Regiospecificity and Isotope Effects Associated with the Methyl–Methylene Eliminations in the Enzyme-Catalyzed Biosynthesis of (*R*)- and (*S*)-Limonene

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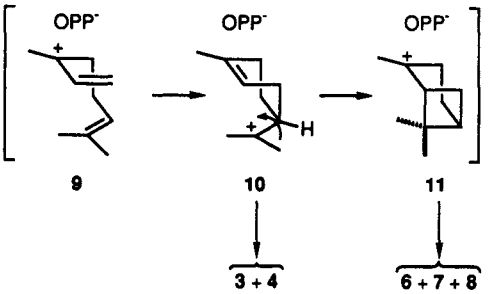
Received January 8, 1993 (Revised Manuscript Received April 26, 1993)

[8-^{3}H]-, [8,8,9,9-$^2H_4$]-, and [1-^{3}H,8,9-$^2H_6$]geranyl diphosphates (1-*t*, 1-d_4, and 1-d_6,*t*) were synthesized and used as substrates for several monoterpene cyclases to determine the regiospecificity and isotope effects attending the terminating proton transfers in the enzyme-catalyzed biosynthesis of (*R*)- and (*S*)-limonene. Degradation of enantiomeric [^{3}H]limonenes produced by cyclization of 1-*t* with the (+)- and (−)-pinene cyclases (synthases) from *Salvia officinalis* demonstrated that the eliminations occur at both the *cis*- (55–65%) and *trans*-methyl (45–35%) groups. In contrast, the terminating eliminations in the formation of (+)- and (−)-limonene catalyzed by limonene cyclases from *Citrus sinensis* and *Perilla frutescens*, respectively, were shown by degradation to occur exclusively (≥97–98%) at the cis terminal methyl group. The intramolecular isotope effects for the methyl–methylene elimination in limonene biosynthesis catalyzed by (+)- and (−)-pinene cyclases from *S. officinalis* were found to be k_H/k_D = 2.3 ± 0.2 and 5.9 ± 0.5, respectively, by GC/MS determinations of [^{2}H]-limonene derived from enzymatic cyclizations of 1-d_4. Similar experiments with (−)-limonene cyclase from *Mentha spicata* resulted in k_H/k_D = 4.0 ± 0.4. Incubations of 1-d_6,*t* with pinene and bornyl PP cyclases from *S. officinalis* exhibited significant remote isotope effects (k_H/k_D = 1.16–1.27) on the total rate of monoterpene formation which suggest that the initial cyclization step of the enzyme-bound linalyl diphosphate intermediate is an important component of the overall rate of the enzymatic reactions. The isotope effects on the partitioning of the α-terpinyl carbocation intermediate between bicyclization and elimination to limonene were determined from the effects of deuterium substitution on the product ratios derived from enzymatic cyclization of 1-d_6,*t*. The small size of these product isotope effects (k_H/k_D = 1.2–1.7) is attributed to a conformational inversion of the α-terpinyl ion to a half-chair conformer prior to proton elimination to limonene, thereby rendering the bicyclizations relatively immune to the intrinsic deuterium isotope effect. The regiospecific proton transfers from the cis terminal methyl group effected by the limonene cyclases from *Citrus* and *Perilla* are attributed to the minimization of charge separation in the transition state.

The widely distributed monoterpene limonene (**3**)[1] is a common precursor to the *p*-menthane family of plant natural products.[2,3] In spite of the important role of limonene in monoterpene biosynthesis, the mechanism and stereospecificity of the enzyme-catalyzed cyclization of geranyl diphosphate (GPP = **1**) to limonene has remained uncertain. Evidence has accumulated that all regular monoterpene cyclases thus far studied (e.g., (−)-pinene cyclase, Scheme I) first isomerize **1** to the transient, enzyme-bound intermediate (*R*)- or (*S*)-linalyl diphosphate (LPP = **2**) which undergoes S_N' cyclizations from the cisoid conformation to produce mono- and bicyclic products such as limonene (**3**), terpinolene (**4**), α- and β-pinenes (**6** and **7**), and camphene (**8**) as well as direct elimination to form small amounts of myrcene (**5**) and *cis*- and *trans*-ocimenes (3,4-double bond isomers of **5**).[3]

Previous labeling experiments concerning the stereochemistry of pinene biosynthesis are consistent with the cyclization mechanism shown in eq 1.[3-7] Thus, the S_N'



(1)

cyclization of **2** is presumed to occur from an anti,endo conformation via **9** producing a transient α-terpinyl carbocation intermediate **10** which undergoes closure of the second ring with a 30° rotation, so that the trans methyl group at the chain terminus becomes the exo methyl on the *gem* dimethyl bridge (eq 1).[7] Although it is reasonable to suppose that limonene is formed by proton elimination from **10**, there does not seem to be any way to predict the regiospecificity of this proton transfer.

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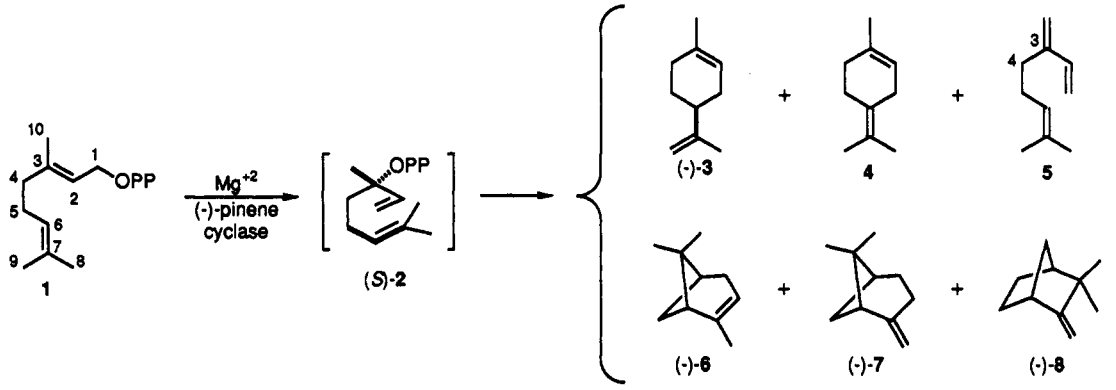
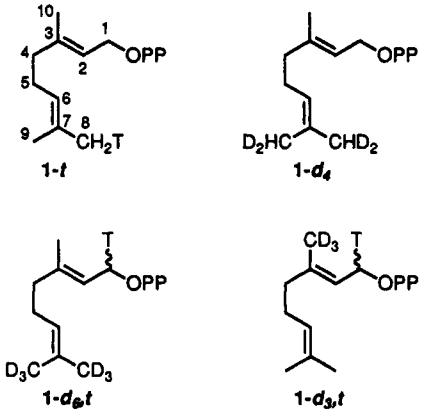
Scheme I



Previous attempts to elucidate the regiospecificity of the terminating methyl–methylene elimination in monoterpene biosynthesis by plant feeding experiments gave inconclusive results owing to low incorporations and a lack of enantiomeric purity of the isolated compounds.[8] More recently, natural abundance ^2H NMR analysis of (+)-limonene from Florida navel oranges indicated that proton elimination occurs at the *cis*-methyl group.[9] The label randomization between the terminal methyl groups frequently observed in investigations of mono- and sesquiterpene biosynthesis in many cases can probably be attributed to secondary transformations occurring subsequent to the initial cyclization.[10,11] However, stereospecific hydride transfers and eliminations forming isopropyl and isopropenyl groups take place in the biosynthesis of the sesquiterpenes avocettin,[12] capsidiol,[13] germacrene-2,3-diol,[14] and aristolochene,[15] and the triterpenes betulonic acid and lupeol.[16]

One objective of this collaborative research was to elucidate the regiospecificity of the terminating eliminations in the biosynthesis of (*R*)- and (*S*)-limonene by means of tritium labeling.[17] Another aim was to assess the effects of deuterium substitution at the distal methyl groups of **1** where the elimination reaction takes place. If the α-terpinyl carbocation **10** undergoes competitive elimination to limonene and cyclization to the pinenes and camphene at a common active site of the cyclases, a primary isotope effect should be observed on the partitioning of this intermediate between monocyclic and bicyclic products. "Isotopically sensitive branching"[18] has been reported previously in monoterpene[19,20] and sesquiterpene[12,21] biosynthesis.



Labeled Substrates and Enzymes. The labeled derivatives of geranyl diphosphate, **1**-*t*, **1**-d_4, and **1**-d_6,*t*, were employed in the present investigation. Substrate bearing tritium in the trans methyl group (**1**-*t*) was prepared as described previously.[7b] Geraniol analogs **15a** (5% d_2, 21% d_3, 67% d_4, 7% d_5) and **15b** (3% d_5, 95% d_6) deuterated in the terminal methyl groups were synthesized by Wittig reactions between aldehyde **12**[22] and deuterium-labeled ylides **13a** and **13b** followed by debenzylation (eq 2). Geraniol-d_6 was also labeled with tritium at C-1 in order to measure kinetic isotope effects on the rates of [^3H]monoterpene formation. The deuterated geraniols were converted to diphosphates by the Cramer procedure ($(Et_3NH)_2HPO_4$, CCl_3CN, CH_3CN).[23] Substrate labeled at the C-3 methyl group (**1**-d_3,*t*) was available from a

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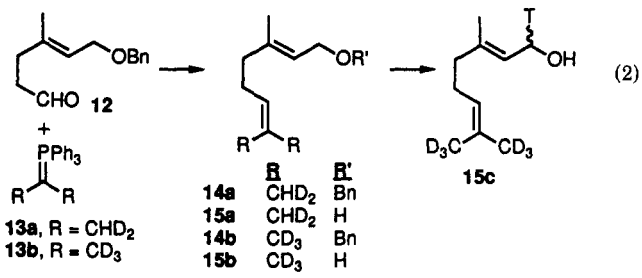

(2)

previous investigation.[19] The specific activity of each tritium-labeled diphosphate ester was verified by radiochromatographic analysis of the corresponding alcohol.

(+)-Pinene cyclase (synthase) from sage (*Salvia officinalis*) leaves catalyzes the conversion of **1** to (+)-**3**, **4**, **5**, (+)-**6**, and (+)-**8**, whereas the (−)-pinene cyclase (synthase) from this tissue transforms the acyclic precursor to (−)-**3**, **4**, **5**, (−)-**6**, (−)-**7**, and (−)-**8** (Scheme I). On the basis of copurification of activities, kinetic properties, and differential inhibition and inactivation studies,[5,20] each set of enantiomerically related olefins is considered to be synthesized at a common site of a single enzyme; however, the production of these multiple products by two or more very similar cyclases, or at two or more very similar sites on the same cyclase, cannot be rigorously excluded. These two pinene cyclase preparations were isolated from sage leaves and assayed according to previously described methods.[4] A soluble limonene cyclase preparation was obtained from Valencia oranges (*Citrus sinensis*) by extraction, ultracentrifugation, and concentration. The conversion of **1**-*t* to limonene as the sole radioactive product by this preparation was demonstrated by radio-GC analysis.[24] That Valencia oranges produce enantiomerically pure (*R*)-(+)-limonene was confirmed by GC analysis on a chiral column of the carvoxime derived from the limonene isolated from the oil.[25] Likewise, *Perilla frutescens* leaves were shown to produce enantiomerically pure (*S*)-(−)-limonene. The partially purified cyclase obtained from the leaves of *Perilla*, by methods previously described for sage and tansy,[26] produces limonene as the sole radioactive product from **1**-*t*, as determined by radio-GC analysis as before.[24] The purification to homogeneity and the characterization of (*S*)-(−)-limonene cyclase from *Mentha spicata* and *Mentha piperita* have been described.[27,28]

Enzymatic Biosynthesis and Chemical Degradation of [^{3}H]Limonene (Scheme II). Enzyme-catalyzed cyclizations of **1** bearing tritium in the trans terminal methyl group (**1**-*t*) to [^{3}H]monoterpenes by the pinene and limonene cyclases were conducted according to previously described procedures.[4] [^{3}H]Limonene produced by the pinene cyclases was separated from the other monoterpenes by preparative TLC on silver nitrate-impregnated silica gel after dilution with the appropriate limonene enantiomer. The diluted (+)- and (−)-[^{3}H]-limonenes, radiochemically pure according to radio-GC[24,25] and TLC, were converted to (−)- and (+)-[^{3}H]carvoximes (e.g., **16**-*t*) which were purified by preparative TLC and partially crystallized. The conversion of limonene to carvoxime via the nitrosyl chloride adduct is known to be stereospecific.[25,29]

The position of the tritium label in the [^{3}H]carvoxime samples was determined by chemical degradation (Scheme II). The reactions in the degradation were optimized in trials with unlabeled compounds, and the purity of all intermediates was established by chromatographic analysis and spectroscopic comparisons. Hydrolysis of the [^{3}H]-carvoximes to [^{3}H]carvone (75–85%)[29] followed by conjugate reduction with K-Selectride (52–65%)[30] and methyllithium addition (77–86%) afforded tertiary alcohol **19**-*t*. Ozonolysis of the isopropenyl group[31] yielded formaldehyde, isolated as the dimedone derivative (**20**-*t*), and [^{3}H]ketol **21**-*t*, which was immediately converted to the crystalline diol **22**-*t* by reaction with methyllithium. A separate portion of **20**-*t* was subjected to base-catalyzed exchange (K_2CO_3, MeOH) prior to reaction with methyllithium.

