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Trichodiene Biosynthesis and the Stereochemistry of the Enzymatic Cyclization of Farnesyl Pyrophosphate

DAVID E. CANE,[1] HYUN-JOON HA, CHRISTOPHER PARGELLIS, FELIX WALDMEIER, STEPHEN SWANSON, AND PUSHPALATHA P. N. MURTHY

Department of Chemistry, Brown University, Providence, Rhode Island 02912

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Cyclization of *trans,trans*-[1-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate (**2a**) by a preparation of trichodiene synthetase isolated from the fungus, *Trichothecium roseum*, gave trichodiene (**5a**), which was shown by chemical degradation to retain both tritium atoms of the precursor at C-11. Incubation of 1*S*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate (**2b**) and 1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate (**2c**) with trichodiene synthetase and degradation of the resulting labeled trichodienes, **5b** and **5c**, established that the displacement of the pyrophosphate moiety from C-1 of the precursor and formation of the new C-C bond in the formation of trichodiene takes place with net retention of configuration. These results are accounted for by an isomerization–cyclization mechanism involving the intermediacy of nerolidyl pyrophosphate (**4**).

INTRODUCTION

Over the last several years, intensive investigations in several laboratories have begun to provide a detailed picture of the mechanism by which the universal isoprenoid precursors, geranyl pyrophosphate (**1**) and farnesyl pyrophosphate (**2**), are converted to cyclic monoterpenes (*1*) and sesquiterpenes (*2*), respectively. Of particular importance in these studies has been the development of cell-free preparations from a variety of microorganisms and higher plants (*3, 4*). The use of these cyclase preparations, in either crude or partially purified form, not only avoids the often substantial barriers to allylic pyrophosphate incorporation which characterize intact cells but alleviates interference by competing hydrolytic and oxidative metabolism which has obscured the results of earlier efforts to elucidate the details of the terpenoid cyclization process. One of the key issues in the study of terpenoid cyclizations has arisen from the recognition that the cyclization of a *trans*-allylic pyrophosphate precursor to a six-membered ring necessarily involves isomerization of the *trans*-2,3 double bond in order to avoid formation of a *trans*-cyclohexene (*2, 5*). It is now evident that the various redox theories for this isomerization which achieved some currency in the early 1970s were largely based on either inadequate or flawed experimental data. Instead, *trans–cis* isomerization–cyclization appears to involve intermediate generation of the corresponding C_{10} and C_{15} tertiary allylic pyrophosphates, linalyl pyrophosphate (**3**) and nerolidyl

[1] To whom correspondence should be addressed.

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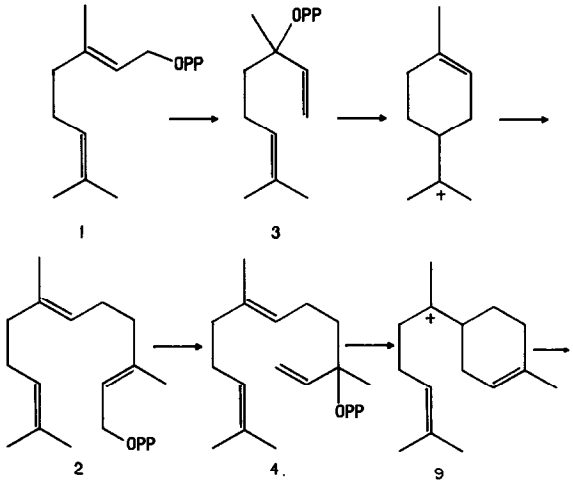


SCHEME 1. Cyclization of geranyl pyrophosphate (**1**) and *trans,trans-farnesyl pyrophosphate* (**2**) to α-terpinyl and bisabolyl cations, respectively, via the corresponding tertiary allylic isomers, linalyl (**3**) and nerolidyl pyrophosphate (**4**).

pyrophosphate (**4**), which in turn can undergo rotation about the newly formed C-2,3 single bond and subsequent cyclization. We describe below our own investigations (*6, 7*) of the enzymatic isomerization–cyclization of farnesyl pyrophosphate to trichodiene (**5**) (*8*), the parent hydrocarbon of the trichothecane family of sesquiterpenes (*2, 9*).

The trichothecanes are a group of nearly 50 fungal metabolites which share a characteristic bicyclic sesquiterpene skeleton and display a variety of potent physiological activities, including antibiotic, antileukemic, phytotoxic, and neurotoxic effects (*10*). Among the latter metabolites, fusariotoxin (T-2 toxin) and nivalenol have recently achieved a certain notoriety in connection with the controversy over "Yellow Rain" in contested areas of Asia (*11*). The mevalonoid origin of trichothecin (**6**), an antibiotic metabolite of the apple mold, *Trichothecium roseum,* was first established by Jones and Lowe (*12*), and subsequent investigations in the laboratories of Hanson, Nozoe, and Tamm have provided a detailed picture of the biosynthesis of **6** and related compounds (*2, 9*). The role of farnesyl pyrophosphate as a precursor has been confirmed (*13*) and the cyclization of **2** has been shown to involve a novel 1,4-hydride shift. The product of this cyclization, trichodiene (**5**), was first isolated by Nozoe from mycelial extracts of *T. roseum* and shown to be an intermediate in the biosynthetic pathway by refeeding of [15-^{3}H]-**5** and incorporation into more oxidized metabolites (*8*). Finally, Hanson has described a cell-free preparation from *T. roseum* which mediates the conversion of *trans,trans*-farnesyl pyrophosphate to trichodiene (*14, 15*). Although several of the detailed conclusions drawn from the latter study have proved to be in error, the development of an active cell-free system was itself an important experimental accomplishment which has provided a powerful tool for the further investigation of the key cyclization mechanism.

The original report on the cell-free cyclization of farnesyl pyrophosphate had

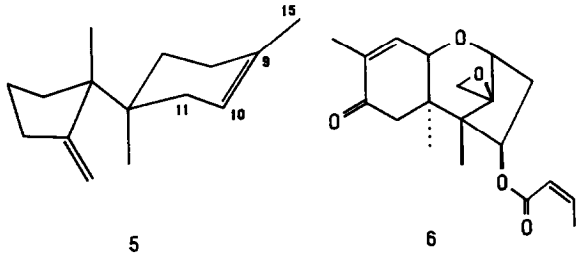
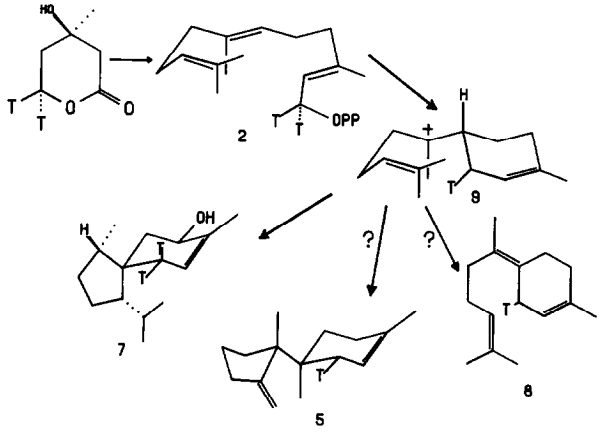


FIG. 1. Trichodiene (**5**) and trichothecin (**6**).

included the claim that incubation of [1,5,9-$^{3}H_6$,4,8,12-$^{14}C_3$]-*trans,trans*-farnesyl pyrophosphate ($^{3}H/^{14}C$ atom ratio, 6:3) had resulted in formation of trichodiene which had lost an equivalent of tritium ($^{3}H/^{14}C$ atom ratio, 5.2:3), consistent with a purported redox mechanism for the isomerization–cyclization process. This result, which was based exclusively on observed isotope ratios without verification of the presumed distribution of label by chemical degradation, did not appear to be consistent with independent studies of the whole cell biosynthesis of the sesquiterpene coccinol (**7**), a metabolite of *Fusidium coccineum* which was reported to be formed without loss of tritium from [5-$^{3}H_2$,2-^{14}C]mevalonate (*2, 16*). On the other hand, earlier studies of the cell-free biosynthesis of a third metabolite, bisabolene (**8**), by extracts of *Andrographis paniculata* tissue cultures, had appeared to establish loss of isotope from C-1 of farnesyl pyrophosphate during cyclization (*17, 18*). Since all three metabolites are believed to be derived from a common bisabolyl cation intermediate (**9**), we chose to reexamine the cell-free biosynthesis of trichodiene from *trans,trans*-farnesyl pyrophosphate. We have now demonstrated that this isomerization–cyclization takes place without loss of

