In both types of holoenzyme complexes, NAD^+ occurs in an extended form with adenine and nicotinamide planes nearly 13 Å separated and roughly perpendicular to each other. It is most striking that in complexes with A-type dehydrogenases the Ψ torsion angles about both C(4′)–C(5′) bonds in NAD^+ are not, as usually preferred, (+)-gauche but rather trans or (−)-gauche (Table V). In the B-type GAPDH and GPDH dehydrogenases, only adenylic acid is trans while the nicotinamide riboside with syn-oriented nicotinamide displays a (−)-gauche arrangement about C(4′)–C(5′). We may conclude that NAD^+ when binding to the enzyme active site has to change its otherwise preferred (+)-gauche conformation, probably in order to render phosphates, sugars, and heterocycles more accessible to the active sites of the enzymes. Some extra energy in the range of ~5 kcal per C(4′)–C(5′) bond has to be put into the system which can easily be provided when NAD^+ binds to the enzyme active site. During this process, NAD^+ has to be dehydrated and the cation removed and replaced by positively charged amino acid side groups lining the NAD^+ binding sites. The side groups do not only compensate for the positive charge, but they also form hydrogen bonds to phosphate, ribose, and nicotinamide while adenine fits into a hydrophobic pocket. In this respect, the extensive hydrogen bonding found in the $Li^+ \cdot NAD^+$ crystal structure is paralleled by hydrogen bonding with the enzyme surface. Since the active sites bind NAD^+ in an extended form, it is reasonable to assume that the dehydrogenases recognize NAD^+ in a similar form, probably close to that observed in $Li^+ \cdot NAD^+$. The folded NAD^+ molecule with nicotinamide stacked on adenine, i.e., both heterocycles at 3.4-Å distance and parallel to each other, looks too different to allow recognition and has to open up in dynamical equilibrium prior to insertion into the active site.

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Supplementary Material Available: A listing of observed and calculated structure amplitudes (10 pages). Ordering information is given on any current masthead page.

Cyclonerodiol Biosynthesis and the Enzymatic Conversion of Farnesyl to Nerolidyl Pyrophosphate

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Abstract: Cell-free enzymes from *Gibberella fujikuroi* convert farnesyl pyrophosphate (**1**) to nerolidyl pyrophosphate (**2**) and thence to cyclonerodiol (**3**) by way of cyclonerodiol pyrophosphate. This pathway is supported by incubation and degradation experiments, competitive incubation studies, trapping and degradation of nerolidyl pyrophosphate, and direct ^{13}C NMR observation of the enzymatic conversion of nerolidyl pyrophosphate to cyclonerodiol. Mass spectrometric analysis of a derivative of cyclonerodiol obtained from an incubation in the presence of [^{18}O]water established that pyrophosphate ester hydrolysis takes place with P–O bond cleavage and that the side-chain hydroxyl of **3** is derived from water. Incubation of a mixture of [12,13-^{14}C]-(3*S*)-**2** and [1-^{3}H]-(3*RS*)-**2** gave cyclonerodiol which was devoid of ^{14}C, establishing that the intermediate nerolidyl pyrophosphate has the 3*R* configuration. Incubation of (*E*)-[1,2-$^{2}H_2$,1-^{3}H]nerolidyl pyrophosphate with the cell-free extract of *G. fujikuroi* gave cyclonerodiol. The labeled cyclonerodiol was subjected to Kuhn–Roth oxidation and the derived acetate shown to be (2*S*)-[2-^{2}H,^{3}H]acetate by the established sequence of conversion to malate with malate synthase and subsequent fumarase incubation. Samples of (5*R*)-[5-^{2}H,^{3}H]- and (5*S*)-[5-^{2}H,^{3}H]mevalonate were fed in separate experiments to cultures of *G. fujikuroi*, and the chirality of the derived C-1 methyl in cyclonerodiol was determined in the usual manner. Thus (5*R*)-[5-^{2}H,^{3}H]mevalonate gave rise to (2*R*)-[2-^{2}H,^{3}H]acetate and (5*S*)-[5-^{2}H,^{3}H]mevalonate yielded (2*S*)-[2-^{2}H,^{3}H]acetate. The results imply that in the biosynthesis of cyclonerodiol, the conversion of farnesyl pyrophosphate to nerolidyl pyrophosphate takes place by a net suprafacial process and that the subsequent cyclization of nerolidyl pyrophosphate involves all trans addition of water across the vinyl and central double bond to form cyclonerodiol. When [1-^{18}O]farnesyl pyrophosphate was converted to cyclonerodiol, the mass spectrum of the derived (trimethylsilyl)oxy lactone (**10**) indicated that one-third of the initial ^{18}O was at C-3 and therefore at C-3 of the intermediate nerolidyl pyrophosphate. The latter experiment supports an ion pair intermediate for the farnesyl–nerolidyl interconversion in which the three nonbridge oxygens of the proximal phosphate are able to scramble.

The central role played by allylic pyrophosphates in the biosynthesis of isoprenoid metabolites has prompted several penetrating investigations of the stereochemistry and mechanism of the biochemical reactions which these substances undergo.[2] Among these transformations, the least well studied have been the allylic transpositions represented by the isomerization of geranyl to linalyl pyrophosphate and farnesyl (**1**) to nerolidyl pyrophosphate (**2**), in spite of the prominence which these isomerizations have been assigned in biogenetic speculations and chemical model studies.[3] In large part, opportunities for studying the metabolism of tertiary allylic alcohols or their pyrophosphate esters have been limited due to a scarcity of suitable biochemical systems. For example, an early suggestion that nerolidyl pyrophosphate might be a precursor of squalene was subsequently disproved by Rilling.[4] Progress in understanding the formation

(1) Fellow of the Alfred P. Sloan Foundation, 1978–1982; National Institutes of Health Research Career Development Award, 1978–1983.

(2) For a recent review of the stereochemistry of allylic pyrophosphate metabolism see D. E. Cane, *Tetrahedron*, **36**, 1109 (1980).

(3) Cf.: D. Arigoni, *Pure Appl. Chem.*, **41**, 219 (1975); N. H. Andersen, Y. Ohta, and D. D. Syrdal, in "Bio-organic Chemistry", Vol. 2, E. E. van Tamelen, Ed., Academic Press, New York, 1977, pp 1–37; N. H. Andersen and D. D. Syrdal, *Tetrahedron Lett.*, 2455 (1972); D. V. Banthorpe, B. V. Charlwood, and M. J. O. Francis, *Chem. Rev.*, **72**, 115 (1972); S. Gotfredsen, J. P. Obrecht, and D. Arigoni, *Chimia*, **31** (2), 62 (1977); C. D. Gutsche, J. R. Maycock, and C. T. Chang, *Tetrahedron*, **24**, 859 (1968); K. Stephan, *J. Prakt. Chem.*, **58**, 109 (1898); W. Rittersdorf and F. Cramer, *Tetrahedron*, **24**, 43 (1968); S. Winstein, G. Valkanas, and C. F. Wilcox, *J. Am. Chem. Soc.*, **94**, 2286 (1972).

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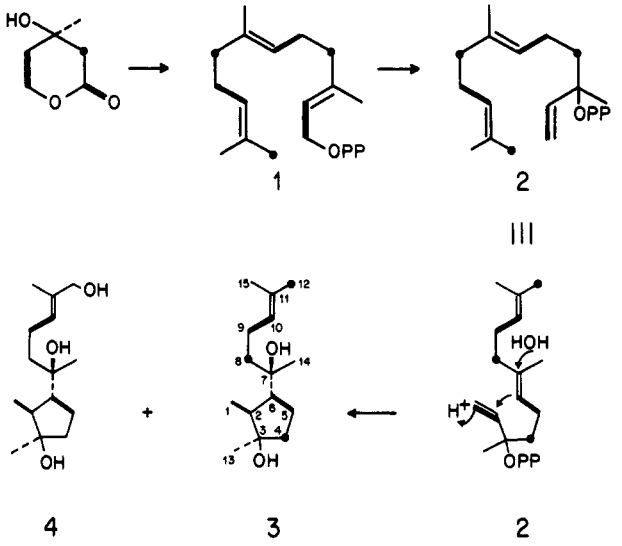
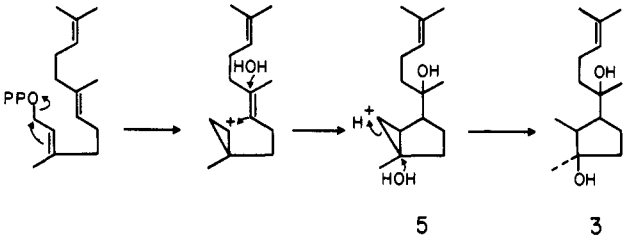
Scheme I



Scheme II



of nerolidyl pyrophosphate has come from studying the biosynthesis of the novel cyclopentanoid sesquiterpene, cyclonerodiol (**3**). We describe below studies which establish that the enzyme-catalyzed isomerization of farnesyl to nerolidyl pyrophosphate takes place with net syn stereochemistry, most probably by an ion-pair mechanism.[5] The ability of this same ion-pair intermediate to account for other allylic pyrophosphate transformations is then discussed.

Nerolidyl Pyrophosphate as an Intermediate in the Biosynthesis of Cyclonerodiol. First isolated in 1970 by Nozoe from cultures of the fungus *Trichothecium roseum*, cyclonerodiol (**3**) was subsequently identified in the extracts of two other commonly studied fungi, *Gibberella fujikuroi*[7] and *Fusarium culmorum*,[8] in the latter case accompanied by an oxidized derivative, cyclonerotriol (**4**). Early biosynthetic incorporation experiments carried out independently by Hanson[9] and in our own laboratories[10] established the mevalonoid origin of **3** and suggested a biosynthetic pathway in which a molecule of water adds across the central double bond and the vinyl group of nerolidyl pyrophosphate, formed by isomerization of farnesyl pyrophosphate. Thus the Sussex investigators found that feeding of [4,5-$^{13}C_2$]mevalonate to *F. culmorum* gave labeled **3** and **4** whose ^{13}C NMR spectra each exhibited three pairs of enhanced and coupled doublets corresponding to C-9 and C-10, C-1 and C-2, and C-5 and C-6. Subsequently we reported incorporation of [2-^{14}C]mevalonate into cyclonerodiol by *G. fujikuroi*. The positions of labeling in **3** were established unambiguously by chemical degradation which confirmed the presence of the expected one-third of the label at C-4, C-8, and C-12.

The proposed biosynthetic scheme was further corroborated by the apparent conversion of farnesyl pyrophosphate to **3**.[9] Hanson also showed that cyclonerodiol is converted by cultures of *F. culmorum* to cyclonerotriol. The failure of *T. roseum* to incorporate nerolidol, however, led Hanson to suggest that either nerolidol or its pyrophosphate ester was formed only as an enzyme-bound intermediate or that cyclization to **3** might occur by an alternative pathway involving a cyclopropyl cation and hydration of an intermediate bicyclo[3.1.0]hexane (**5**).

Subsequent work in our laboratories required the development of a cell-free system from *G. fujikuroi*[11] and provided the first evidence for the intermediacy of nerolidyl pyrophosphate. *G. fujikuroi* was grown in the usual manner for 4 days after which the mycelium (52–57 g wet weight/L of culture) was harvested by filtration and suspended in phosphate buffer (pH 7.6), containing the additives glycerol, dithioerythritol (DTE), and EDTA as stabilizers. Dithiothreitol or 2-mercaptoethanol could be successfully substituted for DTE. The cells were then disrupted by passage of the suspension through a precooled French Press under 10000–15000-psi pressure, and the cell debris was removed immediately by successive centrifugation at 15000*g* and 27000*g* at 4 °C. Treatment of the supernatant with protamine sulfate served to precipitate nucleic acids. Such extracts typically contained 6–8 mg of protein/mL as determined by the method of Lowry.[15] Extracts were assayed for cyclonerodiol synthesizing activity by incubation with [1-$^3H_2$]-*trans,trans*-farnesyl pyrophosphate. Preliminary experiments indicated that magnesium(II) was necessary for activity and hence magnesium chloride was routinely added to the enzyme extract just prior to the incubations. Assays were carried out on 10-mL aliquots of enzyme solution in 20-mL stoppered glass tubes at 26 °C. After periods of 0.5–3.0 h the reactions were stopped by addition of acetone and the mixture was extracted with ether. After addition of 1–2 mg of unlabeled cyclonerodiol as carrier, the ether extracts were purified by preparative thin-layer chromatography. With use of the crude S_{27} enzyme system, the ether extract contained 60–70% of the initial radioactivity, 1% being present as cyclonerodiol and 40–50% in a zone corresponding to C_{15} alcohols, most of which was shown to be farnesol formed by the action of competing phosphatases and pyrophosphatases. The cyclonerodiol radioactivity could be confirmed by conversion to the crystalline bis(dinitrobenzoate) ester[7,10] and recrystallization to constant activity. At this point the enzymatic conversion corresponded to 0.09 nmol of product/mg of protein/3 h. A control incubation with a boiled enzyme preparation resulted in only 0.4% of ether extractable material and inactive cyclonerodiol. Besides being contaminated with the competing phosphatase–pyrophosphatase activities, the enzyme extracts were markedly unstable at this stage, losing all activity after 2–3 h at 27 °C, 1 h at 30 °C, or overnight at 4 °C.

Subsequent experiments eventually succeeded in increasing the enzyme stability while removing the phosphatase activity and improving the yields of cyclonerodiol. Addition of reagents such

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(5) Preliminary communications: (a) D. E. Cane and R. Iyengar, *J. Am. Chem. Soc.*, **101**, 3385 (1979); (b) D. E. Cane, R. Iyengar, and M.-S. Shiao, *ibid.*, **100**, 7122 (1978); (c) D. E. Cane and R. Iyengar, *ibid.*, **100**, 3256 (1978).

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(10) D. E. Cane and M. S. Shiao, *J. Am. Chem. Soc.*, **100**, 3203 (1978).

(11) Other cell-free systems from *G. fujikuroi* have been reported previously. A crude preparation from *G. fujikuroi* which catalyzes the conversion of mevalonate to (−)-kaurene has been described by Hanson.[12] This same enzyme system also converts gibberellin A_{12} aldehyde to the corresponding gibberellin A_{13} and A_{14} 7-aldehydes.[13] West has purified kaurene synthetase from *Fusarium moniliforme* (the imperfect stage of *G. fujikuroi*) some 170-fold.[14]

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(15) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Table I. Partial Purification of *G. fujikuroi* Cell-Free Extract[a]

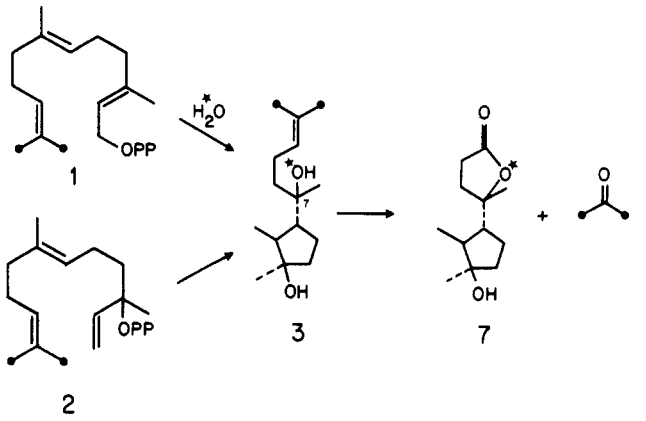
purification step[b]	protein,[c] mg	ether extract[d]	cyclonerodiol[d]
S_{27}	110	5.72	0.09
S_{PS}	92	5.02	0.10
S_{AS}	45	4.22	0.29
G_{150}	22	2.07	0.67
HA gel	12	10.9[e]	1.26[e]

[a] Refers to enzyme from 1 L of 4-day-old *G. fujikuroi* culture. [b] See Experimental Section for definitions. [c] Determined by the method of Lowry.[15] [d] Units in nmol of product/mg of protein/3 h. Assays carried out using [1-$^3H_2$]farnesyl pyrophosphate as substrate in 20-mL stoppered tubes at 25–26 °C for 3 h as described in the Experimental Section. Yields of ether-extractable material and of cyclonerodiol were calculated from the known specific activity of the substrate and the recovered total activity of isolated product. [e] Incubation mixture treated with alkaline phosphatase.

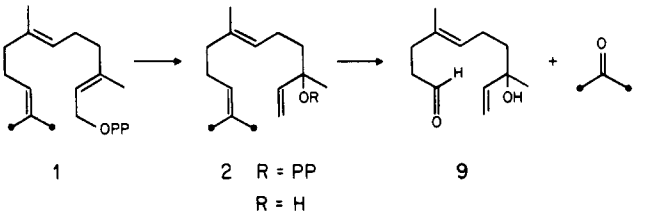
as polyclar AT (3 mg/mL) and polyvinylpyrrolidone (3 mg/mL) to absorb phenolics,[16] use of enzyme stabilizers such as bovine serum albumin (0.25 mg/mL), or addition of the protease inhibitors *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrogen chloride (3 mg/mL) or phenylmethylsulfonyl chloride (2 mg/mL) did not affect the stability of the enzyme preparation. The competing phosphatase activity could not be removed by centrifugation at 4 °C for 1 h at 105000*g* or 200000*g* nor by heat denaturation. Similarly the addition of phosphatase inhibitors such as sodium fluoride (40 mM),[17,18] sodium bromate (40 mM), sucrose (25%, v/v), or ADP[19,20] did not improve the observed yields of cyclonerodiol. In fact sodium fluoride was a marked inhibitor of the normal isomerase–cyclase activity. The phosphatase activity could, however, be removed by further purification of the crude enzyme extracts. All the cyclonerodiol-synthesizing activity was retained in the 40–70% ammonium sulfate precipitate (Table I) which was redissolved and dialyzed overnight against Tris–HCl buffer (pH 8.0), containing DTE and glycerol. Although attempted further purification by ion-exchange chromatography on DEAE- or CM-cellulose resulted in loss of enzyme activity, passage of the dialyzed extract through a Sephadex G-150 column removed 50% of the competing phosphatase activity and improved the conversion to cyclonerodiol to 0.67 nmol/mg of protein/3 h. The resultant lyophilized enzyme preparation could be stored at 4 °C for 2 weeks without loss of activity. Following treatment of the Sephadex eluate with hydroxylapatite, incubation with [1-$^3H_2$]-farnesyl pyrophosphate led to only a low yield of ether extractable material and resulted in formation of a water-soluble, radioactive product, presumably cyclonerodiol pyrophosphate, which could be converted to cyclonerodiol upon subsequent treatment of the heat-denatured mixture with excess alkaline phosphatase.[21] The hydroxylapapatite-treated enzyme-catalyzed conversions yield up to 0.21 nmol of cyclonerodiol/mg of protein in 0.5 h. This latter preparation could be stored as a lyophilisate for 1 month at 4 °C.

The specificity of labeling in the enzymatic formation of cyclonerodiol was established by incubating [12,13-^{14}C]farnesyl pyrophosphate with the crude (S_{27}) enzyme extract from a 2-L culture of *G. fujikuroi*. The resulting cyclonerodiol was diluted with inactive carrier and a portion converted to the bis(dinitrobenzoate) ester. The remaining labeled **3** was treated with osmium tetroxide–sodium periodate to give acetone, isolated as its semicarbazone, and the hemiacetal **6**, which was converted to the corresponding crystalline lactone **7** by Jones oxidation.[7,10] Acetone

Scheme III



Scheme IV



semicarbazone, when recrystallized to constant activity, was found to contain >99% of the activity of the parent bis(dinitrobenzoate) while **7** was inactive (Table II).