The specific radioactivities of [^{3}H]carvoxime (**16**-*t*), tertiary alcohol **19**-*t*, and the ozonolysis products **20**-*t* and **22**-*t* were measured. All samples, except **19**-*t* (oil), were recrystallized repeatedly until the specific activity was constant (Table I). The location of the tritium label in the methyl group of **21**-*t* was confirmed by the complete loss of radioactivity (97–99%, data not shown) from samples that had been subjected to the base-catalyzed exchange.

The data in Table I demonstrate conclusively that the methyl–methylene elimination in limonene formation catalyzed by the two pinene cyclases from sage occurs competitively at both the cis (55–65%) and trans (45–35%) methyl groups. In contrast, regiospecific proton transfer from the cis methyl group (97–98%) occurs in the biosynthesis of (*R*)- and (*S*)-limonene catalyzed by the cyclase enzymes from the *Citrus* and *Perilla* species, respectively.

Intramolecular Deuterium Isotope Effects. The intramolecular isotope effects associated with the $CH_3 \rightarrow CH_2$ eliminations in the formation of limonene were determined by incubating **1**-d_4 with (+)- and (−)-pinene cyclases and with the (−)-limonene cyclase from *M. spicata*. The deuterium content of the limonene and its companion monoterpenes was measured by GC–MS analyses under nearly identical conditions. The isotopic compositions were calculated by the usual method for natural abundance factors and by subtraction of the contribution from the M − H fragmentation (see supplementary material).[32] The intramolecular isotope effects were deduced from the changes in the deuterium content[18a,33] of (+)- and (−)-limonene which lead to values of $k_H/k_D = 2.3 \pm 0.2$ and 5.9 ± 0.5 for the eliminations by (+)- and (−)-pinene cyclases, respectively, and $k_H/k_D = 4.0 \pm 0.4$ for the (−)-limonene cyclase (Table II). The negligible changes in the deuterium content of the bicyclic products are to be expected since no C–D bonds are broken. The small proportion of α- and β-pinenes (ca. 2%) produced by the

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Scheme II

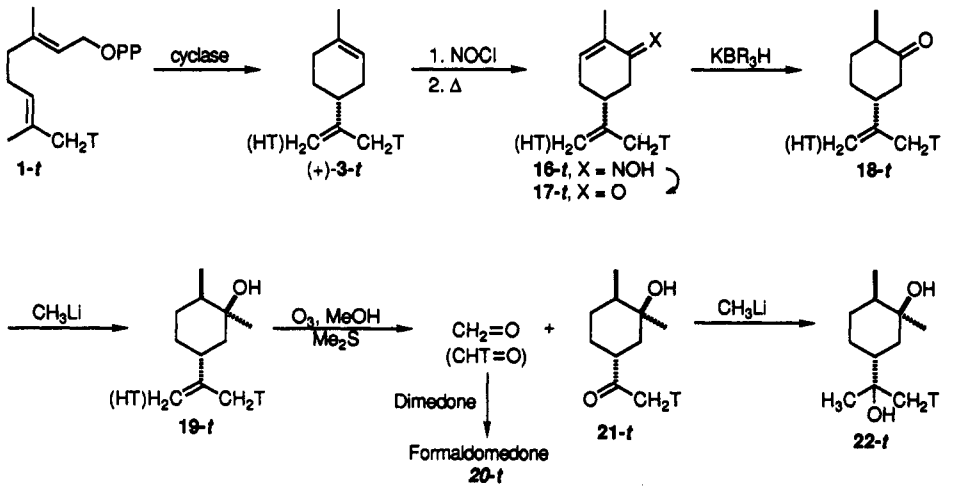


Table I. Radioactivity Data[a] from Degradation of (+)- and (−)-[^{3}H]Limonene (3-*t*) from Enzymatic Cyclizations of [8-^{3}H]Geranyl Diphosphate (1-*t*) (See Scheme II)

					ozonolysis products		
entry	enzyme source	limonene (3-*t*)	oxime (16-t)	alcohol (19-*t*)	20-*t*	22-*t*	total
1	*S. officinalis* (+)-pinene cyclase	*R*-(+)	20 371	9310[b] (100%)	3335 (36%)	4097[c] (44%)	7432 (80%)
2[d]	*S. officinalis* (−)-pinene cyclase	*S*-(−)	9502 (100%)	9426 (99%)	2654 (28%)	5048 (53%)	7702 (81%)
3	*C. sinensis*	*R*-(+)	21 271 (100%)	20 509 (96%)	470 (2%)	20 598 (97%)	21 068 (99%)
4	*P. frutescens*	*S*-(−)	9078 (100%)	8870 (98%)	336 (4%)	8849 (98%)	9185 (102%)

[a] Specific activity (SA) in dpm/mmol. Standard deviations were ±0.2–2.4% unless footnoted otherwise. Numbers in parentheses are relative SAs compared to that of 16-*t*. [b] After dilution with carrier. Calcd SA = 9360 dpm/mmol. [c] ±322. [d] A second degradation of (*S*)-(−)-limonene in which 18-*t* was subjected to ozonolysis gave very similar data.

Table II. Deuterium Content of Cyclic Monoterpenes Biosynthesized by Enzyme-Catalyzed Cyclizations of [8,8,9,9-$^2H_4$]Geranyl Diphosphate (1-d_4). Intramolecular Deuterium Isotope Effects on the Proton Elimination Steps Producing (+)- and (−)-Limonene

			deuterium distribution[a]					
enzyme	monoterpene	GC area (%)	d_1	d_2	d_3	d_4	d_5	k_H/k_D
	geraniol-d_4[b]		0.5	3.9	20.7	66.4	7.5	
(+)-pinene cyclase	(+)-limonene	9.5	1.4	9.7	45.1	40.7	2.9	2.3 ± 0.2[c]
	(+)-α-pinene	30.2	0.5	3.8	19.7	67.6	7.6	
	(+)-camphene	25.2	0.6	4.6	20.5	66.0	7.4	
(−)-pinene cyclase	(−)-limonene	7.9	1.3	6.8	33.9	52.0	5.4	5.9 ± 0.5[c]
	(−)-α-pinene	30.7	0.4	3.6	21.0	66.4	7.8	
	(−)-camphene	29.0	0.7	4.1	21.6	65.4	7.6	
(−)-limonene cyclase	(−)-limonene	94.4	3.5	7.0	36.7	49.3	3.4	4.0 ± 0.4[c]

[a] Determined by GC–MS analyses (selected ion monitoring). [b] Reference compound. [c] 95% confidence level.

limonene cyclase from *M. spicata* did not allow determination of the isotope content.

Kinetic Isotope Effects. The effects of deuterium substitution at the internal (C10) and terminal methyl groups (C8 and C9) on the total rate of conversion of geranyl PP to products were evaluated by careful kinetic measurements with pinene and bornyl PP cyclases from sage. The relative total rates of [^{3}H] monoterpene formation from 1-d_6,*t* and 1-d_3,*t* as a percent of the rate of undeuterated [1-^{3}H]substrate determined simultaneously, together with the apparent total rate isotope effects, are presented in Table III. The rates for 1-d_3,*t* were redetermined in the present study and the data generally agree with those reported earlier.[19] The isotope effects per CD_3 group lie in the range of k_{CH_3}/k_{CD_3} = 1.08–1.13. Although the exact magnitude of these secondary isotope effects is somewhat uncertain owing to the error limits, it is clear that deuterium substitution at the remote methyl groups exerts a significant rate depression which is quite comparable to that observed for deuteration of the proximal methyl group at C10. Similar rate suppression with 1-d_6,*t*

Table III. Effect of Methyl Group Deuteration on the Total Rate of Monoterpene Formation in Incubations of Deuterated GPPs (1-d_6,*t* and 1-d_3,*t*) with Pinene and Bornyl PP Cyclases from sage (*S. officinalis*)

	[1-^{3}H,8,9-$^2H_6$]-GPP(1-d_6,*t*)		[1-^{3}H,10-$^2H_3$]-GPP (1-d_3,*t*)[a]	
enzyme	relative rate (%)[b,c]	k_H/k_D[d]	relative rate (%)[b]	k_H/k_D
(+)-pinene cyclase	85.3 ± 4.65	1.17 (1.08)	85.1 ± 1.2	1.18
(−)-pinene cyclase	78.7 ± 0.71	1.27 (1.13)	75.1 ± 6.9	1.33
(+)-bornyl PP cyclase	86.0 ± 3.2	1.16 (1.08)	91 ± 5.5	1.10
(−)-bornyl PP cyclase	84.0 ± 7.0	1.19 (1.09)	81 ± 5.5	1.23

[a] These rates were redetermined, and the data agree with those reported previously. See ref 19. [b] Relative rates compared to that of incubation with [1-^{3}H]GPP (100). [c] Averages and averge deviations of three separate runs. [d] The values in the parenthesis are k_H/k_D per CD_3 group.