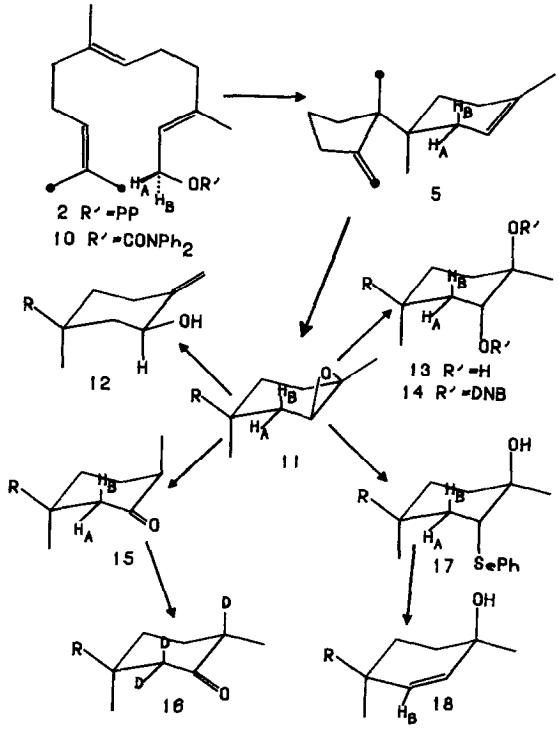


SCHEME 2. Reported retention or loss of tritium from C-1 of farnesyl pyrophosphate in the formation of three bisabolyl cation-derived sesquiterpenes, coccinol (**7**), trichodiene (**5**), and bisabolene (**8**). The formation of both **5** and **8** has subsequently been shown to take place without loss of tritium (*vide infra*).

tritium from C-1 of the substrate and that the displacement of the pyrophosphate moiety and formation of the new C–C bond occur with net retention of configuration.

RESULTS

Our initial investigations of the trichodiene synthetase system focused on the isolation of the cyclase activity and on defining appropriate conditions for preparative-scale incubations. The required sample of *trans,trans*-[1-$^{3}H_2$,12,13-^{14}C]farnesyl pyrophosphate (**2a**) was prepared as previously described (*19*), while a portion of the free farnesol was converted to the corresponding [1-$^{3}H_2$,12,13-^{14}C]farnesyl diphenylurethane (**10a**) which was recrystallized to constant activity. Originally we used an adaptation of the literature procedure (*14, 15*) for the preparation of the cell-free extract; these conditions were significantly modified as we gained more experience with the isolation and manipulation of the synthetase. According to our original procedure (Procedure A), crude trichodiene synthetase was obtained by suspending the mycelium from a 4-day-old culture of *T. roseum* ATCC 8685 in 100 mM potassium phosphate buffer, pH 7.0, and passing the



SCHEME 3. Enzymatic cyclization of [1-$^{3}H_2$,12,13-^{14}C]farnesyl pyrophosphate (**2a**)(H_A=H_B=T), 1*S*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate (**2b**) (H_A=T, H_B=H), and 1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate (**2c**) (H_A=H, H_B=T) to trichodiene and degradation to locate the label.

suspension through a prechilled French pressure cell at 18,000 psi and 4°C. Broken cell debris was removed by centrifugation. The S_{17} fraction, containing 10 mg protein/ml, was extremely unstable and was used immediately for subsequent incubations. Small-scale exploratory experiments established that neither the efficiency of conversion of the substrate farnesyl pyrophosphate nor the $^3H/^{14}C$ ratio of the resultant isolated trichodiene were affected by the presence of a mixture of NAD^+, NADH, and NADPH, in contrast to the role imputed to these coenzymes by the earlier study. Nicotinamides were therefore omitted from all subsequent incubations, which initially were carried out at 30°C in the presence of 10 mM $MgCl_2$, 0.4 mM $MnCl_2$, 0.5 mM DTT,[2] and 50 μM farnesyl pyrophosphate. Typical trichodiene synthetase preparations showed an activity of 0.05 nmol trichodiene mg protein^{-1} h^{-1} with the greater proportion of the farnesyl pyrophosphate substrate being consumed by contaminating phosphatase activity.

Further experimentation eventually led to the development of a superior isolation protocol (Procedure B). Harvested mycelia of 10-day-old cultures of *T. roseum* were washed successively with 1 M KCl and 0.8 M NaCl to remove exogeneous proteases, then suspended in homogenization buffer containing 50 mM potassium phosphate, pH 6.5, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol. The cells were ruptured by rapid stirring with 0.5-mm glass beads in a jacketed bead-beater cell at 4°C. The resultant supernatant was treated with insoluble PVPP and concentrated by precipitation with 80% ammonium sulfate. The protein pellet was redissolved in 20 mM piperizine–HCl, pH 6.5, containing 4 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.02% sodium azide, and 10% glycerol. Specific activities of the resulting trichodiene synthetase preparations were as high as 0.84 nmol trichodiene mg protein^{-1} h^{-1}, but showed significant variation from one culture of *T. roseum* to another. Other resuspension buffer systems which were examined with comparable results included pH 8.1 Tris, pH 7.2 potassium phosphate, pH 5.8 piperazine, and pH 5.05 malate. In each case approximately 50% of the cyclase activity was lost after 44 h at 4°C. The inclusion of 2-mercaptoethanol was essential and glycerol up to 30% (v/v) also improved stability.

In order to verify the specificity of the enzymatic cyclization reaction and to examine the fate of the C-1 hydrogen atoms of farnesyl pyrophosphate, a preparative-scale incubation was carried using trichodiene synthetase obtained from 2.4 liters of *T. roseum* culture according to Procedure A (1.6 g of S_{17} protein) and 10 μmol of [1-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate ($^3H/^{14}C$ atom ratio, 2:2) (Table 1). The resulting labeled trichodiene, after dilution with 5 mg of inactive carrier trichodiene, was extracted with pentane and purified by preparative silica gel TLC. The recovered trichodiene (1.8×10^4 dpm) was further diluted with 105 mg of (+)-trichodiene[3] and treated with 1.05 equiv of *m*-chloroperbenzoic acid in

[2] Abbreviations used: DTT, dithiothreitol; HLADH, horse liver alcohol dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; NADH, dihydronicotinamide adenine dinucleotide; PVPP, polyvinylpolypyrrolidone; THF, tetrahydrofuran.

[3] A persistent logistical obstacle to all the work described here was the limited availability of authentic trichodiene for working out degradation procedures and for use as inactive carrier for the analysis of preparative-scale enzyme incubations. The natural product is normally produced in titers of less than 1 mg/liter of *T. roseum*. On a number of occasions we have succeeded in raising these

TABLE 1

CONVERSION OF [1-$^3H_2$,12,13-^{14}C]FARNESYL PYROPHOSPHATE (**2a**) TO TRICHODIENE (**5a**) BY TRICHODIENE SYNTHETASE AND DISTRIBUTION OF THE LABEL IN **5a**

Compound	^{14}C Specific activity (dpm/mmol)	$^3H/^{14}C$	Atom ratio
2a[a]	1×10^8	9.04 ± 0.12[b]	2:2
5a[c]	5.06×10^4[d]	8.70	
11a	5.32×10^4	9.29	
14a	6.14×10^4	8.23 ± 0.12	1.80:2
16a	6.23×10^4	0.0	0:2
18a	5.84×10^4	4.49 ± 0.09	1.0:2

[a] Amount incubated, 1×10^6 dpm ^{14}C (10 μmol).
[b] Based on recrystallization of farnesyl diphenylurethane (**10a**).
[c] Total recovered activity, 1.8×10^4 dpm ^{14}C.
[d] Diluted to 110 mg (+)-trichodiene.