Repetition of the S_{27} incubation with [12,13-^{14}C]nerolidyl pyrophosphate resulted in a 0.8% conversion to cyclonerodiol. Degradation as above showed that 98% of the label resided in the terminal isopropylidene group, as expected. On the other hand, incubation of the free alcohol [12,13-^{14}C]nerolidol gave cyclonerodiol in less than 0.07% yield, consistent with cyclization of nerolidyl pyrophosphate preceding hydrolysis of the pyrophosphate ester, as suggested above by the results of incubations with phosphatase-free enzyme obtained by treatment with hydroxylapatite.

Direct evidence for the farnesyl–nerolidyl conversion was provided by incubating [12,13-^{14}C]farnesyl pyrophosphate with the AS_{40-70} enzyme system and quenching the reaction after 10 min at 26 °C. Purification of the initial ether extract showed traces of cyclonerodiol (0.4%). The incubation mixture was then extracted with *n*-butyl alcohol, and unlabeled nerolidyl pyrophosphate was added as carrier. The crude mixture of pyrophosphates was purified by ion-exchange chromatography on Dowex 1 (formate form) and the eluted nerolidyl pyrophosphate treated with XAD-2 resin. Subsequent alkaline phosphatase treatment yielded [^{14}C]nerolidol (0.13%). A boiled enzyme control confirmed that the formation of nerolidyl pyrophosphate was enzyme catalyzed (Table III).

The isolated radioactive nerolidol was diluted with additional inactive carrier and then oxidatively degraded by *m*-chloroperbenzoic acid epoxidation followed by acid-catalyzed ring opening to give crystalline 10,11-dihydroxynerolidol (**8**). Sodium periodate cleavage of **8** gave the inactive trinoraldehyde **9** and acetone, the latter isolated as its crystalline semicarbazone and containing 98% of the ^{14}C activity of **2**.

Further evidence for the intermediacy of nerolidyl pyrophosphate came from competition experiments in which a mixture of [1-^{3}H]nerolidyl pyrophosphate and [12,13-^{14}C]farnesyl pyrophosphate was incubated with the hydroxylapatite-treated enzyme at 26 °C. Quenching incubations after various short reaction times and determination of the $^3H/^{14}C$ ratio of the resultant purified cyclonerodiol indicated that nerolidyl pyrophosphate was converted to cyclonerodiol approximately 5 times faster than was farnesyl pyrophosphate (Figure 1).

The cyclization of nerolidyl pyrophosphate by the cell-free extract could also be directly observed by ^{13}C NMR.[22] The

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(17) R. Croteau and F. Karp, *Biochem. Biophys. Res. Commun.*, **72**, 440 (1976).
(18) R. Croteau and F. Karp, *Arch. Biochem. Biophys.*, **176**, 734 (1976).
(19) D. V. Banthorpe, G. A. Bucknall, H. J. Doonan, S. Doonan, and M. G. Rowan, *Phytochemistry*, **15**, 91 (1976).
(20) D. V. Banthorpe, B. V. Charlwood, and M. J. O. Francis, *Chem. Rev.*, **72**, 115 (1972).
(21) D. A. Beller, D. G. Anderson, and J. W. Porter, *Arch. Biochem. Biophys.*, **102**, 26 (1963).

Table II. Conversion of Labeled Substrates to Cyclonerodiol[a]

substrate, dpm (nmol)	3, dpm(nmol)	3-$(DNB)_2$,[b] dpm/mmol	acetone semicarbazone, dpm/mmol	7, dpm/mmol
[12,13-^{14}C]FPP, 2.14×10^6 (4600)	2.03×10^4 (43)	1.99×10^5	1.98×10^5	inactive
[12,13-^{14}C]NPP, 6.48×10^6 (11 100)	4.99×10^4 (85)	4.27×10^5	4.18×10^5	inactive
[12,13-^{14}C]Nerolidol, 4.70×10^5 (900)	[9.1×10][c] (<0.1)	$<7.5 \times 10^3$		

[a] Incubation with S_{27} fraction for 4 h at 26 °C. FPP = farnesyl pyrophosphate. NPP = nerolidyl pyrophosphate. [b] Prepared from 3 after dilution with inactive carrier. [c] Maximum value based on activity of 3-$(DNB)_2$.

Table III

(a) Conversion of Farnesyl Pyrophosphate to Nerolidyl Pyrophosphate

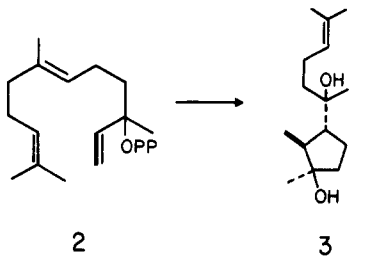
enzyme	[12,13-^{14}C]FPP, dpm	[12,13-^{14}C]NPP, dpm (% yield)
S_{AS}[a]	2.56×10^6	3.28×10^3 (0.13)[b]
S_{AS}-boiled	4.72×10^5	inactive

(b) Degradation of [12,13-^{14}C]Nerolidol

compd	dpm/mmol	compd	dpm/mmol
nerolidol[c]	3.64×10^4	acetone semicarbazone[d]	3.63×10^4
20	3.58×10^4	**9**	inactive
8	3.67×10^4		

[a] Incubation for 10 min at 26 °C. [b] Isolated and purified after dilution with inactive carrier. [c] Diluted to 33 mg with inactive carrier. [d] Recrystallized to constant activity.

Scheme V



2 3

requisite sample of [1,2-$^{13}C_2$]nerolidol was prepared by addition of [1,2-$^{13}C_2$]ethynylmagnesium bromide to *trans*-geranylacetone followed by lithium aluminum hydride–sodium methoxide reduction of the resultant propargylic alcohol.[23,24] Pyrophosphorylation in the usual manner gave [1,2-$^{13}C_2$]nerolidyl pyrophosphate.

The lyophilized Sephadex G-150 eluant derived from 500 mL of *G. fujikuroi* culture (11 mg of protein) was redissolved in Tris–HCl buffer containing 10% D_2O and mixed with [1,2-$^{13}C_2$]nerolidyl pyrophosphate at 0–4 °C. The ^{13}C NMR spectrum, which displayed a pair of satellite doublets (J = 70.6 Hz) superimposed on enhanced singlets at 145.6 and 111.9 ppm, corresponding to C-2 and C-1, respectively, was recorded at 0–4 °C (8300 transients). The NMR tube was quickly warmed to 26 °C, and the incubation was subsequently interrupted after 5-, 10-, and 20-min of total reaction time and cooled each time to 0–4 °C, at which temperature the ^{13}C NMR spectrum was collected. The conversion of [1,2-$^{13}C_2$]nerolidyl pyrophosphate to cyclonerodiol was observed by the decrease in the intensity of the 145.6- and 111.9-ppm doublets and the appearance of a pair of new doublets (J = 38.4 Hz), centered at 14.7 and 44.3 ppm and assigned to C-1 and C-2 of cyclonerodiol (pyrophosphate), respectively[9]

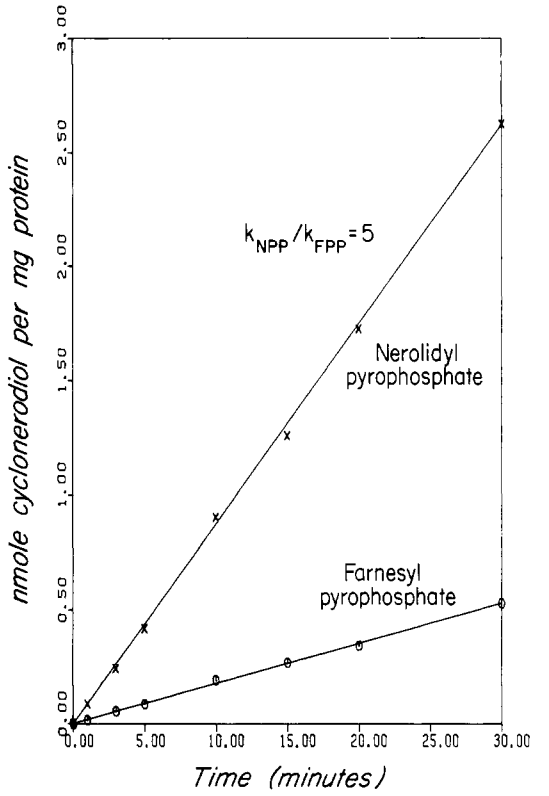


Figure 1. Conversion of farnesyl and nerolidyl pyrophosphates to cyclonerodiol (competitive incubation). A mixture of [12,13-^{14}C]FPP and [1-^{3}H]NPP was incubated at 26 °C with phosphatase-free enzyme extract, obtained after treatment with hydroxylapatite, along with added 0.1 M $MgCl_2$. Aliquots were withdrawn at the indicated time intervals, heat denatured, and treated with alkaline phosphatase. The resulting cyclonerodiol was isolated and purified and the $^3H/^{14}C$ activity determined. The nmol of cyclonerodiol formed from NPP (×) and from FPP (○) were calculated based on the total dpm of ^{3}H and ^{14}C isolated and the known dpm/nmol of each precursor.

(Scheme V). The intensity of the latter doublets increased linearly with time. Interestingly no farnesyl pyrophosphate was detected under these conditions.

The above results are fully consistent with the suggested sequence of isomerization of farnesyl to nerolidyl pyrophosphate, cyclization of nerolidyl pyrophosphate, and hydrolysis of cyclonerodiol pyrophosphate. Although no separation of the isomerase and cyclase activities has yet been achieved, the observed formation of free nerolidyl pyrophosphate is most consistent with the operation of two distinct enzymes. Preliminary experiments have indicated that either nickel(II) or cobalt(II) can substitute for magnesium(II) in the conversion of farnesyl pyrophosphate to cyclonerodiol whereas copper(II), iron(II), and zinc(II) were ineffective. No attempt has yet been made to determine the cofactor requirements of the separate isomerase and cyclase activities.

On the basis of the established mode of action of alkaline phosphatase,[25,26] it was expected that hydrolysis of cyclonerodiol

(22) Similar techniques have recently been used to detect intermediates in enzymatic reactions, for example in the deaminase–cosynthetase-catalyzed conversion of porphobilinogen to urogen III. Cf.: A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham, E. McDonald, and K. E. Gustafson-Potter, *J. Chem. Soc., Chem. Commun.*, 316 (1979); G. Burton, H. Nordlöv, S. Hosozawa, H. Matsumoto, P. M. Jordan, P. E. Fagerness, L. M. Pryde, and A. I. Scott, *J. Am. Chem. Soc.*, **101**, 3114 (1979).

(23) E. J. Corey, J. A. Katzenellenbogen, and G. H. Posner, *J. Am. Chem. Soc.*, **89**, 4245 (1967).

(24) This reaction gives also as a side product [1-^{13}C]geranylactone. For a discussion of the mechanism of this intriguing transformation see D. E. Cane and R. Iyengar, *Tetrahedron Lett.*, 2871 (1979).

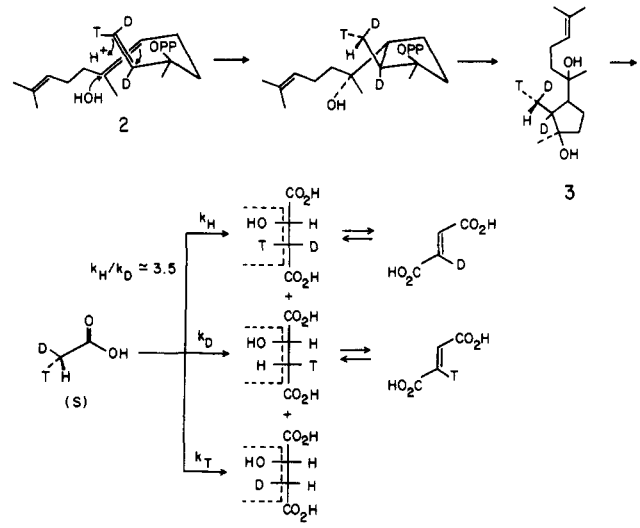
(25) S. S. Stein and D. E. Koshland, *Arch. Biochem. Biophys.*, **39**, 229 (1952). H. N. Fernley in "The Enzymes", Vol. 4, 3rd ed., P. D. Boyer, Ed., Academic Press, New York, 1971, p 417.

pyrophosphate by *G. fujikuroi* phosphatase(s) would take place with P–O rather than C–O bond cleavage. This point was confirmed by converting farnesyl pyrophosphate to cyclonerodiol in ^{18}O-enriched water. Thus [12,13-^{14}C]farnesyl pyrophosphate was incubated at 26 °C with the AS_{40-70} enzymes from 14 L of *G. fujikuroi* culture (630 mg of protein) in 5.0 mL of [^{18}O]water (37.7 atom % excess). The resulting labeled cyclonerodiol was isolated and purified to give 1.08 mg of **3** (2.9% conversion) which was oxidized as before to the corresponding trisnorlactone (**7**) (Scheme III). The latter substance was purified by PLC and recrystallized from chloroform. Examination of the mass spectrum of the hydroxy lactone thus obtained established that only the side-chain hydroxyl group at C-7 of cyclonerodiol is derived from water, as required by the previously established mechanism. The parent (M), as well as M – CH_3, M – H_2O, and M – CH_3, H_2O fragments of labeled **7** each showed an enrichment of 24.8 atom % excess ^{18}O over the corresponding peaks of unlabeled **7**. The fact that no M + 4 peak was detected and that the isotopic enrichment was unchanged in the fragments corresponding to loss of water from the ring confirmed that hydrolysis of the pyrophosphate ester takes place with exclusive P–O bond cleavage. It should also be noted that these results also conclusively rule out the cyclopropyl cation intermediate of Scheme II.

It was also possible to establish the stereochemistry of the nerolidyl pyrophosphate intermediate as 3*R*. Since cyclonerodiol pyrophosphate is hydrolyzed at the phosphorus–oxygen bond, it is reasonable to infer that the stereochemistry at C-3 of nerolidyl pyrophosphate will not be affected by cyclization and subsequent hydrolysis. A direct test of this assumption required a sample of optically pure [^{14}C]nerolidyl pyrophosphate. The requisite (3*S*)-[12,13-^{14}C]nerolidol was prepared from (3*S*)-nerolidol (α_D +15.2°) by oxidation of the distal double bond and treatment of the resulting trisnoraldehyde with [^{14}C]isopropylidenetriphenylphosphorane. Conversion to the corresponding (3*S*)-[12,13-^{14}C]nerolidyl pyrophosphate was effected in the usual manner. The enantiomeric purity of the (3*S*)-pyrophosphate was verified by alkaline phosphatase-catalyzed hydrolysis of a 6-mg sample and examination of the ^1H NMR spectrum of the derived nerolidol in the presence of 40 mol % Eu(tfc)$_3$,[27] confirming that no racemization had occurred. The ^{14}C-labeled (3*S*)-nerolidyl pyrophosphate (0.93 μmol, 1.56 × 10^6 dpm) was mixed with racemic [1-^3H]nerolidyl pyrophosphate (1.35 μmol, 1.23 × 10^6 dpm), $^3H/^{14}C$ = 0.77, and the mixture was incubated with the AS_{40-70} extract from 0.6 L of *G. fujikuroi* (27 mg of protein) for 3 h at 26 °C.[28] If only the 3*S* enantiomer of **2** were utilized the $^3H/^{14}C$ value of the resulting cyclonerodiol would be exactly half the $^3H/^{14}C$ ratio of the nerolidyl pyrophosphate mixture. Were both enantiomers consumed, the $^3H/^{14}C$ ratio would remain unchanged, while conversion of only the 3*R* enantiomer of **2** will give exclusively ^3H-labeled cyclonerodiol. In the event the isolated cyclonerodiol (2.71 × 10^4 dpm, ^3H, 2.98%) was devoid of ^{14}C activity as was the corresponding recrystallized bis(dinitrobenzoate) ester. These results unambiguously demonstrate that the absolute configuration of the nerolidyl pyrophosphate precursor is 3*R*, as previously inferred.

Stereochemistry of the Enzymatic Conversion of Farnesyl to Nerolidyl Pyrophosphate. With the sequence of steps from farnesyl pyrophosphate to cyclonerodiol now clearly defined, an opportunity was at hand to study the enzymatic farnesyl–nerolidyl conversion itself. Our experimental plan was to determine first the stereochemistry of cyclization of the intermediate nerolidyl pyrophosphate. With the results of such a study in hand, we would then be in a position to examine the farnesyl–nerolidyl rearrangement. In the cyclization step, the stereochemistry of folding of the nerolidyl pyrophosphate as well as the direction of attachment of the water oxygen at the side chain is apparent from

Scheme VI



the absolute configuration of cyclonerodiol which we have firmly established by correlation with (–)-(*R*)-linalool[29] (Scheme VI). Protonation of the terminal vinyl carbon of nerolidyl pyrophosphate generates the C-1 methyl group of cyclonerodiol. In order to determine the stereochemistry of this latter protonation, we required a sample of **2** stereospecifically labeled with both deuterium and tritium at C-1. In the resulting cyclonerodiol, the C-1 methyl group would then be chiral by virtue of substitution with the three isotopes of hydrogen protium, deuterium, and tritium—with the sense of chirality a function of both the stereochemistry of the precursor **2** and the direction of protonation at C-1. The requisite sample of (*E*)-[1,2-$^2H_2$,1-^3H]nerolidol was prepared by reduction of [1-^3H]dehydronerolidol with lithium aluminum deuteride in the presence of sodium methoxide followed by quenching with deuterated water.[23,30] Mass spectrometry showed the resultant nerolidol to be 94.8% d_2, 3.4% d_1, and 1.8% d_0, and examination of the ^1H NMR spectrum recorded in the presence of 25 mol % Eu(DPM)$_3$ confirmed that the reduction of the propargyl alcohol had occurred with the expected trans stereochemistry. After conversion to the corresponding pyrophosphate ester the sample of (*E*)-[1,2-$^2H_2$,1-^3H]-**2** was incubated with the S_{27} enzyme extract from 4-day-old cultures of *G. fujikuroi*. The resultant labeled cyclonerodiol was isolated, purified, and then diluted with inactive carrier (15 mg). A portion of this labeled cyclonerodiol was converted to the bis(dinitrobenzoate) which was recrystallized to constant activity, indicating an overall 1.1% conversion of nerolidyl pyrophosphate to **3**. The remainder of the labeled cyclonerodiol was subjected to Kuhn–Roth oxidation,[31] giving rise to ca. 12 mg of potassium acetate. This sample of acetate consisted of chirally labeled acetate derived from C-1 and C-2 of cyclonerodiol, diluted with unlabeled acetate originating from the C-12, C-14, and either the C-12 or C-15 methyls of **3**.[32] The chirality of this sample

(26) H.-L. Ngan and G. Popjak, *Bioorg. Chem.*, **4**, 166 (1975); H. Mackie and K. H. Overton, *Eur. J. Biochem.*, **77**, 101 (1977).
(27) H. L. Goering, J. N. Eikenberry, and G. S. Koermer, *J. Am. Chem. Soc.*, **93**, 5913 (1971).
(28) Cf. E. Leistner, R. N. Gupta, and I. D. Spenser, *J. Am. Chem. Soc.*, **95**, 4040 (1973).
(29) D. Cane and R. Iyengar, *Tetrahedron Lett.*, 3511 (1977).
(30) The sequence of deuteride reduction-deuterated water quench was employed in order to ensure essentially 100% deuteration at C-1, as required by the analytical procedure for chiral methyl.[34] The presence of the extra deuterium at C-2 in no way affects the outcome of subsequent reactions.
(31) E. Wiesenbeger, *Mikrochim. Acta*, **33**, 51 (1948).
(32) A control experiment (see Experimental Section) in which unlabeled **3** was subjected to Kuhn–Roth oxidation in the presence of 5 mCi of [^3H]-water followed by conversion of the derived acetate to the crystalline *p*-bromophenacyl ester indicated that oxidation is accompanied by as much as 26% exchange of the methyl hydrogens. Such small amounts of exchange have been observed before[33] and do not present a serious problem. For example, with the assumption that exchange takes place during oxidation, that the C-1, C-13, C-14, and C-12 (or C-15) methyls contribute equally to the formation of acetate, that there is no isotope effect for exchange, and that each methyl exchanges only once, 26% exchange would correspond to at most 26% racemization of the chiral acetate. This would change the observed retention of tritium in the fumarase assay by only 6%. Furthermore an isotope effect would tend to protect chirally deuterated and tritiated methyl against exchange, with a consequent reduction in the observed extent of racemization.