Table IV. Product Distributions from Incubations of [1-^{3}H]GPP and [1-^{3}H,8,9-^{2}H$_6$]GPP (1-d_6,t) with Pinene and Limonene Cyclases

enzyme	substrate	monoterpene product distribution[a,b] (%)						
		α-P	β-P	C	L	M	T	O
(+)-pinene cyclase	[1-^{3}H]GPP	24.6 ± 1.8	–	28.3 ± 0.6	26.5 ± 1.3	7.1 ± 1.5	11.6 ± 1.3	1.7 ± 0.4
	[1-^{3}H,8,9-^{2}H$_6$]GPP	25.2 ± 2.9	–	29.0 ± 1.8	23.0 ± 0.6	8.5 ± 1.4	12.5 ± 1.6	1.9 ± 0.1
(−)-pinene cyclase	[1-^{3}H]GPP	31.0 ± 1.5	15.9 ± 1.0	35.8 ± 1.5	7.9 ± 1.1	5.1 ± 0.3	2.5 ± 0.8	1.8 ± 0.6
	[1-^{3}H,8,9-^{2}H$_6$]GPP	32.0 ± 0.4	15.3 ± 1.3	35.7 ± 1.1	6.2 ± 0.7	5.5 ± 1.3	2.6 ± 0.6	2.6 ± 0.2
(−)-limonene cyclase[c]	[1-^{3}H]GPP	1.8 ± 0.2	2.0 ± 0.2	–	94.2 ± 9.4	2.0 ± 0.2	–	–
	[1-^{3}H,8,9-^{2}H$_6$]GPP	3.0 ± 0.3	3.2 ± 0.3	–	92.5 ± 9.3	1.3 ± 0.1	–	–

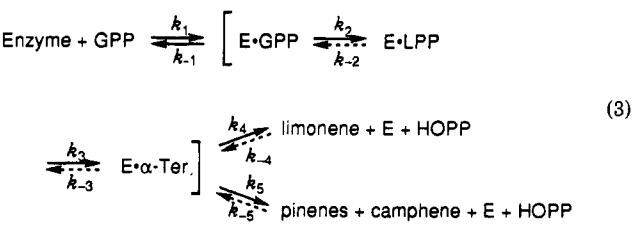
[a] Abbreviations: α-P, α-pinene; β-P, β-pinene; C, camphene; L, limonene; M, myrcene, T, terpinolene; O, ocimenes. [b] Averages and average deviations from three separate runs. [c] These data were taken from ref 28.

as substrate was also observed with the (−)-limonene cyclase from *M. spicata*.[28]

The sizeable secondary isotope effects determined for 1-d_3,t are expected since the initial, and rate-determining, step in the cyclization mechanism involves ionization to geranyl carbocation/MgOPP^{2-} anion pairs at the enzyme active site[3] which would be destabilized by the weaker electron-donating capacity of the deuteriomethyl group.[34] Similar secondary isotope effects (k_H/k_D per CD_3 group = 1.19–1.20) have been observed in the solvolysis of deuterium-labeled geranyl and neryl chlorides.[35]

The substantial total rate isotope effects resulting from deuteration at the terminal methyl groups seemed surprising because the initial geranyl PP → linalyl PP isomerization has been regarded as the rate-determining step of cyclase catalytic activity.[36] Thus, the V_{max} values for linalyl PP are 3–8 times larger than those of geranyl PP for various monoterpene cyclases, and attempts to detect release of the tertiary diphosphate intermediate from the enzyme have been unsuccessful.[3] If a correction is made for the abnormally large amount of acyclic and monocyclic products formed from LPP incubations, V_{max} ratios should be in the range of 2–4. Analysis of the proportional contribution of the isomerization and cyclization steps to the total rate affords an explanation for this apparent inconsistency.

A kinetic scheme for the enzyme-catalyzed cyclization of geranyl PP via the enzyme bound α-terpinyl carbocation (E·α-Ter) is given in eq 3. Derivation of V_{max} according



(3)

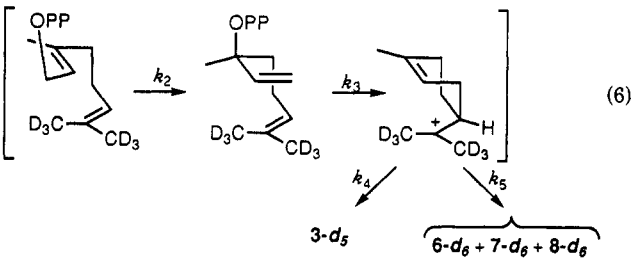
to the steady-state approximation for enzyme catalysis, together with the assumptions that the conversion of E·LPP to E·α-Ter is essentially irreversible [$(k_4 + k_5) \gg k_{-3}$] and that the conversion of E·α-Ter to products is much faster than isomerization or cyclization [$(k_4 + k_5) \gg k_2$ or k_3], leads to the expression in eq 4. The possibility

$$V_{max} = \frac{k_2 k_3}{k_2 + k_3 + k_{-2}}[E]_T \tag{4}$$

$$\frac{(V_{max})_H}{(V_{max})_D} = \frac{(k_3)_H/(k_3)_D + (k_3/k_2)_H}{[(k_3/k_2)_H + 1]} \tag{5}$$

that product release might be the rate-limiting step appears to be excluded by the observation of significantly different rates with GPP and LPP, and with deuterated substrates. Since the reversion of LPP to GPP has never been detected (i.e. k_{-2} and/or k_{-1} are very small) and the rate of conversion of LPP to products is substantially greater than that of GPP (i.e., $k_3 - k_{-2} > k_2$),[3] we also assume that k_{-2} is negligible compared to $(k_2 + k_3)$ which leads to the kinetic expression in equation 5 for the isotope effect on the total rate. According to eq 5, the proportion of the kinetic isotope effect on the cyclization step (k_3) expressed in the total rate $[(V_{max})_H/(V_{max})_D]$ will depend on the $(k_3/k_2)_H$ ratio. Thus, the remote isotope effect on the cyclization rate should be reflected in the total rate.

Isotope Effects on Product Ratios. If limonene, camphene, and pinene are formed competitively at the same active site of pinene cyclase, the primary isotope effect on the proton elimination leading to limonene should manifest itself in reduced monocyclic to bicyclic product ratios from the terminally deuterated substrate (eq 6),



(6)

i.e., the phenomenon of "isotopically sensitive branching."[18a] In fact, the product distribution data in Table IV reveal only slight decreases in the proportion of limonene and no significant changes in the levels of camphene and the pinenes.

The isotope effect $(k_4)_H/(k_4)_D$ can be quantitatively related to the (camphene + pinenes)/limonene product ratios (k_5/k_4) as shown in eq 7

$$(k_4)_H/(k_4)_D = \left(\frac{(k_5)_H}{(k_5)_D}\right)\left(\frac{(k_5)_D}{(k_4)_D}\right)\left(\frac{(k_4)_H}{(k_5)_H}\right) \tag{7}$$

where

$$(k_4)_H/(k_5)_H = 3/(6 + 7 + 8)$$

From the data in Table IV, the branching ratios are as

(34) (a) Shiner, V. J., Jr. *ACS Monogr.* **1970**, *167*, Chapter 2. (b) Sunko, D. E.; Borcic, S. *ACS Monogr.* **1970**, *167*, Chapter 3. (c) Shiner, V. J., Jr.; Humphrey, J. S., Jr. *J. Am. Chem. Soc.* **1963**, *85*, 2416. (d) Sunko, D. E.; Szele, I.; Hehre, W. J. *J. Am. Chem. Soc.* **1977**, *99*, 5000. (e) Shiner, V. J., Jr.; Murr, B. L.; Heinemann, G. *J. Am. Chem. Soc.* **1963**, *85*, 2413.

(35) Bunton, C. A.; Leresche, J. P.; Hachey, D. *Tetrahedron Lett.* **1972**, 2431.

(36) (a) Croteau, R.; Satterwhite, D. M.; Cane, D. E.; Chang, C. C. *J. Biol. Chem.* **1986**, *261*, 13438. (b) Satterwhite, D. M.; Wheeler, C. J.; Croteau, R. *J. Biol. Chem.* **1985**, *260*, 13901.

follows: (+)-pinene cyclase, $(k_5)_H/(k_4)_H = 2.00$ and $(k_5)_D/(k_4)_D = 2.36$; (−)-pinene cyclase, $(k_5)_H/(k_4)_H = 10.5$ and $(k_5)_D/(k_4)_D = 13.4$ and (−)-limonene cyclase (*M. spicata*), $(k_5)_H/(k_4)_H = 0.04$ and $(k_5)_D/(k_4)_D = 0.07$.[28] Thus, $(k_4)_H/(k_4)_D = n(k_5)_H/(k_5)_D$ where $n = 1.18$ ((+)-pinene cyclase), $n = 1.28$ ((−)-pinene cyclase), and $n = 1.66$ ((−)-limonene cyclase). Since the hexadeuterioterpinyl carbocation should cyclize faster than the unlabeled parent ion owing to the greater stabilization of CH_3 over CD_3, the isotope effects $(k_5)_H/(k_5)_D$ should be inverse, and the maximum values for the kinetic isotope effects on limonene formation are estimated as follows:

$$\text{(+)-pinene cyclase, } (k_4)_H/(k_4)_D \leq 1.2 \pm 0.3$$

$$\text{(−)-pinene cyclase, } (k_4)_H/(k_4)_D \leq 1.3 \pm 0.5$$

$$\text{(−)-limonene cyclase, } (k_4)_H/(k_4)_D \leq 1.7 \pm 0.8$$

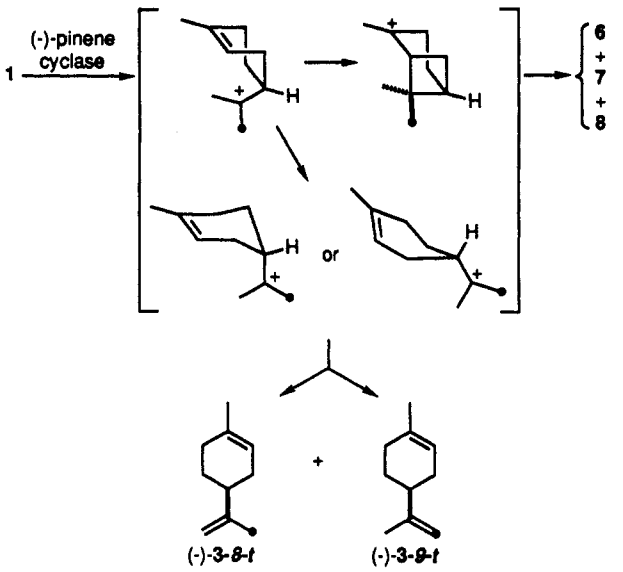
Although there is a significant uncertainty in these values owing to propagated error during the calculations, it is clear from these small isotope effects that the presumed branching between the pathways leading to limonene, pinene, and camphene is actually quite insensitive to deuterium substitution at the terminal methyl groups.

Discussion

The observed intramolecular isotope effects k_H/k_D = 2.3, 4.0, and 5.9 determined for the methyl–methylene eliminations in the enzymatic biosynthesis of (+)- and (−)-limonene are appropriate for primary deuterium kinetic isotope effects (KIE) on E1 proton eliminations.[37,38] The 2.6-fold difference in magnitude for the pinene cyclases deserves some comment. One plausible explanation is simply a difference in the intrinsic primary KIE of the elimination reactions. Differences in primary KIE are generally attributed to varying extents of proton transfer in the transition state, nonlinear transition states, and tunneling.[39] However, the measured intramolecular isotope effects may be the lower limits for the intrinsic isotope effects if the latter are masked, and this raises the possibility that similar intrinsic isotope effects might be masked to different extents in (+)- and (−)-pinene cyclases. Masking of the intrinsic isotope effect would occur if the rates of the elimination reaction were comparable to the rotational rates about the C–CH_3 bond. Although methyl group rotations are usually considered to be quite fast, proton transfer from the α-terpinyl carbocation to a basic group in close proximity would be highly exothermic and might be competitive in the restricted environment of the enzyme active site.

The lower intramolecular isotope effect for (+)-pinene cyclase is similar to values in the literature for (+)-limonene ($k_H/k_D = 3.35$) from *C. sinensis*[9a] and α-pinene (k_H/k_D = 2.1) from a *Pinus* species[40] determined by natural abundance ^2H NMR analysis and for (−)-β-pinene (2.4 and 2.6) determined from the isotope effect on the α-pinene/β-pinene branching ratio in pinene cyclase-catalyzed reactions.[19,20,41]

Scheme III



The substantial differences between the observed intramolecular isotope effects on the enzyme-catalyzed formation of (+)- and (−)-limonene compared to the small isotopic perturbations of the bicyclic olefin/limonene product ratios ($k_H/k_D \leq 1.2$, 1.3, and 1.7) prove that elimination to limonene does not occur competitively with the cyclizations leading to pinene and camphene. One possible explanation for this inconsistency is that the monocyclic and bicyclic terpenes might be produced independently by two or three different cyclase enzymes or at multiple sites on one enzyme. Although this explanation is difficult to disprove rigorously, the behavior of these monoterpene cyclases in chromatographic fractionations, electrophoresis, and differential inactivation and inhibition studies, as well as kinetic evaluations, strongly suggest that each set of enantiomerically-related monoterpenes is actually produced by one enzyme at a single site.[4,5,19,20,27,28]

An alternative explanation for the differences in isotope effects is that the branching between the pathways leading to limonene and the bicyclic products may occur prior to the proton elimination to limonene. That is, the common α-terpinyl carbocation intermediate may actually exist in two forms, one which cyclizes to camphene and/or pinene and another which undergoes elimination to limonene. If the two forms interconvert slowly compared to the rates of their conversion to limonene or to bicyclic products then the intramolecular kinetic isotope effect would be substantially masked. The two forms of the α-terpinyl ion could plausibly be conformational isomers (Scheme III).

