyl ether in the same fraction that contained the putative diterpenol peaks. The observation that these non-polar hydrocarbons eluted with a polar solvent but, unlike isopimaradiene, did not elute with the non-polar solvent is consistent with them not being the initial products of PaLAS in vitro enzyme activity. Instead, they appeared to be the dehydration products of polar products of PaLAS that eluted in this polar fraction and dehydrated into the non-polar diterpenes during the subsequent GC-MS analysis.

LC-MS Analyses—To further corroborate the results from the silica chromatography, we analyzed the assay products of PaLAS by LC-APCI-MS in positive mode. The products from an enzyme assay with PaLAS did not show the presence of any non-polar diterpenes but rather a compound of intermediate polarity. As shown in Fig. 6, the LAS product was a relatively polar product, which eluted between the authentic abietadiene and abietadienol. The elution pattern was consistent with polar products such as alcohols, not hydrocarbons. The chemical ionization mass spectrum of the polar product peak showed a dominating m/z 273, similar to abietadiene. We propose that this ion was the [M + H]+ of the hydrocarbon products of dehydration of the allylic diterpenols, where dehydration occurred after chromatography but during ionization due to the high temperature of the APCI-MS interface.
NMR Analyses—We combined products from several large-scale enzyme assays to obtain material for structure confirmation by NMR. Assay products were prepared in benzene-$d_6$ because the same method in CDCl$_3$ resulted in dehydration of the assay products. The NMR analyses were consistent with 13-hydroxy-8(14)-abietene as the assay product. We observed only one olefinic hydrogen resonance, and the resonances for the single hydrogen atoms on carbon-9 and carbon-15 confirmed that the assay product could not be any of the previously described hydrocarbons (Table 1). The material recovered from the enzyme assays was not sufficient for $^{13}$C NMR. However, heteronuclear multiple-bond coherence was used to make assignments for the carbons. The stereochemistry of carbon-13 could not be determined definitively by NOESY (Fig. 7). The lack of an NOE observed between H11\(\beta\) and H16 or H17 and the very weak NOEs observed between H12\(\beta\) and H16 and between H14 and H17 suggested that the stereochemistry of the hydroxyl group was $\beta$ for the most abundant epimer, but this assignment was not well supported.

Synthesis of the Predicted Product—To confirm the physical and chemical properties with an authentic standard, we and others attempted to obtain \((\pm)-13\text{-hydroxy}-8(14)\text{-abietene} by synthesis from \((\pm)-\alpha\text{-cyclocitral using previously reported methods (19). Unfortunately, despite repeated attempts, the desired compound could not be obtained in a sufficient amount for application as an authentic standard, principally due to dehydration upon final workup and the low yield of an intermediate isomerization step. The previously described method of synthesis (19) proved difficult to reproduce, preventing the direct comparison of the PaLAS assay products with an authentic standard.

Computational Structure Analysis—We modeled the structures of PaLAS and Palso based on the structure of taxadiene synthase from \(T. brevifolia\) (22) to draw insight into the origin of product differences. The most energetically favorable binding located 13-hydroxy-8(14)-abietene in the center of the
active site cavity of both proteins, with the hydroxyl group facing toward the aspartate-rich DDXXD motif on helix D. As to be expected, the cleaved diphosphate group was chelated to the Mg$^{2+}$ cluster. However, it is not known whether or not the diphosphate remains in the active site as a counterion in the course of the PaLAS-catalyzed reaction. Sixteen residues were found within a 7-Å radius from the hydroxyl group of 13-hydroxy-8(14)-abietene. Interestingly, these included three of the four amino acids previously identified to determine the distinct product specificity of PaLAS and PalsO (10), namely Tyr-686 (−5 Å), Ala-713 (−6 Å), and Val-717 (−6 Å). No other significant differences were observed, reinforcing the importance of these specific residues.

**DISCUSSION**

The results of this study provide several lines of evidence suggesting that the initial products formed and released by PaLAS in vitro are unstable alcohols identified here as epimeric thermally unstable allylic tertiary alcohols, 13-hydroxy-8(14)-abietene. Although water capture of a carbocation intermediate and formation of terpenol products are not unusual in terpene synthases, especially monoterpene synthases, they have rarely been described for diterpene synthases. However, one example is the formation of ent-16α-hydroxykaurene as the major product of the ent-kaurene synthase from Physcomitrella patens (29). This product is, however, not an allylic tertiary alcohol and is thus less labile to dehydration. The diterpene alcohols described in this study have not previously been reported to be products of PaLAS or related conifer diterpene synthases.

