Communications to the Editor

Trichodiene Biosynthesis and the Enzymatic Cyclization of Nerolidyl Pyrophosphate

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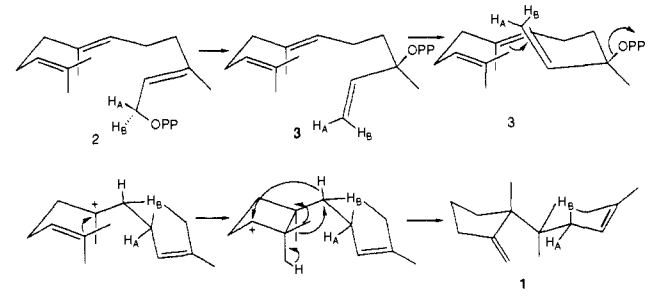
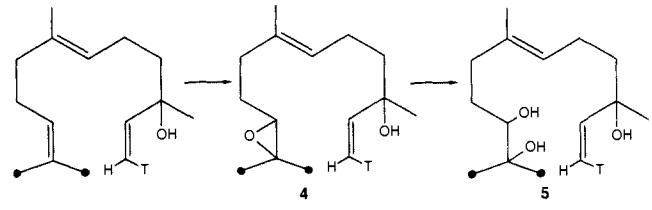
Trichodiene (**1**) is the parent hydrocarbon[1] of the trichothecane family of sesquiterpene antibiotics and toxins.[2] The formation of trichodiene from farnesyl pyrophosphate (**2**) is typical of a general class of cyclizations in which the acyclic trans-allylic pyrophosphate precursor is converted to a cyclic product containing a cis double bond without apparent release of any free intermediates.[3] In recent studies of the cell-free biosynthesis of trichodiene, we have reported that farnesyl pyrophosphate is cyclized to trichodiene by preparations of trichodiene synthetase without loss of either hydrogen atom from C-1 of the acyclic precursor and with net retention of configuration at this center[4] (Scheme I). These results are best explained by an initial isomerization of farnesyl pyrophosphate (**2**) to the corresponding tertiary allylic pyrophosphate ester, nerolidyl pyrophosphate (**3**) which can adopt the appropriate conformation for cyclization to trichodiene, consistent with the results of a significant body of model experiments[5] as well as extensive investigations of monoterpene biosynthesis.[6,7] To date, however, there have been no reports of the conversion of nerolidyl pyrophosphate to cyclic sesquiterpenes.[8] We describe below results that demonstrate the intermediacy of nerolidyl pyrophosphate in the cyclization of farnesyl pyrophosphate to trichodiene and that establish the stereochemical course of this transformation.

A sample of (1*Z*)-6,7-*trans*-[1-^{3}H]nerolidol (5.36 mCi/mmol) was prepared from nerolidol by stereospecific metalation[10] with 2.5 equiv of *n*-butyllithium–TMED followed by quenching with 2.5 equiv of [^{3}H]trifluoroacetic acid. The corresponding (1*Z*)-[1-^{3}H]nerolidyl pyrophosphate was generated in 9% yield by a two-step procedure involving initial conversion to the monophosphate ester by reaction with (1,2-dibromo-1-phenylethyl)-phosphonic acid in the presence of diisopropylethylamine,[11] followed by phosphorylation with bis(triethylammonium) phosphate and trichloroacetonitrile.[9,12] A sample of [12,13-^{14}C]nerolidyl

Scheme I



Scheme II



Scheme III

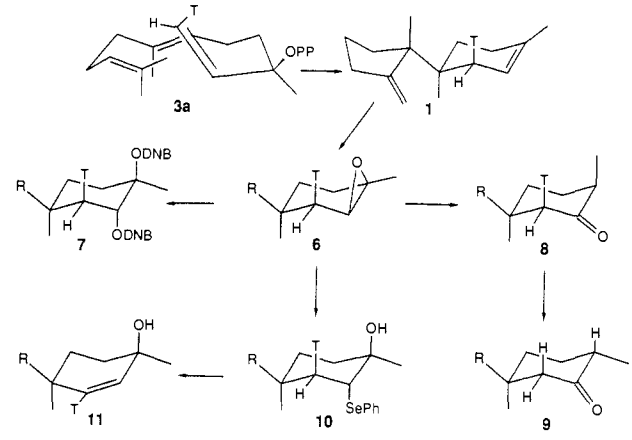


Table I. Conversion of (1*Z*)-[1-^{3}H,12,13-^{14}C]Nerolidyl Pyrophosphate (**3a**) to Trichodiene (**1**) by Trichodiene Synthetase and Distribution of the Label in **1**