The cyclization of the linalyl ion from its cisoid-endo conformation necessarily produces the α-terpinyl ion in a half-boat conformation with the C-4 isopropyl substit-

(37) The observed intramolecular deuterium isotope effect determined by incubating 1-d_4 is not a pure primary kinetic isotope effects (KIE), but is contaminated by an α-secondary KIE due to $sp^3 \rightarrow sp^2$ rehybridization during the elimination reaction. Normal α-secondary KIEs on E1 eliminations are on the order of 1.15 per deuterium atom.[34]

(38) Fry, A. J. *Chem. Soc. Rev.* **1972**, *1*, 163.

(39) Anderson, V. E. In *Enzyme Mechanisms from Isotope Effects*; Cook, P. F., Ed.; CRC: Boca Raton, 1991; pp 393–394.

(40) Pascal, R. A., Jr.; Baum, M. W.; Wagner, C. K.; Rodgers, L. R.; Huang, D.-S. *J. Am. Chem. Soc.* **1986**, *108*, 6477.

(41) Recent natural abundance ^2H NMR analyses of (+)- and (−)-limonene isolated from the same *C. sinensis* and *P. frutescens* plants used for isolation of the limonene cyclases in this work indicated k_H/k_D = 2.3 and 2.4, respectively, for the methyl–methylene eliminations: Leopold, M. F.; Grant, D. M. Unpublished results. We thank Dr. Leopold for permission to cite these results. For a brief description of the procedures and calculations, see: Pyun, H.-J. Ph.D. Thesis, University of Illinois, Urbana–Champaign, 1992, pp 38–40.

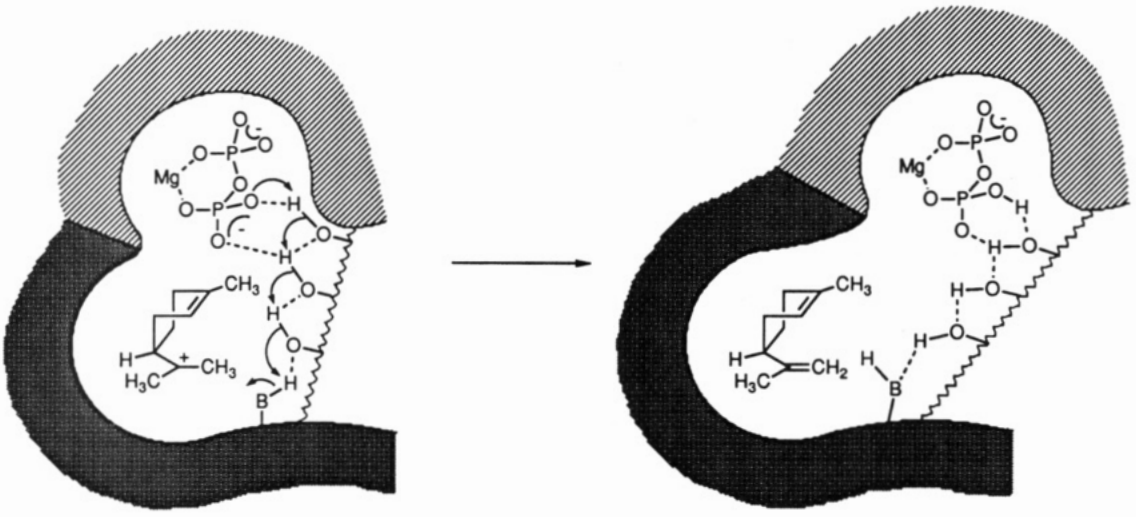
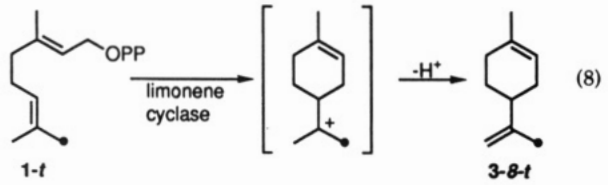


Figure 1.

uent bearing the charge in an axial position. This half-boat, folded conformer could undergo ring inversion to the extended half-chair conformer with an axial or equatorial C4 substituent before deprotonation occurs. Since bicyclization can take place only from the initially formed half-boat conformation of the α-terpinyl carbocation whereas elimination to limonene might occur only or predominantly from the subsequently formed half-chair conformer(s), the product ratios would be governed mainly by the competitive partitioning between bicyclization and conformational inversion. In this mechanism, deuterium substitution would not significantly affect the product ratios, and a reduced isotope effect is expected.

The occurrence of conformational inversion of the α-terpinyl intermediate prior to proton elimination also provides a reasonable explanation for the contrasting regiospecificities at the terminal methyl groups in the biosynthesis of limonene and the pinenes catalyzed by pinene cyclases.[7] It seems plausible that bicyclization of the initially formed half-boat conformer might occur more rapidly than rotation about the exocyclic C4–C8 bond, thus preserving the integrity of the geminal methyl groups in the cyclization to the pinyl carbocation.[7] On the other hand, the half-chair conformer of the α-terpinyl ion resulting from ring inversion may be less tightly bound, and proton elimination may occur more slowly than the rotation that would interconvert the two terminal methyl groups and lead to the observed nonregiospecific elimination. Alternatively, the nonregiospecific eliminations might be caused by competitive proton transfers from both methyl groups to two different bases or a bidentate base (e.g., carboxylate or phosphate) without the requirement of rapid rotation.

The regiospecific elimination at the cis terminal methyl group in the biosynthesis of (+)- and (−)-limonene catalyzed by the limonene cyclases from *C. sinensis* and *P. frutescens* established in this work (eq 8) agrees with the conclusions derived from natural abundance ^2H NMR analyses of limonene samples isolated from *Citrus* (orange) peel and Bergamot oil (*Citrus aurantium*)[9] as well as recent results of enzyme-catalyzed cyclizations carried out independently by Suga and associates at Hiroshima University.[17] Since the main function of these enzymes is to produce limonene, whereas the pinene cyclases produce predominantly camphene and the pinenes, it is perhaps understandable that the dedicated limonene cyclases would bind the α-terpinyl intermediate more tightly and effect regiospecific eliminations.



(8)

It is quite interesting that all regiospecific terminal $CH_3 \rightarrow CH_2$ eliminations investigated thus far occur consistently at the *cis*-methyl group in monoterpene, sesquiterpene,[13–15] and triterpene biosynthesis.[16] A similar terminating elimination in the enzyme-catalyzed cyclization of copalyl PP to the diterpene kaurene takes place with proton transfer toward the sterically more hindered endo region of the kauranyl carbocation intermediate.[42]

With the hope of stimulating further consideration and experimental analysis of the matter, we now speculate briefly upon possible reasons for the consistent *cis*-$CH_3 \rightarrow CH_2$ eliminations in mono- and sesquiterpenes biosynthesis. The enzyme-catalyzed cyclizations of geranyl and farnesyl PPs produce two very different products, the hydrophobic terpene hydrocarbons and the hydrophilic diphosphate·magnesium complex, [HOPP·Mg]$^-$. A simple bond energy calculation indicates that the net conversion of C–H and C–O bonds in the substrates to C–C and O–H bonds in the products is exothermic by $\Delta H_{BE} = -9$ kcal/mol.[43] It is reasonable to suppose that the binding sites for the isoprenoid chain and the $MgOPP^{2-}$ groups in the enzyme·substrate complex move further apart in the enzyme·product complex so that hydrophobic contacts with the hydrocarbon product increase and more effective interaction of the [HOPP·Mg]$^-$ group with the aqueous phase results (see Figure 1).

Minimum charge separation and optimal solvation of the charged groups in the elimination transition state should be important in the evolution of the enzymes' catalytic efficiency. If one assumes a least motion mechanism in the conversion of linalyl PP to the enzyme-bound

(42) Coates, R. M.; Koch, S. C.; Hedge, S. *J. Am. Chem. Soc.* **1986**, *108*, 2762–2764.

(43) Average bond energies from: Streitwieser, A., Jr.; Heathcock, C. H. *Introduction to Organic Chemistry*; MacMillan: New York, 1985; p 1153.

α-terpinyl$^+$/MgOPP^{2-} ion pair intermediate, it is clear that the *cis*-methyl group is positioned nearer to the negatively charged MgOPP^{2-} leaving group (*cis*-CH_3 to C-1 distance is ~2.6 Å compared to ~4.3 Å for the *trans*-CH_3 with Dreiding models). Thus, charge separation should be less in the transition state for proton transfer from the *cis*-methyl group to the basic acceptor group in the enzyme active site.

Plausible candidates for the immediate proton acceptor (B) would be a water molecule, an imidazole group of a histidine residue, a thiol group of cysteine, or an N-terminal amino group (pK_a's for the three amino acid residues = 6.5–8.5).[44] Another possibility would be direct transfer of the proton to the MgOPP^{2-} dianion as proposed previously for the prenyl transferase reaction.[45] In this case considerable movement and reorientation would be necessary to bring the *cis*-CH_3 and MgOPP^{2-} groups into close proximity.

Experimental Section

General Aspects. Melting points were determined in open capillary tubes and are uncorrected. All boiling points are uncorrected. ^1H and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively, using $CDCl_3$ solutions unless specified otherwise. GC analyses were carried out using a 30-m, DU-5, RTX-5, RSL-150, or AT-1000 capillary column. Flash chromatographies were performed according to the procedure of Still *et al.*[46] with Woelm 32-64-μm silica gel. Analytical TLC was carried out on Merck glass plates precoated with 0.25-mm silica gel 60 F_{254}, and preparative TLC was performed on silica gel G plates. TLC plates were visualized with UV light, I_2 vapor, *p*-anisaldehyde spray reagent, and/or phosphomolybdic acid spray reagent.

Radioactivity was determined by liquid scintillation counting. Scintillation fluid was either a solution of 6 g of 2,5-diphenyloxazole in 1 L of toluene, "Syntrex" reagent (J. T. Baker Chemical Co.), or Omnifluor (New England Nuclear). For specific activity (SA) determinations, a minimum of 10 000 counts was accumulated, and background (~31 dpm) was subtracted from each count. During the degradation, each sample was counted five times and averaged, and the counting efficiency for tritium was 30–55%. An average sampling and counting error is estimated to be ±3~7% for all SA determinations. Radio-TLC analyses were performed on Radiomatic Model RS plate scanner. Radio-GC analysis of ^3H-labeled monoterpenes was carried out by procedures previously described on RSL-150 or AT-1000 columns.[24,47] The coupled GC–MS data were acquired (EI, 70 eV) in the SIM mode (selected ion monitoring), and the relative abundances of ions from d_0 to d_6 were recorded using a 75-ms dwell time.

All reactions, except those performed in aqueous solvents, were carried out under N_2. Glassware used was dried at 140 °C for 5 h or flame dried. Technical-grade ethyl acetate and hexane were distilled prior to use for column chromatography. Diethyl ether and THF were distilled from the sodium ketyl of benzophenone, and CH_2Cl_2 was distilled from CaH_2 under N_2. Oxalyl chloride was distilled prior to use. DMSO and pyridine were dried with activated 4-Å molecular sieves. All other reagents and solvents were reagent grade and were used without further purification.