Table IV. Conversion of Labeled Substrates to Chiral Acetates

substrate	$^3H/^{14}C$ acetate[a]	malate[b,c] from malate synthase	malate[d] after fumarase	% tritium retention
(*E*)-[1,2-$^2H_2$,1-^3H]-nerolidyl-PP	4.30	3.38	1.00	29.6
(3*RS*,5*R*)-[5-^2H,^3H]-mevalonate	3.38	2.70	1.86	68.9
(3*RS*,5*S*)-[5-^2H,^3H]-mevalonate	3.88	3.54	1.09	30.8
(2*R*)-[2-^2H,^3H]-acetate[e]	3.95	3.41	2.60	76.2
(2*S*)-[2-^2H,^3H]-acetate[e]	4.10	3.87	0.95	24.5
[2-^3H]acetate	9.71	7.11	3.59	50.5

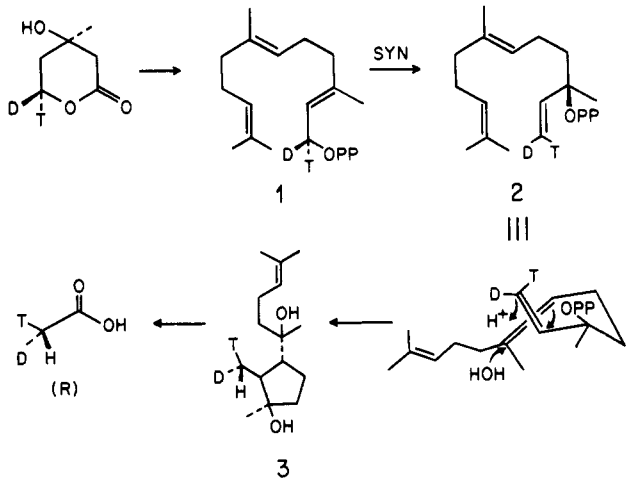
[a] $^3H/^{14}C$ ratio based on *p*-bromophenacyl ester of mixture of [2-^3H]- and [2-^{14}C]acetates. [b] Recrystallized from acetone–hexane after addition of inactive carrier and chromatography on Dowex-1 (formate). [c] The observed retention of tritium in going from acetate to malate is a function of both intra- and intermolecular isotope effects, as well as the extent of conversion of acetyl CoA to malate. On the other hand the retention of tritium after fumarase exchange depends only on an intramolecular isotope effect in the malate synthase reaction, $k_H/k_D \simeq 3.5$. Cf. ref 21, as well as H. Lenz, W. Buckel, P. Wunderwald, G. Biedermann, V. Buschmeier, H. Eggerer, J. W. Cornforth, J. W. Redmond, and R. Mallaby, *Eur. J. Biochem.*, **24**, 207 (1971). [d] Minimum 4-h incubation with fumarase followed by precipitation of barium phosphate, Dowex 50W-X8 chromatography and recrystallization from acetone–hexane. $^3H/^{14}C$ ratio is unchanged upon further fumarase treatment. Cf. Table VIII. [e] Authentic (*R*)- and (*S*)-acetates used as reference samples.

of acetate was determined by using the procedure developed by Arigoni and Cornforth.[34] Thus, a mixture of the [2-^{3}H]acetate and added [2-^{14}C]acetate ($^3H/^{14}C$ = 4.30)[35] was converted to acetyl CoA, and the resultant sample incubated with sodium glyoxylate and freshly purified yeast malate synthase. After addition of carrier L-malate and extraction with ethyl acetate, the derived malate was isolated by ion-exchange chromatography and recrystallized to give $^3H/^{14}C$ = 3.38. Incubation of this latter malate sample with fumarase gave, after isolation and recrystallization, malate $^3H/^{14}C$ = 1.00[36] (Table IV). The observed retention of 29.6% of the tritium activity after fumarase incubation corresponds to (2*S*)-[2-^{2}H,^{3}H]acetate.[34] Since Kuhn–Roth oxidation does not affect the chirality obtained,[32] the C-1 methyl of cyclonerodiol derived from (*E*)-[1,2-$^2H_2$,1-^{3}H]nerolidyl pyrophosphate must also have *S* chirality, and the protonation at C-1 of nerolidyl pyrophosphate therefore must have occurred on the *re* face. The overall stereochemistry of the addition of water across the vinyl and central double bonds is thus trans,trans, as illustrated in Scheme VI.

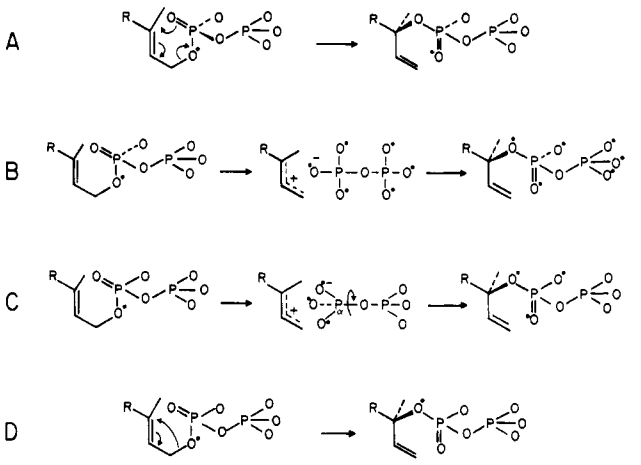
Having established the stereochemistry of the enzymatic conversion of nerolidyl pyrophosphate to cyclonerodiol, it then became possible to use this information to study the farnesyl–nerolidyl isomerization itself. For example isomerization of (1*R*)-[1-^{2}H,^{3}H]farnesyl pyrophosphate by a syn rearrangement would give (3*R*)-(*Z*)-[1-^{2}H,^{3}H]nerolidyl pyrophosphate whose stereochemistry could be determined by conversion to cyclonerodiol and determination of the chirality of the resultant C-1 methyl. In fact, it was found more convenient to feed labeled mevalonates separately to intact cultures of *G. fujikuroi* and to allow the microorganism to carry out all the biosynthetic steps. Isolation of the derived cyclonerodiol and Kuhn–Roth oxidation would give rise to a chiral acetate which could be analyzed by the malate synthetase–fumarase procedure. The requisite (5*R*)-[^{2}H,^{3}H]- and (5*S*)-[^{2}H,^{3}H]mevalonates were prepared from the corresponding (1*R*)- and (1*S*)-[1-^{2}H,^{3}H]isopentenols[37] and fed separately to cultures of *G. fujikuroi*. The derived cyclonerodiol was then isolated and purified in the usual manner and subjected to Kuhn–Roth oxidation. Although the mevalonates utilized label both C-5 and C-9 of **3** as well as C-1, only the latter carbon gives rise to chiral acetate upon oxidation. Analysis of the chirality of each of the resulting acetate samples by the sequence of malate synthase–fumarase incubation described above established that incorporation of (5*R*)-[5-^{2}H,^{3}H]mevalonate gave rise to (2*R*)-[2-^{2}H,^{3}H]acetate, whereas (5*S*)-[5-^{2}H,^{3}H]mevalonate resulted in (2*S*)-[2-^{2}H,^{3}H]-acetate (Table IV). The results imply that the conversion of farnesyl pyrophosphate to nerolidyl pyrophosphate is a net syn process. Thus (5*R*)-[5-^{2}H,^{3}H]mevalonate gives rise to (1*R*)-[1-^{2}H,^{3}H]farnesyl pyrophosphate, which is isomerized to (3*R*)-nerolidyl pyrophosphate. Syn isomerization will give the (*Z*)-[1-^{2}H,^{3}H]nerolidyl stereoisomer which, upon cyclization and pyrophosphate ester hydrolysis with P–O bond cleavage, will yield (1*R*)-[1-^{2}H,^{3}H]cyclonerodiol and therefore the corresponding (2*R*)-[2-^{2}H,^{3}H]acetate upon oxidation, as observed (Scheme VII). The results with (5*S*)-[5-^{2}H,^{3}H]mevalonate are entirely complementary and fully support the above arguments.

Timing of the Rearrangement. The Role of the Pyrophosphate Moiety. The demonstration that the rearrangement of farnesyl to nerolidyl pyrophosphate takes place with syn stereochemistry defines the reaction with respect to the allylic carbon framework

Scheme VII

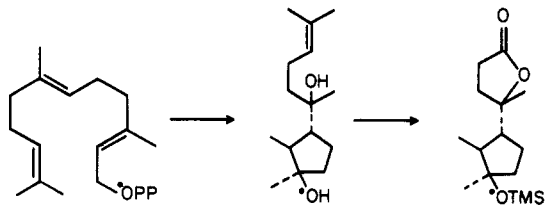


Scheme VIII



(33) D. Arigoni and C. Townsend, unpublished results.
(34) J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Nature (London)*, **221**, 1212 (1969); *Eur. J. Biochem.*, **14**, 1 (1970); J. Luthy, J. Retey, and D. Arigoni, *Nature (London)*, **221**, 1213 (1969).
(35) Determined from the *p*-bromophenacyl ester.
(36) The procedure followed was based on that of Arigoni.[34]
(37) J. W. Cornforth, R. P. Ross, and C. Wakselman, *J. Chem. Soc., Perkin Trans. 1*, 429 (1974); J. W. Cornforth and F. P. Ross, *Chem. Commun.*, 1395 (1970). The synthesis of the necessary [1-^{2}H]- and [1-^{3}H]isopentenals is described in the Experimental Section.

Scheme IX



1 3 10

Table V. Conversion of Farnesyl [1-^{18}O]Pyrophosphate to Cyclonerodiol

substrate	^{14}C, dpm/mmol	^{18}O, excess atom %[40]
[1-^{18}O]farnesyl-OPP	2.85 × 10^6 [a]	70.0 ± 2.3[b]
[1-^{18}O]farnesol[c]		70.2 ± 1.3[b]
cyclonerodiol	2.86 × 10^6 [d]	27.0 ± 0.8[e,f]

[a] Determined on crystalline farnesyl diphenylurethane obtained by alkaline phosphatase hydrolysis of FPP and treatment with diphenyl carbamoyl chloride. [b] Based on farnesyl acetate. [c] Recovered from incubation mixture. [d] Determined on crystalline bis(3,5-dinitrobenzoate). [e] Determined on trimethylsilyl lactone **10**, average of 4 determinations (Table VI). [f] Theoretical for one-third retention: 23.3 ± 0.8%.

and sets strict boundary conditions on any mechanistic proposals. By examining the role of the pyrophosphate moiety during the syn isomerization, we have also established the *timing* of the bond-breaking and bond-making steps. Four mechanisms illustrated in Scheme VIII might be advanced, a priori, to account for this allylic rearrangement: A, a concerted phospho-Claisen or ionic stepover mechanism (in this process it is important to note that the oxygens attached to P_α remain stereochemically distinct in both the forward and reverse reactions and therefore would not be expected to become scrambled by any enzyme-catalyzed process); B, a stabilized allylic cation in which free inorganic pyrophosphate is formed, allowing complete scrambling of all six nonbridge oxygens; C, an ion-pair intermediate in which there is sufficient time for rotation about the P_α–OP_β bond; and D, a 1,3-sigmatropic rearrangement or tight ion pair in which P_α–OP_β rotation is restricted. Each of these mechanisms could be distinguished experimentally by determining the relationship of the ester oxygen of the primary allylic pyrophosphate to the corresponding ester oxygen of the resulting tertiary allylic pyrophosphate. Furthermore, the fact that the hydroxyl oxygen at C-3 of cyclonerodiol corresponds to the C-3 pyrophosphate ester oxygen of the intermediate nerolidyl pyrophosphate allows the analysis to be carried out on a suitable derivative of cyclonerodiol itself (Scheme IX).

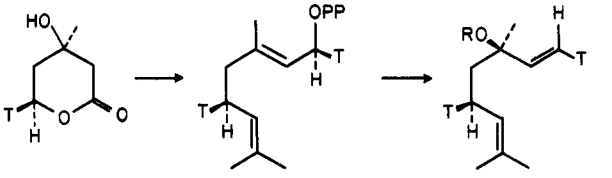
The requisite farnesyl [1-^{18}O]pyrophosphate was prepared by reacting the corresponding chloride[38] with sodium [$^{18}O_2$]acetate,[39] followed by mild base hydrolysis (K_2CO_3, methanol) and pyrophosphorylation in the usual manner. After being mixed with a small quantity of [12,13-^{14}C]farnesyl pyrophosphate, the ^{18}O-enriched **1** (0.16 mmol) was incubated for 3 h at 26 °C with the AS_{40-70} extract derived from a total of 13 L of *G. fujikuroi* culture (585 mg of protein). Extraction with ether and PLC purification gave 1.43 mg of cyclonerodiol and 16 mg of farnesol, the latter formed as a result of the phosphatase activity present in the cell-free enzyme preparation. The mass spectrum of the derived farnesyl acetate indicated the ^{18}O enrichment of the recovered farnesol to be unchanged from that of the precursor (Table V).[40] A small portion of the cyclonerodiol (0.5 mg) was converted to

Table VI. Corrected[a] Relative Peak Intensities of [^{18}O](Trimethylsilyl)oxy Lactone (**10**)

scan	*m/e* 286	*m/e* 284	*m/e* 271	*m/e* 269
run A	27.5	72.5	27.8	72.2
run B	26.4	73.6	26.2	73.8

[a] Mass spectra obtained at 50 eV. The regions of interest were repetitively scanned at slow speed and the areas of the resulting peaks determined by cutting and weighing. Isotope enrichments represent the average of several scans and are corrected by comparison with spectra of unlabeled substrates.

Scheme X



11 12 R=H

the bis(dinitrobenzoate) ester, whose specific activity proved to be identical with that of the precursor [^{14}C]farnesyl [^{18}O]pyrophosphate, indicating that no dilution with endogenous substrates had occurred. Finally the remaining 0.9 mg of cyclonerodiol was converted as before to the hydroxy lactone and thence to the trimethylsilyl ether (**10**). Examination of the parent (M) and M − 15 peaks in the mass spectrum of **10** showed the ^{18}O enrichment at C-3 to be almost exactly one-third the ^{18}O enrichment of the farnesyl [1-^{18}O]pyrophosphate precursor (Tables V and VI). This result conclusively rules out mechanisms A, B, and D for the allylic pyrophosphate rearrangement and strongly supports the ion pair of pathway C.

This experiment, of course, measures only net scrambling of nerolidyl pyrophosphate released from the isomerase. In the absence of purified enzyme it is not possible to determine the scrambling for a single turnover nor the number of times nerolidyl and farnesyl pyrophosphate are interconverted at the active site prior to product release. From results of analysis of the previously described ^{13}C NMR and nerolidyl pyrophosphate trapping experiments, however, it is possible to make a crude estimate that the cyclase will convert nerolidyl pyrophosphate to cyclonerodiol between 2 and 10 times as fast as rebinding of the free tertiary allylic pyrophosphate to the isomerase, making scrambling of nerolidyl pyrophosphate itself unlikely.

The demonstration of an ion-pair intermediate establishes the timing of the allylic rearrangement. The observed scrambling of ^{18}O in the product requires that breaking of the primary allylic C–O bond precede C–O bond making at the tertiary allylic position.

Discussion

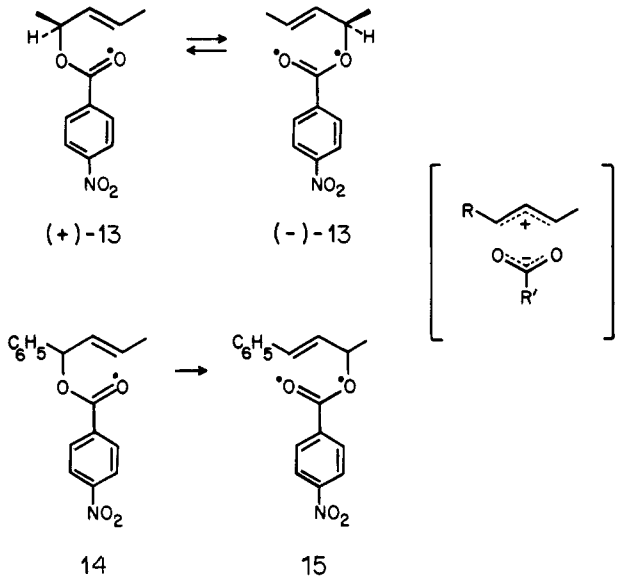
The observed syn stereochemistry of the farnesyl–nerolidyl rearrangement is consistent with the results of an independent study of linalool biosynthesis which has been carried out by Arigoni and Suga.[41] By feeding (3*R*,5*R*)-[5-^{3}H]mevalonate to *Cinnamomon camphora* plants and isolating and degrading the resulting labeled linalool (**12**), these investigators concluded that the formation of linalyl pyrophosphate from geranyl pyrophosphate takes place with exclusive syn stereochemistry, on the basis of the entirely reasonable assumption that subsequent hydrolysis to linalool in the plant takes place with the usual P–O bond cleavage.

The biochemical course of allylic pyrophosphate rearrangements also strongly parallels the results of chemical model studies of allylic phosphonate and carboxylate rearrangements. Herriot has found that the rearrangement of crotyl phenylphosphonate to α-methylallyl phenylphosphonate takes place by an ionic rather

(38) E. W. Collington and A. I. Meyers, *J. Org. Chem.*, **36**, 3044 (1971).
(39) C. R. Hutchinson and C. T. Makune, *J. Labeled Compd. Radiopharm.*, **8**, 571 (1976).
(40) The ^{18}O enrichments of the farnesyl acetate samples have been remeasured and are a few percent lower than that originally reported in Table I of ref 5c. The slight adjustment in no way affects the previously reported conclusions.

