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Isotopically Sensitive Branching as a Tool for Evaluating Multiple Product Formation by Monoterpene Cyclases

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Abstract: The deuterated substrates [4-$^2H_2$,1-^{3}H]geranyl pyrophosphate and [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate were employed to examine isotopically sensitive branching in the biosynthesis of monoterpene olefin isomers. By this method, (-)-α-pinene and (-)-β-pinene were shown to be synthesized via a common intermediate by a single cyclization enzyme from grand fir (*Abies grandis*), as were (-)-α-phellandrene and (-)-β-phellandrene by a single cyclase from lodgepole pine (*Pinus contorta*). Kinetic isotope effects were determined for the various deprotonations leading to the pinenes and phellandrenes.

INTRODUCTION

Several hundred naturally occurring monoterpenes are known, and all are considered to be derived from the ubiquitous isoprenoid precursor geranyl pyrophosphate by the action of a class of enzymes termed the monoterpene cyclases.[1] These enzymes catalyze the formation of the various monoterpene skeletal types by variations on the same basic electrophilic reaction mechanism.[1,2] While the monoterpene cyclases from herbaceous plants have been studied extensively,[2] it is only recently that cell-free systems have been developed to permit examination of the cyclases from conifer species.[3] The monoterpene cyclases of conifers are responsible for the production of the turpentine component of oleoresin that plays an important role in defense against herbivore and pathogen attack.[4,5] Turpentines are very complex mixtures of monoterpenes, and the crude cell-free extracts from conifer stem tissue can produce in excess of a dozen products from geranyl pyrophosphate. The determination of the number of enzymes responsible for the production of these complex mixtures is a formidable problem, requiring detailed and extensive protein purification studies. The task can be further complicated by the presence of enzymes that produce multiple products, a phenomenon now well documented among the terpene cyclases of herbaceous species.[2,6,7] In this context, isotopically sensitive branching experiments can be particularly valuable, since the data gained can reveal whether structurally related

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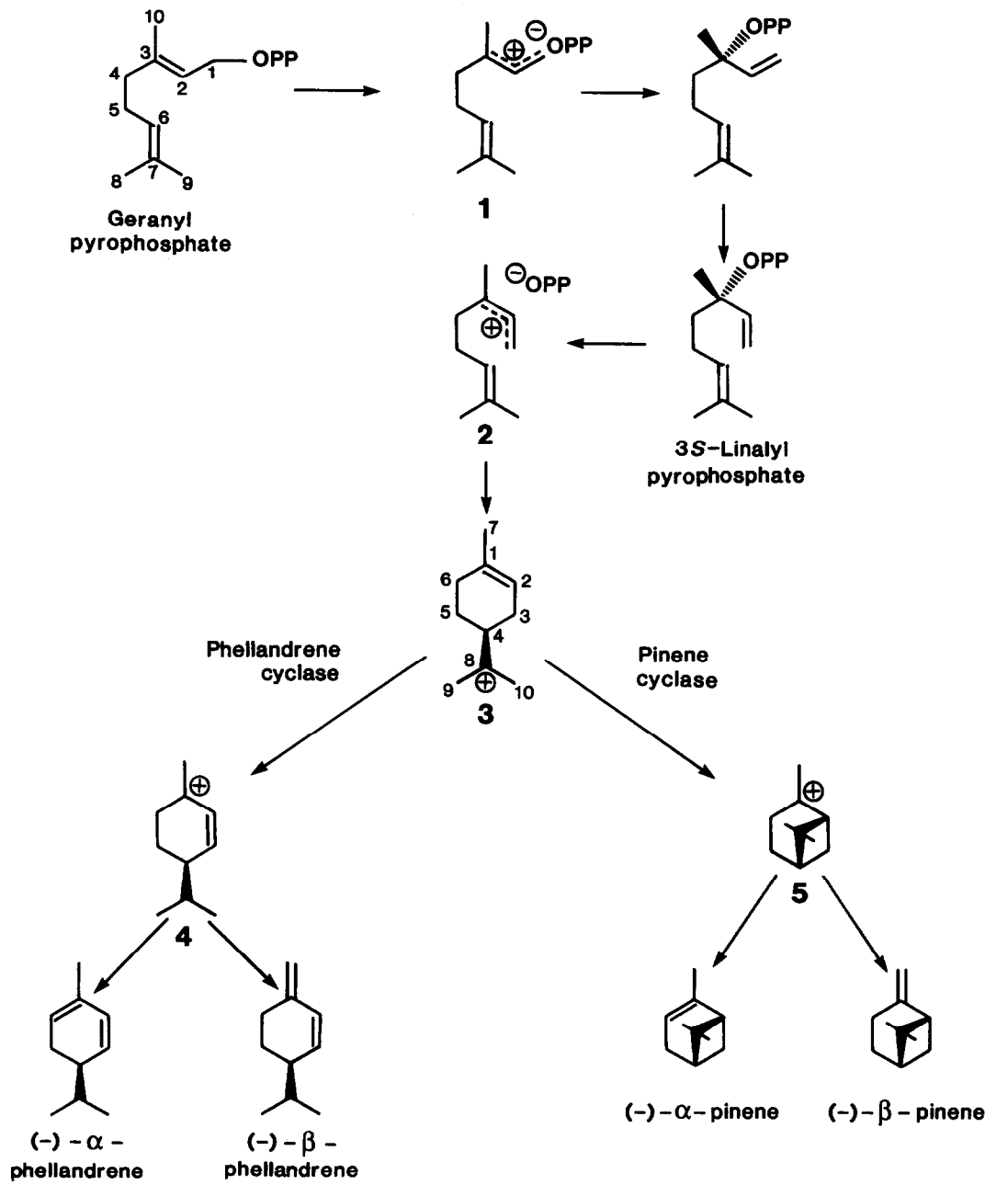
isomers are, in fact, generated by a single cyclase. This information is essential for the design and interpretation of enzyme purification schemes,[8,9] and is often mechanistically revealing.[10]