We suggest that the dehydration of the alcohol products of PaLAS, yielding the well established diterpene products levopimaradiene, abietadiene, neoabietadiene, and palustradiene, occurred due to three conditions of the GC-MS analysis typically used for identification of diterpene synthase products: a hot injector, a hot oven temperature necessary for eluting the compounds, and the high temperature and high energy of the MS and its interface. Using a cold on-column injector rather than a split/splitless injector and using a lower maximum oven temperature, we were able to increase the abundance of the polar products but not prevent at least partial dehydration on the GC column and significant dehydration in the MS. Other conditions at room temperature are likely to promote dehydration, such as evaporating to dryness and the use of drying agents and strong acids.

In prior research, the *in vivo* production of diterpene resin acids in metabolically engineered yeast containing the genes encoding geranylgeranyl diphosphate synthase and PaLAS produced only abietadiene and none of the other diterpenes previously found in the multi-product profiles of LAS-type enzymes (3). At the time, this simpler product profile was rationalized as the lower pH of the yeast culture medium affecting the enzyme product profile. However, given the results presented here, it is likely that the culture conditions, assay extraction, and/or
workup procedures may have dehydrated the initial PaLAS assay products.

To the best of our knowledge, diterpenoids with a hydroxyl group at carbon-13 have not been found in previous analyses of Norway spruce resin or the resin of other spruce species (30, 31). Whether their apparent absence is due to enzymatic or non-enzymatic dehydration in planta or to their sensitivity to dehydration ex planta in the process of resin extraction and analysis is not known and will be difficult to resolve. However, diterpenoids with a hydroxyl or ether group at this same position have recently been described in *Pinus massoniana* resin (32). Analogous compounds with the opposite stereochemistry have also been characterized in *Solidago* spp. and were described as having similar sensitivities to dehydration and similar mass spectral fragmentation patterns ([M – H2O]+ and [M – C3H7]+) (33).

If, indeed, the diterpenol is the initial product of PaLAS, it is possible that there may be an additional enzymatic or non-enzymatic step in the biosynthesis of the known diterpene resin acids of Norway spruce and other conifers. This step may occur prior to the oxidation at carbon-18 by cytochromes P450 of the CYP720B subfamily such as CYP720B1 (3, 8) or after.

The assay product of Palso does not behave chromatographically like that of PaLAS and appears to be truly isopimaradiene (Figs. 1 and 5). The significant differences in the kinetic parameters we previously reported between these two enzymes reflect their differing mechanisms; PaLAS has a catalytic efficiency (kcat/Km) that is 20 times higher compared with Palso, principally due to differences in kcat (10). This difference may originate from the difference in polarity of the products, as the release of non-polar products such as isopimaradiene is thought to be rate-determining for terpene syntheses (34, 35). The release of a polar diterpene alcohol product from the active site of PaLAS may account for the observed higher catalytic efficiency of this enzyme compared with Palso. The kinetic parameters for AgAS (6) are very similar to those of PaLAS, suggesting that AgAS may also produce the diterpenol products rather than the hydrocarbons directly, consistent with the observed variability in product profiles that have been reported previously with this enzyme (6, 9).

Computational structure analyses indicated that three of the four residues we had previously identified to contribute to the different product profiles of Palso and PaLAS (10) were near the hydroxyl group of 13-hydroxy-8(14)-abietene. Although closest to the hydroxyl group, the hydrophobic nature of Tyr-686 in PaLAS compared with the more hydrophilic His-694 in Palso could not support the coordination of a water molecule in this position, and prior reciprocal mutation of this residue is not sufficient alone to change product profiles in PaLAS or Palso (10). However, the corresponding residue in the *P. patens* ent-kaurane synthase, Ala-710, has recently been identified to contribute to the formation of ent-16α-hydroxykaurane (36). This residue determines whether the ent-kauranylation cation is captured by water to produce the alcohol or loses a proton to produce ent-kaurane. In PaLAS, the A713S mutant previously described prevented LAS-type products from forming and instead produced isopimaradiene and sandaracopimaradiene (10). We speculate that Ala-713 in PaLAS generates enough space for a water molecule compared with the respective Ser-721 in Palso. (This residue is Ala-737 in *P. patens* ent-kaurane synthase.) In light of the results of this study, it is now apparent that the four mutations that determine product differences between Palso and PaLAS (10) are likely to determine the presence and position of water molecules in the active site in addition to the stabilization of alternative carbocations. Recent similar work with mutations of the levopimaradiene synthase from *Ginkgo biloba* resulted in the production of abietadiene in addition to levopimaradiene (37). It would be interesting to determine whether these mutant enzymes also produce a diterpenol product or the hydrocarbons directly.

The identification of the *in vitro* product of PaLAS enzyme activity reinforces the caution that must be observed when assuming that the products analyzed by GC-MS, which subjects the analytes to high temperatures, actually reflect the true enzyme products. This notion has precedence in the identification of alcohol products of some sesquiterpene synthases (e.g. Ref. 38). This study may resolve some of the discussion about single- or multi-product profiles of LAS-type diterpene synthases, a topic that was first raised with the variability of product identification of the grand fir AgAS enzyme (6, 9).

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