(41) (a) S. E. Gotfredsen, *Diss. ETH (Zurich)*, **No. 6243** (1978); (b) cf. T. Suga, T. Shishibori, and M. Bukeo, *Bull. Chem. Soc. Jpn.*, **45**, 1480 (1972).

Scheme XI

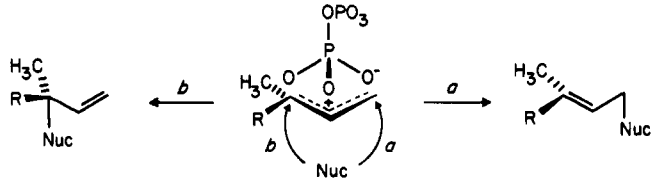


than a concerted mechanism, being strongly subject to acid catalysis and inhibition by added bases such as pyridine.[42] The extensive studies of Goering have established that in polar solvents the rearrangements of allylic carboxylate esters occur by tight ion-pair intermediates.[43] Thorough kinetic and labeling experiments have demonstrated a preference for syn stereochemistry and concurrent scrambling of the carboxylate ester oxygens to an extent determined by the relative stability of the intermediate allylic cation. For example, the rearrangement of optically active *trans*-α,γ-dimethylallyl *p*-nitrobenzoate (**13**) in 90% aqueous acetone resulted in interconversion of the enantiomers by an allylic rearrangement involving a tight ion pair in which the rate of scrambling of the ester oxygens was 1/2.9 times that of racemization (Scheme XI). The analogous transformation of *trans*-α-methyl-γ-phenylallyl *p*-nitrobenzoate (**14**) differed in that it involved a more stable intermediate cation and was essentially irreversible. The latter rearrangement was found to involve almost complete randomization of the carboxyl oxygens accompanied by a partial loss of optical purity, implying a partial anti component to the allylic rearrangement and consistent with a longer lived or looser ion pair.

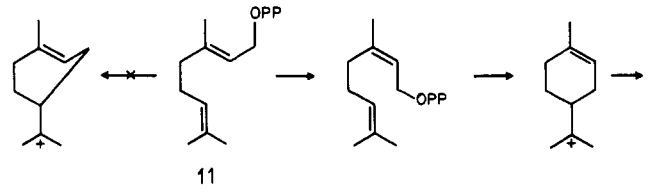
As pointed out in the introduction the central role played by allylic pyrophosphates in isoprenoid metabolism has made these substrates the object of extensive studies. The demonstration of an ion-pair intermediate for allylic pyrophosphate rearrangements is particularly significant since this intermediate can satisfactorily account for the stereochemistry and mechanism of several classes of allylic pyrophosphate transformations.

Direct displacements, typified by the reactions catalyzed by prenyl transferase, have been shown to occur with inversion of configuration at the primary allylic carbon,[44] the rate-determining step being the initial ionization of the allylic substrate.[45] Studies by several groups on the biosynthesis of a variety of metabolites

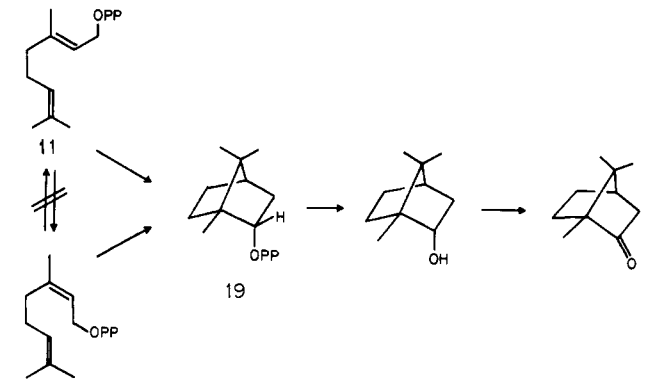
Scheme XII



Scheme XIII



Scheme XIV



including rosenonolactone,[47] virescenol B,[48] sandaracopimaradiene,[2,49] kaurene,[49,50] and pleuromutilin[51] have established that the ring-generating *allylic displacements* by which these diterpenes are formed all occur with overall anti stereochemistry. Although no evidence is as yet available as to the detailed timing of these allylic displacement reactions, the observed anti pathway can be clearly rationalized by initial ionization to an ion pair which then undergoes backside nucleophilic attack at the carbon allylically related to that from which the pyrophosphate originally departed. According to this picture direct displacements and allylic displacements would be mechanistically as well as stereochemically equivalent, with the position of eventual nucleophilic attack being determined by the particular enzyme system involved. Prior rearrangement to a tertiary allylic pyrophosphate followed by direct displacement would therefore be mooted since all these transformations must involve the same ion-pair intermediate.

The same ion-pair model can also be used to account for the *terpenoid cyclizations* and allylic *double-bond isomerizations*. Although the recognition of geranyl and farnesyl pyrophosphate as the universal precursors of cyclized mono- and sesquiterpenes has provided a powerful theoretical tool for the understanding of terpenoid biogenesis, the intimate details of the cyclization reactions are still poorly understood in most cases. A major difficulty stems from the realization that the formation of six-membered

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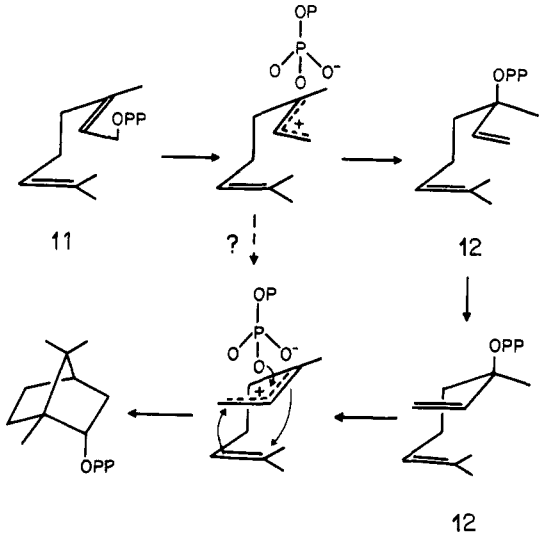
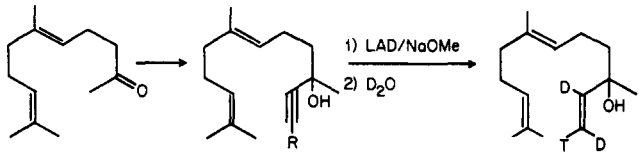
Scheme XV



Scheme XVI



21 R = H
R = T

rings from allylic pyrophosphate requires a prior isomerization of the trans double bond in order to avoid formation of *trans*-cyclohexene. Several theories have in fact been advanced to account for these transformations and much of the experimental evidence bearing on these various hypotheses has been discussed in detail elsewhere.[2] In the last year, however, much of the evidence for a redox isomerization has been eroded while strong experimental support has begun to emerge in favor of an isomerization mechanism involving tertiary allylic pyrophosphates or equivalent structures.[2,41a,52-54] For example, in an extensive and careful study of the monoterpene cyclases of sage (*Salvia officinalis*), Croteau has prepared a cell-free extract which can cyclize either geranyl or neryl pyrophosphate to (+)-bornyl pyrophosphate (**19**) but does not catalyze the interconversion of the two acyclic allylic substrates[55] (Scheme XIV). Moreover, partial purification of this enzyme system to remove contaminating phosphatases and pyrophosphatases established that *geranyl pyrophosphate is the preferred substrate for cyclization*, with a V_{max}/K_m 20 times that of its cis isomer, neryl pyrophosphate.[56] These results are extremely significant since they establish that (a) neryl pyrophosphate is not a mandatory intermediate in the cyclization and (b) inorganic pyrophosphate remains associated with the cationic substrate, being recaptured to form the bornyl pyrophosphate product. In related work Suga has recently shown that a cell-free system from *Mentha spicata* will convert either [1-$^3H_2$,^{14}C]geranyl, -neryl, or -linalyl pyrophosphate to α-terpineol without change in $^3H/^{14}C$ ratio,[57] thereby demonstrating that cyclization does not require loss of the C-1 hydrogen atoms of the acyclic allylic pyrophosphate precursor.

These observations can be satisfactorily explained by the proposed ion-pair model (Scheme XVI). For cyclization of a *trans*-allylic pyrophosphate, the first step would be generation of the corresponding allylic cation–pyrophosphate anion pair. Collapse of this ion pair at the tertiary center would generate the tertiary allylic pyrophosphate in a transoid conformation which is readily converted to its cisoid conformational isomer. Reionization generates the cisoid ion pair which can be attacked from the backside at C-1 by the neighboring double bond with formation of the cyclohexane ring. One obvious consequence of the model is that isomerization–cyclization will take place with net retention of configuration at C-1. Should the cisoid ion pair instead collapse to the primary *cis*-allylic pyrophosphate, further reaction of the latter substrate would still require reionization with regeneration of the same ion pair from whence it had been formed. According to this line of reasoning, formation of the primary *cis*-allylic isomer would occur by a nonproductive equilibrium side reaction of the cisoid ion pair. It is also interesting to contemplate the possibility that isomerization of the transoid to the cisoid ion pair might occur directly without mandatory intervention of the covalent tertiary allylic pyrophosphate intermediate. The (gas-phase) free-energy barrier to such a rotation about the 2–3 bond of a tertiary allylic cation has been calculated to be 13.1 kcal.[46] There is as yet no evidence, however, in support of direct isomerization of the ion pairs in the enzyme-catalyzed reactions. In solution, capture of an initially generated *trans*-allylic cation by solvent nucleophile competes favorably with any rearrangement or isomerization.[3,58] At the active site of the cyclase, however, the allylic cation should be protected on one face by the paired pyrophosphate ion and on the other by the nucleophilic double bond of the substrate. Upon eventual formation of the cisoid ion pair the intermediate can undergo cyclization with eventual recapture of the paired pyrophosphate ion in the case of the bornyl pyrophosphate synthetase.

The ion-pair model of allylic pyrophosphate metabolism, for which we have provided strong evidence in the case of the allylic rearrangement of farnesyl to nerolidyl pyrophosphate, can provide a satisfactory explanation of a variety of other known transformations of these allylic pyrophosphates. Substantiation of this hypothesis in the latter cases will of course require considerably more stereochemical as well as kinetic investigation. On the other hand a coherent picture of allylic pyrophosphate metabolism has already begun to emerge, and the next several years should witness rapid progress in this intriguing area.

Experimental Section

Instrumentation. Proton NMR spectra were obtained on Varian A-60A or Bruker WP 60 spectrometers. ^{13}C NMR spectra were recorded on a Bruker WP 60 spectrometer (15.085 MHz) by using 0.5-mL inserts in 10-mm NMR tubes. All NMR spectra were recorded at ambient temperature in $CDCl_3$ (unless otherwise stated) and are reported as parts per million downfield from Me_4Si (δ 0). Multiplicities are s = singlet, d = doublet, t = triplet, b = broad, and m = multiplet. Infrared spectra were taken by using a Perkin-Elmer Model 257 grating spectrophotometer. Mass spectra were recorded via the direct inlet of a Hitachi Perkin-Elmer RMU-6D instrument at 50-eV ionizing potential and are reported as *m/e*. For determination of isotopic enrichments, the regions of interest were repetitively scanned at slow speed and the areas of the peaks determined by cutting and weighing. Melting points were taken in unsealed capillary tubes in a Hoover melting point apparatus and are uncorrected. Analytical gas chromatography was performed with an Aerograph Model 200 by using a flame ionization detector and nitrogen as the carrier gas. A 6 ft × $^1/_8$ in. column of 5% Carbowax on Chromosorb W was used. All UV measurements were recorded on a Cary Model 14 instrument in 1.0-cm cells. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter at room temperature. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn. Radioactivity measurements were obtained by using a Packard 3330 liquid scintillation counter. The counting was done in 10-mL toluene solutions containing 7.2 g of BuPBD and 0.45 g of PBBO/L of toluene.

Gibberella fujikuroi fermentations were carried out in a New Brunswick Scientific G-25 gyrotory incubator–shaker. The cell-free

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extract was prepared by using a Carver Laboratory Press Model C and a 50-mL French pressure cell.

pH measurements were recorded on a Corning Model 7 pH meter. All enzymatic preparations utilized glass-distilled water, and centrifugations were carried out at 4 °C.

Preparative layer chromatography (PLC) was carried out by using 20 × 20 cm plates, 2-mm thickness of Merck silica gel PF-254 buffered to pH 7, or on Merck precoated silica gel F-254 TLC plates, 0.25-mm thickness. Compounds were visualized by ultraviolet light or by heating to 150 °C after spraying with vanillin in 1:1 ethanol–85% phosphoric acid. Allylic pyrophosphates were analyzed by developing the plates with *n*-propyl alcohol–butyl alcohol–2 N ammonium hydroxide, 5:2:3. After being dried, the plates were sprayed with a visualizing reagent and then heated to 150 °C. The spray reagent was prepared by adding 30 mL of 1 N hydrochloric acid and 15 mL of 60% perchloric acid to 3.0 g of ammonium molybdate in 255 mL of water.

Materials. Tetrahydrofuran was freshly distilled from lithium aluminum hydride. Pentane was stirred overnight over sulfuric acid, decanted, and distilled. Pyridine was freshly distilled from BaO. *n*-Butyllithium (Alfa) was standardized by Gilman titration by using 1,2-dibromoethane, 3,5-dinitrobenzoyl chloride (Aldrich) was recrystallized from CCl_4–pentane, α-*p*-dibromoacetophenone (Aldrich) was recrystallized from methylene chloride, ethyl chloroformate (Aldrich) was distilled, and sodium formate (Mallinckrodt), formic acid (Aldrich), L-malic acid (Aldrich), lithium aluminum deuteride (Merck, Sharp and Dohme), *cis,trans*-geranylacetone (gift of Dr. O. Isler, Hoffman LaRoche Co.), (*S*)-(+)-nerolidol (gift of Dr. N. Anderson, University of Washington, and Dr. V. Herout, Czechoslovakia Academy of Science, Prague, Czechoslovakia), and corn steep liquor (gift of A. E. Staley Manufacturing Co.) were used as received.

Malate synthetase was extracted from baker's yeast by using a modified procedure provided by Dr. H. Eggerer of the Technische Universitat, Munich.[59] NAD^+ and NADH, horse liver alcohol dehydrogenase (crystalline suspension in 20 mM potassium phosphate buffer, pH 7.0, 10% ethanol, specific activity = ca. 2.7 units/mg), acetyl coenzyme A, coenzyme A, and fumarase (crystalline suspension in 3.2 M ammonium sulfate solution, pH 7.5, specific activity ca. 350 units/mg) were products of Boehringer Mannheim. Ammonium sulfate (grade I), Dowex (chloride form), Dowex 50W-X8, and C_γ alumina gel (aged) were purchased from Sigma. Sephadex G-100-120 and G-150 were obtained from Pharmacia, DEAE Cellulose (DE-22) from Whatman, hydroxylapatite gel from Clarkson Chemical Co., Philadelphia, and glycerol (A.R.) from Mallinckrodt.

Sodium borotritide (269.9 mCi/mmol), lithium aluminum [^{3}H]-hydride (171.3 mCi/mmol), sodium [2-^{3}H]acetate (150 mCi/mmol), and sodium [2-^{14}C]acetate (58.8 mCi/mmol) were purchased from New England Nuclear. [^{14}C]Methyl iodide (59.1 mCi/mmol) was a product of Amersham/Searle. [1,2-$^{13}C_2$]Acetylene gas (0.1 L, 90% atom excess) was obtained from Merck, Sharp and Dohme. [^{18}O]Water (90.43% atom excess) was purchased from Mound Laboratory, Miamisburg, Ohio.

General Reaction Procedures. All organic extracts of reaction mixtures were dried over anhydrous sodium sulfate unless otherwise specified. The dried extracts were concentrated by evaporation at 25 °C with a Buchi rotary evaporator evacuated to 10–30 mm by a water aspirator.

Enzyme Assays. The method of Lowry[15] was used to determine the protein content of enzyme solutions.

Alkaline Phosphatase. Calf intestine alkaline phosphatase was purchased as an ammonium sulfate suspension. The enzyme was dialyzed overnight at 4 °C against 4–5 changes of 0.1 M Tris–HCl buffer (pH 8.6) and stored at 4 °C in the same buffer.

The enzyme was assayed by measuring the change in optical density at 405 nm by using *p*-nitrophenyl phosphate as the substrate.[60] To an aqueous solution of *p*-nitrophenyl phosphate (1 mL, 14.5 mM) and Tris–HCl buffer (pH 8.0, 1 M, 1.9 mL) was added 100 μL of alkaline phosphatase solution, and the change in absorbance per minute was used to estimate the enzyme activity.

Yeast Inorganic Pyrophosphatase and Phosphate Determination. Reagents: 3.00 mL of Tris–HCl buffer (pH 7.0, 50 mM), 0.05 mL of $MgCl_2$ (0.1 M), 0.55 mL of $Na_4P_2O_7$ (0.1 M), and 0.02 mL of enzyme solution in buffer.

This mixture[61] was incubated for 15 min at 25 °C and then pipetted into 7 mL of phosphate reagent[62] (10 mL of 5 N H_2SO_4, 10 mL of 2.5% ammonium molybdate, 10 mL of 2.7% sodium pyrosulfite solution, and 1% methyl *p*-aminophenyl sulfate solution, 40 mL of H_2O). The solution was mixed and allowed to stand for 10 min. The absorbance was measured at 578 nm against a blank. The activity of the pyrophosphatase or the amount of inorganic orthophosphate present was calculated by means of an orthophosphate standard.

Malate Synthetase. Assay mixture: 3.00 mL of Tris–HCl buffer (pH 8.0, 0.1 M), 0.10 mL of $MgCl_2$ (0.1 M), 0.10 mL of acetyl coenzyme A solution (0.002 M), and 0.10 mL of enzyme solution. The absorbance of the assay mixture was measured at 232 nm against a blank.[59] Sodium glyoxylate (100 μL, 0.02 M, pH 8) was pipetted into the test cell, and the rate of decrease of optical density at 232 nm was used as a measure of the activity of malate synthetase. All assays were carried out between 22 and 25 °C.

Fumarase. The enzyme was assayed by the change in optical density at 240 nm.[63] Commercially available fumarase was diluted with aqueous bovine serum albumin solution (0.1%). An aliquot was added to the UV cell containing 3 mL of L-malate (50 mM) in 0.1 M phosphate buffer (pH 7.6). The assay was carried out between 22 and 28 °C. The residual fumarase activity after each stage of the malate synthetase purification from baker's yeast was also measured by this assay. With use of the Eggerer purification procedure, no fumarase activity could be detected subsequent to the heat denaturation step.

Production of Cyclonerodiol by *Gibberella fujikuroi*.[10] *Gibberella fujikuroi* (ATCC 12616) was maintained on potato dextrose agar slants. A nutrient solution consisting of 200 g of dextrose, 6.0 g of ammonium succinate, 0.5 g of potassium dihydrogen phosphate, 0.2 g of magnesium sulfate heptahydrate, 0.2 g of potassium sulfate, and 6.0 mL of corn steep liquor/L of distilled water[7] was distributed in 10 500-mL DeLong flasks (100 mL/flask) fitted with Morton closures. The contents were autoclaved at 120 °C for 20 min. Each flask was inoculated with a 5-mm plug of mycelium of *G. fujikuroi* from the slants. The flasks were then incubated for 7 days at 27 °C and 220 rpm in an incubator shaker.