In the present instance, we describe two partially purified enzyme systems from conifers with which we have exploited the isotopically sensitive branching phenomenon. These are a wound-inducible cyclase activity from grand fir (*Abies grandis*) that produces primarily (-)-α-pinene and (-)-β-pinene (pinene cyclase),[11] and a constitutive cyclase activity from lodgepole pine (*Pinus contorta*) that produces mainly (-)-α-phellandrene and (-)-β-phellandrene (phellandrene cyclase).[12] The proposed biosynthetic sequence for the formation of these cyclic monoterpene olefins is initiated by ionization of geranyl pyrophosphate with migration of the pyrophosphate moiety to form the enzyme bound intermediate 3*S*-linalyl pyrophosphate (Scheme 1). Rotation about the newly generated C2-C3 single bond of the linalyl intermediate to the cisoid conformer overcomes the original stereochemical impediment to direct cyclization of geranyl pyrophosphate, and is followed by a second ionization (of the tertiary allylic intermediate) with cyclization to the 4*S*-α-terpinyl cation (**3**). The sequence of steps (or its antipodal counterpart) to this point is believed to be common to all monoterpene cyclases,[1,2] and at this juncture the pathways for the formation of pinenes and phellandrenes diverge. Thus, phellandrene cyclase, by either two 1,2-hydride shifts, or a 1,3-hydride shift in the α-terpinyl cation (**3**), followed by double bond migration, yields the phellandryl cation (**4**), whereas pinene cyclase, by internal addition to the cyclohexenyl double bond of the α-terpinyl cation (**3**) gives rise to the bicyclic pinyl cation (**5**). Both phellandryl and pinyl cations may undergo deprotonation from either of two adjacent carbon atoms to provide the observed isomeric olefin products. If a proton is lost from the adjacent methylene (C4 of the geranyl substrate), the α-isomers are formed, whereas proton loss from the adjacent methyl (C10 of the geranyl substrate) leads to the corresponding β-isomers.

If the pinyl and phellandryl cations are branch-point intermediates formed irreversibly by the respective cyclase enzymes, and each undergoes deprotonation at either the C4 or C10 position (of the geranyl substrate), then it is possible to observe an induced kinetic isotope effect[13] (KIE) on the rate of product formation resulting from reaction with a strategically deuterium-labeled substrate. Thus, an induced KIE occurs when a primary deuterium isotope effect accelerates the rate of formation of one enzymatic product by suppressing the rate of formation of a second enzymatic product. Such an observation indicates that both products arise from a common intermediate generated by the same enzyme. The underlying phenomenon, termed isotopically sensitive branching,[14] has been used to probe the generation of product mixtures in cytochrome P-450-catalyzed oxidations,[14,15] the effect of mechanism-based inhibitors on RTEM β-lactamase,[16] and the formation of isomeric mixtures of sesquiterpenes[17,18] and monoterpenes.[10] In the latter instance, a monoterpene cyclase from common sage (*Salvia officinalis*) that produces (-)-α-pinene, (-)-β-pinene, (-)-camphene and other olefins[19] was examined with [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate, and it was demonstrated that, under saturating substrate conditions, both the relative proportion and absolute rate of (-)-α-pinene formation were enhanced at the expense of (-)-β-pinene formation due to an induced KIE.[10] This observation of isotopically sensitive branching unambiguously established that both pinene products were generated from the same intermediate by a single cyclase, and permitted calculation of an intrinsic KIE for C10-methyl deprotonation by this enzyme of 2.4, a value quite similar to that of 2.1 determined via natural abundance ^{2}H-NMR analysis of samples of (-)-α-pinene and (-)-β-pinene derived from pine turpentine.[20]