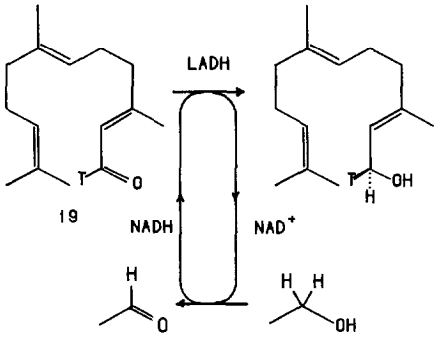
methylene chloride to yield the corresponding 9,10-epoxytrichodiene (**11a**) (*8*), the central intermediate in our planned degradation scheme. The stereochemistry of this epoxide was determined by rearrangement of an unlabeled sample of **11** with diethylaluminum tetramethylpiperidide (*20*) to yield the known allylic alcohol, **12** (*8*). Analysis of **12** by 270-MHz NMR established that the H-10 carbinyl proton was coupled to the vicinal H-11 protons with coupling constants $J = 4.9$ and 11.5 Hz, as expected for an axial proton. Since the bulky 1-methyl-2-methylenecyclopentyl substituent must be equatorial, **11** is therefore the β-epoxide. This conclusion is consistent with earlier observations by Richborn on the stereochemical course of 4-alkylcyclohexene epoxidation (*21*).

Treatment of a portion of the epoxide with 3% $HClO_4$ in aqueous THF gave the 9,10-diol, **13a**, which was converted to the corresponding crystalline 9,10-bis(dinitrobenzoate), **14a**. This crystalline derivative, which served as the reference for subsequent degradations, was recrystallized to constant activity and isotope ratio ($^3H/^{14}C$ atom ratio, 1.8:2). In order to confirm the apparent retention of both equivalents of tritium as well as establish the actual sites of labeling, a further portion of the epoxide was converted to the 10-ketone, **15a**, by exposure to lithium perchlorate in refluxing benzene (*22*). Exchange with sodium deuteroxide in refluxing dioxane-D_2O epimerized **15a** to the more stable equatorial methyl epimer, **16a**, with concomitant loss of >99% of all tritium activity. Since the olefinic H-10

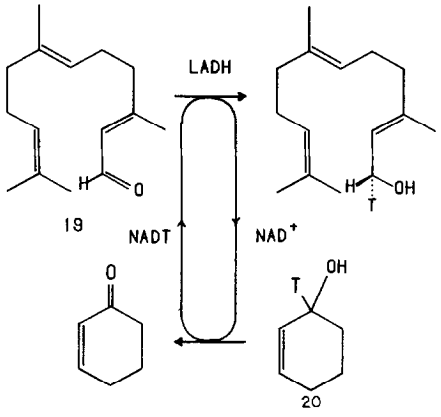
production levels to as much as 20 mg/liter by carrying out incubations over a 4-week period with exclusion of air from the fungal cultures. Whatever success has been achieved by this technique can be ascribed to the fact that there are no oxygen-dependent steps in the formation of trichodiene and that **5** is the last nonoxidized intermediate in the *in vivo* trichothecane biosynthetic pathway. Unfortunately, this modified fermentation method has proven to be not only time-consuming but capricious; in one 2-year period we were never able to accumulate more than 5–10 mg of trichodiene at a time in spite of continuous effort. We were therefore substantially assisted by the generous donation of nearly 1 g of synthetic (±)trichodiene prepared by R. H. Schlessinger and J. A. Schultz of the University of Rochester (*52*).

proton in trichodiene is known to be derived from H_{re}-4 of mevalonate (*2, 9*), all tritium in trichodiene derived from the cyclization of [1-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate was thus shown to be located at the expected site, H-11. It should also be noted that the observed formation of the less stable axial methyl ketone, **15**, by acid-catalyzed epoxide rearrangement reinforces the assigned β configuration of the epoxide moiety in **11**. Further confirmation of the tritium distribution was obtained by treatment of the remaining epoxide with sodium phenylselenide to yield the corresponding 9-hydroxy-10-phenylselenide, **17a**, which was subjected to *syn* selenoxide elimination by oxidation with sodium periodate followed by mild thermolysis in refluxing aqueous THF–MeOH (*23–25*). The derived allylic alcohol, **18a**, retained half the original tritium activity ($^3H/^{14}C$ atom ratio, 1 : 2), indicating an essentially equal distribution of label between H-11α and H-11β.

Having established rigorously that the enzymatic isomerization–cyclization of *trans,trans*-farnesyl pyrophosphate to trichodiene takes place without loss of hydrogen from C-1 of the precursor, we turned our attention to the stereochemical course of this conversion. We therefore required samples of both 1*S*-[1-^{3}H]- and 1*R*-[1-^{3}H]farnesyl pyrophosphate of high enantiomeric purity and high specific radioactivity. The requisite 1*S*-[1-^{3}H]farnesol was readily prepared by stereospecific reduction of [1-^{3}H]farnesal (**19**) using liver alcohol dehydrogenase (HLADH), a catalytic amount of NAD^+, and an excess of ethanol as the hydride source (*26*) (Scheme 4). Synthesis of the enantiomerically tritiated 1*R*-[1-^{3}H]farnesol required considerably more experimentation. With NADH recycling systems in which tritium is to be transferred, isotope effects on both the initial reduction of NAD^+ by the tritiated hydride source and on the coupled reduction of substrate by the transiently generated 4*R*-[4-^{3}H]NADH can severely limit the observed radiochemical yield, especially when the hydride source is used in large stoichiometric excess, as is normally the case when ethanol is used as reductant. To overcome this problem, Battersby has described the use of [1-^{3}H]cyclohexanol, readily prepared with high specific activity by borotritide reduction of cyclohexanone (*27, 28*). The reduction potential of the cyclohexanol–cyclohexanone couple, however, is insufficient to allow effective coupling to the reduction of a conjugated aldehyde to an



SCHEME 4. Preparation of 1*S*[1-^{3}H]farnesol with NADH recycling using ethanol as hydride source.



SCHEME 5. Preparation of 1*R*-[1-^{3}H]farnesol with NADH recycling using [1-^{3}H]cyclohexenol (**20**) as hydride source.

allylic alcohol. For example, by measuring the position of the redox equilibrium for a series of alcohol/NAD$^+$ incubations with HLADH as catalyst, we have estimated that K_{eq} at pH 8.7 for Reaction [1] is ca. 0.2. When a 2° allylic alcohol such as cyclohexenol is substituted as reductant, however, the equilibrium is shifted to favor conjugated aldehyde reduction; we have estimated K_{eq} (pH 8.7) for Reaction [2] to be ca. 6. [cf. Ref. (*28*)]

Cyclohexanol + 3,3-dimethylacrolein → cyclohexanone + dimethylallyl alcohol [1]

Cyclohexenol + farnesal → cyclohexenone + farnesol [2]

Based on the above considerations, we therefore prepared 25 mCi of [1-^{3}H]cyclohexenol (**20**) (0.8 mCi/μmol) by reduction of cyclohexenone with potassium [^{3}H]borohydride (100 mCi, 13.21 mCi/μmol) in aqueous base. After dilution with glycine buffer, pH 8.8, a portion (9 μmol) of the resulting **20** was incubated for 19 h at room temperature with 16 μmol of *trans,trans*-farnesal (**19**) in 0.1 M phosphate buffer, pH 7.2, in the presence of HLADH and 5 mol% NAD$^+$ (Scheme 5). The resulting 1*R*-[1-^{3}H]farnesol was diluted with inactive farnesol and isolated in 26% radiochemical yield, after chromatographic separation from *cis,trans*-1*R*-[1-^{3}H]farnesol, generated by competing reduction of the corresponding *cis,trans*-farnesal. The latter substrate is formed by isomerization of *trans,trans*-farnesal under the incubation conditions. Although shorter incubation times improved the geometric purity of the farnesol product, this improvement was accompanied by an unacceptable reduction in radiochemical yield.[4] To confirm the configurational purity of the 1*R*-[1-^{3}H]farnesol, a portion of this material was mixed with [12,13-^{14}C]farnesol (^{3}H/^{14}C = 69.3) and incubated with HLADH in the presence of excess

[4] Since completion of the work described, we have found that the coupled formate dehydrogenase–liver alcohol dehydrogenase system described by Whitesides (*53*) is considerably more convenient for the preparation of chirally tritiated allylic alcohols, although long reaction times are still necessary for the efficient transfer of tritium from [^{3}H]formate.

NAD^+. The recovered farnesal was essentially devoid of tritium ($^3H/^{14}C = 0.7$), as expected.