For larger growths, Fernbach flasks (2.8 L) each containing 800 mL of culture medium were autoclaved at 120 °C for 30 min. These media were inoculated with 50 mL of 4-day-old culture grown in the DeLong flasks and then incubated at 27 °C and 200 rpm for 10 days.

The contents of the flasks were combined, and a small quantity of Celite (Johns Manville 503) was added before removal of the mycelia by filtration through filter cloth. The filtrate was extracted with ether (3×), and the combined extracts were concentrated to about 500 mL, then washed with water (water back-extracted with ether), 5% sodium hydroxide (until the washings were colorless), water (water back-extracted with ether), and saturated sodium chloride, dried, and concentrated. The resulting yellow oil was partially purified by PLC (ether; R_f 0.3) and then further purified by another PLC (methylene chloride–ether, 3:1; 2×; R_f 0.25). On a large scale, initial column chromatography (silica gel, 70–270 mesh, ether) followed by a second column chromatography (silica gel 60, PF 254, methylene chloride–ether, 3:1) was used to purify the crude extract. Typically about 15–20 mg of cyclonerodiol (**3**) was obtained from 1 L of growth medium: ^{1}H NMR ($CDCl_3$) δ 1.03 (d, J = 7 Hz, CH_3CH, 3 H), 1.16 (s, CH_3, 3 H), 1.25 (s, CH_3, 3 H), 1.65, 1.70 (s, CH_3, 6 H), 1.35–2.3 (b m, CH_2, 12 H), 5.10 (t, J = 7 Hz, CH=, 1 H); ^{13}C NMR ($CDCl_3$,[9] broad-band ^{1}H-decoupled) δ 14.7 (C-1), 17.7 (C-15), 22.7 (C-9), 24.4 (C-5), 25.1 (C-13), 25.8 (C-12), 26.1 (C-14), 40.5 (C-4 and C-8), 44.3 (C-2), 54.3 (C-6), 74.9 (C-7), 81.3 (C-3), 124.6 (C-10), 131.7 (C-11); IR ν_{max} ($CHCl_3$) 3610, 3450 (hydroxyl) cm^{-1}.

(3*RS*)-*trans*-Nerolidol. *trans*-Geranylacetone (428 mg, 2.30 mmol) purified as the semicarbazone[64] was dissolved in 10 mL of methylene chloride at 0 °C, and vinylmagnesium bromide (1.3 M, 7.5 mL, 9.71 mmol) was added dropwise. The solution was stirred at 0 °C for 15 min and then at room temperature for 2–3 h.[65] The excess Grignard reagent was destroyed by the addition of methanol followed by half-saturated ammonium chloride. The reaction mixture was extracted with ether (3×). The combined ether extracts were washed with saturated ammonium chloride, saturated potassium bicarbonate, and saturated sodium chloride, then dried, and concentrated. The resulting crude nerolidol was purified by PLC (benzene–ethyl acetate, 10:1; R_f 0.38) to give 428 mg (87%) of (3*RS*)-*trans*-nerolidol: ^{1}H NMR ($CDCl_3$) δ 1.28 (s, CH_3COH, 3 H), 1.6, 1.7 (s, CH_2, 9 H), 1.92 (s, OH, 1 H), 2.1 (m, CH_2, 8 H), 4.91–5.3 (m, CH=, CH_2=, 4 H), 5.95 (dd, J = 10, 17 Hz, CH=, 1 H); ^{13}C NMR ($CDCl_3$, broad-band decoupled) δ 16.1 (C-13), 17.7 (C-12 cis), 22.8 (C-14), 25.8 (C-12 trans), 26.8 (C-9), 27.9 (C-5), 39.8 (C-8),

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42.2 (C-4), 73.5 (C-3), 111.8 (C-1), 124.5 (C-10 and C-6), 131.4 (C-11), 135.5 (C-7), 145.3 (C-2); IR ν_{max} ($CHCl_3$) 3540 (b, hydroxyl), 1650 (double bond) cm^{-1}.

(3*RS*)-10,11-Epoxynerolidol (20). (3*RS*)-*trans*-Nerolidol (300 mg, 1.35 mmol) was dissolved in 10 mL of methylene chloride at 0 °C and *m*-chloroperbenzoic acid (85%, 328 mg, 1.62 mmol) was added in portions over a period of 2–3 h.[66] The solution was stirred for an additional hour at 0 °C. The excess oxidizing agent was then destroyed by the addition of saturated sodium sulfite, and the solution was extracted with ether (3×). The combined ether extracts were washed successively with saturated sodium sulfite, saturated potassium bicarbonate, and saturated sodium chloride, dried, and concentrated. The crude epoxide was purified by PLC (ether–hexane, 5:1) to give 30 mg (10%) of recovered nerolidol (R_f 0.71) and 226 mg (70%) of 10,11-epoxynerolidol (**20**) (R_f 0.55): ^1H NMR ($CDCl_3$) δ 1.28 (s, CH_3CO, 9 H), 1.65 (b, CH_3, 3 H), 1.8–2.4 (m, CH_2, 8 H), 2.71 (b t, J = 6 Hz, epoxide proton, 1 H), 4.91–5.3 (m, CH=, CH_2=, 3 H), 5.95 (dd, J = 10, 17 Hz, CH=, 1 H). An analytical sample of **20** was prepared by bulb-to-bulb distillation (2×) at 94 °C (0.4 mm). Anal. Calcd for $C_{15}H_{26}O_2$: C, 75.58; H, 10.99. Found: C, 75.37; H, 11.31.

10,11-Dihydroxynerolidol (8). 10,11-Epoxynerolidol (**20**) (226 mg, 0.95 mmol) was dissolved in 5 mL of THF–H_2O (1:1), and 3% perchloric acid (0.55 mL) was added.[67] The solution was stirred at room temperature for 2–3 h, and then saturated sodium chloride was added to separate the phases. The reaction mixture was extracted with ether (3×), and the combined ether extracts were washed with saturated potassium bicarbonate and saturated sodium chloride, dried, and concentrated. The crude triol **8** was purified by PLC (methylene chloride–ether, 1:2, R_f 0.12) to yield 170 mg (71%) of **8** which was crystallized from CCl_4: mp 70–71 °C; ^1H NMR ($CDCl_3$) δ 1.16, 1.2 (s, $(CH_3)_2COH$, 6 H), 1.31 (s, CH_3COH, 3 H), 1.63 (s, allylic CH_3, 3 H), 2.08, 2.83 (m, CH_2, 8 H), 4.9–5.3 (m, CH=, CH_2=, 3 H), 5.95 (dd, J = 10, 17 Hz, CH=, 1 H); IR ν_{max} ($CHCl_3$) 3600 (b, hydroxyl) cm^{-1}. Anal. Calcd for $C_{15}H_{28}O_3$: C, 70.27; H, 11.01. Found: C, 70.34; H, 11.21.

Nerolidol Trisnoraldehyde (9). 10,11-Dihydroxynerolidol (**8**) (110 mg, 0.43 mmol) was dissolved in 5 mL of THF–H_2O (1:1), and sodium periodate (111 mg, 0.52 mmol) was added in portions over a period of 2 h.[67] The solution was left stirring for another 4 h after which solid sodium chloride was added to separate the phases and then the reaction mixture was extracted with ether (3×). The combined organic extracts were washed with water and saturated sodium chloride, then dried, and concentrated. The crude aldehyde **9** was purified by PLC (ether–hexane, 2:1; R_f 0.46) to give 83 mg (80%) of nerolidol trisnoraldehyde **9**: ^1H NMR ($CDCl_3$) δ 1.33 (s, CH_3COH, 3 H), 1.77 (b s, CH_3, 3 H), 2.08 (m, CH_2, 4 H), 2.5 (m, CH_2, 4 H), 4.9–5.3 (m, CH=, CH_2=, 3 H), 5.95 (dd, J = 10, 17 Hz, CH=, 1 H), 9.8 (t, J = 1.5 Hz, CHO, 1 H); IR ν_{max} ($CHCl_3$) 2740, 1760 (aldehyde) cm^{-1}.

(3*RS*)-[12,13-^{14}C]-*trans*-Nerolidol. *n*-Butyllithium (2.01 M, 0.51 mL, 1.03 mmol) was added to ethyltriphenylphosphonium bromide (392 mg, 1.06 mmol) in 2 mL of THF at 0 °C under nitrogen. The yellow-orange ethyl ylide was stirred for 20 min followed by the addition of [^{14}C]methyl iodide (1 mCi) in 1 mL of hexane. Unlabeled methyl iodide (0.5 mL, freshly distilled over CaH_2 and stored over copper wire) was diluted with 4.5 mL of THF, and 0.65 mL of this solution was added to the reaction flask and the mixture was stirred for 1 h. *n*-Butyllithium (0.51 mL, 2.01 M) was added to the white solid, and the blood-red ylide produced was stirred for an additional hour at 0 °C. Nerolidol trisnoraldehyde (**9**) (83 mg, 0.42 mmol) dissolved in 1 mL of THF was added to the reaction mixture and the solution stirred at 0 °C for 2 h and then overnight at room temperature. The resulting yellow solution was poured into 40% methanol in half-saturated ammonium chloride, and the aqueous layer was extracted with hexane (3×). The combined hexane extracts were washed with 40% methanol–ammonium chloride solution, followed by water and saturated sodium chloride, then dried, and concentrated. The crude product was purified by PLC (ether–hexane, 1:1; R_f 0.52) to yield 67 mg (70%) of (3*RS*)-[12,13-^{14}C]-*trans*-nerolidol (activity = 2.35 × 10^6 dpm/mg); ^1H NMR was identical with that reported for *trans*-nerolidol.

Dioxane Diphosphate. Aqueous phosphoric acid (90%, 13.6 mL) was added to 10.5 mL of freshly distilled dioxane. The warm syrupy liquid was cooled in ice. Crystallization was induced by scratching the walls of the flask. The mother liquor was removed by a quick suction filtration, and the wet precipitate was dried under high vacuum over sodium hydroxide. The hydroscopic solid was recrystallized from ether to yield 25 g of dioxane diphosphate, mp 82–86 °C (lit.[68] mp 83–87 °C).

Bis(triethylammonium) Hydrogen Phosphate.[69] Dioxane diphosphate (5 g, 17.6 mmol) was dissolved in 5 mL of freshly distilled acetonitrile under nitrogen, and distilled triethylamine (9.5 mL, 60.0 mmol) was added slowly. The resulting two-phase reaction mixture was stirred for 15 min and then left standing overnight at room temperature. The precipitated solid was removed by filtration and washed with cold acetonitrile to yield 6.07 g of bis(triethylammonium) hydrogen phosphate, mp 105–107 °C.

Washing of Ion-Exchange Resins. Dowex 1-8. Dowex 1-8 (200–400 mesh), chloride form (25 g), was suspended in 100 mL of absolute ethanol–acetone (1:1) and allowed to stand with occasional stirring at room temperature for at least 30 min. The resin was filtered in a sintered glass funnel and washed thoroughly with distilled water followed by 8.8% formic acid or 1.1 N sodium formate until the effluent was free from chloride (negative silver nitrate test). The prewashed resin was stored in a glass-stoppered bottle in distilled water. Just prior to use the resin was washed with increasing concentrations of methanol–water solutions and finally with 100% methanol. The column was packed and equilibrated by elution with at least 15 column volumes of 0.053 M ammonium formate in methanol.

Amberlite XAD-2 Resin. Amberlite XAD-2 resin was washed with water until the washings were neutral (pH paper). The resin was then washed several times alternately with 0.01 M aqueous ammonia and 0.01 M ammonia in methanol; the last wash was aqueous. The resin was stored over 0.01 M aqueous ammonia in a stoppered bottle.

(3*RS*)-[12,13-^{14}C]-*trans*-Nerolidyl Pyrophosphate (2).[4,69] Bis(triethylammonium) phosphate (270 mg, 0.91 mmol) was dissolved in 4 mL of acetonitrile with heating under nitrogen. The solution was cooled and over a period of 1–2 h added to a stirred solution of (3*RS*)-[12,13-^{14}C]-*trans*-nerolidol (67 mg, 0.296 mmol, 2.35 × 10^6 dpm/mg) and trichloroacetonitrile (0.27 mL, 1.09 mmol) under nitrogen. The yellow solution was then left stirring overnight at room temperature. After evaporation of the solvent, the residual oil was dissolved in the minimum amount of 0.053 M ammonium formate in methanol and applied to a preequilibrated 1 × 16-cm column of Dowex 1-8 (200–400 mesh) ion-exchange resin in the formate form. The column was eluted with 500 mL of a 0.053–0.43 M linear gradient of ammonium formate in methanol. Forty fractions of about 7 mL each were collected.

An aliquot of every third fraction was counted, and, on the basis of the radioactivity, fractions 6–15 (monophosphate) and 21–26 (pyrophosphate) were combined. The solution was lyophilized to remove the methanol until the ammonium formate began to crystallize out. Ammonium hydroxide (0.01 M, 2–3 mL) was added and the lyophilization was continued. The process of lyophilization and addition of ammonium hydroxide was repeated until all the methanol was driven off and the final volume was 2–3 mL.

The holdup volume from the Dowex 1-8 column was concentrated on the rotary evaporator. The residue was poured into 5 mL of distilled water, and the recovered trichloroacetamide (20 mg) was removed by filtration. The aqueous solution was extracted with hexane (3×), and the combined organic extracts were washed with saturated sodium chloride, dried, and concentrated to yield 21 mg (33%) of nerolidol.

The lyophilized fraction dissolved in 0.01 M ammonium hydroxide was stirred for 4 h with 30 g of pretreated Amberlite XAD-2 resin (greater than 90% of the radioactivity was adsorbed by the resin). The resin was washed with 5 × 40 mL of 0.01 M aqueous ammonia to elute inorganic salts and then with 4 × 30 mL of 0.01 M ammonia in methanol to elute the pyrophosphate ester. The methanol solution was lyophilized to give 12 mg (10%) of (3*RS*)-[12,13-^{14}C]-*trans*-nerolidyl pyrophosphate (activity = 1.35 × 10^6 dpm/mg): TLC (silica gel) *n*-propyl alcohol–butyl alcohol–2 M ammonium hydroxide, 5:2:3; phosphate spray reagent, monophosphate R_f 0.39; pyrophosphate R_f 0.31.

Characterization of Nerolidyl Pyrophosphate. The experiments described below were carried out on unlabeled samples of (3*RS*)-*trans*-nerolidyl pyrophosphate.

Identification of Acid Labile Derivative as a Pyrophosphate Ester. An aliquot of *trans*-nerolidyl pyrophosphate dissolved in ammoniacal methanol was treated with 5% trichloroacetic acid for 10 min, and then the solution was neutralized with potassium hydroxide.[4] Extraction of the alcohols with hexane and analysis by GC using a 5% Carbowax column at 155 °C with octadecane as the internal standard established that 0.9 equiv of nerolidol had been released. Under these conditions nerolidol had a retention time of 2.1 min.

Half of the aqueous phase that had been extracted with hexane was analyzed directly for inorganic orthophosphate.[62] (Background inorganic orthophosphate was always detected in the initial solution; hence it was necessary to do this control.) An aliquot (200 μL) was pipetted into 3

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mL of the phosphate reagent described above and allowed to stand at 70 °C for 10 min. The absorbance measured at 578 nm against a blank corresponded to 1.4 equiv of orthophosphate on the basis of standard calibration solutions.

The other half of the aqueous layer was incubated at 25 °C for 15 min with yeast inorganic pyrophosphatase to hydrolyze inorganic pyrophosphate to orthophosphate.[61] An aliquot was pipetted into an incubation mixture containing 2 units of yeast inorganic pyrophosphatase in 3 mL of Tris–HCl buffer (pH 7.0) and 0.5 mL of 0.1 M $MgCl_2$. At the end of the incubation the solution was pipetted into 7 mL of phosphate reagent and allowed to stand at 70 °C for 10 min, and the absorbance at 578 nm was measured against a blank. Incubation with the pyrophosphatase released 3.25 equiv of orthophosphate which corresponded to 1.85 equiv of orthophosphate in excess of the control and therefore 0.92 equiv of inorganic pyrophosphate.

A third aliquot of the nerolidyl pyrophosphate solution was incubated with yeast inorganic pyrophosphatase without prior acidification. Analysis indicated that the resulting solution contained 1.64 equiv of orthophosphate, an amount comparable to the previously determined blank of 1.4 equiv. Thus acidification of nerolidyl pyrophosphate releases between 0.8 and 0.9 equiv of additional inorganic pyrophosphate/1 equiv of alcohol, establishing that the original sample was an allylic pyrophosphate ester.

Nerolidyl pyrophosphate was dissolved in D_2O and lyophilized to dryness. The residue was dissolved in deuterated methanol with Me_4Si as the internal standard: ^1H NMR (CD_3OD) δ 1.26 (s, CH_3COH, 3 H), 1.6, 1.7 (s, CH_3, 9 H), 2.1 (m, CH_2, 8 H), 4.9–5.3 (m, CH=, CH_2=, 4 H), 5.95 (dd, J = 10, 17 Hz, CH=, 1 H).

Partial Purification of *trans,trans*-Farnesol. Commercial farnesol (4.5 g), available as a mixture of isomers, cis,cis–trans,cis–cis,trans–trans,trans (1:4:6:5), was partially purified on a Silica gel column (70–270 mesh, 1:50) by using hexane–ethyl acetate (2:1) as the eluting solvent. The separation was monitored by GC using a 5% Carbowax column, temperature 180 °C: t_R = 5.2 min for cis,cis, 5.8 min for trans,cis, 7 min for cis,trans, and 7.8 min for trans,trans. The fractions enriched in the trans,trans isomer were combined to give 1.34 g of farnesol (trans,-trans–cis,trans, 2:1).

***trans,trans*-Farnesyl Diphenylurethane.**[70] Diphenylcarbamyl chloride (2.9 g, 12.61 mmol) was added to a solution of farnesol (trans,trans–cis,trans, 2:1) (1.34 g, 6.01 mmol) in 1.5 mL of pyridine, and the mixture was heated at 100–105 °C for 4 h. The brown reaction mixture was cooled, water was added, and the solution was stirred for 15 min followed by extraction with ether (3×). The combined ether extracts were washed successively with 5% sulfuric acid, water, saturated potassium bicarbonate, and saturated sodium chloride, then dried, and concentrated. The crude urethane was purified on a short silica gel column (70–270 mesh, 1:20) using hexane–ethyl acetate (10:1) as the eluting solvent. The diphenylurethane (2.68 g, 99%) was recrystallized from methanol five times; mp 61–62 °C (lit.[70] 61–63 °C). The separation of the isomers was monitored by GC of the farnesol obtained by cleaving the diphenylurethane with potassium hydroxide: ^1H NMR ($CDCl_3$) δ 1.61, 1.68 (s, CH_3, 12 H), 2.05 (b, CH_2, 8 H), 4.66 (d, J = 7 Hz, CH_2OCO, 2 H), 4.9–5.4 (overlapping t, CH=, 3 H), 6.4 (m, aromatic, 10 H); IR ν_{max} ($CHCl_3$) 1690, 1749 (urethane) cm^{-1}.