In this paper, we expand these studies to cyclases of conifer species, employing both [10-$^2H_3$,1-^{3}H]- and [4-$^2H_2$,1-^{3}H]-labeled geranyl pyrophosphates as substrates. Evidence based on the deuterium isotope effects observed indicates that a single cyclase from grand fir catalyzes the formation of both (-)-α- and (-)-β-pinene, and that a single cyclase from lodgepole pine catalyzes the formation of both (-)-α- and (-)-β-phellandrene. Additionally, the intrinsic primary kinetic isotope effects associated with the various deprotonations leading to



Scheme 1. Proposed reaction mechanisms for the formation of pinene and phellandrene isomers.

the olefinic products are described, and the implications of deuterium isotope effects are discussed with regard to transition state geometries in these multistep cyclization reactions.

RESULTS AND DISCUSSION

Isotopically sensitive branching describes the rate enhancement in the formation of one product of a multiple product enzyme caused by a reduction of the rate constant for the formation of a second product due to isotopic substitution in the substrate.[13] Such an observation indicates that the two products arise from a common intermediate formed by the same enzyme. For the monoterpene cyclase reaction, a simplified kinetic model for the generation of two products can be described as,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES\,I \xrightarrow{k_2} ES\,II \begin{cases} \xrightarrow{k_3} E + P_1 \\ \xrightarrow{k_4} E + P_2 \end{cases}$$

where **ESI** is the enzyme-substrate complex, k_2 is taken as the rate constant for the irreversible ionization-isomerization-ionization-cyclization sequence leading to the branch-point intermediate **ESII** (either the pinyl cation or the phellandryl cation), and k_3 and k_4 are the rate constants for the terminating deprotonations (and the dissociation steps) leading to the isomeric olefin products, $\mathbf{P_1}$ and $\mathbf{P_2}$. Therefore, the steady state rate equation for the formation of $\mathbf{P_1}$ is

$$V_1 = k_3[\mathbf{ES\,II}] = \frac{k_3}{k_3 + k_4} \cdot \frac{k_2\,[E_0]}{1 + \dfrac{k_2}{k_3 + k_4} + \dfrac{k_{-1} + k_2}{k_1\,[S]}} = \rho \cdot V_{TOTAL} \qquad (1)$$

where V_{total} is the total velocity of the reaction (the sum of the rates of formation of **P1** and **P2**; that is V_1 and V_2), and ρ is the proportionality constant $k_3/(k_3+k_4)$. Thus, isotopically sensitive branching arises from an increase in the concentration of **ESII** due to a decrease in k_4, so that k_3[**ESII**], and thus V_1, is enhanced. It is this rate enhancement, that would not occur if the two products were formed by different enzymes, which is essential in determining whether the two products observed arise from the same enzyme. However, evaluation of the rate equation for the branching sequence reveals that the ability of [**ESII**] (and thus V_1) to increase with a reduction in k_4 depends upon both the substrate concentration and the relative size of k_2 compared to k_3+k_4. Changes in k_4 in the steady-state rate equation, due to isotopic substitution, can affect V_1 by influencing both ρ and V_{total}. While reduction in k_4 increases ρ simply by increasing the relative size if k_3 to k_3+k_4, this reduction in k_4 can also reduce V_{total}, thereby off-setting the increase in ρ so that no enhancement of V_1 is observed. However, the reduction of V_{total} with a reduction in k_4 depends upon [**S**] and the relative size of k_2 to k_3+k_4, such that if [**S**] is subsaturating, or if $k_2<(k_3+k_4)$, the reduction of k_4 will have little influence on

V_{total}, and branching will be observed. This analysis also underscores the importance of overall velocity on isotopically sensitive branching; any reduction in velocity due to binding or secondary isotope effects may greatly influence the ability to detect branching.

The dependence of isotopically sensitive branching on subsaturating [S], or on the presence of a rate-limiting step prior to the branch-point, develops from the requirement for [ESII] to increase with a decrease in k_4. [ESII] can increase at the expense of either [ESI] or [E], and it is the substrate concentration and $k_2/(k_3+k_4)$ that determines whether enzyme is available in these pools to allow an increase in [ESII]. When $[S]<<K_m$, binding equilibrium favors dissociation of [ESI] and the concentration of free enzyme is sufficiently large that enzyme can be shifted from [E] to increase [ESII]. Similarly, when $k_2<<(k_3+k_4)$ (i.e., k_2 is rate-limiting), a large pool of enzyme exists as ESI and enzyme can be shifted from [ESI] to increase [ESII]. However, when [S] is saturating and k_2 is fast, nearly all the enzyme in solution exists as ESII, so that a decrease in k_4 cannot further increase [ESII] because [ESII] is essentially at maximum. In this case, reduction in k_4 results in a drop in overall velocity equal to the increase in $k_3/(k_3+k_4)$, and branching cannot be observed. Previous studies with monoterpene cyclases have shown that the initial ionization-isomerization of geranyl pyrophosphate is rate-limiting,[2,21,22] so that branching experiments can be performed at saturating levels of substrate without significant reductions in overall rate due to these effects.