The tritium-labeled substrate 1-*t* (62 Ci/mol >95%) was prepared according to the procedure described previously as was [1-^3H]geranyl diphosphate (120 Ci/mol, >95%) and unlabeled substrate.[7] The specific activity of these and all other tritium-labeled diphosphate esters was determined by enzymatic hydrolysis to the corresponding alcohol followed by aliquot counting and mass determination by GC analysis. Multiple determinations of this type gave the values reported with standard error of less than 5%. All geranyl diphosphate substrates contained less than 4% of the *cis*-isomer, NPP, and all kinetic assays were carried out in the presence of one unit of inorganic pyrophosphatase to eliminate any possible interference from minor contamination of the substrate with inorganic pyrophosphate. Isotope ratios of the synthetic deuterated samples were measured by field ionization mass spectrometry (FIMS).

2-(2-Butoxyethoxy)ethyl Formate. A solution of 50.0 g (308 mmol) of 2-(2-butoxyethoxy)ethanol and 50 mL of pyridine was stirred and cooled below 0 °C with an ice–salt bath as 43 g (490 mmol) of acetic formic anhydride[48] was added over 30 min. After 5 h at 0 °C and 12 h at rt, the solution was washed with water (400 mL), and the aqueous layer was extracted with ether (2 × 150 mL). The combined organic fractions were successively washed with water (400 mL), 3 N HCl (2 × 400 mL), saturated $NaHCO_3$ (400 mL), and saturated NaCl (400 mL), dried ($MgSO_4$), and concentrated. Distillation of the residue at 82–83 °C (1.3 mm) gave 52.79 g (90%) of the formate ester: IR (neat) ν_{max} 2959 (CH), 2934 (CH), 2870 (CH), 1727 (C=O), 1181 (CO), 1121 (CO) cm^{-1}; ^1H NMR δ 0.92 (t, 3H, J = 7.3 Hz, CH_3), 1.36 (sextet, 2H, J = 7.3 Hz, CH_3CH_2), 1.57 (septet, 2H, J = 7.3 Hz, $CH_3CH_2CH_2$), 3.47 (t, 2H, J = 6.7 Hz, CH_2CH_2O), 3.59 (m, 2H, CH_2O), 3.66 (m, 2H, CH_2O), 3.75 (m, 2H, CH_2O), 4.33 (m, 2H, CH_2OCHO), 8.09 (s, 1H, CHO).

($^2H_2$)Iodomethane was prepared according to a modified literature procedure.[49] To a 250-mL three-necked flask were attached a thermometer, a N_2 inlet, and a 15-cm Vigreux column, which was connected to the distillation head with a dry ice–2-propanol cold finger condenser and a receiver flask (50 mL) which was cooled with dry ice–2-propanol. A dry ice trap, N_2 inlet, and oil bubbler were linearly connected to the distillation head. The N_2 inlet at the flask was closed and 97 mL (735 mmol) of 57% HI and 4.87 g (143 mmol) of methanol-d_2 (Cambridge Isotope Lab.; 0.4% d_0, 8.6% d_1, 90.3% d_2, 0.7% d_3 by FIMS) were placed in the flask. Most of the ($^2H_2$)iodomethane produced was distilled while the solution was stirred and heated gradually from 50 to 85 °C for 5.5 h. The remaining product in the solution was collected by sweeping with N_2 for 2 h at 85 °C. The remaining product in the pot residue was collected in a second flask by sweeping with N_2 for 5 h at rt. The contents of the two receiving flasks and the dry ice trap were combined and dried ($MgSO_4$). A cannula transfer of the product gave 16.78 g (116.6 mmol, 82%) of ($^2H_2$)iodomethane. A portion (12 mL) of ether was added to the flask containing $MgSO_4$, and the rinse was also collected by a cannula transfer. This ethereal solution was estimated to contain 1.47 g (10.2 mmol, 7%) of the product: ^1H NMR δ 2.14 (quintet, 1H, J = 1.3 Hz, CHD_2).

(1,1,3,3-$^2H_4$)-2-Propanol was prepared by the reaction of ($^2H_2$)-methylmagnesium iodide and 2-(2-butoxyethoxy)ethyl formate. The Grignard reagent was formed as described in a literature procedure[50] by reaction of 15.17 g (105 mmol) of ($^2H_2$)iodomethane and 2.60 g (107 mmol) of Mg turnings in 25 mL of ether. The ethereal solution of the Grignard reagent was stirred and cooled in an ice bath as 10.46 g (55 mmol) of the formate ester in 10 mL of ether was added slowly to allow a gentle reflux. After the addition was completed, the solution was refluxed for 2 h. The bath temperature was maintained at 45 °C, while 30 mL of 2-ethoxyethyl ether was added, which turned the solution into a slurry. A dry ice trap was connected to the top of the dropping funnel, and N_2 was swept into the flask and through the reflux condenser for 12 h to remove diethyl ether. The remaining thick residue was cooled to rt, and the dropping funnel was replaced with a 10-cm Vigreux column. The column was fitted with a distillation head connected to a dry ice–2-propanol cold finger condenser, a receiving flask (25 mL) cooled at −78 °C, and an oil bubbler. The remaining residue in the reaction flask was dissolved by adding 30 mL of 2-(2-butoxyethoxy)ethanol at rt. The N_2 sweep into the flask was resumed, and the solution was stirred

(44) Stryer, L. *Biochemistry*; W. H. Freeman: New York, 1988; p 21.
(45) Poulter, C. D.; Rilling, H. C. *Acc. Chem. Res.* **1978**, *11*, 307.
(46) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
(47) Croteau, R.; Cane, D. E. *Methods Enzymol.* **1985**, *110*, 352–405.
(48) Krimen, L. I. *Organic Syntheses*; Wiley: New York, 1988; Collect. Vol. VI, p 8.
(49) Ott, D. G. *Syntheses with Stable Isotopes of Carbon, Nitrogen, and Oxygen*; Wiley-Interscience: New York, 1981; p 126.
(50) *Vogel's A Textbook of Practical Organic Chemistry*, 4th ed; Longman: London, 1978; p 371.

and heated at 50 °C for 0.5 h, at 60 °C for 1 h, and at 120 °C for 4 h. Distillation of the collected product at 60–82 °C at atmospheric pressure afforded 1.38 g of (1,1,3,3-$^2H_4$)-2-propanol. Integration of the ^1H NMR spectrum showed that the product contained about 0.16 g of diethyl ether: corrected yield, 1.22 g (36%); ^1H NMR δ 1.19 (nine lines d quintent, 2H, J = 7, 2 Hz, (CHD_2)$_2$CH), 1.36 (d, 1H, J = 4.0 Hz, OH), 4.01 (br d, 1H, J = 4.0 Hz, CHOH). This mixture was used for the preparation of (1,1,3,3-$^2H_4$)-2-bromopropane without further purification.

(1,1,3,3-$^2H_4$)-2-Bromopropane. PBr_3 (1.5 mL, 15.8 mmol) was slowly added to 1.22 g (19.0 mmol) of (1,1,3,3-$^2H_4$)-2-propanol while the temperature of the solution was kept below 5 °C.[51] The solution was stirred at rt for 17 h and cooled at −18 °C as 5 mL of water was slowly added. The lower organic layer was separated, washed with saturated $NaHCO_3$, and dried ($MgSO_4$). Distillation at 54 °C at atmospheric pressure gave 945 mg of (1,1,3,3-$^2H_4$)-2-bromopropane. According to the ^1H NMR spectrum, the product contained about 114 mg of diethyl ether: corrected yield, 831 mg (34%); ^1H NMR δ 1.67 (m, 2H, (CHD_2)$_2$CH), 4.27 (t, 1H, J = 6.5 Hz, CHBr). This mixture was used for the preparation of [1-($^2H_2$)methyl-(2-$^2H_2$)ethyl]triphenylphosphonium bromide without further purification.

[1-($^2H_2$)Methyl(2-$^2H_2$)ethyl]triphenylphosphonium Bromide. A mixture of 1.99 g (7.58 mmol) of triphenylphosphine and 932 mg (7.34 mmol) of (1,3-$^2H_6$)-2-bromopropane was heated at 150 °C for 2 days in a thick wall tube with a tightly sealed screw cap.[52] The resulting solid was triturated with 10 mL of ethyl acetate and collected by filtration. Recrystallization of the crude product gave 1.82 g (64%) of the title compound; mp 233–236 °C (lit.[52] mp for the unlabeled compound, 238–239 °C). The ^1H NMR spectrum indicated the presence of some impurities, but it was used for the Wittig reaction without further purification: ^1H NMR (CD_3OD) δ 1.36 (br m, 2.3H, (CHD_2)$_2$CH), 3.44 (m, 0.2H, impurity), 4.15 (br m, 1H, CH), 4.89 (s, 0.5H, impurity), 7.73–7.94 (m, 15H, PhH).

(2*E*)-(8-$^2H_2$)-1-(Benzyloxy)-3-methyl-7-($^2H_2$)methyl-2,6-octadiene ((8,8,9,9-$^2H_4$)Geranyl Benzyl Ether, 14a). A suspension of 1.63 g (4.2 mmol) of [1-($^2H_2$)methyl-(2-$^2H_2$)ethyl-triphenylphosphonium bromide in 25 mL of THF was stirred and cooled at 0 °C as 1.38 M *n*-butyllithium in hexane (2.9 mL, 4.00 mmol) was added.[52,53] After 1 h at rt, 828 mg (3.8 mmol) of 12 was added to the blood red solution of **13a** at 0 °C, and the resulting solution was stirred for 3 h at rt. After the excess phosphorane was quenched by adding 0.8 mL of methanol, the reaction mixture was concentrated under reduced pressure, and the residue was extracted with hexane (3 × 40 mL) by refluxing for 2 h each. The combined extracts were concentrated, and the residue was purified by flash chromatography with ethyl acetate–hexane (1:50) as eluent. The product was 94.5% pure as analyzed by GC and was found to contain three impurities. (1.5%, 3.0%, and 1.0% each by GC peak integrations). Attempts to remove these impurities by flash column chromatography were unsuccessful. Kugelrohr distillation at 180 °C (1.5 mm) provided 809 mg (86%) of **14a** (94.5% purity by GC): IR (neat) ν_{max} 3031 (=CH), 2911 (CH), 2853 (CH), 2136 (CD), 1669, 1453 (C=C), 1069, 1028 cm^{-1}; ^1H NMR δ 1.56 (br, 1H, CHD_2), 1.64 (s, 4H, CH_3 and CHD_2), 2.01–2.14 (m, 4H, CH_2CH_2), 4.03 (d, 2H, J = 6.7 Hz, =$CHCH_2O$), 4.50 (s, 2H, OCH_2Ph), 5.10 (t, 1H, J = 6.5 Hz, =$CHCH_2CH_2$), 5.40 (t, 1H, J = 6.7 Hz, =$CHCH_2O$), 7.25–7.35 (m, 5H, PhH); ^{13}C NMR δ 16.5, 17.4 (quintet, J = 19.4 Hz, CHD_2), 25.1 (quintet, J = 18.6 Hz, CHD_2), 26.3, 39.6, 66.6, 71.9, 120.8, 124.0, 127.5, 127.8, 128.3, 131.5, 138.6, 140.4.

