inner-sphere mode to the phosphate group, one may conclude that probably the larger part (or even all) of the 46 ± 10% of Cu(AMP)$_{cl}$ (Table VI) is present as a pure inner-sphere macrochelate, i.e., in the form of structure (iii).

The possible occurrence of structure (ii) with N-7 outer sphere coordinated via a water molecule and direct metal ion–phosphate binding should not lightly be dismissed. There is evidence for the formation of such species in M(ATP) systems,[7,12,13] and it is well known[60,63] that N-7 has a pronounced tendency to form hydrogen bonds to water molecules. Should there be traces of closed species present in the alkaline earth ion systems, like Mg(AMP)$_{cl}$ (Table VI) or Mg(ε-AMP)$_{cl}$ (Table VII), then N-7 binding may occur outer sphere as suggested[12,13] for the small fraction of $Mg(ATP)^{2-}_{cl}$. The question if structure (ii) is also playing a role in closed M(NMP) complexes of transition-metal ions has to be left open for the present; it can only be solved with additional experiments like those carried out[7] for Ni(AMP), and (as concluded above) in this latter case structure (ii) is not significant.

To conclude, the structures to be considered for M(NMP)$_{cl}$ species comprise the three described structures, (i), (ii), and (iii). Equilibria between these different forms have to be assumed, though depending on the metal ion, the one or other closed isomer may form only in traces or not at all. It is now evident that equilibrium 1 is a simplification as M(NMP)$_{cl}$ is not a well defined single isomer; hence, the K_I values and the percentages for M(NMP)$_{cl}$ given in Tables VI and VII with regard to equations 2 and 6 are actually overall values which quantify the *total* formation of all closed species, and the macrochelate formation may be inner sphere or outer sphere at both the base and phosphate moieties.

Acknowledgment. We thank Rita Baumbusch for technical assistance. A research grant from the Swiss National Science Foundation (H.S.), support from the Stiftung der Portlandcementfabrik Laufen (H.S.), and a fellowship to S.S.M. from the Amt für Ausbildungsbeiträge des Kantons Basel-Stadt are gratefully acknowledged.

Registry No. H_2(TuMP), 16719-46-3; H_2(AMP), 61-19-8; H_2(ε-AMP), 37482-16-9; $H(TuMP)^-$, 116129-94-3; $H(AMP)^-$, 47287-36-5; $H(ε\text{-}AMP)^-$, 116129-95-4; Mg(TuMP), 116129-96-5; Ca(TuMP), 116129-97-6; Sr(TuMP), 116129-98-7; Ba(TuMP), 116129-99-8; Mn(TuMP), 116130-00-8; Co(TuMP), 116130-01-9; Ni(TuMP), 116130-02-0; Cu(TuMP), 116130-03-1; Zn(TuMP), 116130-04-2; Cd(TuMP), 116130-05-3; Mg(AMP), 73077-74-4; Ca(AMP), 63573-27-3; Sr(AMP), 116130-06-4; Ba(AMP), 3249-91-0; Mn(AMP), 75389-10-5; Co(AMP), 18839-80-0; Ni(AMP), 18839-81-1; Cu(AMP), 18839-82-2; Zn(AMP), 18839-83-3; Cd(AMP), 116130-07-5; Mg(ε-AMP), 70824-97-4; Mn(ε-AMP), 116149-00-9; Co(ε-AMP), 116149-01-0; Ni(ε-AMP), 116149-02-1; Cu(ε-AMP), 116149-03-2; Zn(ε-AMP), 116149-04-3.

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Trichodiene Biosynthesis and the Role of Nerolidyl Pyrophosphate in the Enzymatic Cyclization of Farnesyl Pyrophosphate†

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Contribution from the Department of Chemistry, Brown University, Providence, Rhode Island 02912. *Received February 4, 1988*

Abstract: Incubation of (1*Z*)-[1-^{3}H,12,13-^{14}C]nerolidyl pyrophosphate (**4a**) with a preparation of trichodiene synthetase isolated from the fungus *Trichothecium roseum* gave labeled trichodiene (**3**), which was shown by chemical degradation to carry the tritium label exclusively at H-11β. These results were consistent with a previously proposed isomerization–cyclization mechanism for the formation of trichodiene from farnesyl pyrophosphate (**1**). The absolute configuration of the enzymatically active enantiomer of nerolidyl pyrophosphate was determined by incubating a mixture of (3*S*)-(1*Z*)-[1-^{3}H]nerolidyl pyrophosphate and (3*RS*)-[12,13-^{14}C]nerolidyl pyrophosphate with trichodiene synthetase. The finding that the resulting trichodiene (**3**) was labeled only with ^{14}C established that the cyclase utilized only (3*R*)-nerolidyl pyrophosphate. Further support for the proposed cyclization mechanism was obtained by carrying out a competitive incubation of [1-^{3}H]farnesyl pyrophosphate (**1**) and [12,13-^{14}C]nerolidyl pyrophosphate (**4**) with trichodiene synthetase and examining the $^3H/^{14}C$ ratio of the resulting trichodiene as well as that of remaining farnesyl pyrophosphate and nerolidyl pyrophosphate as a function of time. The results of the latter experiment indicated that both allylic pyrophosphate substrates compete for the same active site in trichodiene synthetase, with one enantiomer of nerolidyl pyrophosphate having a V_{max}/K_m approximately 1.5–2 times that of farnesyl pyrophosphate.

Sesquiterpene synthetases provide a striking example of Nature's synthetic virtuosity and economy of catalytic design. On the basis of the cyclization of a single substrate, farnesyl pyrophosphate (**1**), these remarkable enzymes are able to mediate the formation of more than 200 distinct sesquiterpene hydrocarbons and alcohols.[1,2] According to the currently accepted hypothesis, all these cyclizations take place by variations on a simple mechanism involving ionization of the allylic pyrophosphate and intramolecular electrophilic attack of the resulting carbocation on either the central or distal double bond of the farnesyl skeleton (Scheme I). Subsequent cyclization or rearrangement processes followed by quenching of the cationic intermediates by deprotonation or capture of an external nucleophile such as water can, in principle, account for the formation of all the known sesquiterpene carbon skeletons. Completely analogous schemes have been invoked to account for the formation of cyclic monoterpenes from the universal acyclic precursor geranyl pyrophosphate (**2**).[3]

Over the last 10 years, substantial progress in the understanding of terpenoid cyclizations has come from the isolation and study of a variety of monoterpene and sesquiterpene cyclases.[1,3,4] One

† This paper is dedicated to Professor Duilio Arigoni on the occasion of his 60th birthday.