For the monoterpene cyclases at focus here, preliminary purification was carried out by three chromatographic steps and electrophoresis. For the enzyme from grand fir, the activities for the cyclization to α-pinene and β-pinene copurified without alteration in product distribution, as did the activities for the cyclization to α-phellandrene and β-phellandrene by the enzyme preparation from lodgepole pine. Moreover, chromatographic resolution of these biosynthetic products on a β-cyclodextrin column indicated that each pair of positional isomers was of the same absolute configuration ((-)-α-[1*S*:5*S*]-pinene with (-)-β-[1*S*:5*S*]-pinene, and (-)-α-[4*R*]-phellandrene with (-)-β-[4*R*]-phellandrene). The copurification of cyclization activities to isomers of the same antipodal series suggested the presence of a multiproduct pinene cyclase (grand fir) and a multiproduct phellandrene cyclase (lodgepole pine), each of which operates by alternative deprotonations at C4 or C10 of the geranyl substrate (Scheme 1). To test this possibility, isotopically sensitive branching experiments were carried out with each enzyme by using three differently labeled substrates as probes; [1-^{3}H]-, [4-$^2H_2$,1-^{3}H]; and [10-$^2H_3$,1-^{3}H]-geranyl pyrophosphate. If branching via a common intermediate does occur, then incubation of the pinene and phellandrene cyclases with the C10-perdeuteriomethyl substrate would be expected to increase the rate of formation of the α-isomers resulting from the alternate C4-deprotonation, whereas incubation with the C4-dideuteriomethylene-labeled substrate would be expected to promote formation of the β-isomers derived by the C10-deprotonation.

Overall reaction rates with each substrate were determined by isolation of the olefin fraction (generated under linear conditions) followed by radio-GLC analysis of this material to determine product composition, from which rates for each enzymatic product were calculated. The shifts in product distribution observed with each substrate probe were dramatic, as illustrated in Figure 1 for the pinene cyclase. In the case of the pinene cyclase, both the percentage composition of the product mixture and the absolute rate of formation of (-)-α-pinene increased relative to that of the control substrate when incubated with [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate, whereas both the percentage composition and the absolute rate of formation of (-)-β-pinene increased relative to that of the control substrate when the enzyme was incubated with [4-$^2H_2$,1-^{3}H]geranyl pyrophosphate (Table 1). The summation of these results clearly indicates that both (-)-α-pinene and (-)-β-pinene arise from the same intermediate (the pinyl cation) and are produced by a single cyclization enzyme.

Very similar branching was observed when the phellandrene cyclase from *Pinus contorta* was incubated with the deuterated substrate probes (Table 1), although the lopsided product distribution generated by this enzyme, and the overall rate reductions noted, limited the isotopically-induced rate enhancements.

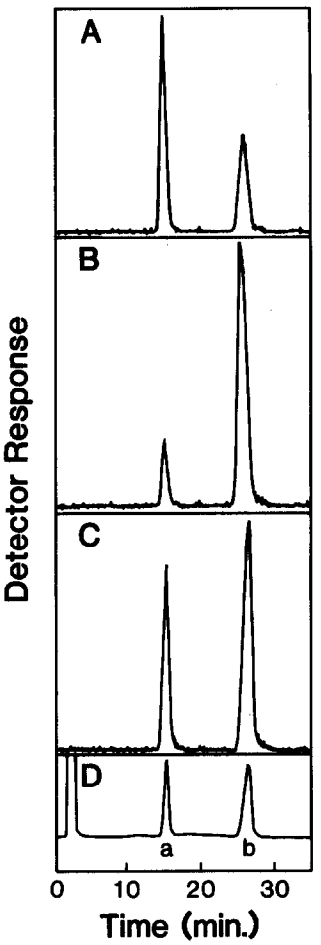


Figure 1. Radio-GLC of the α-pinene (a) and β-pinene (b) mixtures derived from [10-$^2H_3$,1-^3H](A), [4-$^2H_2$,1-^3H](B) and [1-^3H](C) labeled geranyl pyrophosphate. Panel (D) illustrates the elution pattern of authentic α-pinene (a) and β-pinene (b).