Each of the chirally tritiated farnesol samples, was separately mixed with *trans,trans*-[12,13-^{14}C]farnesol as internal standard and, after removal of a portion of the resultant mixture for preparation of farnesyl diphenylurethanes, (**10b**) and (**10c**) ($^3H/^{14}C$ atom ratio, 1 : 2), was converted to the corresponding *trans,trans*-1*S*-[1-^{3}H,12,13-^{14}C]- or 1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate, **2b** or **c**. Incubation with freshly prepared trichodiene synthetase, isolated by either Procedure A (**2b**) or Procedure B (**2c**), gave labeled trichodienes (**5b** and **5c**), which were diluted with inactive carrier and subjected to the previously developed degradation sequence in order to establish the location and stereochemistry of tritium labeling. As summarized in Table 2 and illustrated in Scheme 3, the complete retention of precursor tritium was confirmed for both enantiomeric series, as evidenced by the isotope ratios of the derived bis(*p*-nitrobenzoate) esters **14b** ($^3H/^{14}C$ atom ratio, 0.90 : 2) and **14c** ($^3H/^{14}C$ atom ratio, 0.95 : 2). Tritium in trichodiene (**5b**) derived from 1*S*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate was shown to reside exclusively in the 11α position, as demonstrated by the absence of tritium in the allylic alcohol, **18b**, obtained by *syn* elimination of the selenoxide corresponding to **17b**. Conversely, the allylic alcohol, **18c**, retained its tritium label ($^3H/^{14}C$ atom ratio, 0.95 : 1), consistent with the expected 11β configuration in trichodiene (**5c**) derived from 1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate. The location of tritium label in **5c** was unambiguously established by exchange of the derived ketone **15c** with sodium hydroxide in aqueous dioxane to give the equatorial ketone **16c** ($^3H/^{14}C$ atom ratio, 0.05 : 2).

DISCUSSION

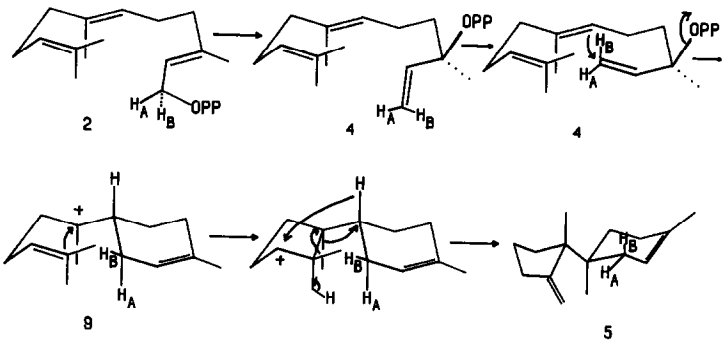
The observation that the enzymatic isomerization–cyclization of *trans,trans*-farnesyl pyrophosphate to trichodiene takes place without loss of either C-1 hydrogen atom of the precursor, despite earlier claims to the contrary, rules out all

TABLE 2

ENZYMATIC CONVERSION OF 1*S*-[1-^{3}H,12,13-^{14}C]FARNESYL PYROPHOSPHATE (**2b**) AND 1*S*-[1-^{3}H,12,13-^{14}C]FARNESYL PYROPHOSPHATE (**2c**) TO TRICHODIENES **5d** AND **5c** AND DISTRIBUTION OF THE LABEL

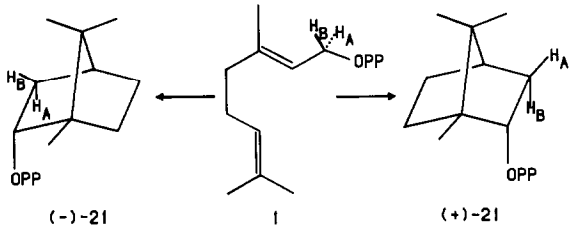
Compound	^{14}C Specific activity (dpm/mmol)	$^3H/^{14}C$	Atom ratio	Compound	^{14}C Specific activity (dpm/mmol)	$^3H/^{14}C$	Atom ratio
2b	1.3×10^9	3.46 ± 0.10^a	1 : 2	**2c**	4.35×10^9	3.46 ± 0.07^a	1 : 2
5b	$2.48 \times 10^{5\,b}$	3.49		**5c**	$2.37 \times 10^{4\,c}$	3.52	
11b	$1.08 \times 10^{5\,d}$	3.30		**11c**	1.75×10^4	3.41	
14b	1.34×10^5	3.14 ± 0.09	0.90 : 2	**14c**	2.31×10^4	3.22 ± 0.09	0.95 : 2
18b	0.89×10^5	0.32 ± 0.01	0.10 : 2	**18c**	1.92×10^4	3.29 ± 0.10	0.95 : 2
				16c	1.96×10^4	0.24 ± 0.01	0.05 : 2

[a] Based on recrystallization of farnesyl diphenylurethane.
[b] Diluted to 21 mg with (+)-trichodiene.
[c] Diluted to 55 mg with (±)-trichodiene.
[d] Diluted to 32 mg with unlabeled **11**.



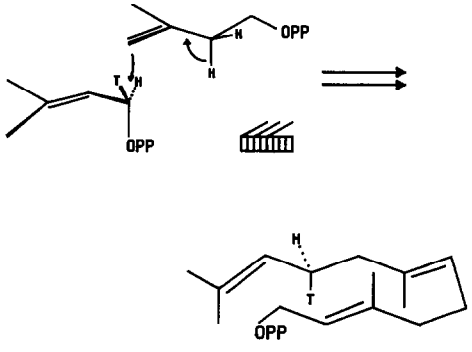
SCHEME 6. Mechanism of enzymatic isomerization–cyclization of farnesyl pyrophosphate to trichodiene via nerolidyl pyrophosphate.

redox mechanisms proposed to date, and strongly favors the mechanism illustrated in Scheme 6 involving the intermediacy of the tertiary allylic isomer nerolidyl pyrophosphate (**4**). Following the publication of our initial report on the complete retention of tritium label in the enzymatic formation of trichodiene from [1-$^{3}H_2$,12,13-^{14}C]farnesyl pyrophosphate (*6*), Overton and his collaborators reexamined their earlier work on bisabolene biosynthesis. Using a sample of [1-$^{3}H_2$,12,13-^{14}C]farnesyl pyrophosphate prepared in our laboratories, these workers concluded that their earlier observations had been in error and that, in fact, enzymatic cyclization of farnesyl pyrophosphate to bisabolene takes place without loss of label from C-1 of the allylic pyrophosphate precursor (*29, 30*). The postulated intermediacy of nerolidyl pyrophosphate is further supported by the finding that, in the biosynthesis of trichodiene, nucleophilic displacement of the pyrophosphate moiety by the C-6,7 double bond of the acyclic precursor takes place with net retention of configuration. Completely analogous results have been obtained by Croteau for the enzymatic isomerization–cyclization of geranyl pyrophosphate to both (+)- and (−)-bornyl pyrophosphate (**21**), mediated by cyclases isolated from sage and tansy, respectively (*31, 32*) (Scheme 7). The observed stereochemical results in both the monoterpene and the sesquiterpene series are a direct consequence of the required 2,3 double isomerization of the *trans,trans*-allylic pyrophosphate substrates, geranyl pyrophosphate and farnesyl pyrophosphate. Analogous, albeit indirect, evidence for the intermediacy of nerolidyl pyro-



SCHEME 7. Enzymatic conversion of 1*S*-[1-^{3}H]geranyl pyrophosphate (H_A=T, H_B=H) and 1*R*-[1-^{3}H]geranyl pyrophosphate (H_A=H, H_B=T) to (+)- and (−)bornyl pyrophosphate (**21**) with net retention of configuration.