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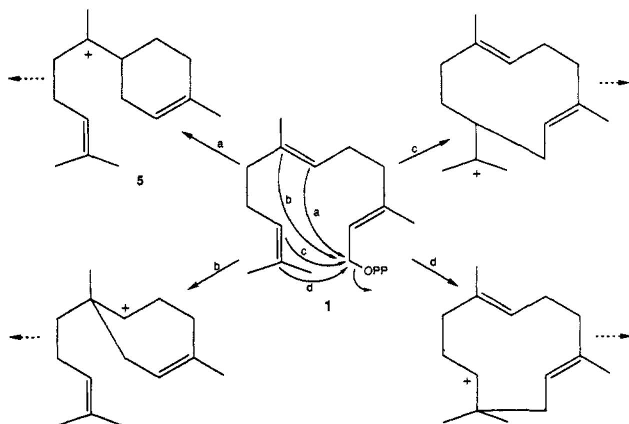
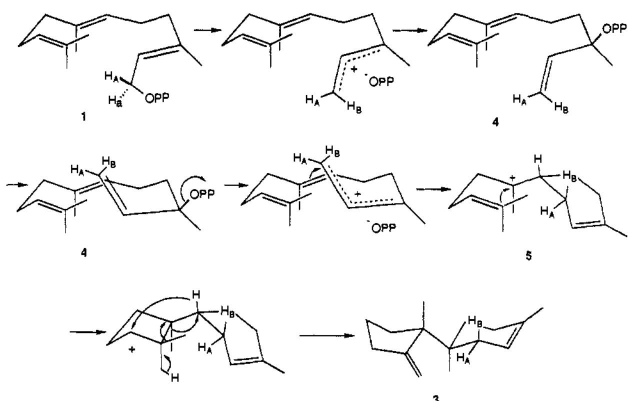
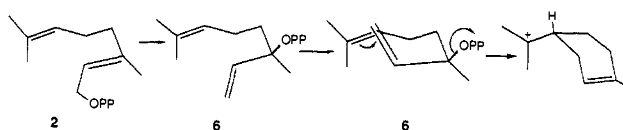
Scheme I



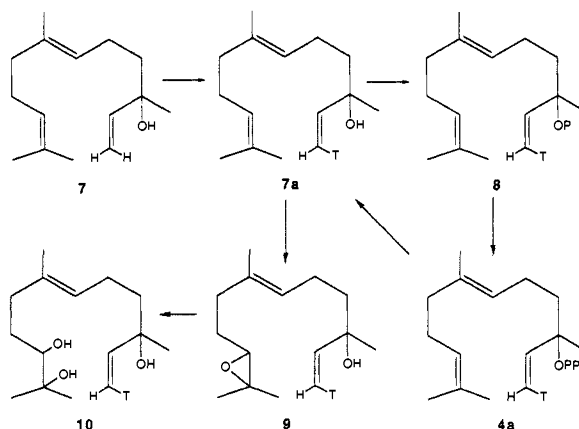
Scheme II



Scheme III



Scheme IV



of the best studied of the sesquiterpene cyclases has been trichodiene synthetase,[5] an enzyme isolated from a variety of fungal sources, which catalyzes the conversion of *trans,trans*-farnesyl pyrophosphate to trichodiene (**3**), the parent hydrocarbon of the trichothecane family of sesquiterpene antibiotics. Within the last 2 years, Hohn has purified trichodiene synthetase to homogeneity from *Fusarium trichosporioides* and has determined that the enzyme is a dimer consisting of two identical subunits of M_r 45 000.[6] As with all other known sesquiterpene and monoterpene cyclases, trichodiene synthetase requires no cofactors other than a divalent cation, Mg^{2+} being preferred. The observed K_m for farnesyl pyrophosphate, 25 nM, is considerably lower than the values that have been reported for other sesquiterpene synthetases, typically 0.5–2.0 μM.

We have reported studies that establish that the cyclization of farnesyl pyrophosphate to trichodiene takes place without loss of either of the original C-1 hydrogen atoms of the precursor and with net retention of configuration at this center.[5] These results are completely consistent with a cyclization mechanism, illustrated in Scheme II, involving initial isomerization of farnesyl pyrophosphate (**1**) to the corresponding tertiary allylic pyrophosphate ester nerolidyl pyrophosphate (**4**). Rotation about the newly formed 2,3 single bond allows nerolidyl pyrophosphate to adopt a conformation appropriate for cyclization to the bisabolyl cation (**5**) and thence to trichodiene itself by a series of well-established hydride shifts and methyl migrations. The proposed intermediacy of nerolidyl pyrophosphate is supported by the analogous role of linalyl pyrophosphate (**6**), which has been established beyond doubt in the formation of a wide variety of monoterpene cyclizations[3] (Scheme III). On the other hand, direct evidence for the intermediacy of nerolidyl pyrophosphate in the formation of trichodiene, or indeed any other cyclized sesquiterpene, has been lacking. We now present evidence that demonstrates conclusively the intermediacy of nerolidyl pyrophosphate in the enzymatic conversion of farnesyl pyrophosphate to trichodiene and which establishes the complete stereochemical course of this transformation.[7]

Results

As a first step in testing the viability of nerolidyl pyrophosphate as a precursor of trichodiene, we required a suitably labeled sample of **4**. To this end, 6,7-*trans*-nerolidol (**7**) was treated with 2.5 equiv of *n*-butyllithium–TMED,[8] and the resulting (1*Z*)-vinyllithium derivative was quenched with 2.5 equiv of [^{3}H]trifluoroacetic acid to give (1*Z*)-6,7-*trans*-[1-^{3}H]nerolidol (**7a**) (5.36 mCi/mmol) (Scheme IV). To verify the stereospecificity of the reaction, the metalation sequence was carried out under identical conditions followed by quenching with D_2O. Analysis of the resulting nerolidol by 250-MHz ^{1}H NMR in the presence of 30 mol % Eu(dpm)$_3$ established that the corresponding deuteriated product consisted of >99% of the 1*Z* isomer of [1-^{2}H]nerolidol. Preparation of the pyrophosphate esters of tertiary allylic alcohols by the traditional sequential phosphorylation method of Cramer and Boehm normally results in exceedingly low yields of the desired product.[9] Unfortunately, the otherwise excellent procedure introduced recently by Poulter,[10] based on nucleophilic substitution of the allylic halides by inorganic pyrophosphate, is inapplicable to tertiary allylic pyrophosphates. We therefore developed a modification of the Cramer–Boehm method, based on the stepwise phosphorylation of nerolidol. Treatment of (1*Z*)-[1-^{3}H]nerolidol (**7a**) with 1 equiv of (1,2-dibromo-1-phenylethyl)phosphonic acid in methylene chloride in the presence of diisopropylethylamine[11] gave nerolidyl phosphate (**8**), which, after evaporation of the solvent, was reacted directly with bis(triethylammonium) phosphate and trichloroacetonitrile in acetonitrile[12] to provide a 9%