The percentage composition and absolute rate of formation of (-)-α-phellandrene increased when the enzyme was incubated with [10-$^2H_3$,1-^3H]geranyl pyrophosphate, thus demonstrating that both phellandrene isomers were generated from a common phellandryl cation by this cyclase; however, only the percentage composition, but not the absolute rate of formation, of (-)-β-phellandrene increased with [4-$^2H_2$,1-^3H]geranyl pyrophosphate as substrate. The lack of rate enhancement in the case of (-)-β-phellandrene with the C4-deuterated substrate can be attributed both to the large overall rate reduction noted in this instance, as well as to the analytical difficulty of detecting a small absolute velocity increase for the major product (β-phellandrene)

resulting from a small absolute (but clearly significant) decrease in the rate of formation of the minor product (α-phellandrene).

Overall rate reductions (relative to the control) occurred with the deuterated substrates in all cases (Table 1), and were most pronounced for the phellandrene cyclase and for the C4-deuterated substrate. Thus, with [4-$^2H_2$,1-^{3}H]geranyl pyrophosphate, the total rate reduction for the phellandrene cyclase was 45%, and for the pinene cyclase was 15%. With [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate, the overall rate reduction was 18% for the phellandrene cyclase and 7% for the pinene cyclase. Because the ability to detect isotopically sensitive branching depends greatly upon total reaction rate, the source of these rate reductions was considered in detail. Such rate reductions with the deuterated precursors could arise from primary isotope effects, secondary isotope effects, or differences in binding affinities of the cyclases for the differently labeled substrates. Differences in binding affinities are unlikely to represent a significant source of rate reductions since the reactions were performed with substrate concentrations that were 4- to 5-times higher than the K_m for [1-^{3}H]geranyl pyrophosphate. Thus, the K_m values for the deuterated substrates could vary by as much as 25% relative to the control substrate (this seems extremely unlikely) without significant influence on overall velocity.

Table 1. Percentage Composition and Absolute Velocities of Product Formation by Pinene Cyclase (Grand Fir) and Phellandrene Cyclase (Lodgepole Pine) with Deuterium Labeled Geranyl Substrates.

Enzyme and Products	Geranyl Pyrophosphate Substrates					
	[1-^{3}H]		[4-$^2H_2$,1-^{3}H]		[10-$^2H_3$,1-^{3}H]	
	%	V[1]	%	V[1]	%	V[1]
Pinene Cyclase [2]						
(-)-α-pinene	35	816	14	287	57	1272
(-)-β-pinene	65	1526	86	1702	41	900
Total	100	2342	100	1989	100	2172
Phellandrene Cyclase [2]						
(-)-α-phellandrene	6	74	1	10	18	188
(-)-β-phellandrene	94	1220	99	699	82	871
Total	100	1294	100	709	100	1059

[1] V is given in pmol/h, and represents an average of 3 or 4 independent determinations with standard error < 4%.

[2] Each enzyme preparation produced minor amounts of other products including myrcene, limonene and terpinolene.

To determine if the rate reductions may be due to primary isotope effects (i.e., that the deprotonation step itself could be rate-limiting), both deuterium-labeled substrates were examined with other cyclases that do not involve deprotonation at either C4 or C10. These were the (-)-limonene cyclase from *Mentha piperita* (that involves deprotonation at C9 of the α-terpinyl cation) and a terpinolene cyclase preparation from lodgepole pine (that involves deprotonation at C6 of the α-terpinyl cation).[12] The latter activity is distinct from the phellandrene cyclase and also produces (-)-sabinene (C10-deprotonation); however, preliminary experiments established the lack of isotopically sensitive branching for these products (either they are synthesized by different enzymes or, as likely, they do not share a common branch-point intermediate). With the limonene cyclase, the rate suppression with [4-$^2H_2$, 1-^{3}H]geranyl pyrophosphate was 28%, while with [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate the rate suppression was negligible. For terpinolene cyclization with [4-$^2H_2$, 1-^{3}H]geranyl pyrophosphate, the rate suppression observed was 22%, and with [10-$^2H_3$, 1-^{3}H]geranyl pyrophosphate the rate suppression was again insignificant. In the case of (-)-sabinene cyclization, the rate suppression with the C4-perdeuterio substrate (15%) exceeded that with the C10-perdeuterio substrate (9%) for which the primary isotope effect might be anticipated. These results clearly suggest, at least in the case of [4-$^2H_2$,1-^{3}H]geranyl pyrophosphate, that the overall rate reductions noted were not due to primary isotope effects on the terminating deprotonations, but rather were the result of secondary isotope effects, almost certainly upon the initial, rate limiting ionization step of the reaction (Scheme 1).[2] This proposal is supported by previous enzymatic rate suppression studies with [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate[10] and by model studies with [4-$^2H_2$,10-$^2H_3$]geranyl chloride[23] in which solvolytic rate suppressions were observed.