compd	^{14}C specific activity, dpm/mmol	^{3}H/^{14}C	atom ratio
3a[a]	1.01×10^9	3.95 ± 0.02[b]	1:2
1[c]	2.90×10^4[d]	3.85 ± 0.04	0.97:2
7	2.59×10^4	3.73 ± 0.05	0.94:2
9	2.05×10^4	0.10 ± 0.01	0.03:2
11	2.25×10^4	3.41 ± 0.05	0.86:2

[a] Amount incubated, 1.05×10^6 dpm ^{14}C (1.04 μmol); 244 mg of F_0[80] trichodiene synthetase, 30 °C, 2 h. [b] Based on recrystallization of 10,11-dihydroxynerolidol (**5**). [c] Total recovered activity, 7.1×10^3 dpm ^{14}C; 0.014 (nmol of trichodiene/mg of protein)/h. [d] Diluted with 50 mg of (±)-trichodiene.

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(8) The cyclization of nerolidyl pyrophosphate to cyclonerodiol pyrophosphate[9] does not involve the ionization of the allylic pyrophosphate moiety and is therefore unrelated to the mechanism of trichodiene formation.
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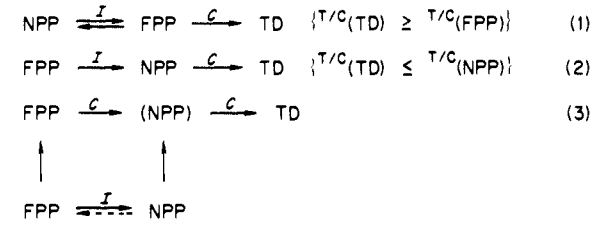
pyrophosphate (0.64 mCi/mmol) was obtained in an analogous manner from [12,13-^{14}C]nerolidol, prepared as previously described.[9] The two labeled pyrophosphate esters were mixed so as to give (1*Z*)-[1-^{3}H,12,13-^{14}C]nerolidyl pyrophosphate (**3a**) and the sample was incubated with trichodiene synthetase.[4] At the end of the incubation period, the resulting labeled trichodiene (**1**) was extracted with pentane and the extracts were subjected to purification by flash column chromatography after addition of 3 mg of synthetic[13] unlabeled (±)-trichodiene as carrier. The remaining aqueous incubation mixture was incubated with alkaline phosphatase to generate free nerolidol which was diluted with 100 mg of unlabeled nerolidol and treated with *m*-chloroperbenzoic acid[9] to give the corresponding 10,11-epoxynerolidol (**4**). Hydrolysis of **4** by treatment with 3% perchloric acid in THF–water gave 10,11-dihydroxynerolidol (**5**) which was recrystallized from $CHCl_3$–hexane for determination of the precise $^{3}H/^{14}C$ ratio of the nerolidyl pyrophosphate sample (3.95, $^{3}H/^{14}C$ atom ratio 1:2) (Scheme II).

The position and stereochemistry of tritium labeling in the trichodiene was established by using the previously developed chemical degradation sequence.[4] (Scheme III and Table I). Thus the labeled trichodiene was further diluted to 50 mg and converted to 9,10-epoxytrichodiene (**6**) by oxidation with *m*-chloroperbenzoic acid. A portion of the latter substrate was converted to the crystalline bis(dinitrobenzoate) derivative **7** ($^{3}H/^{14}C$ atom ratio 0.94:2). Acid-catalyzed rearrangement ($LiClO_4$, benzene) of **6** followed by base-catalyzed exchange of the resulting ketone **8** resulted in formation of the 10-ketotrichodiene derivative **9** which was devoid of tritium label ($^{3}H/^{14}C$ atom ratio 0.03:2), consistent with the presence of tritium at the expected site, C-11. Finally, conversion of the remaining portion of the epoxide to the corresponding hydroxyphenyl selenide **10** and oxidative syn elimination gave the allylic alcohol **11** which retained essentially all the tritium of the parent trichodiene ($^{3}H/^{14}C$ atom ratio 0.86:2), thereby establishing the 11β stereochemistry for the tritium label.