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Scheme V

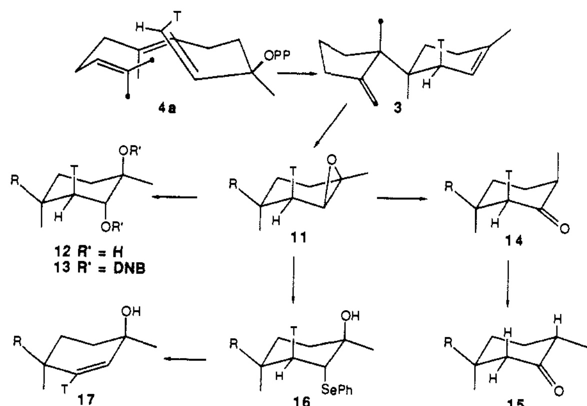

Table I. Conversion of (1*Z*)-[1-^{3}H,12,13-^{14}C]Nerolidyl Pyrophosphate (**4a**) to Trichodiene (**3**) by Trichodiene Synthetase and Distribution of the Label in **3**

compd	^{14}C specific activity dpm/mmol	^{3}H/^{14}C	atom ratio
4a[a]	1.01 × 10^9	3.90 ± 0.08[b]	1:2
3	2.90 × 10^4	3.85 ± 0.04	0.99:2
13	2.59 × 10^4	3.73 ± 0.05	0.96:2
15	2.05 × 10^4	0.10 ± 0.01	0.03:2
17	2.25 × 10^4	3.41 ± 0.05	0.87:2

[a] Amount nerolidyl pyrophosphate incubated, 1.05 × 10^6 dpm (1.04 μmol). [b] Ratio based on recrystallization of 10,11-dihydroxynerolidol (**10**).

yield of (1*Z*)-[1-^{3}H]nerolidyl pyrophosphate after purification by ion-exchange chromatography. In like manner, [12,13-^{14}C]nerolidyl pyrophosphate was prepared from [12,13-^{14}C]nerolidol, which was synthesized as previously described. After mixing of the two labeled pyrophosphate esters, (1*Z*)-[1-^{3}H,12,13-^{14}C]-nerolidyl pyrophosphate (**4a**) was incubated with freshly prepared trichodiene synthetase. The resulting labeled trichodiene (**2**) was extracted into pentane and purified by flash column chromatography after addition of 3 mg of synthetic (±)-trichodiene[13] as unlabeled carrier. Treatment of the residual aqueous phase with alkaline phosphatase liberated nerolidol, which was diluted with 100 mg of inactive nerolidol and converted to the corresponding 10,11-epoxynerolidol (**9**) by reaction with *m*-chloroperbenzoic acid[12] (Scheme IV). Exposure of **9** to 3% perchloric acid in THF–water generated 10,11-dihydroxynerolidol (**10**), which was recrystallized from $CHCl_3$–hexane to constant activity and isotope ratio.

In order to establish the position and stereochemistry of tritium labeling, the trichodiene (**3**) derived from [1-^{3}H,12,13-^{14}C]nerolidyl pyrophosphate (**4a**) was subjected to the previously described chemical degradation sequence[5] after dilution with a further 50 mg of trichodiene (Scheme V, Table I). Selective epoxidation with *m*-chloroperbenzoic acid gave 9,10β-epoxytrichodiene (**11**), a portion of which was hydrolyzed to the 9,10-diol **12** and thence derivatized to the crystalline bis(dinitrobenzoate) **13** for determination of the precise ^{3}H/^{14}C ratio. The tritium label in **3** was shown to be at H-11 by Lewis acid catalyzed rearrangement of the epoxide ($LiClO_4$, benzene) to yield the axial methyl ketone **14**, which lost all tritium upon base-catalyzed exchange and conversion to the more stable equatorial epimer **15**. When the epoxide was treated with sodium phenylselenide and the derived hydroxy phenylselenide **16** was oxidized with periodate, the allylic alcohol **17**, which resulted from syn elimination, retained essentially all the tritium of the parent trichodiene, thereby demonstrating the 11β stereochemistry of the tritium label.

Having demonstrated that nerolidyl pyrophosphate can serve as a substrate for trichodiene synthetase, we turned our attention to establishing which enantiomer of **4** is involved in the cyclization.

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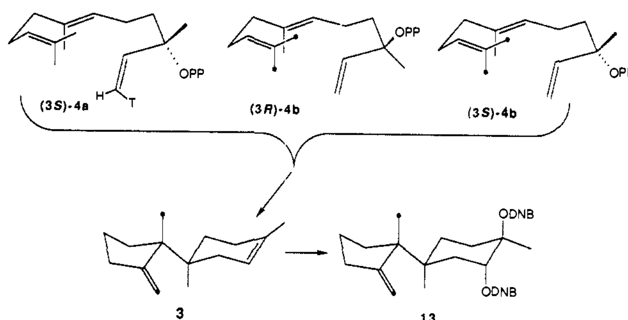
Scheme VI



Scheme VII

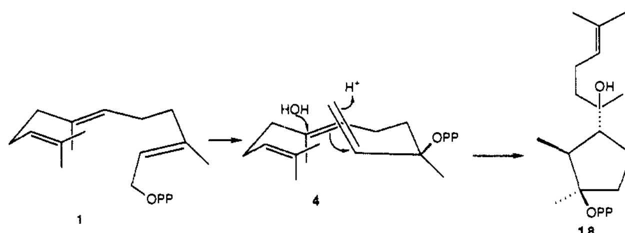


Table II. Conversion of (3*S*)-(1*Z*)-[1-^{3}H]Nerolidyl Pyrophosphate and (3*RS*)-[12,13-^{14}C]Nerolidyl Pyrophosphate (**4**) to Trichodiene (**3**) by Trichodiene Synthetase

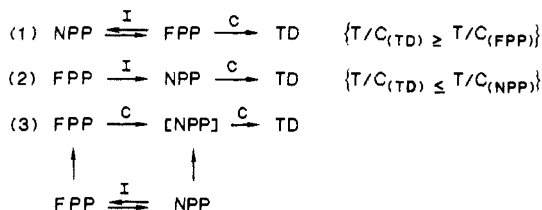
compd	^{14}C specific activity dpm/mmol	^{3}H/^{14}C
4[a]	1.23 × 10^6	0.95 ± 0.02[b]
13	4.93 ± 0.04 × 10^4	0.05 ± 0.01

[a] Amount nerolidyl pyrophosphate incubated: 2.6 × 10^6 dpm ^{3}H (0.27 μmol); 2.43 × 10^6 dpm ^{14}C (1.71 μmol). [b] Ratio based on recrystallization of 10,11-dihydroxynerolidol (**10**).