Cleavage of *trans,trans*-Farnesyl Diphenylurethane. A mixture of *trans,trans*-farnesyl diphenylurethane (485 mg, 1.16 mmol), potassium hydroxide pellets (290 mg, 5.23 mmol), 16 mL of ethanol, and 2 mL of water was refluxed under nitrogen for 6 h and then allowed to stir overnight at room temperature. Five percent sulfuric acid was added to the reaction mixture, and the solution was stirred for 15 min. The aqueous layer was extracted with ether (3×), and the combined organic extracts were washed with water, saturated potassium bicarbonate, and saturated sodium chloride, then dried, and concentrated. The crude product was purified by column chromatography (hexane–ethyl acetate, 5:1) to yield 240 mg (93%) of *trans,trans*-farnesol: ^1H NMR ($CDCl_3$) δ 1.61, 1.68 (s, CH_3, 12 H), 2.05 (b s, CH_2, 8 H), 2.4 (s, OH, 1 H), 4.1 (d, J = 7 Hz, CH_2OH, 2 H), 4.9–5.6 (overlapping t, vinyl, 3 H); ^{13}C NMR ($CDCl_3$, broad-band decoupled) δ 16.0 (C-14), 16.3 (C-13), 17.7 (C-12 cis), 25.7 (C-12 trans), 26.4 (C-5), 26.8 (C-9), 39.7 (C-8), 39.8 (C-4), 59.3 (C-1), 123.5 (C-2), 123.9 (C-10), 124.5 (C-6), 131.4 (C-11), 135.5 (C-7), 139.8 (C-3); IR ν_{max} ($CHCl_3$) 3600 (hydroxyl), 1669 (double bond) cm^{-1}.

***trans,trans*-Farnesal.** *trans,trans*-Farnesol (183 mg, 0.96 mmol) was dissolved in 3.5 mL of hexane at 0 °C, and active manganese dioxide[71] (1.74 g, 19.9 mmol) was added in portions. The mixture was stirred for 2–3 h at 0 °C. The solid was removed by filtration and washed thoroughly with hexane, and the hexane filtrate was concentrated to yield 175 mg (97%) of *trans,trans*-farnesal: ^1H NMR ($CDCl_3$) δ 1.61, 1.66 (s, CH_3, 9 H), 2.0 (b s, CH_2, 4 H), 2.1 (s, CH_3, 3 H), 2.09, 2.2 (s, CH_2, 4 H), 5.08 (b, vinyl, 2 H), 5.86 (d, J = 7 Hz, $CHCHO$, 1 H), 9.93 (d, J = 8 Hz, CHO, 1 H); IR ν_{max} ($CHCl_3$) 2730, 1660 (aldehyde) cm^{-1}.

[1-$^3H_2$]-*trans,trans*-Farnesol. Sodium borotritide (1 mg, 25 mCi) was added to *trans,trans*-farnesal (175 mg, 0.80 mmol) dissolved in 3 mL of ethanol at 0 °C. The mixture was stirred for 1 h, and sodium borohydride (37 mg, 0.81 mmol) was added in portions. The solution was stirred for 4 h at room temperature whereupon saturated sodium chloride was added and the aqueous layer was extracted with ether (3×). The combined ether extracts were washed with saturated sodium chloride, dried, and concentrated. The crude farnesol was purified by PLC (hexane–ethyl acetate, 2:1; R_f 0.4) to yield 120 mg (70%) of [1-$^3H_2$]-*trans,trans*-farnesol (activity of 3.28×10^7 dpm/mg). ^1H NMR was identical with that reported for *trans,trans*-farnesol.

[1-$^3H_2$]-*trans,trans*-Farnesyl Pyrophosphate.[4,69] Bis(triethylammonium) hydrogen phosphate (389 mg, 1.29 mmol) was dissolved in 7 mL of acetonitrile with heating under nitrogen, then cooled, and added over a period of 1–2 h to a stirred solution of [1-$^3H_2$]-*trans,trans*-farnesol (120 mg, 0.54 mmol, 3.28×10^7 dpm/mg) in trichloroacetonitrile (0.32 mL, 1.29 mmol). The resulting yellow solution was stirred overnight at room temperature. The solvent was removed on the rotary evaporator, and the residual oil was purified by the Dowex 1-8 column as described for the preparation of nerolidyl pyrophosphate. On the basis of their radioactivity, fractions 5–12 (monophosphate) and 27–39 (pyrophosphate) were combined followed by the usual Amberlite XAD-2 resin treatment to yield 15 mg of farnesyl monophosphate and 38 mg (18%) of [1-$^3H_2$]-*trans,trans*-farnesyl pyrophosphate (activity of 1.5×10^7 dpm/mg).

Farnesyl Acetate. Acetic anhydride (3 mL) was added to *trans,-trans*-farnesol (245 mg, 1.10 mmol) dissolved in 3 mL of pyridine, and the mixture was stirred overnight at room temperature. The solution was poured into ice cold water, and the aqueous layer was extracted with ether (3×). The combined organic extracts were washed successively with 5% sulfuric acid, cold water, saturated potassium bicarbonate, and saturated sodium chloride, then dried, and concentrated to yield 288 mg (98%) of farnesyl acetate (TLC hexane–ethyl acetate, 5:1; R_f 0.53). MS, m/e 264 (parent peak); ^1H NMR ($CDCl_3$) δ 1.6, 1.66 (s, CH_3, 12 H), 2.0 (s, CH_3CO, 3 H), 2.1 (b, CH_2, 8 H), 4.5 (d, J = 7 Hz, CH_2OAc, 2 H), 4.9–5.5 (overlapping t, vinyl, 3 H); IR ν_{max} ($CHCl_3$) 1720 (acetate) cm^{-1}.

10,11-Epoxyfarnesyl Acetate.[66] Farnesyl acetate (285 mg, 1.08 mmol) was dissolved in 10 mL of methylene chloride at 0 °C and *m*-chloroperbenzoic acid (85%, 262 mg, 1.30 mmol) was added in portions. The reaction mixture was stirred for 3 h. The excess oxidizing agent was destroyed by the addition of 10% sodium sulfite, followed by extraction with ether (3×). The combined ethereal extracts were washed with saturated sodium sulfite, saturated potassium bicarbonate, and saturated sodium chloride, then dried, and concentrated. The crude epoxide was purified by PLC (hexane–ether, 1:1) to yield 190 mg (63%) of a ca. 2–3:1 mixture of 10,11-epoxyfarnesyl acetate and 6,7-epoxyfarnesyl acetate (R_f 0.53), 40 mg (14%) of farnesyl acetate (R_f 0.64), and 30 mg (10%) of 6,7:10,11-diepoxyfarnesyl acetate (R_f 0.26). The mixture of monoepoxides was usually not separated at this stage but instead converted to the corresponding mixture of diols. ^1H NMR (monoepoxide mixture) ($CDCl_3$) δ 1.23, 1.28 (s, epoxy CH_3), 1.6, 1.66 (s, CH_3), (total 12 H), 2.01 (s, CH_3CO, 3 H), 2.05 (b, CH_2, 8 H), 2.7 (t, J = 6 Hz, epoxide proton, 1 H), 4.6 (d, J = 7 Hz, CH_2OAc, 2 H), 4.9–5.5 (overlapping t, vinyl, 2 H); IR ν_{max} ($CHCl_3$) 1720 (acetate) cm^{-1}.

10,11-Dihydroxyfarnesyl Acetate. The above isolated mixture of 10,11-epoxyfarnesyl acetate and 6,7-epoxyfarnesyl acetate (190 mg, 0.68 mmol) was dissolved in 10 mL of THF–H_2O (1:1), and 3% perchloric acid (0.5 mL) was added.[67] The reaction mixture was stirred for 3 h at room temperature after which solid sodium chloride was added to separate the phases, and the aqueous layer was extracted with ether (3×). The combined organic extracts were washed with saturated potassium bicarbonate and saturated sodium chloride, then dried, and concentrated. The crude diol was purified by PLC (methylene chloride–ether, 1:1) to yield 144 mg (71%) of the desired 10,11-dihydroxyfarnesyl acetate (R_f 0.25). The corresponding 6,7-diol had an R_f of 0.35. 10,11-Dihydroxyfarnesyl acetate: ^1H NMR ($CDCl_3$) δ 1.15, 1.2 (s, CH_3, 6 H), 1.63, 1.71 (s, CH_3, 6 H), 2.07 (s, CH_3CO, 3 H), 1.5–2.4 (m, CH_2, 8 H),

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2.85 (b s, OH, 2 H), 3.35 (dd, J = 3, 8 Hz, C*H*OH, 1 H), 4.6 (d, J = 7 Hz, CH_2OAc, 2 H), 4.9–5.5 (b m, vinyl, 2 H); IR ν_{max} ($CHCl_3$) 3500 (hydroxyl), 1720 (acetate) cm^{-1}. 6,7-Dihydroxyfarnesyl acetate: ^1H NMR ($CDCl_3$) δ 1.18 (s, CH_3, 3 H), 1.63, 1.71 (s, CH_3, 9 H), 2.08 (s, CH_3CO, 3 H), 1.5–2.5 (m, CH_2, OH, 10 H), 3.37 (dd, J = 3.8 Hz, C*H*OH, 1 H), 4.59 (d, J = 7 Hz, CH_2OAc, 2 H), 5.1–5.4 (m, vinyl, 2 H); IR ν_{max} ($CHCl_3$) 3550 (OH), 1725 (acetate) cm^{-1}. The structure of the 6,7-dihydroxyfarnesyl acetate was confirmed by cleavage with sodium periodate to 6-methylhept-5-en-2-one, by direct comparison identical with an authentic sample.[72]

Farnesyl Acetate Trisnoraldehyde. Solid sodium periodate (124 mg, 0.58 mmol) was added in portions to 10,11-dihydroxyfarnesyl acetate (144 mg, 0.48 mmol) dissolved in 12 mL of THF–H_2O (1:1), and the solution was stirred at room temperature for 3 h.[67] Solid sodium chloride was added to separate the phases, and the reaction mixture was extracted with ether (3×). The combined organic extracts were washed with water and saturated sodium chloride, then dried, and concentrated. The crude product was purified by PLC (hexane–ether, 2:1; R_f 0.32) to yield 85 mg (75%) of farnesyl acetate trisnoraldehyde: ^1H NMR ($CDCl_3$) δ 1.63, 1.7 (s, CH_3, 6 H), 2.01 (s, CH_3CO, 3 H), 2.02 (b, CH_2, 6 H), 2.4 (b t, J = 5 Hz, CH_2CHO, 2 H), 4.55 (d, J = 7 Hz, CH_2OAc, 2 H), 4.9–5.6 (b m, vinyl, 2 H), 9.7 (t, J = 1 Hz, CHO, 1 H); IR ν_{max} ($CHCl_3$) 2740 (aldehyde), 1720 (acetate) cm^{-1}.

[12,13-^{14}C]-*trans,trans*-Farnesol. *n*-Butyllithium (2.02 M, 0.20 mL) was added to ethyltriphenylphosphonium bromide (158 mg, 0.43 mmol) in 3 mL of THF at 0 °C under nitrogen. The yellow-orange solution of the ethyl ylide was stirred for 30 min, and then [^{14}C]methyl iodide (1 mCi) in 1 mL of hexane was added. Unlabeled methyl iodide (0.5 mL, freshly distilled from CaH_2 and stored over Cu wire) was diluted with 4.5 mL of THF. From this solution 0.56 mL was added to the reaction flask and the mixture was stirred for 1 h. *n*-Butyllithium (2.03 M, 0.2 mL) was then added, and the blood-red ylide was stirred for an additional hour at 0 °C. Farnesyl acetate trisnoraldehyde (85 mg, 0.36 mmol) in 1 mL of THF was added to the reaction mixture, and the solution was stirred at 0 °C for 2 h and then overnight at room temperature. The resulting yellow solution was poured into 40% methanol in half-saturated ammonium chloride and the aqueous layer was extracted with hexane (3×). The combined organic extracts were washed with 40% methanol–ammonium chloride solution, water, and saturated sodium chloride, then dried, and concentrated. The crude product (120 mg) was hydrolyzed to the free alcohol by stirring with anhydrous potassium carbonate (71 mg, 0.52 mmol) in 1 mL of methanol for 30 min at room temperature. After addition of saturated sodium chloride, the aqueous layer was extracted with hexane (3×) and the combined hexane extracts were washed with water and saturated sodium chloride, then dried, and concentrated. The recovered farnesol was purified by PLC (hexane–ethyl acetate, 2:1; R_f 0.45) to yield 67 mg (75%) of [12,13-^{14}C]-*trans,trans*-farnesol (activity = 3.52×10^6 dpm/mg). ^1H NMR was identical with that reported for *trans,trans*-farnesol.

[12,13-^{14}C]-*trans,trans*-Farnesol (67 mg, 0.30 mmol) was phosphorylated in the usual manner to yield 15 mg (15%) of [12,13-^{14}C]-*trans,trans*-farnesyl pyrophosphate (activity = 1.11×10^6 dpm/mg).

Feeding of (3*RS*)-[12,13-^{14}C]-*trans*-Nerolidol to *G. fujikuroi* Cultures. *G. fujikuroi* was grown at 27 °C for 5 days. (3*RS*)-[12,13-^{14}C]-*trans*-nerolidol (2 mg, 2.35×10^6 dpm/mg) was dissolved in 2.5 mL of 80% aqueous ethanol and distributed over five flasks by sterile filtration using Swinnex-13 Millipore filter units. After an additional 2 days the cultures were harvested and 7 mg of cyclonerodiol was obtained after purification in the usual manner. A mixture of this cyclonerodiol (7 mg, 0.029 mmol) and 3,5-dinitrobenzoyl chloride (21 mg, 0.091 mmol) in 150 μL of dry pyridine was stirred at room temperature for 5 days[7] after which the reaction was quenched by the addition of water. The mixture was stirred for 15 min and then ether was added. The aqueous layer was extracted with ether (3×) and the combined ether extracts were washed with 5% sulfuric acid, water, saturated potassium bicarbonate, water, and saturated sodium chloride, then dried, and concentrated. The crude ester was purified by PLC (benzene–ethyl acetate, 3:1; R_f 0.78) followed by recrystallization from carbon tetrachloride to give the 3,7-bis(3,5-dinitrobenzoate) ester: mp 141–143 °C (lit.[7] mp 141–143 °C); activity = 5 dpm/mg; ^1H NMR ($CDCl_3$) δ 1.28 (d, J = 7 Hz, C*H*$_3$CH, 3 H), 1.67 (b s, CH_3, 6 H), 1.73 (s, CH_3, 3 H), 1.78 (s, CH_3, 3 H), 1.6–3.2 (m, CH_2, 10 H); IR ν_{max} ($CHCl_3$) 1720 (carbonyl) cm^{-1}.

Feeding of (3*RS*)-[12,13-^{14}C]-*trans*-Nerolidyl Pyrophosphate to *T. roseum* Cultures. *Trichothecium roseum* was grown at 25 °C for 4 days.[8] (3*RS*)-[12,13-^{14}C]-*trans*-nerolidyl pyrophosphate (1 mg, 1.35×10^6 dpm/mg) was dissolved in 4 mL of 0.01 M methanolic ammonia, and 1-mL portions of this solution were distributed to 10 cultures using Swinnex-13 Millipore filter units on the 4th, 5th, 6th, and 7th day of growth. After a total of 11 days the cultures were harvested. The mycelium was worked up in the usual manner to yield 65 mg of rosenonolactone: mp 213–214 °C (lit.[73] mp 214 °C); activity = 3 dpm/mg. Extraction and purification of the filtrate yielded 25 mg of cyclonerodiol which was converted to 40 mg of 3,7-bis(3,5-dinitrobenzoate) ester: mp 141–143 °C; activity = 17 dpm/mg.

Cell-Free Extract of *G. fujikuroi*. *G. fujikuroi* was grown as described above. When the growth was carried out in the DeLong flasks, the cell-free system was prepared on the 4th day. On a larger scale, 2.4-L Fernbach flasks were used. These flasks were inoculated with 200 mL of 4-day-old *G. fujikuroi* culture and incubated an additional 5 days. The mycelium was separated by filtration and washed with cold, glass-distilled water followed by potassium phosphate buffer (0.1 M, pH 7.6). The wet mycelium (52–57 g) was suspended in 10 mL of 0.1 M potassium phosphate buffer (pH 7.6), containing 2 mM dithioerythritol (DTE), 5 mM EDTA, and 25% glycerol (v/v), and then passed through a precooled French press under 10 000–15 000 psi of pressure. The crushed cell mass was centrifuged immediately at 15000*g* for 20 min. The supernatant fraction S_{15} was separated from the cell debris and recentrifuged at 27000*g* for 60 min. The resulting cloudy supernatant S_{27} was filtered through cotton wool to remove the floating lipid. All centrifugations were conducted at 0–4 °C and the extracts kept at ice bath temperature.

(a) Protamine Sulfate Precipitation. The S_{27} extract was treated with 1% protamine sulfate (0.5 mg of protamine sulfate/mg of protein), and the turbid mixture was centrifuged at 27000*g* for 20 min. The clear supernatant fraction S_{PS} contained the desired enzyme activity.

(b) Ammonium Sulfate Precipitation. The stirred S_{PS} extract at 4 °C was brought to 40% saturation with ammonium sulfate by the slow addition of solid ammonium sulfate (controlling the pH of the supernatant during the addition of the ammonium sulfate did not affect the results). After the addition the mixture was stirred for 1–2 h and then centrifuged at 27000*g* for 20 min. The pellet was discarded, and the pale yellow supernatant fraction was again treated with solid ammonium sulfate (70% saturation). After the centrifugation the supernatant was discarded and the 40–70% $(NH_4)_2SO_4$ pellet (S_{AS}) was dissolved in the minimum amount of 0.02 M Tris–HCl buffer (pH 8.0), containing 2 mM DTE and 10% glycerol (v/v) and dialyzed overnight at 4 °C against 4–5 changes of the same buffer.

(c) Sephadex Column Chromatography. Sephadex G-150 resin was allowed to swell in glass distilled water for 72 h at room temperature. The swollen resin was stored in glass-distilled water at 4 °C and a few drops of 0.02% NaN_3 added to prevent microbial growth. Just prior to packing the column the resin was washed thoroughly with water followed by the buffer (0.02 M Tris–HCl, pH 8.0/DTE/glycerol), the fines were removed and the column was packed (resin–protein, 10:1). The packed column was equilibrated with buffer (at least 10 column volumes). The homogeneity of the column and the holdup volume were checked by using Blue Dextran 2000 at a concentration of 2 mg/mL.

The dialysate from the previous step was applied to the column at a flow rate of 35 mL/h, and fractions were collected by column volumes. The column was eluted with the same buffer. The holdup volume was discarded (containing 40–50% of the protein applied to the column). The next two column volumes contained the desired enzyme activity and were combined (G_{150}). The lyophilized enzyme could be stored for 2 weeks at 4 °C without appreciable loss in activity.