phosphate has been obtained by Arigoni in the course of extensive stereochemical studies of the whole cell biosynthesis of members of the longifolene- and cadalane-derived families of sesquiterpenes (*33–35*). The observed retention of configuration in the enzyme-catalyzed cyclizations can be contrasted with the inversion of configuration which occurs in the prenyl transferase-catalyzed condensation of dimethylallyl pyrophosphate with two equivalents of isopentenyl pyrophosphate, leading to the formation of farnesyl pyrophosphate itself (*36, 37*) (Scheme 8). The latter stereochemical course was for many years ascribed to the presumed simultaneity of C–O bond breaking and C–C bond formation. More recently, elegant mechanistic studies by Poulter and Rilling have established that prenyl chain elongation involves initial ionization of the pyrophosphate moiety to generate an allylic cation which in turn undergoes electrophilic attack on the 3,4 double bond of isopentenyl pyrophosphate (*38*). We have previously pointed out that the observed stereochemical course of all these transformations can be accounted for by the intermediacy of allylic cation–pyrophosphate anion pairs (*19*). Thus, initial isomerization of *trans,trans*-farnesyl pyrophosphate to nerolidyl pyrophosphate will take place via the corresponding transoid ion pair, a conversion which we have shown takes place with net syn stereochemistry. Rotation about the newly formed 2,3 single bond and ionization to the cisoid allylic cation–pyrophosphate anion pair allows net anti displacement by the now juxtaposed 6,7 double bond. The postulated anti-boat conformation of the cyclizing nerolidyl pyrophosphate intermediate is based on (a) the known stereochemistry of the solvolytic cyclization of linalyl *p*-nitrobenzoate to α-terpineol (*39, 40*), (b) the observation by Poulter that the individual enantiomers of α-terpineol generated by solvolysis of chirally deuterated derivatives of nerol are generated in a completely stereospecific manner with inversion of configuration (*41*), and (c) the demonstrated anti stereochemistry of allylic displacement reactions of allylic pyrophosphates (*5*). Since the 6,7 double bond of nerolidyl pyrophosphate does not participate in the ionization of the allylic ester, the observed stereochemical results are best accounted for by a preassociation mechanism (*42*) in which the initially generated allylic cation–anion pair is captured from the opposite face by the proximally placed nucleo-



SCHEME 8. Formation of farnesyl pyrophosphate from isopentenyl pyrophosphate and dimethylallyl pyrophosphate catalyzed by prenyl transferase with inversion of configuration at C-1 of the allylic pyrophosphate.

philic double bond at a rate greater than competing dissociation of the anion from the ion pair. In the trichodiene synthetase reaction, therefore, the mechanism of double bond isomerization and the mechanism of the subsequent cyclization reaction are assumed to be essentially identical: generation of an allylic cation–pyrophosphate anion pair which is quenched either by recapture of the pyrophosphate moiety or displacement by the proximal double bond of the substrate. Although direct evidence for the intermediacy of nerolidyl pyrophosphate in the isomerization–cyclization process is not yet available, the analogous role for linalyl pyrophosphate (**3**) has been established in the biosynthesis of several families of monoterpenes (*43, 44*). Further studies of the trichodiene synthetase reaction are underway, including attempts to study the individual isomerization and cyclization reactions and to elucidate the role played by the cyclase in enforcing the formation of a single cyclized sesquiterpene product.

EXPERIMENTAL PROCEDURES

Materials and Methods. The preparation of [1-$^3H_2$]farnesol and of [12,13-^{14}C]farnesol have been described previously (*19*). (+)-Trichodiene was produced by partially anaerobic fermentation of *T. roseum* ATCC 8685. An additional sample of (+)-trichodiene was a gift from Professor K. H. Overton of the University of Glasgow. Synthetic (±)-trichodiene was a gift of Professor R. H. Schlessinger of the University of Rochester. *trans,trans*-Farnesol was separated from the four-component mixture as previously described (*19*), except that the preliminary separation was carried out on a Waters Prep 500 LC. The purity of the farnesol isomers was monitored by GC on a 6-ft 10% Carbowax 20M column; temperature, 165°C; t_R = 20.15 min (*cis,cis*), 23.28 min (*cis,trans*), 24.65 min (*trans,cis*), and 27.20 min (*trans,trans*). [The assignments of the *cis,trans* and *trans,cis* isomers are reversed in Reference (*19*).] Corn steep liquor was provided by CPC International. Horse liver alcohol dehydrogenase (EC 1.1.1.1) was obtained from Sigma or Boehringer-Mannheim. NAD, grade I, was from Boehringer-Mannheim. Glycerol (>99%) was obtained from Sigma. Potassium [^{3}H]borohydride (13 mCi/μmol) was purchased from Amersham. Sodium [^{3}H]borohydride (250–400 mCi/mmol) was purchased from New England Nuclear.

Flash chromatography on silica gel was carried out according to Still (*45*). Radioactivity measurements were performed as previously described (*46*). Protein concentration was determined by the method of Lowry (*47*) or by the dye binding assay, Bio-Rad Laboratories. (*48, 49*). High-resolution chemical ionization (CI) and electron impact (EI) mass spectra were obtained on a VG Micromass 7070H at the University of Pennsylvania. NMR spectra were recorded on a Bruker WM-250 spectrometer at 250 MHz (^{1}H) and 62.9 MHz (^{13}C).

Fermentation of T. roseum. (*50*) A lyophilized spore suspension of *T. roseum* ATCC 8685 was rehydrated with 0.4 ml of sterile water and used to inoculate eight potato–dextrose agar slants. The slants were incubated at 24°C for 4 days, after

which each slant exhibited the characteristic rose-colored pigment. A vegetative inoculum was prepared by adding 2.0 ml of sterile water to a slant and using 0.2 ml of the resulting spore suspension to inoculate a 500-ml flask containing 100 ml of culture medium composed of 2.0 g ammonium tartrate, 1.0 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 50 g Bacto-Dextrose, and 10 ml Corn steep liquor per liter, pH adjusted to 6.5 with 1 N NaOH. After incubation at 24°C and 250 rpm for 4 days, the resulting vegetative culture was used to inoculate 100-ml cultures (2 ml of inoculum per flask) which were incubated under the same conditions for 4–10 days. Cultures of *T. roseum* were maintained by mixing 2- to 4-day-old vegetative inocula with sterile glycerol (2/1, v/v) and dispensing 2.0-ml aliquots of the mixture into Pro-Vials which were stored in an Orion ET-34 dewar over liquid nitrogen. Fresh vegetative inocula were routinely prepared by adding the contents of a freshly thawed Pro-Vial to 100 ml of fermentation medium.

Trichodiene synthetase. Procedure A. Mycelia from 2.4 liters of a 4-day-old fermentation culture of *T. roseum* were collected by filtration and washed thoroughly with glass-distilled water. The mycelia were suspended in 30 ml of 0.1 M potassium phosphate buffer, pH 7.0, at 4°C and partially homogenized in a blender before cell disruption by passage through a prechilled French press at 15,000–18,000 psi and 4°C. Broken cell debris was removed by centrifugation at 4°C, first at 10,000*g* for 20 min and then at 17,000*g* for 90 min. The resultant 200 ml of supernatant (10 mg protein/ml) was mixed with an additional 10 ml of 0.1 M phosphate buffer, pH 7.0, along with 2.0 mmol $MgCl_2$, 0.8 mmol $MnCl_2$, 0.1 mmol DTT, and 10 μmol farnesyl pyrophosphate, and the mixture was incubated at 30°C for 2 h. The reaction was quenched by addition of 12 N NaOH (25 ml) and 95% ethanol (25 ml), allowed to stand for 30 min, and then extracted with 120 ml of *n*-pentane containing 5 mg of carrier trichodiene. Typical activities corresponded to 0.05 nmol of trichodiene mg protein^{-1} h^{-1}.

Trichodiene synthetase. Procedure B. The mycelia from 20 100-ml cultures of *T. roseum* were harvested after 10 days by centrifugation at 10,800*g* for 20 min. All subsequent steps were carried out at 4°C. The mycelial mass was resuspended in cold water and recentrifuged at 10,800*g* for 15 min, followed by consecutive washes with 1.0 M KCl, 0.8 M NaCl, and homogenization buffer, containing 50 mM potassium phosphate, pH 6.5, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol. The washed mycelia were resuspended in ca. 200 ml of homogenization buffer and the cells were disrupted by rapid stirring with 0.5 mm glass beads in a 350-ml ice/water-jacketed stainless-steel bead-beater cell (Biospec Products) using a 30-s on, 30-s off cycle for a total of 6 min. The homogeneous slurry from the beater was centrifuged at 27,500*g* for 5 min and the resulting supernatant was treated with insoluble PVPP (3 mg/ml) before recentrifugation at 47,800*g* for 60 min. The S_{48} preparation was carefully decanted and concentrated by precipitation by slow addition of solid ammonium sulfate to 80% saturation (51.6 g/100 ml). The F_o^{80} pellet was collected by centrifugation at 10,400*g* for 10 min, then redissolved in 10 ml of resuspension buffer. For the preparative experiment with 1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate this resuspension buffer was composed of 20 mM piperazine–HCl, pH 6.5, 4 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.02% so-

dium azide, and 10% glycerol. Final protein concentration was 1.3 mg protein/ml with specific activity ranging up to 0.84 nmol trichodiene mg protein^{-1} h^{-1}.