While a detailed description of secondary kinetic isotope effects is complex, and should take into account possible inductive, steric, anchimeric and solvent influences, it is hyperconjugation that appears to be of major significance in most cases.[24] The precise magnitude of β-deuterium isotope effects will reflect the angular dependence of C-H bond overlap with incipient β-cations[25] as well as transition state electron demand.[24,26] Moreover, in the solvolyses of tertiary alkyl chlorides it has been found that methyl substitution on the carbon carrying the deuterium atom(s) increases the isotope effect;[27] thus, the substituent influence on β-deuterium isotope effects behaves as expected for hyperconjugative electron release.[28] If the rate suppressions observed are due to β-secondary isotope effects, as they appear to be, then the magnitude of suppression is a reflection of C-H bond weakening due to electron demand during the rate-determining ionization step and, as such, proper analysis of the data may afford insight into the relative contribution of C4 and C10 C-H bond hyperconjugative stabilization of the incipient allylic cation.

Data from solvolyses of geranyl pyrophosphate and linalyl pyrophosphate support differential stabilization of the incipient allylic cations by the C4 and C10 C-H bonds.[29] In the olefin fractions obtained in these reactions, the level of ocimenes (via C4-deprotonation) considerably exceeds the level of myrcene (via C10-deprotonation). This observation suggests that, in the generation of the corresponding allylic cations, the C4 C-H bond is weakened by hyperconjugative electron donation more so than the C10 C-H bond and is, therefore, more likely to undergo deprotonation (to ocimenes). Such preferential weakening of the C4 C-H bond is consistent with established substituent effects on hyperconjugation.[27,28]

Direct evidence for differential hyperconjugative stabilization of the initial cation formed by the pinene and phellandrene cyclases may be obtained by analyzing the rate suppressions observed with the respective C4 and C10 deuterated substrates. Dividing the total rate reductions by the number of deuterium atoms reveals that for the pinene cyclase there is a 7% rate reduction per ^{2}H atom at the C4 position, and only a 2.3% rate reduction per ^{2}H atom at the C10 position. Likewise, the rate reduction for the phellandrene cyclase is 23% per ^{2}H atom at the C4 position, and 7% per ^{2}H atom at the C10 position. β-Deuterium isotope effects in S_N1 solvolyses are on the order of 5 to 15% per deuterium atom.[28] However, for optimum conformational influences, β-effects as high as 31% per ^{2}H atom may be expected.[30]

The greater rate reduction per deuterium atom at C4 is consistent with the predicted substituent effect and, for both cyclases, the rate suppression per ^2H atom at C4 is about three times that at C10. The enzyme may attenuate the substituent effect by conformational folding of the substrate to increase the overlap of the carbon-hydrogen bond (at either the C4 or C10 position) and the *p*-orbital of the cation, thereby decreasing electron demand on the other site. Although the conformational and substituent effects cannot be evaluated separately in these experiments, the substituent effects would be expected to be the same between these enzymes since the same substrates are used for both. Because of the large conformational dependence of β-deuterium isotope effects in model studies,[24] the three-fold difference in rate reductions per ^2H atom at the C4 and C10 positions for both the pinene and phellandrene cyclases suggests a similar transition state geometry for both enzymes. The overall rate reductions observed with the phellandrene cyclase are about three times those of the pinene cyclase with both deuterated substrates. This observation suggests a relatively greater overall demand for hyperconjugative electron donation to the incipient allylic cation in the phellandrene cyclase case.

Isotopically sensitive branching is also a powerful tool for determining the magnitude of intrinsic isotope effects for terminal deprotonations in enzyme-mediated catalysis.[14] Historically, determination of intrinsic isotope effects in single-product enzymatic reactions has been hampered by the presence of preceding rate-limiting steps, so that V_H/V_D is a poor measure of k_H/k_D for a single subsequent deprotonation event.[31] In isotopically sensitive branching experiments, the kinetics of the terminating deprotonation can be separated from the kinetics of the earlier steps in the reaction pathway by relating the velocity for the formation of one product to the velocity for the formation of the other product. The ratios of the velocities are equivalent to the ratios of the corresponding rate constants because both products experience the same concentration of the branch point intermediate. Thus,

$$\frac{V_2}{V_1} = \frac{k_4[\text{ES II}]}{k_3[\text{ES II}]} = \frac{k_4}{k_3} \tag{2}$$

The kinetic isotope effect for the production of $\mathbf{P_2}$, as k_{4H}/k_{4D}, can then be calculated from the relative velocities of the reactions with the deuterated substrate and control (for the inhibition of V_2) because for the deuterium substitution that influences k_4, k_{3H} equals k_{3D}. Thus,

$$\frac{k_{4H}}{k_{4D}} = \frac{k_{4H}}{k_{3H}} \cdot \frac{k_{3D}}{k_{4D}} \tag{3}$$