(d) Hydroxylapatite Gel Treatment. Hydroxylapatite gel was washed with glass-distilled water followed by 0.02 M Tris–HCl buffer (pH 8.0/DTE/glycerol). The G_{150} fraction was stirred with the equilibrated hydroxylapatite gel (gel–protein, 2:1; based on the dry weight of the gel) for 15 min at 4 °C and then centrifuged at 10000*g* for 10 min. The supernatant was used as the source of the purified enzyme. After incubation with the labeled substrates, the heat-denatured enzyme solution was further incubated with alkaline phosphatase (4 units) for 3 h at 37 °C and then overnight at room temperature.[21] The lyophilized enzyme could be stored at 4 °C for 1 month without appreciable loss of activity.

Assay Procedure. The enzyme extract (10 mL) was supplemented with 0.1 M magnesium chloride (100 μL) and then incubated with the substrate. The conversions were monitored with [1-$^3H_2$]-*trans,trans*-farnesyl pyrophosphate (3.28×10^7 dpm/mg) in 20-mL stoppered tubes at 25–26 °C for 3 h unless otherwise stated. The enzymatic reaction was stopped by the addition of an equivalent volume of acetone. The solution was extracted with ether (3×), and the combined ether extracts were

(72) The isolation and identification of 6,7-dihydroxyfarnesyl acetate was carried out by Dr. Stephen Swanson.

(73) A. Harris, A. Robertson, and W. B. Whalley, *J. Chem. Soc.*, 1799, 1807 (1958). D. Arigoni in "CIBA Foundation Symposium on the Biosynthesis of Terpenes and Sterols", G. E. W. Wolstenholme and M. O. Conner, Eds., J. A. Churchill, London, 1959, pp 239–242. A. J. Birch, R. W. Richards, H. Smith, A. Harris, and W. B. Whalley, *Tetrahedron*, 7, 241 (1959).

washed with saturated sodium chloride, dried, and concentrated. The crude material was diluted with inactive cyclonerodiol (**3**) as a carrier (1–2 mg) and then purified by PLC (methylene chloride–ether, 3:1; 2×; R_f 0.25). All conversions were monitored by measuring the radioactivity of the purified compounds and are expressed as the total nanomoles of the compound produced per milligram of protein (Table I). Controls established that the specific activity of the purified cyclonerodiol was within 5% of the specific activity of the corresponding crystalline bis(dinitrobenzoate) ester. (See below.)

Incubation of [12,13-^{14}C]-*trans,trans*-Farnesyl Pyrophosphate, (3*RS*)-[12,13-^{14}C]-*trans*-Nerolidyl Pyrophosphate and Nerolidol with the Cell-Free Extract of *G. fujikuroi*. The [12,13-^{14}C]-labeled substrates were incubated with the S_{27} fraction for 4 h at 26 °C. At the end of the incubation the labeled cyclonerodiol was extracted and purified by PLC, after the addition of inactive cyclonerodiol as carrier. A portion of the purified cyclonerodiol was converted to the crystalline 3,7-bis(3,5-dinitrobenzoate) ester and recrystallized to constant activity. The remainder of the cyclonerodiol was degraded as described below (Table II).

Degradation of ^{14}C-Labeled Cyclonerodiol. Acetone Semicarbazone. Osmium tetroxide (2.5 mg, 9.8 μmol) was dissolved in 0.1 mL of THF and added to cyclonerodiol (**3**) (20 mg, 0.084 mmol) dissolved in 0.2 mL of THF and 0.1 mL of water. The solution was stirred for 15 min in the dark at room temperature.[7] Sodium periodate (64 mg, 0.30 mmol) in 0.2 mL of water was added in portions over a period of 2 h, and then the reaction mixture was left stirring overnight at room temperature.

A mixture of semicarbazide hydrochloride (18 mg, 0.16 mmol), sodium acetate (12 mg, 0.15 mmol), and 0.1 mL of water in a trap was cooled to −78 °C. The organic solvents from the osmium tetroxide–sodium periodate oxidation of cyclonerodiol were distilled in vacuo and collected in the cooled trap. The contents of the trap were allowed to come to room temperature, and then the solution was lyophilized to dryness. The residue was treated with the minimum amount of water to remove the excess unreacted semicarbazide hydrochloride and the remaining solid lyophilized to dryness. The acetone semicarbazone (5 mg, 52%) was recrystallized from ethanol to constant activity, mp 190–192 °C (lit.[74] mp 190–191 °C).

Jones Oxidation of Cyclonerodiol Hemiacetal (6). The aqueous phase from the above reaction (solution remaining in the reaction flask after the organic solvents were collected in the trap) was extracted with ethyl acetate (3×). The combined organic extracts were washed with saturated sodium chloride, dried, and concentrated. The residue was dissolved in 1 mL of acetone, and then 8 N Jones reagent[75] was added dropwise until the yellow color persisted (approximately 2–3 drops).[7] After 10–15 min the excess oxidizing agent was destroyed by the addition of 2-propanol and then the acetone was removed by rotary evaporation. The residue was dissolved in water and extracted with ethyl acetate (3×). The combined ethyl acetate extracts were washed with saturated sodium chloride, dried, and concentrated. The crude product was purified by PLC (methylene chloride–ether, 3:1; 2×; R_f 0.43) and recrystallized from carbon tetrachloride–pentane to give the trisnorlactone (**7**), mp 82–84 °C (lit.[7,10] mp 82–83 °C).

(a) Isolation of Nerolidyl Pyrophosphate. [12,13-^{14}C]-*trans,trans*-Farnesyl pyrophosphate (2.4 mg, 1.07×10^6 dpm/mg) was incubated with the S_{AS} cell-free extract from 2 L of *G. fujikuroi* (90 mg of protein) for 10 min at 26 °C. After the usual ether extraction the aqueous layer was extracted with n-butyl alcohol (3×). The combined butanol extracts were lyophilized to dryness, and unlabeled nerolidyl pyrophosphate (5 mg) was added as a carrier. The crude labeled nerolidyl pyrophosphate was purified by a Dowex 1-8 column (formate form) followed by treatment with Amberlite XAD-2 resin. As a control experiment the S_{AS} enzyme system from 400 mL of growth medium was boiled on a water bath for 15 min and incubated with [12,13-^{14}C]-*trans,trans*-farnesyl pyrophosphate (0.4 mg, 1.07×10^6 dpm/mg) for 10 min at 26 °C. The solution was then treated as above, and the nerolidyl pyrophosphate was isolated (Table III).

(b) Alkaline Phosphatase Hydrolysis of [12,13-^{14}C]Nerolidyl Pyrophosphate. The purified [12,13-^{14}C]nerolidyl pyrophosphate isolated from the above incubation (3.28×10^3 dpm) was dissolved in 5 mL of Tris–HCl buffer (0.1 M, pH 8.6), and 2.5 mL of magnesium chloride (0.1 M) was added, followed by alkaline phosphatase (3 units). The solution was incubated at 37 °C for 3 h and then overnight at room temperature. The enzyme reaction was quenched by the addition of acetone and unlabeled (3*RS*)-*trans*-nerolidol (10 mg) was added as carrier. The solution was extracted with hexane (3×). The combined organic extracts were washed with saturated sodium chloride, dried, and concentrated. The recovered nerolidol was purified by PLC (hexane–ether, 2:1; 2×; R_f 0.59). An additional 25 mg of inactive nerolidol was then added, and the oil was bulb-to-bulb distilled at 94 °C (0.04mmHg) to give 33 mg of [12,13-^{14}C]nerolidol (3.65×10^4 dpm/mmol).

(c) Degradation of [12,13-^{14}C]Nerolidol. [12,13-^{14}C]Nerolidol (33 mg, 3.65×10^4 dpm/mmol) was converted to the crystalline 10,11-dihydroxynerolidol (**8**) in the usual manner. The 10,11-dihydroxynerolidol (**8**) (20 mg, 0.079 mmol, 3.67×10^4 dpm/mmol) was dissolved in 1 mL of THF–H_2O (1:1), and sodium periodate (19 mg, 0.089 mmol) was added. The solution was stirred at room temperature for 3 h, following which the solvent in the flask was distilled in vacuo and collected in a trap cooled in liquid nitrogen containing sodium acetate (12 mg, 0.15 mmol) and semicarbazide hydrochloride (16 mg, 0.14 mmol) in 0.1 mL of water. The trap was allowed to come to room temperature, and the solution was lyophilized to dryness. The residue was treated with the minimum amount of water to remove the unreacted semicarbazide hydrochloride and then lyophilized to dryness. The recovered acetone semicarbazone was recrystallized from ethanol to constant activity; mp 190–192 °C (lit.[75] mp 190–191 °C).

The aqueous phase from the same periodate oxidation (solution remaining in the reaction flask after the organic solvent was removed) was extracted with ether (3×). The combined ethereal extracts were washed with saturated potassium bicarbonate and saturated sodium chloride, then dried, and concentrated. The crude product was purified by PLC (ether–hexane, 2:1; R_f 0.46) to yield 9 mg of inactive nerolidol trisnoraldehyde (**9**) (Table III).

(3*RS*)-[1,2-$^{13}C_2$]-*trans*-Dehydronerolidol. [1,2-$^{13}C_2$]Acetylene (90% atom excess, 0.1 L) was transferred on a high vacuum line into a two-necked flask containing 3 mL of THF and equipped with a nitrogen balloon. Ethyl bromide (0.21 mL, 2.88 mmol) was added to crushed magnesium turnings (75 mg, 3 mmol) in 1 mL of THF, and the reaction mixture was refluxed for 1 h. The resulting black solution was slowly added at room temperature to the acetylene–THF solution, and after the addition was completed, the solution was left stirring for 1 h. *trans*-Geranylacetone (170 mg, 0.87 mmol) dissolved in 1 mL of methylene chloride was added to the reaction mixture. The solution was stirred for 2 h at room temperature. Excess Grignard reagent was destroyed by the addition of methanol followed by extraction with ether (3×). The combined ethereal extracts were washed with saturated ammonium chloride, saturated potassium bicarbonate, and saturated sodium chloride, dried, and concentrated. The crude product was purified by PLC (hexane–ether, 4:1; 2×; R_f 0.38) to yield 90 mg (50%) of (3*RS*)-[1,2-$^{13}C_2$]-*trans*-dehydronerolidiol: ^{13}C NMR ($CDCl_3$) δ 87.88 (d, J = 167.8 Hz, C-2), 71.69 (d, J = 167.8 Hz, C-1) (spectrum of unlabeled **21** reported below).

(3*RS*)-[1,2-$^{13}C_2$]-*trans*-Nerolidyl Pyrophosphate. [1,2-$^{13}C_2$]-*trans*-Dehydronerolidol (90 mg, 0.41 mmol) was reduced with sodium methoxide–lithium aluminum hydride by the procedure described below to yield 55 mg of [1,2-$^{13}C_2$]-*trans*-nerolidol. The latter compound was phosphorylated in the usual manner to give 8 mg of (3*RS*)-[1,2-$^{13}C_2$]-*trans*-nerolidyl pyrophosphate.

Incubation of (3*RS*)-[1,2-$^{13}C_2$]-*trans*-Nerolidyl Pyrophosphate. The eluant from the Sephadex G-150 column (G_{150}) (11 mg of protein) was lyophilized to dryness, and the residue was redissolved in the minimum amount of 0.02 M Tris–HCl buffer (pH 8.0), containing 2 mM DTE and 10% glycerol with 10% D_2O added to provide the NMR lock. (3*RS*)-[1,2-$^{13}C_2$]-*trans*-Nerolidyl pyrophosphate (10.5 μmol) was added and the ^{13}C spectrum recorded in a 10-mm NMR tube with a 500-μL insert, the temperature of the probe being maintained at 0–4 °C while the spectrum was recorded (8300 transients). The NMR tube was incubated at 26 °C for 5, 10, and 20 min total reaction time, and the spectrum was recorded after each incremental incubation period. The conversion of the nerolidyl pyrophosphate to cyclonerodiol was observed by the appearance of two new doublets (J_{CC} = 38.4 Hz) centered at 14.7 and 44.3 ppm (C-1 and C-2, respectively). Farnesyl pyrophosphate was not detected. The decrease in intensity of the signals of [1,2-$^{13}C_2$]nerolidyl pyrophosphate (two doublets (J_{CC} = 70.57 Hz) centered at 145.6 and 111.9 ppm) corresponded to its conversion to cyclonerodiol (pyrophosphate). The formation of the product was linear with time.

Competitive Incubation Study. The 0.02 M hydroxylapatite fraction (HA-gel) supplemented with 0.1 M $MgCl_2$ was incubated with a mixture of 20.22 nmol of (3*RS*)-[1,2-$^2H_2$,1-^{3}H]-*trans*-nerolidyl pyrophosphate and 20.16 nmol [12,13-^{14}C]-*trans,trans*-farnesyl pyrophosphate at 26 °C for different time intervals (Figure 1). The enzyme was heat denatured and then treated with an excess of alkaline phosphatase. The resulting cyclonerodiol was isolated and purified and the $^3H/^{14}C$ ratio determined.

Incubation in the Presence of [^{18}O]Water. The S_{AS} cell-free extract from 14 L of *G. fujikuroi* (630 mg of protein) was lyophilized to dryness and then dissolved in 1 mL of [^{18}O]water (37.68% ^{18}O atom excess). This water was recovered by lyophilization, and the residue was redis-

(74) "CRC Handbook of Tables for Organic Compound Identification", 3rd ed., CRC Press, Cleveland, 1967.

(75) R. G. Curtis, I. Heilbron, E. R. H. Jones, and G. F. Wood, *J. Chem. Soc.*, 457 (1953).

Table VII. Mass Spectra of [7-^{18}O]Lactone 7

substrate	m/e 214 (M + 2)	m/e 212 (M)	m/e 199 (M − 13)	m/e 197 (M − 15)	m/e 196 (M − 16)	m/e 194 (M − 18)	m/e 181 (M − 31)	m/e 179 (M − 33)
7	2.51	97.48	2.82	97.18	2.20	97.80	2.24	97.76
	2.21	97.79	2.51	97.49	2.40	97.60	2.26	97.74
[^{18}O]-7[a,b]	25.17	74.83	24.58	75.42	23.19	76.81	25.00	75.00
	25.49	74.51	24.60	75.40	23.19	76.81	25.42	74.59

[a] Derived from 3 obtained by incubation of FPP with S_{AS} in [^{18}O]water. [b] Calculated enrichment, 24.8 atom excess % ^{18}O.

solved in 5 mL of [^{18}O]water and then incubated with a total of 62 mg of [12,13-^{14}C]-*trans,trans*-farnesyl pyrophosphate (total activity = 9.17 × 10^4 dpm) for 3 h at 26 °C. At the end of the incubation the [^{18}O]water was recovered by lyophilization. The labeled cyclonerodiol was isolated and purified by PLC to give 1.08 mg (2.89% conversion, total activity 2.65· × 10^4 dpm).

[^{18}O]Hydroxy Lactone. The cyclonerodiol (**3**) (1.08 mg) isolated from the above incubation was treated with osmium tetroxide–sodium periodate, and the crude product was then oxidized with 8 N Jones reagent. PLC purification of the crude product (methylene chloride–ether, 3:1; 2×; R_f 0.43) yielded [^{18}O]lactone **7** which was recrystallized from chloroform, and its mass spectrum was recorded (Table VII).

(3*S*)-[12,13-^{14}C]-*trans,trans*-Nerolidyl Pyrophosphate. (3*S*)-*trans*-Nerolidol [200 mg, 0.9 mmol; $[\alpha]_D$ +15.2° (*c* = 1.8, CCl_4) (lit.[76] $[\alpha]_D$ +15.1°)] was converted by the same procedure used for the synthesis of isopropylidene-labeled (3*RS*)-*trans*-nerolidol to [12,13-^{14}C]nerolidol (activity = 6.68 × 10^6 dpm/mg) and then phosphorylated in the usual manner to give 12 mg of (3*S*)-[12,13-^{14}C]-*trans*-nerolidyl pyrophosphate (3.89 × 10^6 dpm/mg). Just prior to the incubation with the cell system from *G. fujikuroi*, 6 mg of (3*S*)-[12,13-^{14}C]-*trans*-nerolidyl pyrophosphate was incubated with alkaline phosphatase. The isolated nerolidol was purified by PLC (hexane–ether, 2:1; R_f 0.5). The ^{1}H NMR signal corresponding to the C(3) methyl of recovered (3*S*)-*trans*-nerolidol recorded in the presence of 33 mol % Eu(tfc)$_3$ was a singlet at δ 4.7. Doping the sample with (3*RS*)-*trans*-nerolidol confirmed that no racemization had occurred during the pyrophosphorylation.

Incubation of (3*S*)-[12,13-^{14}C]- and (3*RS*)-[1,2-^{2}H$_2$,1-^{3}H]-*trans*-Nerolidyl Pyrophosphate. A mixture of (3*S*)-[12,13-^{14}C]-*trans*-nerolidyl pyrophosphate (0.40 mg, ^{14}C, 3.89 × 10^6 dpm/mg) and (3*RS*)-[1,2-^{2}H$_2$,1-^{3}H]-*trans*-nerolidyl pyrophosphate (0.58 mg, ^{3}H, 2.12 × 10^7 dpm/mg) (see below) was incubated with the S_{AS} cell-free enzyme preparation from *G. fujikuroi* for 3 h at 26 °C. Unlabeled cyclonerodiol was added as carrier, and the isolated cyclonerodiol was converted to its crystalline bis(dinitrobenzoate) ester which was recrystallized to constant activity (4.10 × 10^6 dpm/mmol, ^{14}C).

The exact ^{3}H/^{14}C ratio of the nerolidyl pyrophosphate used in the above incubation was determined by incubating a portion of the nerolidyl pyrophosphate mixture with alkaline phosphatase. After dilution of the resulting nerolidol with 10 mg of inactive carrier, the nerolidol was bulb-to-bulb distilled and then converted to 10,11-dihydroxynerolidol (**8**) which was recrystallized from carbon tetrachloride to constant activity (^{3}H/^{14}C = 0.77).

(3*RS*)-*trans*-Dehydronerolidol (21) (Scheme XVI). Ethyl bromide (0.63 mL, 8.65 mmol) was added to crushed magnesium (226 mg, 9.07 mmol) in 3 mL of THF under nitrogen, and the resulting solution was refluxed for 1 h. Acetylene gas (purified by passage through a dry ice–acetone trap, concentrated sulfuric acid, and potassium hydroxide pellets in succession) was bubbled through 4 mL of THF. The ethylmagnesium bromide was then added slowly, and the acetylene gas was bubbled through the solution for 4 h. The resulting brown solution of ethynylmagnesium bromide was cooled and *trans*-geranylacetone (400 mg, 2.06 mmol) dissolved in 1 mL of methylene chloride was added after which the reaction mixture was stirred for 1 h at room temperature under nitrogen. The excess Grignard reagent was destroyed by the addition of methanol, and the solution was poured into half-saturated ammonium chloride and extracted with ether (3×). The combined ether extracts were washed with saturated potassium bicarbonate and saturated sodium chloride, dried, and concentrated. The crude product was purified by PLC (hexane–ether, 4:1; 2×; R_f 0.38) to yield 408 mg (90%) of (3*RS*)-*trans*-dehydronerolidol (**21**): ^{1}H NMR ($CDCl_3$) δ 1.5 (s, CH_3C-OH, 3 H), 1.61, 1.66 (s, CH_3, 9 H), 2.01–2.1 (m, CH_2, 8 H), 2.38 (s, OH, 1 H), 2.45 (s, acetylenic proton, 1 H), 4.9–5.4 (m, vinyl, 2 H); ^{13}C NMR ($CDCl_3$, broad-band decoupled) δ 16.1 (C-13), 17.7 (C-12 cis), 23.6 (C-14), 25.8 (C-12 trans), 26.8 (C-9), 29.8 (C-5), 39.8 (C-8), 43.4 (C-4), 68.2 (C-3), 71.6 (C-1), 87.9 (C-2), 123.9 (C-10), 124.5 (C-6), 131.4 (C-11), 135.9 (C-7); IR ν_{max} ($CHCl_3$) 3410 (b, hydroxyl), 3300 (acetylenic) cm^{-1}; GC 155 °C, t_R = 9 min.