Trichodiene synthetase assay. Assays were carried out in 13 × 100-mm disposable test tubes by addition of 0.10 ml of trichodiene synthetase preparation to 0.250 ml of assay buffer, 0.140 ml of water or resuspension buffer, and 0.010 ml of *trans,trans*-[1^{3}H$_2$]farnesyl pyrophosphate (3.65 × 10^5 dpm; final concentration, 5 μM) and incubation at 30°C for 60 min. The assay buffer consisted of 50 mM Tris-HCl, 50 mM malate (pH 6.8), 4 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM 2-mercaptoethanol, and 2 mM ammonium molybdate (as phosphatase inhibitor) (*4*). The enzymatic reaction was terminated by addition of 0.5 ml of ethanol, and the mixture was extracted with a single 2-ml portion of hexane. The hexane extract was passed through a short (2 cm) column of TLC-grade silica gel in a Pasteur pipet and eluted directly into a plastic scintillation vial for counting. Controls established that the ^{3}H activity of the eluant corresponded only to trichodiene. The assay tube was further extracted with a 2-ml volume of ether which was eluted through the same silica column and counted, thereby giving a measure of the net phosphatase activity.

trans,trans-1S-[1-^{3}H]-Farnesol. [1-^{3}H]Farnesal (**19**) (20 mg, 90 μmol), prepared by oxidation of *trans,trans*-[1-^{3}H$_2$]farnesol with activated manganese dioxide (*19*), was dissolved in 4.0 ml of ethanol and mixed with 25 mg (38 μmol) of NAD^+ in 10 ml of 0.01 M phosphate buffer, pH 7.0, containing 2 drops of Tween-80. The mixture was equilibrated at 30°C in a water bath before addition of 6 mg of HLADH (6 units, 2.7 units/mg) and incubation for 24 h. Extraction with ethyl acetate, followed by washing of the organic extracts with saturated aqueous sodium chloride, drying over anhydrous $MgSO_4$, and concentration gave crude farnesol which was purified by column chromatography (silica; 3 : 1 hexane : ethyl acetate) to give 14 mg (70%) of *trans,trans*-1S-[1-^{3}H]farnesol (3.66 × 10^{10} dpm/mmol).

[1-^{3}H]Cyclohexenol. Cyclohexenone (2.91 mg, 30.3 μmol) was dissolved in 2.0 ml of 0.005 N NaOH and the mixture was reacted with the contents of a single ampoule of potassium [^{3}H]borohydride (100 mCi, 0.409 mg, 7.57 μmol) for 2 h at room temperature. Potassium borohydride (0.4 mg) was then added and the reaction mixture was stirred for an additional 30 min, after which 0.1 ml of 1 M phosphoric acid was added to bring the pH to 1 and decompose excess borohydride. The pH was adjusted to 8 by addition of 50 μl of concentrated ammonia, and the resulting solution was diluted with 0.35 ml of water and 2.5 ml of 0.2 M glycine buffer, pH 8.8 (KOH), to a final volume of 5.0 ml. Extraction of an aliquot and analysis of the isolated cyclohexenol showed that the solution contained 30 μmol of [1-^{3}H]cyclohexenol (25 mCi, 0.83 mCi/μmol).

trans,trans-1R-[1-^{3}H]Farnesol. trans,trans-Farnesal (**19**) (3.52 mg, 16 μmol) was mixed with 2 drops of Tween-80 and dissolved in 2.0 ml of water. HLADH (3.61 mg, 5.78 units) and 1.14 mg (1.72 μmol) of NAD^+ in 5.0 ml of 0.1 M phosphate, pH 7.2, were mixed with 1.5 ml (9.0 μmol, 7.5 mCi) of the above-prepared solution of [1-^{3}H]cyclohexenol, followed by the solution of farnesal, and the reaction mixture was stirred at 25°C. After 4.5 h an additional 2.1 mg (3.4 units) of HLADH was added and the mixture was incubated overnight (total, 19 h). The

remaining farnesal was reduced by reaction for 10 min with a few milligrams of potassium borohydride, after which 5 ml of 2-propanol was added to break the resultant foam. After a further 20 min, 1 M sulfuric acid was added dropwise until evolution of hydrogen ceased (pH 2.5) and the pH was readjusted to neutrality by addition of 1 M ammonia. Solid ammonium chloride and 6.3 mg of *trans,trans*-farnesol in pentane were added, followed by addition of 50 ml of brine and extraction with pentane. The dried and concentrated organic layer was purified by column chromatography on silica (5 : 1 hexane : ethyl acetate) to yield 7 mg (70%) of *trans,trans*-1*R*-[-1-^{3}H]farnesol (1.94 mCi, 26% radiochemical yield) and <1 mg of *cis,trans*-1*R*-[^{1}^{3}H]farnesol (1 mCi, 13% radiochemical yield.) A second column purification removed all traces of the *cis,trans* isomer from the *trans,trans*-1*R*-[1-^{3}H]farnesol.

The configurational purity of the 1*R*-[1-^{3}H]farnesol was confirmed by mixing ca. 1 mg (5 μmol, 20 μCi) with ca. 0.25 μCi of [12,13-^{14}C]farnesol, ^{3}H/^{14}C = 69.3. The resulting 1*R*-[1-^{3}H,12,13-^{14}C]farnesol was mixed with 1 drop of Tween-80, suspended in 1 ml of water, and added to a mixture of 2.41 mg of HLADH (3.85 units) and 6.0 mg (9.1 μmol) of NAD^+ in 3.0 ml of 0.1 M phosphate, pH 7.2. After incubation for 0.5 h at room temperature, 6 mg of carrier *trans,trans*-farnesal was added in pentane. Extraction with pentane and column chromatographic purification (7 : 1 hexane : ethyl acetate) of the isolated recovered mixture of farnesal and unreacted farnesol gave *trans,trans*-[12,13-^{14}C]farnesal, ^{3}H/^{14}C = 0.69.

trans,trans-Farnesyl diphenylurethanes. The individual tritiated farnesol samples were mixed with [12,13-^{14}C]farnesol and a portion of each mixture was diluted with *trans,trans*-farnesol and converted to the corresponding farnesyl diphenylurethane derivative for recrystallization to constant activity, as previously described (*19*).

Purification of farnesyl pyrophosphates. The various [^{3}H,^{14}C]farnesol mixtures were converted to the corresponding pyrophosphate esters by the previously described (*19*) modification of the method of Cramer and Boehm (*51*). The resulting samples of farnesyl pyrophosphate were purified either as previously described (*19*) or by ion-exchange chromatography on DEAE-Sephadex A-25 using a gradient of triethylammonium bicarbonate. The latter procedure was not only more convenient but avoided substantial losses associated with the use of Amberlite XAD-2 resin. Using the revised purification procedure, the lyophilized ammonium salts of the crude product derived from 4.5 μmol of *trans,trans*-1R-[1-^{3}H,12,13-^{14}C]farnesol was dissolved in 0.05 M triethylammonium bicarbonate, pH 7.5, and applied to a 15 × 1.5-cm column of DEAE-Sephadex A-25 preequilibrated with the same buffer. The column was developed with a linear gradient (180 ml × 180 ml) of 0.05 to 1.0 M triethylammonium bicarbonate, pH 7.5, which was applied immediately following sample application. Fractions of 5.0 ml each were collected, farnesyl pyrophosphate eluting in factions 23 to 27 and being identified by comigration with authentic [1-$^3H_2$]farnesyl pyrophosphate on TLC. The combined farnesyl pyrophosphate fractions were lyophilized and taken up in 0.01 N NH_4OH.

Cyclization of trans,trans[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate (2) to trichodiene. Preparative-scale incubations of [1-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate

and 1*S*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate were carried out using trichodiene synthetase prepared by Procedure A while cyclization of 1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate was carried out using synthetase prepared by Procedure B. A detailed description follows for the conversion of 1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate to trichodiene.