(2*E*)-(8-$^2H_2$)-3-Methyl-7-($^2H_2$)methyl-2,6-octadien-1-ol ((8,8,9,9-$^2H_4$)Geraniol, 15a). A solution of 113 mg (16.3 mmol) of Li in 50 mL of liquid NH_3 was stirred at −78 °C as 745 mg (3.0 mmol) of **14a** in 10 mL of THF was added.[7b] A rinse with 2 mL of THF was added to the reaction mixture. The solution was refluxed for 0.5 h and cooled to −78 °C before the excess Li was destroyed by adding several drops of 3-hexyne followed by 1.5 mL of methanol. NH_3 was evaporated by N_2 sweeping, and the remaining solution was diluted in water (50 mL). The aqueous solution was extracted with hexane (4 × 35 mL). The combined organic extracts were washed with water (2 × 50 mL) and saturated NaCl (100 mL), dried ($MgSO_4$), and concentrated. After the residue was purified by flash chromatography four times using ether–hexane (1:5) as eluent, a pure fraction was collected. Kugelrohr distillation of the purified product at 150 °C (7.5 mm) afforded 361 mg (76%) of **15a** (>99% pure by GC): IR (neat) ν_{max} 3322 (OH), 2967 (CH), 2909 (CH), 2222 (CD), 2137 (CD), 1669, 1441 (C=C), 1381, 1279, 1003 (CO) cm^{-1}; ^1H NMR δ 1.31 (br, 1H, OH), 1.56 (br, 1H, CHD_2), 1.65 (br, 1H, CHD_2), 1.67 (s, 3H, CH_3), 1.99–2.13 (m, 4H, CH_2CH_2), 4.15 (d, 2H, J = 7.0 Hz, CH_2O), 5.09 (t, 1H, J = 6.6 Hz, =CH–), 5.41 (t, 1H, J = 7.0 Hz, =$CHCH_2O$–); ^{13}C NMR δ 16.2, 25.1 (quintet, CHD_2), 26.3, 39.5, 59.4 (CH_2OH), 123.3, 123.9, 131.6, 139.8; isotope ratio by FIMS analysis, 0.8% d_0, 0.7% d_1, 4.6% d_2, 20.2% d_3, 65.8% d_4, 7.2% d_5, 0.7% d_6. The missing quintet for the other CHD_2 group in the ^{13}C NMR spectrum was apparently obscured by the noise.

(1,3-$^2H_6$)-2-Propanol. A suspension of 4.5 g (119 mmol) of $LiAlH_4$ in 200 mL of 2-ethoxyethyl ether was mechanically stirred and cooled in an ice–salt bath as 20 g (311 mmol) of acetone-d_6 (99.5% D, Aldrich Chemical Co.) was added over 20 min.[51] After 16 h at rt, 100 mL of 2-(2-butoxyethoxy)ethanol was slowly added at 0 °C. The product was distilled from the reaction mixture at 82–84 °C at atmospheric pressure.[54] Redistillation of the product at 81–83 °C at atmospheric pressure gave 14.3 g (69%) of (1,3-$^2H_6$)-2-propanol: IR (CCl_4) ν_{max} 3629 (OH), 3337 (OH), 2228 (CD), 1134, 1044 cm^{-1}; ^1H NMR δ 3.38 (s, 1H, OH), 3.97 (s, 1H, CH); ^{13}C NMR δ 24.1 (septet, J = 19.3 Hz, CD_3), 63.5 (CH).

(1,3-$^2H_6$)-2-Bromopropane was prepared by reaction of 13.2 g (200 mmol) of (1,3-$^2H_6$)-2-propanol with 11.4 mL (120 mmol) of PBr_3 as described above for (1,1,3,3-$^2H_4$)-2-bromopropane.[51] The solution was stirred at rt for 8 h after which 15 g of ice and 50 mL of water was slowly added. The lower organic layer was separated, washed with saturated $NaHCO_3$, and dried ($MgSO_4$). Distillation at atmospheric pressure afforded 10.2 g (39%) of (1,3-$^2H_6$)-2-bromopropane: bp 58–59.5 °C; IR (CCl_4) ν_{max} 2967 (CH), 2236 (CD), 2220 (CD), 2136, 2070, 1202, 1059, 1049, 893 cm^{-1}; ^1H NMR δ 4.26 (s, 1H, CH); ^{13}C NMR δ 27.5 (heptet, J = 19.3 Hz, CD_3), 63.5 (CH).

[1-($^2H_3$)Methyl(2-$^2H_3$)ethyl]triphenylphosphonium bromide was prepared according to the procedure described previously for [1-($^2H_2$)methyl(2-$^2H_2$)ethyl]triphenylphosphonium bromide. Recrystallization from ethanol–ethyl acetate (3:20) provided 4.2 g (36%) of the title compound: mp 238–239.5 °C (lit.[52] mp for the unlabeled compound, 238–239 °C); IR (KBr) ν_{max} 3034 (=CH), 2979 (CH), 2861 (CH), 2226 (CD), 1433 (C=C), 1109, 994 cm^{-1}; ^1H NMR (CD_3OD) δ 4.16 (d, 1H, J = 11.2 Hz, CH), 7.73–7.94 (m, 15H, PhH); ^{13}C NMR (CD_3OD) δ 20.6 (d, J = 48.0 Hz, CH), 117.6 (d, J = 83.3 Hz, PC), 130.2 (d, J = 12.3 Hz, *o*-CH), 133.6 (d, J = 9.0 Hz, *m*-CH), 134.8 (d, J = 3.2 Hz, *p*-CH).

(2*E*)-(8-$^2H_3$)-1-(Benzyloxy)-3-methyl-7-($^2H_3$)methyl-2,6-octadiene ((8,9-$^2H_6$)geranyl benzyl ether, 14b) was prepared according to the procedure described previously for **14a**. The crude product was purified by flash chromatography with ethyl acetate–hexane (1:50) as eluent and Kugelrohr distillation at 163 °C (8.5 mm) to give 1.1 g (88%) of **14b**: IR (neat) ν_{max} 3301, (=CH), 2923 (CH), 2851 (CH), 2191 (CD), 1455 (C=C), 1071 (CO) cm^{-1}; ^1H NMR δ 1.64 (s, 3H, CH_3), 2.02–2.14 (m, 4H, CH_2CH_2), 4.03 (d, 2H, J = 6.5 Hz, CH_2O), 4.50 (s, 2H, CH_2Ph), 5.10 (t, 1H, J = 6.5 Hz, =CH–), 5.40 (t, 1H, J = 6.5 Hz, =CH–), 7.25–7.35 (m, 5H, PhH); ^{13}C NMR δ 16.4, 26.3, 39.6, 66.5, 71.9, 120.7, 124.0, 127.4, 127.8, 128.3, 131.4, 138.5, 140.3.

(2*E*)-(8-$^2H_3$)-3-Methyl-7-($^2H_3$)methyl-2,6-octadien-1-ol ((8,9-$^2H_6$)geraniol, 15b) was prepared according to the procedure described previously for **15a**. The crude product was purified by flash chromatography using ethyl acetate–hexane (1:5) as eluent, and Kugelrohr distillation of the product at 136 °C (8.5 mm) gave 619 mg (97%) of (8,9-$^2H_6$)geraniol **15b**: IR (neat) ν_{max} 3326 (OH), 2924 (CH), 2224 (CD), 2191 (CD), 2112 (CD), 2064 (CD), 1443 (C=C), 1381, 1001 cm^{-1}; ^1H NMR δ 1.58 (br, 1H, OH), 1.68 (s, 3H, CH_3) 2.03–2.15 (m, 4H, CH_2CH_2), 4.14 (d, 2H, J = 6.6 Hz, CH_2O), 5.09 (t, 1H, J = 6.6 Hz, =CH–), 5.41 (t, 1H, J = 6.8 Hz,

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=CH–); ^{13}C NMR δ 16.2, 26.3, 39.5, 59.3 (CH_2OH), 123.3, 123.9, 131.5, 139.6; Isotope ratio by FIMS analysis, 1.6% d_0, 3.2% d_5, 95.2% d_6.

(2*E*)-[1-^{3}H,8-$^2H_3$]-3-Methyl-7-($^2H_3$)methyl-2,6-octadien-1-ol ([1-^{3}H,8,9-$^2H_6$]Geraniol, 15c). Oxidation of **15b** was carried out according to a literature procedure.[55] Purification by flash chromatography using ether–hexane (1:8) as eluent afforded 81.7 mg (89%) of (2*E*)-(8-$^2H_3$)-3-methyl-7-($^2H_3$)methyl-2,6-octadien-1-al [(8,9-$^2H_6$)citral].

The reduction of (8,9-$^2H_6$)citral with NaB^3H_4 was performed according to a literature procedure with some modifications.[7b] To the vial containing ca. 24 mCi of NaB^3H_4 (New England Nuclear, 386 Ci/mol, 0.063 mmol) and a magnetic stirring bar was added 36.5 mg (0.23 mmol) of (8,9-$^2H_6$)citral in 1 mL of ethanol. A rinse with 1 mL of ethanol was added to the reaction vial. This solution was stirred at rt for 1 h under N_2 and transferred to a separatory funnel using ether (10 mL) and water (10 mL). The separated aqueous layer was extracted with ether (10 mL). The combined organic extracts were washed with saturated NaCl (10 mL), dried ($MgSO_4$), and concentrated. The crude product was purified by flash chromatography with ether–hexane (1:6) as eluent with aliquot counting. The fractions containing radioactive product were concentrated by distillation at atmospheric pressure, and the remaining solvent was removed under reduced pressure (~20 mm) at 40 °C for 1 h to afford 18.4 mg (50%, 7.5 mCi, 64.8 Ci/mol, 31% radiochemical yield) of [1-^{3}H,8,9-$^2H_6$]geraniol **15c**. The product was >95% radiochemically pure as judged by radio-TLC (ether:hexane = 1:6, R_f = 0.16). The chemical identity of the product was confirmed by TLC comparison to unlabeled geraniol.

The radiochemical purity of **15c** was further confirmed by dilution and derivatization as the 3,5-dinitrobenzoate. An aliquot containing 0.75 μCi of **15c** was diluted with 495 mg (3.21 mmol) of carrier geraniol and dissolved in 5 mL of pyridine before 3,5-dinitrobenzoyl chloride (1.12 g, 4.86 mmol) was added. After 1.5 h of stirring at rt, the solution was diluted with 3 N HCl (30 mL) and extracted with ether (2 × 30 mL). The combined ether extracts were sequentially washed with 3 N HCl (30 mL), saturated $NaHCO_3$ (30 mL), and saturated NaCl (30 mL). The organic fraction was dried ($MgSO_4$) and concentrated, and the remaining residue was crystallized from 20 mL of ethanol to yield 850 mg (76%) of white crystals: mp 61.5–62.5 °C (lit.[56a] mp 62 °C). After five additional recrystallizations the average specific activity was 67.7 ± 1.5 mCi/mol for [1-^{3}H,8,9-$^2H_6$]geraniol (**15c**).

Preparation of Enzymes. The sources and propagation of sage (*S. officinalis*), the *Mentha* species, and perilla (*P. frutescens*) have been described.[27,57] Cyclase isolation from leaf tissue employed mechanized techniques either to extract selectively these enzymes from the epidermal oil grands while still on the leaf surface[26] or to remove (by abrasion) and collect the oil grands (by filtration) prior to extraction of the enzymes from these structures.[58] In either case, 10–20 g of leaf tissue was employed for most preparations, and the cyclases were isolated in dilute sodium phosphate buffer (15–20 mM, pH 6.5) containing 5 mM $Na_2S_2O_5$, 5 mM sodium ascorbate, 5 mM dithiothreitol, and 15% (v/v) glycerol, as well as XAD-4 (polystyrene) and polyvinylpyrrolidone as adsorbents.[47] Following filtration and centrifugation, the soluble enzyme preparation from perilla was concentrated about 20-fold by ultrafiltration and used as the cyclase source without further purification. In the case of sage oil gland extracts, the soluble enzyme preparation was partially purified by step gradient elution from Sephadex A-25 (500 mM KCl in assay buffer consisting of 10 mM Mes–5 mM sodium phosphate, pH 6.8, 1 mM dithiothreitol, and 10% glycerol) and by gel permeation chromatography of the concentrated eluate on a 2.5 × 110 cm column of Sephacryl S-200 previously equilibrated with assay buffer. Column fractions were assayed for pinene cyclase activity,[5] and fractions at the leading edge of the (+)-pinene cyclase peak were pooled, as were fractions at the trailing edge of the later-eluting (–)-pinene cyclase peak, in order to obtain a (+)-pinene cyclase fraction free of (–)-pinene cyclase contamination and vice versa. The pH of the (+)-pinene cyclase fraction was adjusted to the optimum of 6.1, and the pH of the (–)-pinene cyclase fraction was adjusted to the optimum of 7.1, prior to assay.