We therefore required a sample of optically pure [^{3}H]nerolidyl pyrophosphate. To this end, (3*S*)-(1*Z*)-[1-^{3}H]nerolidol ((3*S*)-**7a**) was prepared from naturally occurring (3*S*)-nerolidol ($[\alpha]_D$ +15.2°) by regiospecific deprotonation with 2.5 molar equiv of *n*-butyllithium–TMED followed by treatment of the resulting lithiated species with [^{3}H]trifluoroacetic acid. This (3*S*)-(1*Z*)-[1-^{3}H]nerolidol was mixed with racemic [12,13-^{14}C]nerolidol, and the combined samples were converted in 12% yield to the pyrophosphate ester (3*S*)-**4a** as before. Unreacted nerolidol was derivatized as 10,11-dihydroxynerolidol (**10**), which was recrystallized to constant activity and isotope ratio. The mixture of (3*S*)-(1*Z*)-[1-^{3}H]nerolidyl pyrophosphate (0.27 μmol, 2.5 × 10^6 dpm ^{3}H) and (3*RS*)-[12,13-^{14}C]nerolidyl pyrophosphate (1.71 μmol, 2.43 × 10^6 dpm ^{14}C) was incubated with trichodiene synthetase for 2 h at 30 °C (Scheme VI). Were only the 3*S* enantiomer of nerolidyl pyrophosphate to be utilized, the ^{3}H/^{14}C value of the resulting trichodiene would be exactly twice the isotope ratio of the parent nerolidyl pyrophosphate mixture. If, on the other hand, only the 3*R* enantiomer were consumed, the derived trichodiene would contain only ^{14}C, while equal conversion of both enantiomers would give trichodiene with an unchanged ^{3}H/^{14}C ratio. In the event, the isolated trichodiene (9.2 × 10^3 dpm ^{14}C, 0.4% of total nerolidyl pyrophosphate ^{14}C activity) was devoid of tritium, as was the corresponding recrystallized bis(dinitrobenzoate) ester **13** (^{3}H/^{14}C = 0.06) (Table II), thereby unambiguously establishing (3*R*)-nerolidyl pyrophosphate as the enantiomer utilized by trichodiene synthetase.

We next set out to establish that nerolidyl pyrophosphate is in fact an *intermediate* in the conversion of farnesyl pyrophosphate to trichodiene. Since, at the time, we did not have access to homogeneous trichodiene synthetase, our task was complicated by the knowledge that the crude preparations of trichodiene synthetase from *Trichothecium roseum* that we were using were contaminated with a discrete isomerase capable of catalyzing the interconversion of farnesyl to nerolidyl pyrophosphate, as well as with a third enzyme, cyclonerodiol pyrophosphate synthetase,

which generates cyclonerodiol pyrophosphate (**18**) by addition of 1 equiv of water across the vinyl and central double bonds of nerolidyl pyrophosphate[14] (Scheme VII). In principle, therefore, we had to consider the possibility that the contaminating isomerase (I) might simply be converting nerolidyl pyrophosphate (NPP) to farnesyl pyrophosphate (FPP), which would then be cyclized to trichodiene (TD) by an as yet unrecognized mechanism.[15] Alternatively, either the isomerase or trichodiene synthetase itself (*C*) might be generating free nerolidyl pyrophosphate, which would serve as the true substrate for trichodiene synthetase. These two alternatives are summarized by mechanisms 1 and 2 below. The third possibility, implicit in the mechanism of Scheme II and illustrated by mechanism 3, is based on the assumption that isomerization and cyclization of the allylic pyrophosphate substrates occur at the same active site of trichodiene synthetase without release of free intermediates.



To distinguish among these three mechanistic alternatives, we carried out a competition experiment in which the relative rate of formation of trichodiene from [^{3}H]farnesyl pyrophosphate (**1**) and [^{14}C]nerolidyl pyrophosphate (**4**) was determined by measuring the ^{3}H/^{14}C ratio in the product **3** and comparing this value with the corresponding isotope ratio in each recovered substrate as a function of time. Thus, according to mechanism 1, trichodiene would be formed faster from (1*Z*)-[1-^{3}H]farnesyl pyrophosphate than from nerolidyl pyrophosphate, which must first undergo isomerization to the primary allylic pyrophosphate. Put in other terms, tritium will appear in trichodiene faster than does ^{14}C, while ^{14}C will begin to enrich farnesyl pyrophosphate before it reaches trichodiene. It is readily seen that at any given time the resulting ^{3}H/^{14}C (T/C) ratio of the trichodiene would always exceed that of unreacted farnesyl pyrophosphate. By contrast, mechanism 2 requires trichodiene to be enriched more rapidly with ^{14}C from nerolidyl pyrophosphate than with ^{3}H from farnesyl pyrophosphate. In this instance, the ^{3}H/^{14}C ratio of the resulting trichodiene would always be less than the ^{3}H/^{14}C ratio of the recovered nerolidyl pyrophosphate. According to mechanism 3, the transformation of farnesyl pyrophosphate to trichodiene involves the intermediacy of enzyme-bound nerolidyl pyrophosphate. In the competition experiment, both farnesyl and nerolidyl pyrophosphate would compete for the same active site of the cyclase, and the proportion of trichodiene formed from each substrate will be a function of the relative V_{max}/K_m and concentration of each substrate. The observed ^{3}H/^{14}C ratio of the resulting trichodiene at low conversions will therefore be given by eq 1:

$$^3\text{H}/^{14}\text{C (TD)} = \left(\frac{V_{max}/K_m(\text{FPP})}{V_{max}/K_m(3R\text{-NPP})}\right) \times \left(\frac{[\text{FPP}]}{[3R\text{-NPP}]}\right)\left(\frac{^3\text{H}/\mu\text{mol (FPP)}}{^{14}\text{C}/\mu\text{mol }(3R\text{-NPP})}\right) = \frac{V_{max}/K_m(\text{FPP})(0.5)(1.48 \times 10^8)}{V_{max}/K_m(3R\text{-NPP})(2.5)(1.42 \times 10^6)} \quad (1)$$

As the reaction progresses, the observed ratio is expected to vary as the individual substrate concentrations change due to the action of the contaminating isomerase, phosphatase, and cyclonerodiol synthetase activities.