In a 125-ml Erlenmeyer flask were combined 19 ml of the F_0^{80} fraction of trichodiene synthetase obtained by Procedure B (1.3 mg protein/ml), 25 ml of assay buffer, 5 ml of water, and 1 ml of a 0.01 N NH_4OH solution of *trans,trans*-1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate (1.5×10^6 dpm ^{14}C; final concentration, 6.9 μM) and the contents were incubated for 120 min at 30°C. The reaction was terminated by the addition of 50 ml of ethanol and the mixture was extracted with 80 ml of pentane containing 4 mg of carrier trichodiene. The aqueous layer was extracted with two additional 80-ml portions of pentane and the combined extracts were washed with saturated NaCl, dried, and concentrated at ambient pressure to 2–3 ml. The sample was purified by flash chromatography on silica gel 60 using pentane as eluant. The total ^{14}C activity of the recovered trichodiene fractions represented a net conversion of 0.5% (7.3×10^3 dpm ^{14}C, ^{3}H/^{14}C = 3.46), which corresponded to a specific activity for trichodiene synthetase of 0.034 nmol trichodiene mg protein^{-1} h^{-1}. The sample was concentrated at ambient pressure to 1–2 ml and diluted with 5 ml of benzene before storage at −20°C.

Degradation of Trichodiene. General procedures. The samples of labeled trichodiene obtained from the individual preparative-scale incubations with [1-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate, 1*S*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate, and 1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate were diluted to 110 mg and 21 mg of (+)-trichodiene and 55 mg of (±)trichodiene, respectively, and subjected to the chemical degradation sequence described below. Details are given for the degradation of trichodiene derived from 1*R*-[1-^{3}H,12,13-^{14}C]farnesyl pyrophosphate. Specific activities and isotope ratios for the intermediates in each degradation sequence are summarized in Tables 1 and 2. Spectroscopic data, including high-field NMR and accurate mass analyses, were obtained on unlabeled samples of the various intermediates.

9,10-Epoxytrichodiene (**11c**) (*8*). Trichodiene (55 mg, 0.27 mmol) in 3 ml of benzene was mixed with a solution of 55 mg (0.32 mmol) of 85% *m*-chloroperbenzoic acid in 1 ml of benzene and the reaction mixture was stirred at room temperature with monitoring by TLC. Normally, if after 5 min the reaction was not complete a further small portion of *m*-chloroperbenzoic acid was added and the progress of the reaction was monitored after an additional 3 min. The reaction was quenched by addition of 10 ml of saturated Na_2SO_3 and the resulting mixture was extracted three times with ether. The ethereal extracts were washed with saturated $NaHCO_3$ and saturated NaCl, dried, and concentrated and the crude epoxide was purified by flash column chromatography (4 : 1 hexane : ether) to afford 36.5 mg (62%) of 9,10-epoxytrichodiene (**11c**). ^{1}H NMR ($CDCl_3$) δ 0.86 (s, CH_3, 3H), 1.01 (s, CH_3, 3H), 1.31 (s, CH_3CO, 3H), 0.88-2.37 (m, 12H), 2.93 (d, J = 5.2 Hz, HCO, 1H), 4.70 (s, exomethylene H, 1H), 4.97 (s, exomethylene H, 1H); ^{13}C NMR ($CDCl_3$) δ 20.0, 23.1, 23.2, 24.2, 24.8, 26.6, 32.3, 36.1, 37.4, 38.7, 50.4, 57.0, 58.8, 107.2, 159.4.

9,15-Dehydro-9,10-dihydro-10-hydroxytrichodiene (**12**) (*8*). To 68 mg (0.48 mmol) of 2,2,6,6-tetramethylpiperidine under N_2 at 0°C was added 0.48 mmol of *n*-butyllithium and the mixture was stirred for 10 min. Upon addition of diethylaluminum chloride (58 mg, 0.48 mmol) a white precipitate formed. The mixture was stirred for 45 min after which 26 mg (0.12 mmol) of trichodiene (**5**) in benzene was added and the resulting solution was stirred for 6 h. The reaction was terminated by addition of 6 **N** HCl and the mixture was extracted with ether. The organic layer was washed successively with water and saturated NaCl, dried, and concentrated to yield, after chromatographic purification, 15 mg (60%) of **12** as an oil. ^{1}H NMR ($CDCl_3$) δ 1.02 (s, CH_3, 3H), 1.04 (s, CH_3, 3H), 1.2-2.3 (m, CH_2, 12H), 4.19 (dd, J = 5, 12 Hz, CHOH, 1H), 4.74 (s, C=CH_2, 2H), 4.86 (s, C=CH_2, 1H), 4.96 (s, C=CH_2, 1H); IR λ_{max} ($CHCl_3$) 3350, 3450 (-OH), 1650 cm^{-1} (C=C).

trans-9,10-Dihydroxy-9,10-dihydrotrichodiene (**13**). 9,10-Epoxytrichodiene (**11c**) (15 mg, 68.1 μmol) was dissolved in 2 ml of THF : water (1 : 1). An additional 3 drops of THF was added to give a homogeneous solution which was cooled to 0°C before addition of 0.45 ml of 3% aqueous $HClO_4$, corresponding to 2 equivalents of acid. The mixture was maintained at 0°C for 16 h, after which excess solid $NaHCO_3$ and NaCl were added. Water was added and the mixture was extracted twice with ether. The ethereal extracts were washed with saturated NaCl, dried over $MgSO_4$, filtered, and concentrated to an oil. Purification by TLC (1 : 3 hexane : ether, R_f 0.32) gave 11.9 mg (73%) of diol **13c.** ^{1}H NMR ($CDCl_3$) δ 1.03 (s, CH_3, 3H), 1.12 (s, CH_3, 3H), 1.17 (s, CH_3CO, 3H), 1.14-2.37 (m, 12H), 1.89 (s, OH, 1H), 1.92 (s, OH, 1H), 3.64 (bs, HCO, 1H), 4.80 (s, exomethylene, 1H), 4.98 (s, exomethylene, 1H); ^{13}C NMR ($CDCl_3$) δ 20.5, 23.4, 23.7, 26.5, 27.1, 29.7, 30.1, 34.5, 37.2, 39.1, 51.2, 71.4, 75.2, 106.9, 160.0; IR λ_{max} ($CHCl_3$) 3640 (OH), 1645 cm^{-1} (C=C); MS (CI, isobutane) *m/e* M^+ calcd for $C_{15}H_{26}O_2$: 238.1932, found: 238.1894; *m/e* M^+-H calcd for $C_{15}H_{25}O_2$: 237.1855, found: 237.1846.

trans-9,10-Dihydroxy-9,10-dihydrotrichodiene bis(dinitrobenzoate) (**14c**). Diol **13c** (10 mg, 42.0 μmol) was mixed with 77 mg (336 μmol, 8 equiv) of 3,5-dinitrobenzoylchloride and 20 mg (168 μmol, 4 equiv) of *N,N*-dimethyl-4-aminopyridine followed by 8 drops of acetonitrile (dried by distillation from CaH_2 and stored under argon) and an equal volume of triethylamine (distilled and stored over KOH). The semisolid mixture was stirred for 20 h under argon. (If the mixture became dry, more acetonitrile and triethylamine were added.) At the end of the reaction period, 2 ml of acetonitrile was added and the suspension was evaporated to dryness prior to addition of further acetonitrile, a few drops of pyridine, and water. After 5 min stirring, the solution was reevaporated and the residue was taken up in CH_2Cl_2 and saturated $NaHCO_3$. The resulting mixture was extracted with three portions of CH_2Cl_2, the organic layer being washed with saturated $NaHCO_3$ and saturated NaCl. Drying over $MgSO_4$ and evaporation gave crude bis(dinitrobenzoate) which was purified by TLC (1 : 3 hexane : ether, R_f 0.64) to afford 17 mg (65%) of bis(dinitrobenzoate) **14c.** **14c** was recrystallized to constant activity from $CHCl_3$: ether. mp (rac) 194°C, mp [opt act, from (+)-**5**] 220°C; ^{1}H NMR ($CDCl_3$) δ 1.01 (s, CH_3, 3H), 1.16 (s, CH_3, 3H), 1.72 (s, CH_3CO, 3H), 1.18–2.35 (m, 11H), 2.65 (bd, J = 12 Hz, CH, 1H) 4.66 (s, exomethylene, 1H), 4.69 (s, exomethylene, 1H), 5.79 (d, J = 0.7 Hz, HCO, 1H), 9.11 (d, J = 2.1 Hz, aromatic,

2H), 9.17 (d, J = 2.0 Hz, aromatic, 2H), 9.27 (m, aromatic, 2H); IR λ_{max} ($CHCl_3$) 3120 (arom CH), 1730 (C=O), 1630 (C=C), 1545 cm^{-1} (arom); MS (CI, isobutane) *m/e* M^+-$C_7H_3N_2O_6$ calcd for $C_{22}H_{27}N_2O_6$: 415.1869, found: 415.1855.