Using this approach, the kinetic isotope effects for the deprotonations leading to (-)-α-pinene and (-)-β-pinene were calculated as 3.0 and 2.6, respectively, and the kinetic isotope effects for the deprotonations leading to (-)-α-phellandrene and (-)-β-phellandrene were determined as 4.3 and 3.7, respectively. These values are only slightly higher than k_H/k_D = 2.4 for the deprotonation leading to (-)-β-pinene calculated from similar isotopically sensitive branching experiments with the cyclase enzyme from sage,[10] and the k_H/k_D of 2.1 for the generation of β-pinene in *Pinus* determined from natural abundance ^2H-NMR,[20] but all are considerably lower than the theoretical maximum k_H/k_D of 7 for C-H bond breaking.[32] Nevertheless, primary kinetic isotope

effects in the range of 2-5 are not uncommon in model (non-enzymatic) deprotonations where an asymmetric transition state exists between the proton donor and acceptor.[33] From the Hammond postulate, it seems likely that the transition state for the deprotonation in the cyclase reaction more closely resembles the high-energy cationic intermediate than the deprotonated olefin product; that is, at the transition state, the hydrogen atom is closer to the donating carbon than to the receiving base. This asymmetry would lead to lowered primary kinetic isotope effects due to participation of the hydrogen (deuterium) in a vibrational mode at the transition state.[32] Any stabilization (such as by resonance in the phellandryl cation) should lead to a more symmetric transition state and a greater primary KIE. This may explain the observation that the primary kinetic isotope effects at both the C4 and C10 positions for the phellandrene cyclase are 40% higher than those for the pinene cyclase.

EXPERIMENTAL

Substrates. [1-^{3}H]Geranyl pyrophosphate (140 Ci/mol) and [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate (99+ atom %, 25 Ci/mol) were prepared as previously described.[10,34] [4-$^2H_2$,1-^{3}H]Geranyl pyrophosphate (98+ atom %, 140 Ci/mol) was prepared from [1-$^2H_2$]-4-methyl-3-penten-1-ol, via 6-methyl-5-hepten-2-one and methyl geranoate, by a procedure to be described in detail elsewhere.

Enzyme isolation and assay. Enzyme isolation from stem tissue of conifer saplings has been described.[3] The crude enzyme extracts were fractionated by step gradient elution from *O*-diethylaminoethyl-cellulose (Whatman DE-52) and hydroxyapatite (Bio-Rad laboratories), and then separated into six or more distinct cyclase activities by linear gradient elution from a quaternary ammonium anion-exchange column (Mono Q 10/10, Pharmacia FPLC). All cyclase assays were run in triplicate using standard procedures to ensure linearity.[1] The reaction was initiated by addition of 25 μM geranyl pyrophosphate (4 x K_m) and the aqueous mixture was overlayed with pentane (1:1, v/v) to trap the volatile olefinic products. Following incubation for 2 h at 30°C, the solution was chilled and mixed thoroughly, and the phases separated by centrifugation. The pentane layer and an additional pentane extract were then passed through a short column of silica gel overlaid with $MgSO_4$ to afford the olefin fraction. Total radioactivity in this fraction was determined by aliquot counting, and product distribution was determined by radio-GLC following the addition of authentic carriers.[1,35]

Product stereochemistry. For determination of product stereochemistry, preparative-scale incubations were carried out and the pentane extracts from each enzyme preparation were steam distilled (J&W Scientific, 320-1000 simultaneous steam distillation-solvent extraction apparatus), and the distillate passed through silica gel as before. The product mixture was then separated by capillary GLC on a β-cyclodextrin column (J&W Scientific Cyclodex-B, 0.25 mm x 30 m, 70°C isothermal, 10 psi H_2), and the identification of enantiomers established by comparison of retention times with those of authentic standards (the identity of the pinenes and phellandrene products had been previously determined by combined GLC-MS on a normal phase column). Authentic pinene standards were obtained as previously described,[36] and the (+)- and (-)-phellandrene standards (α- and β-isomers) were prepared from parsley oil and lodgepole pine turpentine, respectively.