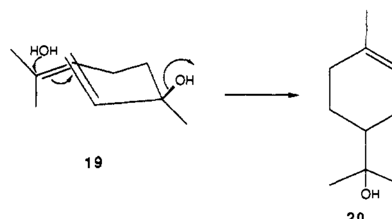
In the actual experiment, a mixture of [1-^{3}H]farnesyl pyrophosphate and [12,13-^{14}C]nerolidyl pyrophosphate was incubated with trichodiene synthetase, aliquots being withdrawn at appropriate intervals, beginning 2 min after the start of the incubation. Each aliquot was first extracted with hexane to recover the labeled trichodiene, and the remaining aqueous phase was then treated with alkaline phosphatase. The resulting nerolidol and farnesol were combined with the allylic alcohols generated by endogenous phosphatase activity and purified by preparative TLC. The results summarized in Table III clearly rule out both mechanisms 1 and 2, since at each time point the ^{3}H/^{14}C ratio of the recovered trichodiene was significantly less than that of the corresponding residual farnesol but far in excess of that of the recovered nerolidol. On the other hand, the observed isotope ratios are completely consistent with mechanism 3 in which both substrates compete for the same active site with neglible release of free intermediates. Since only the (3*R*)-nerolidyl pyrophosphate serves as a substrate for trichodiene synthetase, the apparent V_{max}/K_m of (3*R*)-nerolidyl pyrophosphate can be calculated from eq 1 to be at least 1.5–2.0 times the apparent V_{max}/K_m of farnesyl pyrophosphate.

Table III. Competitive Incubation of [1-^{3}H]Farnesyl Pyrophosphate (**1**) and [12,13-^{14}C]Nerolidyl Pyrophosphate (**4**) with Trichodiene Synthetase[a]

incubation time, min	^{3}H/^{14}C		
	farnesol	nerolidol	trichodiene
2	≥200	0.09	10.2
4	≥200	0.07	13.8
8	≥200	0.06	14.1
15	≥200	0.07	14.6
30	≥200	0.06	15.5
60	≥200	0.04	17.4

[a] Incubation carried out with 24.7 nmol (0.5 μM) of [1-^{3}H]farnesyl pyrophosphate (1.48 × 10^5 dpm/nmol) and 250 nmol (5 μM) [12,13-^{14}C]nerolidyl pyrophosphate (1.42 × 10^3 dpm/nmol).

Scheme VIII



Discussion

The above-described experiments have established the intermediacy of nerolidyl pyrophosphate in the enzymatic conversion of farnesyl pyrophosphate to trichodiene. These results are fully consistent with the mechanism illustrated in Scheme II. It has long been recognized that direct formation of six-membered rings from trans allylic pyrophosphate precursors such as geranyl and farnesyl pyrophosphate is geometrically forbidden, since the double bond of the product cyclohexene must of necessity be cis.[1-4] This obstacle to cyclization is readily overcome by initial isomerization of the precursor to the corresponding tertiary allylic pyrophosphate, linalyl or nerolidyl pyrophosphate, which can undergo rotation about the 2,3 single bond as a prelude to further ionization and cyclization. Indeed, the solvolytic cyclization of linalool (**19**) to α-terpineol (**20**) (Scheme VIII), a reaction which was first reported 90 years ago by Stephan,[16a] has been shown to involve an anti-endo conformation of the substrate.[16b] Andersen has exploited these basic ideas in an elegant series of investigations of biomimetic cyclization reactions.[17] Moreover, extensive studies of mono-

(14) *T. roseum* produces cyclonerodiol (cf. Nozoe, S.; Morisaki, N. *Tetrahedron Lett.* **1970**, 1293) and therefore must contain the relevant farnesyl pyrophosphate–nerolidyl pyrophosphate isomerase.[12]

(15) The possibility that cyclization of farnesyl pyrophosphate might involve a redox mechanism has been conclusively excluded for the formation of trichodiene[5] as well as for all other sesquiterpene and monoterpene cyclases which have been estimated to date.[1-4]

(16) (a) Stephan, K. *J. Prakt. Chem.* **1898**, *58*, 109. (b) Gotfredsen, S.; Obrecht, J. P.; Arigoni, D. *Chimia* **1977**, *31*(2), 62.

(17) Andersen, N. H.; Ohta, Y.; Syrdal, D. D. In *Bio-Organic Chemistry*; van Tamelen, E. E., Ed.; Academic: New York, 1977; Vol. 2, pp 1–37. Andersen, N. H.; Syrdal, D. D. *Tetrahedron Lett.* **1972**, 2455.

terpene cyclases have confirmed the intermediacy of linalyl pyrophosphate (**6**) in the formation of all the major classes of cyclic monoterpenes.[3] The demonstration that nerolidyl pyrophosphate plays an analogous role in the formation of trichodiene is the first explicit demonstration of the intermediacy of a teriary allylic pyrophosphate in the formation of a six-membered ring sesquiterpene and emphasizes the essential similarity of monoterpene and sesquiterpene cyclizations.

On the basis of the established intermediacy of nerolidyl pyrophosphate in the cyclization of farnesyl pyrophosphate to trichodiene, trichodiene synthetase is seen to be an isomerase–cyclase. Thus initial isomerization of farnesyl pyrophosphate (**1**) will generate nerolidyl pyrophosphate (**4**), presumably by way of the transoid allylic cation–pyrophosphate anion pair. Rotation about the newly generated 2,3-single bond and ionization will generate the corresponding cisoid ion pair, which can now undergo backside attack by the neighboring 6,7 double bond. Both isomerization and cyclization are therefore presumed to occur at the same active site and by the same general ionization mechanism. The calculated value of 2.0 for the ratio of V_{max}/K_m for nerolidyl pyrophosphate to that of farnesyl pyrophosphate can be compared with values of 10–20 for the relative V_{max}/K_m of linalyl and geranyl pyrophosphate, which have been determined for a variety of monoterpene cyclases.[3] Neglecting possible differences in the rate of substrate binding, the 2-fold or greater value of the relative V_{max}/K_m for the tertiary pyrophosphate implies that the initial isomerization step is rate-determining.