Having established the viability of nerolidyl pyrophosphate as a substrate for trichodiene synthetase, it was necessary to demonstrate that nerolidyl pyrophosphate is in fact an *intermediate* in the conversion of farnesyl pyrophosphate to trichodiene. This task was complicated by the knowledge that cell-free preparations from *T. roseum* contain a separate isomerase which mediates the simple isomerization of farnesyl to nerolidyl pyrophosphate.[14] In principle, therefore, we had to distinguish among three alternatives:



These three alternatives could be readily distinguished by carrying out a competition experiment involving [^{3}H]farnesyl pyrophosphate and [^{14}C]nerolidyl pyrophosphate and measuring the $^{3}H/^{14}C$ of the resulting trichodiene as well as recovered farnesol and nerolidol as a function of time. In eq 1, farnesyl pyrophosphate will be converted by the cyclase (*C*) to trichodiene faster than is nerolidyl pyrophosphate. At any given time, therefore, the $^{3}H/^{14}C$ (T/C) ratio of the trichodiene will exceed that of the farnesyl pyrophosphate. In eq 2, on the other hand, nerolidyl pyrophosphate must be converted to trichodiene faster than is farnesyl pyrophosphate. As a consequence, the $^{3}H/^{14}C$ ratio of the resulting trichodiene will always be less than the corresponding $^{3}H/^{14}C$ ratio of recovered nerolidyl pyrophosphate. In eq 3, trichodiene synthetase mediates the conversion of farnesyl pyrophosphate to trichodiene by way of enzyme-bound nerolidyl pyrophosphate, while a separate isomerase present in the crude cell-free extract catalyzes the competing interconversion of the primary and tertiary allylic pyrophosphate esters. Since both farnesyl pyrophosphate and nerolidyl pyrophosphate compete for the same active site of the cyclase, the relative proportion of trichodiene formed from the two allylic pyrophosphate esters will therefore be a function of the relative V_{max}/K_m for each substrate, corrected for the relative concentrations of the two substrates.[15] In the event, a mixture of [1-$^{3}H_2$]farnesyl pyrophosphate and [12,13-$^{14}C_2$]nerolidyl pyrophosphate was incubated with trichodiene synthetase. Aliquots were withdrawn at appropriate intervals, beginning 2 min after incubation, and extracted with hexane to recover the trichodiene. Each aliquot was then treated with alkaline phosphatase and the recovered farnesol and nerolidol were combined with the allylic alcohols released by endogenous phosphatase activity. As summarized in Table II, the $^{3}H/^{14}C$ of the trichodiene generated was significantly less for each sample than that of the recovered farnesol but greater than that of the recovered nerolidol. The two allylic pyrophosphate substrates are therefore competing for the same cyclase (eq 3), with negligible release of free intermediates. Assuming that only one enantiomer of nerolidyl pyrophosphate serves as a substrate for trichodiene synthetase, the apparent V_{max}/K_m for nerolidyl pyrophosphate is estimated to be 1.5–2.0 times the apparent V_{max}/K_m of farnesyl pyrophosphate.[15]

Table II. Competitive Incubation of [1-$^{3}H_2$]Farnesyl Pyrophosphate and [12,13-^{14}C]Nerolidyl Pyrophosphate with Trichodiene Synthetase[a]

	$^{3}H/^{14}C$		
incubation time, min	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] The incubation mixture consisted of 24.7 nmol (0.5 μM) of [1-$^{3}H_2$]farnesyl pyrophosphate (66.7 mCi/mmol, 3.65 × 10^6 dpm) and 250 nmol (5.0 μM) of [12,13-^{14}C]nerolidyl pyrophosphate (0.64 mCi/mmol, 3.55 × 10^5 dpm) in 25 mL of 50 mM potassium phosphate buffer, pH 7.2, containing 10.1 mg/mL of trichodiene synthetase (F_0^{80}), 5 mM DTE, 1 mM EDTA, and 10% glycerol plus 24.5 mL of assay buffer: pH 6.8, 50 mM Tris-HCl, 50 mM malate, 4mM $MgCl_2$, 2mM $MnCl_2$, and 5mM mercaptoethanol. The incubation was carried out at 30 °C and aliquots of 7 mL were withdrawn at the indicated intervals, chilled in ice, and extracted with hexane, which was passed through a short column of silica[4] for recovery of trichodiene. The remaining pyrophosphate esters were hydrolyzed by treatment with 3 units of alkaline phosphatase after addition of 1.0 mL of 0.1 M Tris-HCl, pH 8.6, and the resulting allylic alcohols were purified by TLC (hexane–ethyl acetate, 2:1, two developments.)