To prepare limonene cyclase from Valenica oranges (*C. sinensis*; Dole), approximately 200 g of slightly green peel (obtained locally) was cut into small pieces and squeezed while submerged in 400 mL of isolation buffer in order to rupture and to extract the contents of the subepidermal oil grands. The suspension of crushed peel in buffer was mixed on ice for 1 h and then filtered and centrifuged at 27000*g*. The resulting supernatant was used as the enzyme source without concentration since ultrafiltration was inefficient due, probably, to the presence of pectinaceous material.

The purification and assay of (*S*)-(–)-limonene cyclase from *M. spicata* and *M. piperita* have been described in detail.[27]

Incubation of (6*E*)-[8-^{3}H]Geranyl Diphosphate (1-*t*) and Conversion of [^{3}H]Limonene to [^{3}H]Carvoxime. The cyclization reactions were initiated by addition of $MgCl_2$ (11 mM) and 1-*t* (40 μM) to the various enzyme preparations which were incubated for 2 h at 32 °C. Following chilling and the addition of salt to saturation, the incubation mixtures were extracted with three volumes of pentane and the extract was passed through a short column of silica gel overlaid with $MgSO_4$ to afford the terpene hydrocarbon fraction.[5] An aliquot of this fraction was taken for determination of radioactivity and another for radio-GC analysis. Boiled controls were included in all experiments and evidenced negligible nonenzymatic conversion.

Since radio-GC analysis indicated the presence of essentially pure [^{3}H]limonene in the product extracts from the *Perilla* and *Citrus* cyclase preparations, these radiolabeled substances were diluted with 3 μmol of authentic carrier (for perilla, (*S*)-(–)-limonene, 92%, Aldrich; for orange, (*R*)-(+)-limonene, 97%, Aldrich) and were converted directly to carvoxime by slight modification of literature procedures.[29] Thus, following preparative TLC (silica gel G, hexane) to remove unlabeled contaminants, the dry sample was dissolved in anhydrous ethanol (1 mL) to which 2.1 equiv of isoamyl nitrite was added while the sample was stirred and cooled in an ice bath. Following stirring for an additional 10 min, concd HCl (2.1 equiv) was added, and the mixture was stirred for 1 h and then placed in the freezer overnight. The solid nitroso chloride was collected by filtration (and by repeated concentration and chilling of the filtrate), and the combined sample was dissolved in 3 mL of acetone containing 0.2 mL pyridine and heated on a steam bath for 20 min to afford the corresponding oxime. Following removal of solvents, the crude oxime was purified by preparative TLC (silica gel G with hexane/ether (7:3, v/v)), diluted 2 μmol of authentic (*R*)- or (*S*)-carvoxime as carrier, and crystallized once from hexane.

Since the (+)-pinene cyclase and (–)-pinene cyclase from sage produce several olefins from geranyl diphosphate,[5] [^{3}H]limonene was separated from the other radioactive products by preparative TLC on silica gel G-8% $AgNO_3$ with hexane as eluent. To further avoid any possible cross-contamination of products from the antipodal (+)- and (–)-pinene cyclases, the oximes were generated as above but with unlabeled carrier intentionally diluted to ~90% ee so that enantiomer purification could be more easily monitored. Thus, because the racemate of carvoxime is less soluble than the pure enantiomers, the final solutions were partially crystallized and the mother liquor was used for subsequent rounds of crystallization until the carvoxime remaining in solution was >99% ee.

Radiochemical Degradation of [^{3}H]Carvoximes (16-*t*). The procedures were optimized, and the products were fully characterized using nonradioactive material. The procedures and product characterization data for these nonradioactive reactions are described in detail first. The reactions with the tritium-labeled compounds were conducted in a similar manner, and only the chemical yields and, when determined, the specific activities are given in abbreviated form for runs 1–4 (Table I, entries 1–4).

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Dilution and Specific Activity Determination of [^{3}H]-Carvoximes. The [^{3}H]carvoximes **16**-*t* were diluted with enantiomerically pure carrier **16**, and the SAs were determined prior to degradation. Optically pure carvoximes were obtained from the mother liquor by successive crystallizations of the material from the mother liquor in 80% aqueous methanol. The carvoximes were obtained from the corresponding carvones by a general procedure.[56b] The progress of the purifications was monitored by measuring mp and optical rotation, and enantiomeric purity of the final (*R*)-(−)-carvoxime [≥99% ee; mp 71.5–72.5 °C, $[\alpha]^{26}_D$ −44.0° (*c* 1.00, $CHCl_3$) [lit.[59] mp 72 °C, $[\alpha]^{18}_D$ −39.3° (EtOH)]] was determined by comparing the optical rotation value of (−)-carvone which was prepared by hydrolysis as described in the degradation procedure below. (*S*)-(+)-Carvoxime [mp 71.5–72.5 °C, $[\alpha]^{25}_D$ +44.0° (*c* 1.00, $CHCl_3$) [lit.[59] mp 72 °C; $[\alpha]^{18}_D$ +39.5°]] was ≥98% ee based on the comparison of the optical rotations with its enantiomer. Counting and recrystallization were repeated until the SA of the sample was constant. Usually, two recrystallizations were sufficient for this purpose.

Run 1. Samples of (−)-**16**-*t* (171 mg; total activity, 127 000 dpm) and 853 mg of (−)-**16** were dissolved in hexane, and the solution was concentrated. The diluted (−)-**16**-*t* was purified by chromatography using ether/hexane (1:10) as eluent, and the collected sample was crystallized in 50% aqueous ethanol (831 mg). Ten mg of the crystals was used for counting. The remainder was recrystallized, and 10 mg of the new crystals was used for counting. The averge SA was 20 371 ± 183 dpm/mmol: yield, 709 mg. **Run 2**: yield, 759 mg; average SA = 13 675 ± 294 dpm/mmol. The diluted and purified (+)-**16**-*t* was further diluted was 451 mg of the carrier: yield, 1203 mg; SA = 9502 dpm/mmol. **Run 3**: yield, 1230 mg; average SA = 21 271 ± 227 dpm/mmol. **Run 4**: yield, 986 mg; average SA = 9078 ± 146 dpm/mmol.

2-Methyl-5-(1-methylethenyl)-2-cyclohexen-1-one (Carvone, 17). Oxime (−)-**16** was hydrolyzed according to literature procedures[29] with substantial modification. A solution of 826 mg (5 mmol) of (−)-**16**, 2.2 g (25 mmol) of pyruvic acid, and 32 mg (0.33 mmol) of concd H_2SO_4 in 12.5 mL of ethanol was refluxed for 24 h. After evaporation of the ethanol, the residue was dissolved in hexane (20 mL) and washed with saturated $NaHCO_3$ (2 × 20 mL) and saturated NaCl (20 mL). After the organic fraction was dried ($MgSO_4$) and concentrated, the residue was purified by flash chromatography with ether/hexane (1:25) as eluent. Kugelrohr distillation at 130 °C (14 mm) gave 597 mg (80%) of (5*R*)-(−)-**17** as a colorless liquid. The IR spectral data are identical with those of commercial (−)-**17**: $[\alpha]^{26}_D$ −62.2° (neat) [lit.[60a] $[\alpha]^{20}_D$ −62.46° (neat)].

The chemical yields for the [^{3}H]carvone samples (**17**-*t*) for runs 1–4 were as follows: 519 mg (82%); 829 mg (76%); 875 mg (79%); 760 mg (85%).

2-Methyl-5-(1-methylethenyl)cyclohexan-1-one (Dihydrocarvone, 18). The reduction of (5*R*)-(−)-**17** (533 mg) was carried out with 1 equiv of K-Selectride solution in THF according to the procedure of Fortunato and Ganem.[30] The two isomers formed were separated by flash chromatography using ether–hexane (1:40) as eluent. *cis*-Dihydrocarvone [(2*S*,5*R*)-**18**] obtained as a minor product was reequilibrated in 6 mL of methanol–10% aqeuous NaOH (2:1) for 5 h at 25 °C and chromatographed again to obtain additional trans product. Kugelrohr distillation of the trans product at 120 °C (14 mm) afforded 303 mg (54%) of (2*R*,5*R*)-(+)-**18**: $[\alpha]^{26}_D$ +16.6° (*c* 1.00, $CHCl_3$); ^{1}H-NMR δ 1.03 (d, 3H, *J* = 6.5 Hz, C*H*$_3$CH), 1.37 (qd, 1H, *J* = 13.0, 3.4 Hz), 1.5–1.8 (m, 1H), 1.74 (s, 3H, CH_3), 1.9 (m, 1H), 2.1 (m, 1H), 2.2–2.5 (m, 4H), 4.73, 4.76 (2s, 2H, CH_2=). The IR spectral data agree with the literature values.[61]

The radioactive [^{3}H]dihydrocarvone (**18**-*t*) samples were also Kugelrohr distilled after chromatographic purification, and the chemical yields for runs 1–4 were as follows: 300 mg (57%); 436 mg (52%), 550 mg (62%); 501 mg (65%), respectively.

1,2-Dimethyl-5-(1-methylethenyl)cyclohexan-1-ol (19). A solution of 609 mg (4.0 mmol) of (+)-**18** in 10 mL of ether was stirred and cooled at −78 °C as 5 mL of 1.24 M CH_3Li in ether was added. After 1 h at −78 °C, 10 mL of saturated NH_4Cl and 6 mL of water were added. The aqueous layer was extracted with hexane (3 × 10 mL). The combined organic extracts were washed with water (20 mL) and saturated NaCl (20 mL), dried ($MgSO_4$), and concentrated. Purification by flash chromatography with ether/hexane (1:5) as eluent and subsequent Kugelrohr distillation at 130 °C (10 mm) gave 581 mg (86%) of (1*S*,2*R*,5*R*)-(+)-**19**: $[\alpha]^{25}_D$ +26.4° (*c* 1.00, $CHCl_3$); IR (neat) ν_{max} 3486 (OH), 2965 (CH), 2926 (CH), 1645, 1451 (C=C), 1372, 1150, 930, 885 cm^{-1}; ^{1}H NMR δ 0.92 (d, 3H, *J* = 6.0 Hz, =(C*H*$_3$)CH–), 1.20 (s, 3H, –(C*H*$_3$)C(OH)–), 1.1–1.6 (m, 7H, cyclohexyl-H), 1.71 (s, 3H, C*H*$_3$C(=CH$_2$)–), 1.7–1.8 (m, 1H, –(CH$_3$)C*H*–), 2.23 (tt, 1H, *J* = 12.4, 3.2 Hz, CH$_2$=C(CH$_3$)C*H*–), 4.68 (s, 2H, C*H*$_2$=C-(CH$_3$)–); ^{13} NMR δ 14.9, 21.0, 28.9, 30.6, 31.5, 40.0, 40.3, 45.3, 71.4 (COH), 108.3 (CH_2=), 150.2 (CH_2=*C*). Anal. Calcd for $C_{11}H_{20}O$: C, 78.51; H, 11.98. Found; C, 78.66; H, 11.74.