Combined with earlier studies of the stereochemistry of the cyclization of farnesyl pyrophosphate to trichodiene,[5] a complete stereochemical picture of the trichodiene synthetase reaction is now available. Thus the demonstrated net retention of configuration at C-1 of farnesyl pyrophosphate in the displacement of the pyrophosphate moiety and formation of the new C–C bond is the inevitable consequence of the coupled isomerization–rotation–cyclization process. The observed conversion of (1*Z*)-[1-^{3}H]nerolidyl pyrophosphate to [11β-^{3}H]trichodiene establishes that cyclization occurs on the 1-*re* face of the vinyl double bond. Combined with the demonstration that trichodiene synthetase utilizes exclusively the 3*R* enantiomer of nerolidyl pyrophosphate, it can be inferred that the cyclization takes place by way of the anti-endo conformation of **4**. The latter conclusion is completely consistent with not only the aforementioned studies of nonenzymatic linalool solvolysis but the results of extensive studies of monoterpene cyclizations as well.[3] Finally, with a knowledge of the stereochemical course of the conversion of both farnesyl and nerolidyl pyrophosphate to trichodiene, it can be inferred that the isomerization of **1** to **4** catalyzed by trichodiene synthetase is a suprafacial rearrangement as expected from the results of our earlier studies of farnesyl to nerolidyl pyrophosphate isomerization,[12] which also strongly implicated an intermediate allylic cation–pyrophosphate ion pair in this rearrangement. Further studies designed to uncouple the isomerization and cyclization events of trichodiene biosynthesis are in progress.

Experimental Section

General Methods. High-field NMR spectra were recorded on a Bruker WM250 spectrometer at 250 MHz (^{1}H), 62.9 MHz (^{13}C), and 101.2 MHz (^{31}P). High-resolution chemical-ionization and electron-impact mass spectra were obtained on a Kratos MS80 mass spectrometer. Melting points are uncorrected. Liquid scintillation counting was carried out in a Beckman Model LS 5801 in 5-mL solutions of OptiFluor (United Technology Packard). Quench corrections were established with commercial standards containing increasing amounts of nitromethane, and backgrounds were automatically corrected to zero. Background activity of all cocktails was measured before addition of samples, and the final values were corrected. Fermentations were carried out in a New Brunswick G-25 gyrotory incubator shaker. Nanopure water obtained from a Sybron Barnstead Nanopure II Ion Exchange system was used for all enzyme preparations.

Materials and Methods. [1-^{3}H]Farnesyl pyrophosphate and [12,13-^{14}C]nerolidol were prepared as previously described.[12] Synthetic (±)-trichodiene was prepared by the method of Harding.[13] *trans*-Nerolidol was synthesized as previously described[12] from *trans*-geranyl acetone, which was purchased from Fluka. (3*S*)-(+)-*trans*-Nerolidol was a gift from Prof. N. H. Andersen of the University of Washington. [^{14}C]-Methyl iodide (5–10 mCi/mmol) and [^{3}H]water (18 mCi/mmol) were purchased from New England Nuclear. Alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa was purchased from Sigma. All solvents for reactions were distilled prior to use, and reagents were of the highest grade available. Alkyllithium reagents were titrated with 2,5-dimethoxybenzyl alcohol[18] under argon atmosphere. Flash column chromatography was carried out according to the method of Still[19] with Merck silica gel 60 (40–60 μm; 230–400 mesh.) Silver nitrate impregnated silica gel for column chromatography was prepared by mixing 100 g of Merck silica gel 60 with 100 mL of acetonitrile containing 15 g of silver nitrate. The solvent was removed from the resulting slurry on the rotary evaporator, and the silica was dried overnight in a vacuum oven at 50 °C. Protein concentrations were determined by dye binding assay[20] by using standard curves based on Protein Standard II (bovine albumin) purchased from Bio-Rad. Maintenance and fermentation of *T. roseum* ATCC 8685 was carried out as previously described.[5] Trichodiene synthetase (F_0^{80} fraction, 0.05–0.8 nmol of trichodiene mg of protein^{-1} h^{-1}) was prepared (procedure B) and assayed as previously described.[5]

(1*Z*)-*trans*-[1-^{3}H]Nerolidol (7a). To 146 mg of tetramethylethylenediamine (TMED, 1.25 mmol) and 111.2 mg (0.5 mmol) of *trans*-nerolidol (**7**) in 2 mL of hexane was added 0.48 mL of 2.6 M *n*-butyllithium (1.25 mmol) in hexane at room temperature under argon. The solution was refluxed for 1 h under positive argon pressure. The resulting dark red solution was cooled quickly to room temperature with a water bath, and the vinyllithium was quenched with [^{3}H]trifluoroacetic acid prepared from 0.63 mmol of tritiated water (11 μL, 1 mCi/μL) and 0.63 mmol of trifluoroacetic anhydride (90 μL). After 10 min of further stirring, the pale yellow reaction mixture was poured into saturated NH_4Cl solution and extracted with two 10-mL portions of ether. The extracts were combined, washed successively with saturated $NaHCO_3$ and saturated NaCl solution, dried over $MgSO_4$, and concentrated under reduced pressure to afford 109 mg of crude [1-^{3}H]-**7**, which was purified by flash column chromatography (1 × 25 cm column, 2:1 *n*-hexane–ether) to give 70.3 mg (63% yield) of (1*Z*)-[1-^{3}H]nerolidol (5.41 mCi/mmol, 1.19 × 10^{10} dpm/mmol, 31.5% radiochemical yield).

(3*S*)-(1*Z*)-*trans*-[1-^{3}H]Nerolidol ((3S)-7a). (3*S*)-*trans*-Nerolidol ([α]$_D$ +15.2°, 189 mg, 0.85 mmol) was tritiated in the manner described above to give 113 mg of (3*S*)-(1*Z*)-*trans*-[1-^{3}H]nerolidol (60% yield, 9.30 × 10^9 dpm/mmol, 11% radiochemical yield).