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REFERENCES

1. Croteau, R.; Cane, D.E. *Methods Enzymol.* **1985**, *110*, 383-405.
2. Croteau, R. *Chem. Rev.* **1987**, *87*, 929-954.
3. Lewinsohn, E.; Gijzen, M.; Savage, T.J.; Croteau, R. *Plant Physiol.* **1991**, *95*, in press.
4. Croteau, R.; Johnson, M.A. In *Biosynthesis and Biodegradation of Wood Components*; Higuchi, T., Ed.; Academic Press: New York, **1985**, 379-439.
5. Johnson, M.A.; Croteau, R. In *Ecology and Metabolism of Plant Lipids*; Fuller, G., Nes, W.D., Eds., ACS Symp. Ser. 325: Washington, D.C., **1987**, 76-82.
6. Cane. D.E. *Chem. Rev.* **1990**, *90*, 1089-1103.
7. West, C.A. In *Biosynthesis of Isoprenoid Compounds*; Porter, J.W., Spurgeon, S.L., Eds.; John Wiley: New York, **1981**, 375-411.
8. Munck, S.L.; Croteau, R. *Arch. Biochem. Biophys.* **1990**, *282*, 58-64.
9. Alonso, W.R.; Croteau, R. *Arch. Biochem. Biophys.* **1991**, *286*, 511-517.
10. Croteau, R.B.; Wheeler, C.J.; Cane, D.E.; Ebert, R.; Ha,H.-J. *Biochemistry* **1987**, *26*, 5383-5389.
11. Gijzen, M.;Lewinsohn, E.; Croteau, R. *Arch. Biochem. Biophys.* **1991**, *287*, in press.
12. Detailed characterization of these enzymes by T.J. Savage, J.I. Rajaonarivony and R. Croteau is unpublished. These enzymes have been briefly described in ref. 3 and Kjonaas, R.; Croteau, R. *Arch. Biochem. Biophys.* **1983**, *220*, 79-89.
13. Samuelson, A.G.; Carpenter, B.K. *J. Chem. Soc., Chem. Commun.* **1981**, 354-356.
14. Jones, J.P,; Korzekwa, K.R.; Rettie, A.E.; Trager, W.F. *J. Am. Chem. Soc.* **1986**, *108*, 7074-7078.
15. Harada, N.; Miwa, G.T.; Walsh, J.S.; Lu, A.Y.H. *J. Biol. Chem.* **1984**, *259*, 3005-3010.
16. Brenner, D.G.; Knowles, J.R.; Rihs, G. *Biochemistry* **1981**, *20*, 3680-3687.
17. Arigoni, D. *Pure Appl. Chem.* **1975**, *41*, 219-245.
18. Dorn, F.; Bernasconi, P.; Arigoni, D. *Chimia* **1975**, *29*, 24-25.
19. Gambliel, H.; Croteau, R. *J. Biol. Chem.* **1984**, *259*, 740-748.
20. Pascal, R.A., Jr.; Baum, M.W.; Wagner, C.K.; Rodgers, L.R.; Huang, D.-S. *J. Am. Chem. Soc.* **1986**, *108*, 6477-6482.
21. Satterwhite, D.M.; Wheeler, C.J.; Croteau, R. *J. Biol. Chem.* **1985**, *260*, 13901-13908.
22. Croteau, R. *Arch. Biochem. Biophys.* **1986**, *251*, 777-782.
23. Bunton, C.A.; Leresche, J.-P.; Hachey, D. *Tetrahedron Lett.*, **1972**, 2431-2434.
24. Shiner, V.J., Jr. *ACS Monogr.* **1970**, *167*, Cpt. 2. See also Sunko, D.E.; Borcic, S., *ibid* **1970**, 167, Cpt. 3.
25. Sunko, D.E.; Szele, I.; Hehre, W.J. *J. Am. Chem. Soc.* **1977**, *99*, 5000-5005.
26. Liu, K.-T.; Wu, Y.W. *Tetrahedron Lett.* **1986**, 3623-3626.
27. Shiner, V.J., Jr. *Tetrahedron* **1959**, 243-252.
28. Melander, L.; Saunders, W.H. In *Reaction Rates of Isotopic Molecules*; John Wiley: New York, **1980**, Cpt. 6.
29. Cramer, F.; Rittersdorf, W. *Tetrahedron* **1967**, 3015-3022.
30. Shiner, V.J., Jr.; Humphrey, J.S., Jr. *J. Am. Chem. Soc.* **1963**, *85*, 2416-2419.
31. Northrop, D.B. *Biochemistry* **1975**, *14*, 2644-2651.
32. Westheimer, F.H. *Chem. Rev.* **1961**, *61*, 265-273.
33. Knesge, A.J. In *Isotope Effects on Enzyme-Catalyzed Reactions*; Cleland, W.W.; O'Leary, M.H.; Northrop, D.E., Eds.; University Press: Baltimore, **1977**, 37-63.
34. Croteau, R.; Karp, F. *Arch. Biochem. Biophys.* **1976**, *176*, 734-746.

35. Satterwhite, D.M.; Croteau, R. *J. Chromatogr.* **1988**, *452*, 61-73.
36. Satterwhite, D.M.; Croteau, R. *J. Chromatogr.* **1987**, *407*, 243-252.