9β-9,10-Dihydro-10-ketotrichodiene (**15c**). Trichodiene epoxide (**11c**) (7.2 mg, 32.7 μmol) was dissolved in 2 ml of dry benzene and stirred under argon while 9.3 mg (87.4 μmol, 2.7 equiv) of $LiClO_4$ was added. Since after 4 h of reflux TLC analysis showed little conversion, a further 7 mg (65.7 μmol) of $LiClO_4$ was added and refluxing was continued for an additional 23 h. Saturated NaCl was added and the mixture was extracted with two portions of ether. Washing of the ether extract with saturated NaCl, drying over $MgSO_4$, and evaporation of the solvent, followed by TLC purification (2:1 hexane:ether, R_f 0.48) gave 2.7 mg (38%) of ketone **15c**. ^1H NMR ($CDCl_3$) δ 0.89 (s, CH_3, 3H), 1.07 (s, CH_3, 3H), 1.16 (d, CH_3CH, 3H), 0.98–2.51 (m, 13H), 4.83 (s, exomethylene, 1H), 5.03 (s, exomethylene, 1H); ^{13}C NMR ($CDCl_3$) δ 17.2, 20.0, 23.3, 23.9, 25.5, 28.3, 37.3, 38.7, 43.4, 44.1, 45.0, 50.7, 107.5, 159.0, 217.1; IR λ_{max} ($CHCl_3$) 1695 (C=O), 1650 cm^{-1} (C=C); MS (CI, isobutane) *m/e* M^+ calcd for $C_{15}H_{24}O$: 220.1827, found: 220.1821.

9α-9,10-Dihydro-10-ketotrichodiene (**16c**). A solution of 2.7 mg (11.9 μmol) of **15c** in 270 μl of dioxane was mixed with 51 μl (0.118 mmol) of 3.5 M NaOH and the mixture was further diluted with 168 μl of dioxane and 236 μl of water to give a clear solution, which was refluxed for 20 h. Ether and solid NaCl were added and the mixture was extracted twice with ether. The combined organic extracts were washed with saturated NaCl, dried over $MgSO_4$, and concentrated. The recovered crude ketone was purified by TLC (2:1 hexane:ether, R_f 0.55) to give 0.8 mg (30%) of exchanged, equatorial-methyl ketone **16c**. ^1H NMR ($CDCl_3$) δ 0.84 (s, CH_3, 3H), 1.00 (s, CH_3, 3H), 1.06 (d, J = 6.4 Hz, CH_3CH, 3H), 1.08–2.43 (m, 13 H), 4.80 (s, exomethylene, 1H), 5.02 (s, exomethylene, 1H); ^{13}C NMR ($CDCl_3$) δ 14.2, 18.6, 23.3, 23.9, 30.7, 31.3, 37.3, 38.7, 44.5, 45.0, 48.9, 50.6, 107.6, 158.8, 214.1; IR λ_{max} ($CHCl_3$) 1705 (C=O), 1650 cm^{-1} (C=C); MS (CI, isobutane) *m/e* M^+ +H calcd for $C_{15}H_{25}O$: 221.1905, found 221.1902.

trans-9-Hydroxy-10-phenylseleno-9,10-dihydrotrichodiene (**17c**). A solution of 7.4 mg (23.9 μmol) of diphenyldiselenide in 450 μl of absolute ethanol was added to a two-neck flask equipped with a reflux condenser and $CaCl_2$ tube and under a positive flow of argon. To this solution was added 0.2 ml (50 μmol) of an ethanolic solution of $NaBH_4$, prepared by dissolving 18.8 mg of $NaBH_4$ in 2.0 ml of absolute ethanol. An additional 2 drops of $NaBH_4$ solution was added to discharge the yellow color, after which 7.5 mg (34.1 μmol) of epoxytrichodiene (**11c**) in 1 ml of ethanol was added and the mixture was refluxed for 18 h under argon. Additional ethanol was added from time to time to prevent the reaction from going dry. At the end of the reaction period, 2.5 ml of 10% HCl was added, leading to the formation of a small quantity of precipitate. Following neutralization with saturated $NaHCO_3$, the resulting suspension was extracted three times with ether, and the organic extracts were washed with saturated $NaHCO_3$ and saturated NaCl, dried over $MgSO_4$, filtered, and evaporated. The crude product was purified by TLC (2:1 hexane:ether, R_f 0.35) to yield 5.8 mg of hydroxyphenylselenide **17c**. ^1H NMR ($CDCl_3$) δ 0.98 (s, CH_3, 3H), 1.03 (s, CH_3, 3H), 1.28 (s, CH_3CO, 3H), 1.09–2.3 (m, 12H), 2.45 (dd, J = 5.1 14.5 Hz, 1H), 3.49 (bt, J = ca. 5.5 Hz, CHSePh,

1H), 4.76 (s, exomethylene, 1H), 4.93 (s, exomethylene, 1H), 7.25 (m, aromatic, 3H), 7.59 (m, aromatic, 2H); ^{13}C NMR ($CDCl_3$) δ 21.8, 23.2, 24.2, 27.2, 29.2, 32.7, 36.7, 37.4, 38.7, 39.0, 51.2, 54.4, 73.4, 107.2, 127.3, 129.1, 132.2, 134.2, 160.0; IR λ_{max} ($CHCl_3$) 3580 (OH), 1640 (C=C), 1580 cm^{-1} (arom); MS (EI) *m/e* M^+ calcd for $C_{21}H_{30}O^{80}Se$: 378.1461, found: 378.1473.

9-Hydroxy-10,11-dehydro-9,10-dihydrotrichodiene (**18c**). A solution of 9.8 mg (51.6 μmol) of $NaIO_4$ in 0.25 ml of 70% aqueous methanol was added to 5.8 mg (17.2 μmol) of β-hydroxyphenylselenide **17c** in 0.3 ml of THF at 0°C. After stirring for 1.5 h at 0°C, the reaction mixture was refluxed for 2–3 h, THF being added as necessary to make up for losses due to evaporation. Saturated NaCl was added and the mixture was extracted twice with ether. The combined ethereal extracts were washed with saturated NaCl, dried over $MgSO_4$, filtered, and evaporated. The resulting crude product was purified by TLC (1 : 3 hexane : ether, R_f 0.42) to yield 1.8 mg (53%) of allylic alcohol **18c**. A portion of the alcohol was withdrawn for radioactivity counting while the remainder was subjected to capillary microdistillation in order to remove traces of volatile and involatile impurities. ^1H NMR ($CDCl_3$) δ 0.97 (s, CH_3, 3H), 1.07 (s, CH_3, 3H), 1.27 (s, CH_3CO, 3H), 1.13–2.0 (m, 9H), 2.3 (bd, J = 6.5 Hz, 2H), 4.83 (s, exomethylene, 1H), 4.98 (s, exomethylene, 1H), 5.59 (d, J = 10.1 Hz, vinyl, 1H), 5.77 (d, J = 10.1 Hz, vinyl, 1H); ^{13}C NMR ($CDCl_3$) δ 20.3, 23.3, 24.4, 26.3, 29.8, 34.2, 37.3, 38.6, 40.6, 49.6, 66.6, 106.6, 131.7, 136.5, 159.8; IR λ_{max} ($CHCl_3$) 3580 (OH), 1640 cm^{-1} (C=C); MS (CI, isobutane) *m/e* M^+ +H calcd for $C_{15}H_{25}O$: 221.1905, found 221.1891; M^+ calcd for $C_{15}H_{24}O$: 220.1827, found 220.1811.

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