(3*RS*)-*trans*-[12,13-^{14}C]Nerolidyl Pyrophosphate (4). Solid (1-phenyl-1,2-dibromoethyl)phosphonic acid (6.6 mg, 18.9 μmol) was added to a solution of (3*RS*)-*trans*-[12,13-^{14}C]nerolidol (4.0 mg, 18.0 μmol, 1.42 × 10^9 dpm/mmol) in 0.4 mL of anhydrous CH_2Cl_2 under a nitrogen atmosphere. Diisopropylethylamine (5.7 mg, 6.6 μL, 38 μmol) was added via syringe to the well-stirred solution to give a homogeneous mixture. The resulting solution was stirred for 5 h at room temperature in the dark and then concentrated under a gentle stream of nitrogen to remove CH_2Cl_2. Bis(triethylammonium) hydrogen phosphate (17 mg, 57 μmol) in 0.3 mL of CH_3CN was added followed by 17 μL of trichloroacetonitrile (68 μmol). The homogeneous solution was stirred for 5 h at room temperature under nitrogen. The phosphorylated products were extracted with four 0.5-mL portions of 0.01 N NH_4OH and applied to a DEAE Sephadex A-25 column (1.5 cm × 15 cm), which was eluted with a linear gradient of 0.05–1.0 M triethylammonium bicarbonate at 4 °C with a flow rate of 1 drop/5 s and collecting 4.8-mL fractions. Fractions 21–24 were lyophilized and checked by comigration on TLC (*n*-propanol–1-butanol–2 M aqueous NH_4OH, 5:2:3; R_f nerolidyl monophosphate, 0.47; R_f nerolidyl pyrophosphate, 0.38) with an authentic sample to give chromatographically pure (3*RS*)-*trans*-[12,13-^{14}C]nerolidyl pyrophosphate (**4**) in 7 % yield: ^{31}P NMR (D_2O, pH adjusted to 8.0 with ND_4OD, 1 drop of 1% w/v EDTA in D_2O), δ −4.70 (d, J_{PP} = 23 Hz, 1 P, P(2)), −8.83 (d, J_{PP} = 23 Hz, 1 P, P(1); δ ext H_3PO_4 0.00).

(3*RS*)-(1*Z*)-*trans*-[1-^{3}H]Nerolidyl Pyrophosphate (4a). Solid (1-phenyl-1,2-dibromoethyl)phosphonic acid (12.2 mg, 37.8 μmol) was added to a solution of (3*RS*)-(1*Z*)-*trans*-[1-^{3}H]nerolidol (8.0 mg, 36.0 μmol) in 0.7 mL of anhydrous CH_2Cl_2 under nitrogen atmosphere. Diisopropylethylamine (11.4 mg, 13.2 μL, 76 μmol) was added, and the resulting solution was stirred for 5 h at room temperature in the dark and then concentrated under a gentle stream of nitrogen to remove CH_2Cl_2. Bis(triethylammonium) hydrogen phosphate (34 mg, 114 μmol) in 0.5 mL of CH_3CN was added followed by 34 μL of trichloroacetonitrile (136 μmol), and the solution was stirred for 5 h at room temperature under nitrogen. The phosphorylated products were extracted and purified by

(18) Winkle, M. R.; Lansinger, J. M.; Ronald, R. C. *J. Chem. Soc., Chem. Commun.* **1980**, 87.
(19) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
(20) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248. Spector, T. *Anal. Biochem.* **1978**, *86*, 142.

ion-exchange chromatography as above, collecting fractions of 3.2 mL. Fractions 60–70 were combined and lyophilized, taken up in 0.01 N NH_4OH, and identified by ^{31}P NMR comparison with an authentic sample of nerolidyl pyrophosphate.

(3*S*)-(1*Z*)-*trans*-[1-^{3}H]- and (3*RS*)-*trans*-[12,13-^{14}C]Nerolidyl Pyrophosphate. A sample of 7.7 mg (35 μmol) of nerolidol composed of 1 mg of (3*S*)-(1*Z*)-*trans*-[1-^{3}H]nerolidol and 6.7 mg of (3*RS*)-*trans*-[12,13-^{14}C]nerolidol was converted to the corresponding pyrophosphate ester by the same two-stage phosphorylation procedure. Purification by ion-exchange chromatography as before gave a 12% yield of (3*S*)-(1*Z*)-*trans*-[1-^{3}H]- and (3*RS*)-*trans*-[12,13-^{14}C]nerolidyl pyrophosphate. Unreacted nerolidol was extracted with three 5-mL portions of ether and mixed with 150 mg of unlabeled nerolidol for conversion to 10,11-dihydroxynerolidol (**10**) by the procedure described below for (3*RS*)-(1*Z*)-*trans*-[1-^{13}H,12,13-^{14}C]nerolidol. The derived 10,11-dihydroxynerolidol displayed a $^3H/^{14}C$ ratio of 0.95.

Incubation of (3*RS*)-(1*Z*)-*trans*-[1-^{3}H,12,13-^{14}C]Nerolidyl Pyrophosphate with Trichodiene Synthetase. Trichodiene synthetase (F_0^{80} fraction, 5.05 mg of protein/mL) in 48.3 mL of 50 mM potassium phosphate buffer, pH 7.2, containing 5 mM dithioerythritol (DTE), 1 mM EDTA, and 10% glycerol was mixed with 50 mL of Tris-malate buffer (consisting of 50 mM Tris·HCl, 50 mM malate, pH 6.8, 4 mM $MgCl_2$, 2 mM $MnCl_2$,[21] and 5 mM DTE) and 1.7 mL of a 0.01 N NH_4OH solution of (3*RS*)-(1*Z*)-[1-^{3}H,12,13-^{14}C]nerolidyl pyrophosphate (sp act after mixing, 1.01×10^9 dpm/mmol ^{14}C, total act 1.05×10^6 dpm ^{14}C, 1.04 μmol). The resulting mixture was incubated for 2 h at 30 °C. The aqueous layer was extracted with two 50-mL portions of pentane, and the combined extracts were washed with saturated NaCl, dried over $MgSO_4$, and concentrated to 1–2 mL. Synthetic (±)-trichodiene was added as carrier, and the sample was purified by flash column chromatography (1 × 5 cm column, pentane) to give trichodiene (7.1×10^3 dpm ^{14}C, 1.4% yield). The yield of trichodiene produced corresponded to a net turnover for trichodiene synthetase of 0.014 nmol of trichodiene mg of protein^{-1} h^{-1}. The labeled trichodiene was diluted with a further 50 mg of (±)-trichodiene (**3**) and subjected to the previously described chemical degradation procedure[5] to locate the site of tritium labeling. The aqueous phase remaining after extraction of the incubation mixture with pentane was incubated with alkaline phosphatase (30 units) in 35 mL of 0.1 M Tris·HCl (pH 8.6) at 30 °C for 24 h to hydrolyze the remaining pyrophosphate ester. The resulting nerolidol (**7**) was extracted with ether (2 × 50 mL), and the ethereal extracts were washed with saturated NaCl, dried over anhydrous $MgSO_4$, and filtered.