The above results are fully consistent with the postulated intermediacy of nerolidyl pyrophosphate in the enzymatic conversion of farnesyl pyrophosphate to trichodiene. 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. Taken together with the demonstrated retention of configuration at this center in the cyclization of farnesyl pyrophosphate, as well as our earlier finding that isomerization of farnesyl pyrophosphate to nerolidyl pyrophosphate takes place in a suprafacial manner,[9] these results imply that the formation of trichodiene involves initial generation of (3*R*)-nerolidyl pyro-

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(15) The observed $^{3}H/^{14}C$ ratio of trichodiene at low conversions (10.2–15.5) is given by the equation:

$$^{3}H/^{14}C(TD) = \left(\frac{V_{max}/K_m(FPP)}{V_{max}/K_m(R\text{-}NPP)}\right)\left(\frac{[FPP]}{[NPP]}\right)\left(\frac{^{3}H/\mu mol(FPP)}{^{14}C/\mu mol(R\text{-}NPP)}\right) = \frac{V_{max}/K_m(FPP)(0.5)(66.7)}{V_{max}/K_m(R\text{-}NPP)(2.5)(0.64)}$$

phosphate which cyclizes via the illustrated anti-boat conformation. Further experiments to demonstrate this point explicitly are in progress.[16]

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(16) **Note Added in Proof**: We have now experimentally confirmed that trichodiene synthetase cyclizes (3*R*)-nerolidyl pyrophosphate.

$Pd(\eta^1\text{-}H_2)$ and $Pd(\eta^2\text{-}H_2)$: Ligand-Free End-on and Side-on Bonded Molecular Dihydrogen Complexes

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As part of our program on H_2 and CH_4 activation on single metal atoms,[1] we have discovered a system that yields molecular dihydrogen complexes rather than hydrides. Following Kubas' seminal discovery of $W(CO)_3(PR_3)_2(\eta^2\text{-}H_2)$[2] containing side-on bound molecular dihydrogen a flurry of reports appeared on other dihydrogen complexes including $Cr(CO)_5(H_2)$,[3-5] $Cr(CO)_4(H_2)_2$,[3] $[Ir(H)(H_2)(PPh_3)_2(C_{13}H_8N)]^+$,[6] *trans*-$[M(H)(H_2)(PPh_2CH_2CH_2PPh_2)_2]BF_4$,[7] and $[IrH_2(H_2)_2L_2]^+$, where L = $P(C_6H_{11})_3$.[8] Interestingly, some reports of intrazeolite dihydrogen complexes of Ni^+ and Pd^+ exist in the earlier literature.[9,10] In conjunction with ab initio quantum chemical calculations on model $M + H_2$ potential energy surfaces,[11] a clearer insight into the H_2 activation process and the role and properties of precoordinated molecular dihydrogen is developing.

Despite this impressive progress there still did not exist any experimental information on the most basic of all systems, namely, $M(H_2)$, a "ligand-free molecular dihydrogen complex".

As a direct outcome of our work with the Pd/H_2 system at low temperatures we have discovered that ground-state $4d^{10}$ Pd atoms "spontaneously" react with H_2 in rare gas matrices to produce molecular dihydrogen complexes.