Radioactive Degradations. Run 1: yield, 258 mg (78%). The product **19**-*t* was further diluted with 303 mg of the carrier (+)-**19**. Kugelrohr distillation gave 543 mg of the diluted (+)-**19**-*t*: SA = 9310 dpm/mmol. **Run 2**: yield, 407 mg (85%); SA = 9426 dpm/mmol. **Run 3**: yield, 479 mg (79%); SA = 20 509 dpm/mmol. **Run 4**: yield, 428 mg (77%); SA = 8870 dpm/mmol.

Ozonolysis of 19. Bis(4,4-dimethyl-2,6-dioxocyclohexyl)-methane (Formaldomedone, 20). A solution of 168 mg (1.0 mmol) of (+)-**19** and 202 μL (5.0 mmol) of methanol in 10 mL of CH_2Cl_2 was magnetically stirred at −65 to −70 °C as a stream of ozone in oxygen was bubbled through it until a blue color appeared. After purging with oxygen until the blue color disappeared, N_2 was bubbled into the solution for 3–5 min. After 1.5 mL of dimethyl sulfide was added, the solution was stirred at rt for 1 h and extracted with water (1 × 10 mL, 2 × 5 mL). The combined aqueous extracts were stirred with 2 drops of pyridine and 282 mg (2.00 mmol) of dimedone at rt for 2 h and at 0 °C for 2 h.[56c] The precipitate was filtered, washed with cold water, and crystallized from methanol to give 228 mg (78%) of **20** as white crystals: mp 188–189 °C (lit.[60b] 189–190 °C); IR (KBr) ν_{max} 2963 (CH), 2629 (br, OH), 1611 (C=O), 1580 (C=O), 1377, 1248, 1086, 876 cm^{-1}; ^{1}H NMR δ 1.04 (s, 12H, (CH$_3$)$_2$C), 2.28 (s, 8H, 4CH$_2$CO), 3.15 (s, 2H, CH$_2$).

Radioactive Degradations. Run 1: yield, 750 mg (82%); average SA = 3335 ± 19 dpm/mmol (36% of SA of (−)-**19**-*t*). **Run 2**: yield, 493 mg (72%); average SA = 2654 ± 42 dpm/mmol (28% of SA of (+)-**16**-*t*). **Run 3**: yield, 641 mg (79%); average SA = 470 ± 7 dpm/mmol (2.2% of SA of (−)-**16**-*t*). **Run 4**: yield, 415 mg (64%); average SA = 336 ± 8 dpm/mmol (3.7% of SA of (+)-**16**-*t*).

1,2-Dimethyl-5-(1-oxoethyl)cyclohexan-1-ol (21). The combined CH_2Cl_2 layers from the preceding ozonolysis were dried ($MgSO_4$) and concentrated. Purification by chromatography using ether–hexane (1:7) as eluent gave 132 mg (78%) of (1*S*,2*R*,5*R*)-(+)-**21**: $[\alpha]^{25}_D$ +10.7° (*c* 1.00, $CHCl_3$); IR (neat) ν_{max} 3474 (OH), 2965 (CH), 2932 (CH), 2859 (CH), 1701 (C=O), 1453, 1374, 1354, 1177, 1152 cm^{-1}; ^{1}H NMR δ 0.92 (d, 3H, *J* = 6.3 Hz, C*H*$_3$CH), 1.23 (s, 3H, CH_3), 1.2–1.6 (m, 5H, cyclohexyl-H), 1.8–2.0 (m, 3H, cyclohexyl-H), 2.14 (s, 3H, CH_3CO–), 2.76 (tt, 1H, *J* = 12.3, 3.3 Hz, COCH). Anal. Calcd for $C_{10}H_{18}O_2$: C, 70.55; H, 10.66. Found: C, 70.15; H, 10.77.

For the degradation with labeled products, a portion (50–60%) of crude **21**-*t* was treated with CH_3Li without purification as described below.

5-(2-Hydroxy-2-propyl)-1,2-dimethylcyclohexan-1-ol (22). Crude **21** in CH_2Cl_2 from the preceding ozonolysis of **19** was dried ($MgSO_4$) and concentrated. A solution of the residue in 5 mL of ether was stirred at 0 °C as 3 mL (4.2 mmol) of 1.4 M CH_3Li in ether was added. After 0.5 h at 0 °C and 1 h at rt, 5 mL of saturated NH_4Cl and 2 mL of water were added at 0 °C, and the aqueous fraction was extracted with hexane (3 × 10 mL). The combined organic fractions were washed with water (2 × 20 mL) and saturated NaCl, dried ($MgSO_4$), and concentrated. Purification of the residue by flash chromatography with ethyl acetate–hexane (2:3) as eluent followed by crystallization from hexane gave 114 mg (61%) of (1*S*,2*R*,5*R*)-(+)-**22** as white crystals: mp 77–78.5 °C; $[\alpha]^{26}_D$ +32.4° (*c* 1.00, $CHCl_3$); IR (KBr) ν_{max} 3414 (OH), 2969 (CH), 1462, 1374, 1246, 1159, 1132, 932, 922 cm^{-1}; ^{1}H NMR δ 0.91 (d, 3H, *J* = 6.1 Hz, C*H*$_3$CH), 1.15, 1.17 (2s,

(59) *The Merck Index*, 11th ed.; Merck: Rahway, NJ, 1989; p 287.
(60) (a) *CRC Handbook of Chemistry and Physics*, 70th ed.; Weast, R. C., Ed.; CRC: Boca Raton, FL, 1989; p C-198; (b) p C-278.
(61) Vig, O. P.; Chugh, P.; Matta, K. L. *J. Ind. Chem. Soc.* **1968**, *45*, 748.

6H, $2CH_3$), 1.20 (s, 3H, $(CH_3)C(OH)$), 1.0–1.9 (m, 10H, cyclohexyl-H and 2 OH); ^{13}C NMR δ 14.9, 26.6, 27.2, 27.5, 29.1, 30.6, 40.0, 41.1, 43.9, 71.4 (CCH_3OH), 72.5 ($(CH_3)_2COH$); MS (FI, 70 °C) *m/z* (rel int) 186 (M^+, 48.6), 171 (54.1), 168 (71.6), 150 (52.0), 128 (58.8), 110 (100.0). Anal. Calcd for $C_{11}H_{22}O_2$: C, 70.92; H, 11.90. Found: C, 70.90; H, 11.93.

Radioactive Degradations. Run 1: yield 204 mg (70% from (+)-19-*t*); average SA = 4097 ± 322 dpm/mmol (44% of SA (of (+)-19-*t*). **Run 2**: yield, 159 mg (61% from (−)-19-*t*); average SA = 5048 ± 8 dpm/mmol (53% of SA of (−)-16-*t*). **Run 3**: yield, 200 mg (64% from (+)-19-*t*); average SA = 20 598 ± 192 dpm/mmol (97% of SA of (+)-16-*t*). **Run 4**: yield, 159 mg (58% from (−)-19-*t*); average SA = 8849 ± 74 dpm/mmol (98% of SA of (−)-16-*t*).

Measurement of the Total Activity in Crude 21 and Tritium Washout Experiment. A solution of purified **21** (1 mg) and 30 mg of anhydrous K_2CO_3 in 2 mL of ($^2H_4$)methanol was stirred at rt for 10 min. GC–MS analysis of the exchanged product showed no M^+ peak; an (M + 5) peak (presumed to arise from OD, CD, and CD_3 exchange) was observed as the molecular ion. The same experiment was performed, and the solution was analyzed by ^1H NMR spectroscopy after the undissolved K_2CO_3 was filtered off. The integrations indicated that all of the methyl protons ($COCH_3$) were exchanged within 10 min, whereas two thirds of the methine proton ($CHCOCH_3$) were exchanged. After treatment with K_2CO_3, **21** was purified by flash chromatography using ether–hexane (3:5) as eluent and treated with CH_3Li to afford (+)-**22**. Purification of the crude product and subsequent recrystallization gave crystalline (+)-**22**. For the experiments with tritium-labeled product, the exchanges were performed with K_2CO_3 in methanol for 4 h to allow for the possibility of slow exchange due to an isotope effect.

Radioactive Degradations. An aliquot of the remaining crude **21**-*t* (2–3%) was used to measure the total radioactivity, and the rest was used for the tritium washout experiment described above. **Run 1**: total activity in the crude (+)-**21**-*t*, 19 067 dpm (65% of (+)-19-*t*). After the tritium washout experiment, yield of (+)-**22**, 133 mg (47% from (+)-19-*t*); SA = 71 dpm (0.8% of SA of (+)-19-*t*). **Run 2**: total activity in the crude (−)-**21**-*t*, 12 900 dpm (59% of (−)-19-*t*). After the tritium washout experiment, yield of (−)-**22**, 81 mg (51% from (−)-19-*t*); SA = 280 ± 30 dpm (2.9% of SA of (+)-16-*t*). **Run 3**: total activity in the crude (+)-**21**-*t*, 53 153 dpm (93% of (+)-19-*t*). After the tritium washout experiment, yield of (+)-**22**, 89 mg (56% from (+)-19-*t*); SA = 167 ± 1 dpm (0.8% of SA (−)-16-*t*). **Run 4**: total activity in the crude (−)-**21**-*t*, 19,665 dpm (90% of (−)-19-*t*). After the tritium washout experiment, yield of (−)-**22**, 89 mg (53% from (−)-19-*t*); SA = 94 ± 1 dpm (1.0% of SA of (+)-16-*t*).

Incubation and Determination of Isotope Content of Olefinic Products. All assays with 1 and 1-d_4 were done in duplicate. To each sample (6 mL) in a screw cap tube at 0 °C was added the substrate to saturation (70 μM), and the resulting mixture was extracted three times with 1-mL portions of pentane to remove any potentially interferring materials arising from either the enzyme preparation or substrate. The reaction was initiated by adding $MgCl_2$ to 15 mM, and 1 mL of pentane was added as an overlay to trap volatile products. Samples were incubated at 32 °C for 2 h, and the reaction was stopped by rapid chilling. *p*-Cymene (21.2 nmol) was then added as an internal standard, and the sample was thoroughly mixed and the phases were separated by centrifugation. The pentane layer and two additional 1-mL pentane extracts were then passed through a short column of silica gel overlaid with $MgSO_4$ to yield the terpene hydrocarbon fraction. The combined pentane eluate was concentrated to about 0.4 mL by short-path distillation to minimize losses of volatile products and analyzed by GC and combined GC–MS.

Incubation and Determination of Isotope Effects and Product Distributions. All assays with 1-d_6,*t* and 1-^3H-labeled control substrate were performed in triplicate, generally at the 1-mL scale. The enzymatic reaction was initiated by the addition of 15 mM $MgCl_2$ and the substrate (at 25 μM, with one set of assays at 50 μM), and 1 mL of pentane was overlaid the aqueous reaction mixture as a trap. Following incubation at 32 °C for 1 h (<15% substrate conversion to ensure linearity), the pentane-soluble products were isolated as before, and the extracts were passed through silica gel to obtain the olefin fraction to which authentic carrier standards were added.

For the assay of (+)- or (−)-bornyl pyrophosphate cyclases (sage)[57a] the aqueous phase remaining after enzyme incubation was treated with phosphatase and apyrase to release the corresponding borneol which was extracted into ether following the addition of authentic carrier. The ether extracts were treated with OsO_4 to remove interfering unsaturated materials (e.g., geraniol) and the borneol was purified by TLC.[57a]

In all cases, radioactivity in the final extracts was determined by aliquot counting, and the samples were concentrated under a Vigreux column in preparation for radio-GC analysis. By comparing the relative composition of the olefin fractions (with carrier) before and after the concentration step, it was possible to compensate in the calculations for differential product loss during this step.

Acknowledgment. This investigation was supported in part by grants GM 13956 (R.M.C.) at the University of Illinois and GM 31354 (R.B.C.) at Washington State Univerity from the National Institutes of Health. We thank the reviewers for their constructive criticisms and useful comments.

Supplementary Material Available: Calculations of observed intramolecular isotope effects and derivations of eqs 4 and 5 (7 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.