(3*RS*)-(1*Z*)-10,11-Epoxy[1-^{3}H,12,13-^{14}C]nerolidol (9). Unlabeled nerolidol (100 mg, 0.45 mmol) was added to the ether solution of labeled nerolidol recovered from the foregoing incubation of (3*RS*)-(1*Z*)-[1-^{3}H,12,13-^{14}C]nerolidyl pyrophosphate, and the mixture was concentrated under reduced pressure. The residue was taken up in 5 mL of CH_2Cl_2 and reacted at 0 °C with 93 mg (0.46 mmol) of 85% *m*-chloroperbenzoic, which was added in portions over a period of 3 h. The reaction mixture was stirred for an additional hour at 0 °C before neutralization of excess oxidant by addition of saturated Na_2SO_3 and extraction with ether. The combined ethereal extracts were washed successively with saturated Na_2SO_3, saturated K_2CO_3, and saturated NaCl, dried over $MgSO_4$, and concentrated to give 98 mg of crude oil, which contained 10,11-epoxynerolidol (**9**) as the major product.[12] Purification by flash column chromatography (hexane–ether, 1:1) afforded 72 mg of 10,11-epoxynerolidol (R_f 0.47, hexane–ether, 3:1) in 67% yield.

(3*RS*)-(1*Z*)-[1-^{3}H,12,13-^{14}C]-10,11-Dihydroxynerolidol (10).[12] 10,11-Epoxynerolidol (**9**) (72 mg, 0.32 mmol) was dissolved in 3 mL of 1:1 THF–water, and 0.2 mL of 3% perchloric acid was added. The solution was stirred for 3 h at room temperature, and then saturated NaCl was added to separate the phases. The reaction mixture was extracted with ether, and the combined ethereal extracts were washed with saturated K_2CO_3 and saturated NaCl, dried over $MgSO_4$, and concentrated to give 49 mg of crude triol. Purification by flash column chromatography (ether) afforded 35 mg of **10** (R_f 0.23, ether), which was recrystallized to constant activity and isotope ratio from $CHCl_3$–hexane ($^3H/^{14}C$ 3.90 ± 0.08).

Incubation of (3*S*)-(1*Z*)-*trans*-[1-^{3}H]- and (3*RS*)-*trans*-[12,13-^{14}C]-Nerolidyl Pyrophosphate with Trichodiene Synthetase. The mixture of (3*S*)-(1*Z*)-*trans*-[1-^{3}H]nerolidyl pyrophosphate (0.27 μmol, 1.25×10^6 dpm/mmol, 2.5×10^6 dpm ^3H) and (3*RS*)-*trans*-[12,13-^{14}C]nerolidyl pyrophosphate (1.71 μmol, 1.23×10^6 dpm/mmol, 2.43×10^6 dpm ^{14}C) in 2.5 mL of 0.01 N NH_4OH was combined with 197.5 mL of the F_0^{80} fraction of trichodiene synthetase (2.55 mg protein/mL in 50 mM potassium phosphate buffer, pH 7.2, containing 5 mM DTE, 1 mM EDTA, and 10% glycerol) and 200 mL of buffer consisting 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 of ammonium molybdate. The mixture was incubated for 2 h at 30 °C and then extracted with two 50-mL portions of pentane. The combined pentane extracts were washed with saturated NaCl, dried over $MgSO_4$, and concentrated to 1–2 mL. Trichodiene (3 mg) was added, and the labeled product was purified by flash column chromatography (1.5 × 5 cm column, hexane) to yield 9.2×10^3 dpm ^{14}C of trichodiene (0.8% yield based on single enantiomer of nerolidyl pyrophosphate). An additional 17 mg (77 μmol) of unlabeled trichodiene was added for conversion to *trans*-9,10-dihydroxy-9,10-dihydrotrichodiene bis(dinitrobenzoate) ester (**13**) by the usual method, and the crystalline derivative was recrystallized to constant activity and isotope ratio (4.93 ± 0.04 × 10^4 dpm/mmol ^{14}C, $^3H/^{14}C$ 0.06).

Competitive Incubation of *trans*,*trans*-[1-^{3}H]Farnesyl Pyrophosphate and (3*RS*)-*trans*-[12,13-^{14}C]Nerolidyl Pyrophosphate with Trichodiene Synthetase. Trichodiene synthetase (25 mL of F_0^{80} protein in 50 mM potassium phosphate buffer, pH 7.2, containing 5 mM DTE, 1 mM EDTA, and 10% glycerol, 10.1 mg protein/mL) was added to a solution containing 24.46 mL of Tris-malate buffer (50 mM Tris·HCl, 50 mM malate, pH 6.8, 4 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM 2-mercaptoethanol), 100 μL of [1-^{3}H]farnesyl pyrophosphate in 0.01 N NH_4OH (1.48×10^5 dpm/nmol, total activity 3.65×10^6 dpm ^3H, final concentration 0.5 μM), and 440 μL of (3*RS*)-[12,13-^{14}C]nerolidyl pyrophosphate in 0.01 N NH_4OH (1.42×10^3 dpm/nmol, total activity 3.55×10^5 dpm ^{14}C, final concentration 5 μM), and the mixture was incubated at 30 °C. At intervals of 2, 4, 8, 15, 30, and 60 min, 7-mL aliquots were withdrawn and transferred to a precooled test tube (1.5 × 15 cm) chilled in ice. Precooled hexane (2 mL) was added to extract-labeled trichodiene, and the hexane extract was passed through a short (2 cm) column of TLC grade silica gel overlayed with $MgSO_4$ in a Pasteur pipette. The total hexane extract was collected in a scintillation vial for counting. The residual aqueous solution was purged with stream of nitrogen at 0 °C for 2 min to remove any remaining organic solvent. Tris·HCl (1 mL, 0.1 M, pH 8.6) and 3 units of alkaline phosphatase were added, and the mixture was incubated for 30 h at 30 °C. The liberated farnesol and nerolidol were extracted into ether (2 × 2 mL), and the ether extract was dried and passed through the same column as had been used for the earlier hexane extract. Carrier farnesol and nerolidol (40 μL of an 80 mM ether solution) were added to the eluate, and the mixture was purified by preparative TLC (*n*-hexane–ethyl acetate, two developments; R_f farnesol, 0.38; R_f nerolidol, 0.52.) The two components were visualized with iodine vapor, the bands were scraped off each plate and eluted with ether, and each sample of nerolidol and farnesol was counted directly.

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(21) Hohn has reported that Mn^{2+} inhibits trichodiene synthetase at mM concentrations; the use of $MnCl_2$ is therefore undesirable.