The work can be briefly described as follows. Pd atoms react on deposition with H_2-doped Kr and and Xe matrices at 12 K (Figures 1 and 2). Optical and IR spectroscopy, in conjunction with Pd concentration studies, H_2, HD, and D_2 isotope substitution experiments, thermal annealing, and wavelength-selective photochemistry, define the products to be $Pd(\eta^1\text{-}H_2)$ coexisting with $Pd(\eta^2\text{-}H_2)$ in Kr, while $Pd(\eta^2\text{-}H_2)$ exclusively exists in Xe,[12] (Figures 1 and 2). The fact that small matrix changes (Kr to Xe) can induce such alterations in the mode of coordination of dihydrogen, from $\eta^1\text{-}H_2$ to $\eta^2\text{-}H_2$, implies that the energy differences between $Pd(\eta^1\text{-}H_2)$ and $Pd(\eta^2\text{-}H_2)$ are of the same order of magnitude as matrix interactions. The lack of observable isotope scrambling in H_2/D_2 and HD experiments and the nonobservation

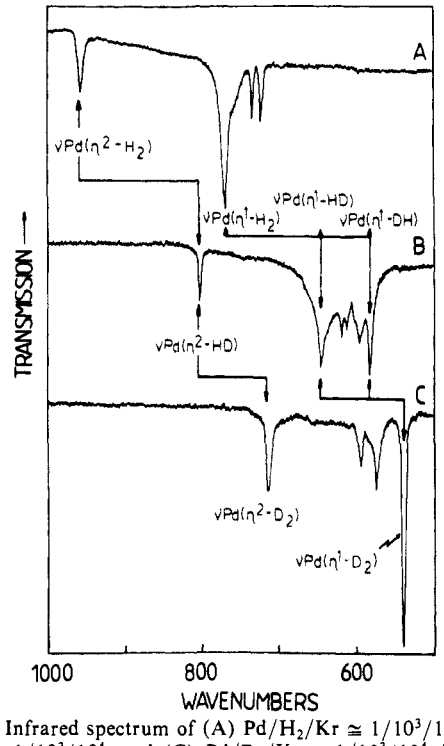

Figure 1. Infrared spectrum of (A) $Pd/H_2/Kr \cong 1/10^3/10^4$, (B) $Pd/HD/Kr \cong 1/10^3/10^4$, and (C) $Pd/D_2/Kr \cong 1/10^3/10^4$ deposited at 10-12 K. The vibrational assignments are indicated. Resolution, 2 cm^{-1}. A preliminary N.C.A. of $Pd(\eta^1\text{-}H_2)/Pd(\eta^1\text{-}HD)/Pd(\eta^1\text{-}DH)/Pd(\eta^1\text{-}D_2)$ for a linear configuration favors the assignments denoted in the figure for this species.

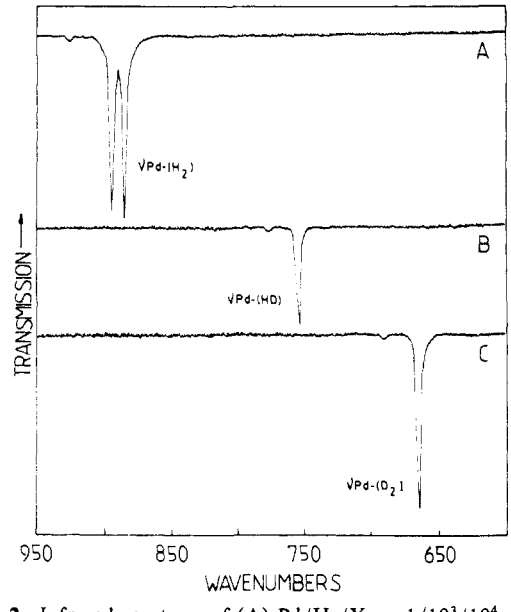

Figure 2. Infrared spectrum of (A) $Pd/H_2/Xe \cong 1/10^3/10^4$, (B) $Pd/HD/Xe \cong 1/10^3/10^4$, and (C) $Pd/D_2/Xe \cong 1/10^3/10^4$ deposited at 10-12 K. The vibrational assignments are indicated. Resolution, 2 cm^{-1}.

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(12) Thermal annealing up to 45 K shows the growth and decay of an additional *unsplit* band in each Pd/H_2, HD, or D_2/Xe sample, blue-shifted by 37.5, 24.0, or 24.5 cm^{-1} with respect to the band observed on deposition. Two distinct trapping sites for $Pd(\eta^2\text{-}H_2)$ are therefore defined.

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