Lithium Aluminum Hydride–Sodium Methoxide Reduction of [1,2-^{13}C$_2$]-*trans*-Dehydronerolidol.[23,24] [1,2-^{13}C$_2$]-*trans*-Dehydronerolidol (**21**) (90 mg, 0.41 mmol) was added to a suspension of lithium aluminum hydride (50 mg, 1.30 mmol) and freshly prepared sodium methoxide (144 mg, 2.66 mmol) in 1.5 mL of THF under nitrogen, and the heterogeneous mixture was refluxed overnight. The reaction was quenched by the successive addition of 50 μL of H_2O, 50 μL of 5% NaOH, and 150 μL of H_2O, followed by the addition of ether, and the precipitated solids were removed by filtration through Celite. After concentration of the filtrate the residual oil was purified by PLC (hexane–ether, 4:1; 2×) to give 55 mg (55%) of [1,2-^{13}C$_2$]nerolidol (R_f 0.38) and 15 mg (18%) of [1-^{13}C]geranylacetone (R_f 0.48). [1,2-^{13}C$_2$]Nerolidol: ^{13}C NMR ($CDCl_3$) δ 111.9 (d, *J* = 67.6 Hz, C-1), 145.4 (d, *J* = 67.6 Hz, C-2). [1-^{13}C]-Geranylacetone: ^{1}H NMR ($CDCl_3$) δ 2.1 (d, $J_{^{13}CH}$ = 120 Hz, CH_3CO, 3 H); ^{13}C NMR ($CDCl_3$) δ 30.0 (^{13}C-enriched singlet, C-1).

[1-^{3}H]-*trans*-Dehydronerolidol (21). *n*-Butyllithium (2.01 M, 1 mL) was added to *trans*-dehydronerolidol (**21**) (370 mg, 1.68 mmol) in 0.5 mL of THF at 0 °C under nitrogen, and the solution was stirred for 1 h at room temperature. Tritiated water (200 μL, 20 mCi) was added over a period of 2 h followed by H_2O (100 μL), and the reaction mixture was stirred for an additional hour. The solution was poured into saturated ammonium chloride, and the aqueous layer was extracted with ether (3×). The combined ether extracts were washed with saturated sodium chloride, dried, and concentrated. The crude product was purified by PLC (hexane–ether, 4:1; 2×; R_f 0.38) to yield 276 mg (75%) of (3*RS*)-[1-^{3}H]-*trans*-dehydronerolidol (**21**) (3.82 × 10^7 dpm/mg): ^{1}H NMR ($CDCl_3$) (D_2O quench) identical with that of **21** except signal at δ 2.45 (s, acetylenic proton, 1 H) no longer present; MS *m/e* 221 (M + 1).

(*E*)-[1,2-^{2}H$_2$,1-^{3}H]-*trans*-Nerolidol.[23,24] [1-^{3}H]-*trans*-Dehydronerolidol (**21**) (276 mg, 1.25 mmol) was added to a suspension of lithium aluminum deuteride (158 mg, 3.76 mmol) and sodium methoxide (406 mg, 7.52 mmol) in 2 mL of THF under nitrogen, and the mixture was refluxed overnight. The reaction was quenched by the successive addition of 160 μL of D_2O, 160 μL of 5% NaOD, and 480 μL of D_2O, followed by the addition of ether. After removal of the precipitated solids by filtration through Celite and concentration of the filtrate, the crude product was purified by PLC (hexane–ether, 4:1; 2×) to give 159 mg (57%) of (*E*)-[1,2-^{2}H,1-^{3}H]nerolidol (R_f 0.38; 3.58 × 10^7 dpm/mg), 40 mg (16%) of [1-^{2}H$_3$]geranylacetone (R_f 0.48), and 10 mg (3%) of methyl homogeranylallene (R_f 0.9); MS, nerolidol (M − H_2O fragment) d_2 94.8%, d_1 3.4%, d_0 1.8%; ^{1}H NMR ($CDCl_3$) δ 1.28 (s, CH_3COH, 3 H), 1.6, 1.7 (s, CH_3, 9 H), 1.92 (s, OH, 1 H), 2.1 (m, CH_2, 8 H), 4.9–5.15 (m, vinyl, 3 H). A lanthanide shift study of (*E*)-[1,2-^{2}H$_2$,1-^{3}H]nerolidol in CCl_4 using up to 25 mol % of Eu(dpm)$_3$ confirmed that the reduction had occurred with trans stereochemistry and that the remaining vinylic proton occupied the 1*E*-position. The mass spectrum of the purified geranyl acetone gave a parent peak cluster corresponding to 98.1% d_3, 0.8% d_2, 0.6% d_1, and 0.5% d_0. In the ^{1}H NMR the signal at δ 2.1 (s, CH_3CO, 3 H) was absent while the rest of the spectrum was identical with that of authentic geranyl acetone. The broad-band decoupled ^{13}C NMR spectrum displayed a septuplet at δ 28.8 (*J* = 19 Hz) in place of the normal singlet at 29.4 ppm (C-1).

(*E*)-[1,2-^{2}H$_2$,1-^{3}H]-*trans*-Nerolidyl Pyrophosphate. (*E*)-[1,2-^{2}H$_2$,1-^{3}H]-*trans*-Nerolidol (159 mg, 0.70 mmol) was pyrophosphorylated in the usual manner to give 25 mg (10%) of nerolidyl pyrophosphate (**2**) (2.12 × 10^7 dpm/mg).

Incubation of (*E*)-[1,2-^{2}H$_2$,1-^{3}H]-*trans*-Nerolidyl Pyrophosphate. The S_{27} cell-free extract of *G. fujikuroi* (143 mg of protein) was incubated with (*E*)-[1,2-^{2}H$_2$,1-^{3}H]-*trans*-nerolidyl pyrophosphate (1.5 mg, 3.17 × 10^7 dpm/mg) for 4 h at 26 °C. The isolated cyclonerodiol (**3**) (1.1% conversion, 2.32 × 10^4 dpm/mg) was diluted to 15 mg (5.59 × 10^6 dpm/mmol) with inactive cyclonerodiol as carrier. A portion (4 mg) of this [^{3}H]cyclonerodiol was used to prepare the crystalline bis(dinitrobenzoate) ester (6.11 × 10^6 dpm/mmol) while the remaining **3** was used for the Kuhn–Roth oxidation.

(a) Kuhn–Roth Oxidation of Cyclonerodiol. [1-^{2}H,^{3}H]Cyclonerodiol (11 mg, 6.10 × 10^6 dpm/mmol) and 5 mL of oxidizing reagent (prepared

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from 16.7 g of chromium trioxide, 100 mL of water, and 25 mL of concentrated sulfuric acid) was refluxed at 165 °C for 1.5 h in a standard Kuhn–Roth apparatus.[31] The reaction mixture was cooled to room temperature, and then acetic acid was steam distilled by using the flame of a bunsen burner. The distillate (50 mL) was treated with 20.2 mL of 7.4 mM potassium hydroxide (phenolphthalein indicator). The resulting potassium acetate solution was evaporated to dryness on a rotary evaporator, and an aliquot of the potassium acetate was converted to its crystalline *p*-bromophenacyl acetate (procedure described below). On the basis of the crystalline derivative, 12 mg (81%) of potassium acetate (1.48×10^4 dpm/mg) had been obtained.

(b) *p*-Bromophenacyl [2-^{2}H,^{3}H,^{14}C]Acetate. Potassium [2-^{2}H,^{3}H]-acetate from the Kuhn–Roth oxidation and a known activity of sodium [2-^{14}C]acetate were dissolved in distilled water and evaporated to dryness. A small sample of the resulting dry salt was converted to the corresponding *p*-bromophenacyl acetate by reaction with 2 equiv of α-*p*-dibromoacetophenone and 20 mol % 18-crown-6 in 1 mL of acetonitrile overnight at room temperature.[77] The solvent was removed by rotary evaporation, and the residue was purified by PLC (benzene–hexane–ethyl acetate, 5:5:1; R_f 0.28) to yield *p*-bromophenacyl acetate (96%). The latter compound was recrystallized from methylene chloride–pentane to constant activity (3.42×10^5 dpm/mmol, ^{14}C; ^{3}H/^{14}C = 4.30): mp 84–85 °C (lit.[74] 86 °C). ^{1}H NMR ($CDCl_3$) δ 2.2 (s, CH_3, 3 H), 5.28 (s, OCH_2CO, 2 H), 7.7 (m, aromatic, 4 H); IR ν_{max} ($CHCl_3$) 1750, 1730 (carbonyl), 1590 (aromatic) cm^{-1}.

(c) Estimation of Maximum Proton Exchange during Kuhn–Roth Oxidation of Cyclonerodiol.[32,33] Inactive cyclonerodiol (3) (7.8 mg, 0.032 mmol) was mixed with 5 mL of the standard Kuhn–Roth oxidizing reagent containing 5 mCi of tritiated water. This mixture was refluxed at 165 °C for 2 h, a time which corresponded to the upper limit of the oxidation period used for all labeled cyclonerodiol samples. After steam distillation, the recovered acetic acid was titrated with 7.8 mM KOH and the resultant potassium acetate (87% yield) was converted to its *p*-bromophenacyl ester in the usual manner. After PLC, the *p*-bromophenacyl acetate was recrystallized to constant activity (1.56×10^7 dpm/mmol). The extent of exchange could be calculated by using eq 1–3, taking into account that each molecule of water has two exchangeable protons while the product, acetate, has three: (1) specific activity of water = $(5.22 \times 10^9$ dpm$)/(5.0$ mL$/0.018$ mL$/$mol$) = 4 \times 10^7$ dpm/mmol; (2) specific activity of acetate = 1.56×10^7 dpm/mol; (3) % exchange = $[(1.56 \times 10^7/3)/(4 \times 10^7/2)] \times 100 = 26.1\%$.[32]

(d) [2-^{2}H,^{3}H,^{14}C]Acetyl CoA.[34,78] Potassium [2-^{2}H,^{3}H,^{14}C]acetate (5 mg) in 1 mL of ether was acidified with 2 drops of 10 N sulfuric acid, and the solution was dried with sodium sulfate followed by extraction with 1 mL of ether (5×). To the combined ethereal extracts was added 6 drops of triethylamine and the solution was first dried over sodium sulfate and then filtered through glass wool. The filtrate was concentrated under reduced pressure to a volume of 1 mL and then diluted with 3.5 mL of THF. All the above operations were carried out at 0 °C. After the solution was cooled to −10 °C, 10 mg of ethylchloroformate (as a 10% solution in THF) was added in portions. The reaction mixture was stirred vigorously for 40 min after which the ethereal solution was filtered directly into a solution of 50 mg of coenzyme A in 2 mL of water (pH adjusted to 7.5 with solid sodium bicarbonate). The resulting mixture was stirred at 0 °C for 30 min. After the pH of the aqueous layer was adjusted to 1 with 1 N HCl, the resulting mixture was continuously extracted with 100 mL of ether overnight at 0 °C. The aqueous layer containing the acetyl CoA was filtered through glass wool and then lyophilized to dryness. The yield of the reaction was estimated to be 45%, on the basis of the UV absorption at 232 nm.

(e) Incubation of [2-^{2}H,^{3}H,^{14}C]Acetyl CoA with Malate Synthetase.[34,78] The crude [2-^{2}H,^{3}H,^{14}C]acetyl coenzyme A from the previous reaction was dissolved in 20 mL of Tris–HCl buffer (0.1 M, pH 8.0) containing 2 mL of 0.1 M magnesium chloride. Malate synthetase (4 units) was added, and the enzyme reaction was initiated by the addition of 1 mL of 0.1 M sodium glyoxylate. The progress of the incubation was followed by a UV assay at 232 nm and 25 °C. After 1 h an additional 4 units of malate synthetase and 1 mL of 0.1 M sodium glyoxylate were added, and the solution was stirred for two more hours. A further 2 units of malate synthetase and 1 mL of 0.1 M sodium glyoxylate were again added, and the incubation was continued for another 1.5 h. The enzyme was denatured by freezing the solution to −78 °C, and the reaction mixture was concentrated to 2 mL by lyophilization. The resulting solution was acidified with 6 N HCl to pH 1, and 6.3 mg of inactive L-malic acid was added as carrier. The aqueous solution was saturated with solid ammonium sulfate and then continuously extracted with ethyl acetate for 30 h. The organic extract was concentrated, and the residue was dissolved in anhydrous methanol. The methanolic solution was dried, filtered, and evaporated to dryness, and the residue was redissolved in 1 mL of 1.1 N formic acid, mixed with an additional 6.3 mg of inactive L-malic acid, and applied to a 1.8 × 16-cm Dowex 1-8 (200–400 mesh) ion-exchange column in the formate form. The column was eluted with 1.1 N formic acid and 35 fractions of 5–7 mL each were collected. The column effluent was monitored by TLC (silica gel, chloroform–acetic acid, 1:2; R_f (malic acid) 0.39; R_f (glyoxylate) 0.22; spray reagent 1% $KMnO_4$ solution), and the tubes containing malic acid (22–25) were combined. The solution was evaporated to dryness on the rotary evaporator, and the malic acid was recrystallized from acetone–hexane: ^{3}H/^{14}C = 3.38; 2.83×10^4 dpm/mmol, ^{14}C; 78.6% retention of ^{3}H based on potassium [2-^{2}H,^{3}H,^{14}C]acetate.

Table VIII. Incubation of L-Malate[a,b] with Fumarase

tube	time of incubation	^{3}H, dpm/mmol	^{14}C, dpm/mmol	^{3}H/^{14}C	% ^{3}H retention
A	15 min	2.97×10^4	2.79×10^4	1.06	31.4
	7 h	2.78×10^4	2.77×10^4	1.00	29.6
B	4 h	2.79×10^4	2.78×10^4	1.00	29.6

[a] Derived from incubation of (*E*)-[1,2-^{2}H$_2$,1-^{3}H]NPP. [b] Initial ^{3}H/^{14}C = 3.38.

(f) Incubation of Malic Acid with Fumarase. Malic acid (9.5 mg, 0.070 mmol) was dissolved in 2 mL of potassium phosphate buffer (0.1 M, pH 7.6), and the pH was adjusted to 7.3 by addition of 0.1 N sodium hydroxide. The solution was divided equally between two 15-mL centrifuge tubes and the final volume in each brought to 2 mL with buffer. To each tube was added 12 units of fumarase, and the tubes were incubated at 25 °C for the times indicated in Table VIII. The reaction was stopped by freezing the solution to −78 °C, and the enzyme was thermally denatured (two exposures to a 95 °C bath for 2 min) followed by the addition of 1 mL of 0.2 N barium acetate. The precipitated barium sulfate and phosphate were centrifuged, and the supernatant was filtered through glass wool and then acidified with 1 N HCl to pH 2.0. The solution was concentrated on the rotary evaporator, and the residue was dissolved in glass-distilled water and applied to a pretreated Dowex 50W-X8 column (acidic form, 1 × 4 cm, resin pretreated by washing with 1% HCl followed by water). This column was eluted with water, and the effluent (14 mL) was evaporated to dryness. After the residue was redissolved in ethanol–water (1:1), the solution filtered, and the solvent reevaporated, the reisolated L-malic acid was recrystallized from acetone–hexane.

An aliquot of the malic acid from the 15-min incubation was used to measure the radioactivity. The remaining solution was concentrated and reincubated with fumarase followed by reisolation and purification of the malic acid.

Preparation of Chirally Labeled Mevalonolactones.[37] **(5*R*)-[5-^{2}H,^{3}H]Mevalonolactone. (a) 3-Methylbut-3-enoic Acid.** Magnesium turnings (5.8 g, 0.24 mol), washed with THF and dried by flushing with nitrogen, were covered with 100 mL of anhydrous THF in a 250-mL two-neck round-bottom flask connected to a nitrogen balloon and a serum stopper. Distilled 3-chloro-2-methylpropene (5 mL, 0.051 mol) was added to the flask by syringe. This heterogeneous suspension was stirred vigorously at 0–10 °C for 5 h. A second 250-mL three-neck round-bottom flask was fitted with a drying tube, serum stopper, and glass tube with one end extending about 0.5 cm higher than the bottom of the flask and the other end connected to a carbon dioxide source. The glass tube was bent in a U-shape to allow trapping of any moisture in the CO_2. After flushing of the three-neck round-bottom flask with carbon dioxide for a few minutes, (methylallyl)magnesium chloride solution was transferred in three portions by syringe to this flask over a 1-h period. Carbon dioxide was continuously bubbled into the reaction mixture until the Grignard reagent had been consumed. The reaction was terminated by removing the carbon dioxide source and evaporating most of the solvent at 0 °C. Water (20 mL) was added to the remaining mixture followed by dropwise addition of 6 N HCl to adjust the pH to 2. The aqueous solution was immediately extracted with ether (3 × 100 mL), and the combined ethereal extract was washed with saturated sodium chloride solution and dried over sodium sulfate. After evaporation of solvent at 0 °C, short-path distillation under reduced pressure gave a 62% yield as 3-methylbut-3-enoic acid (3.16 g, 0.032 mol), contaminated with ca. 10% of its conjugated isomer: ^{1}H NMR ($CDCl_3$) δ 1.82 (s, CH_3, 3 H), 3.0 (s, CH_2, 2 H), 4.9 (b s, CH_2=C, 2 H), 9.1 (s, COOH, 1 H).

(b) [1,1-^{2}H$_2$]-3-Methylbut-3-en-1-ol (Isopentenol). 3-Methylbut-3-enoic acid (213.7 mg, 2.14 mmol) was dissolved in 50 mL of anhydrous tetrahydrofuran. Lithium aluminum deuteride (87 mg, 2.1 mmol) was added in 3 portions to the reaction mixture which was stirred at room

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temperature under nitrogen. After 5 h the reaction was terminated by adding 4 drops of water and 4 drops of 1 N NaOH and stirring for 30 min. The resulting precipitate was removed by centrifugation and thoroughly washed with anhydrous ether (3 × 3 mL). The combined solution was evaporated under reduced pressure at 0 °C to give [1,1-$^2H_2$]-3-methylbut-3-en-1-ol (isopentenol) (168 mg, 1.95 mmol). The corresponding [1,1-$^2H_2$]dimethylallyl alcohol constituted 10% of the reaction product. The quantitative deuteration of C-1 was established by ^1H NMR. This [1,1-$^2H_2$]isopentenol was completely devoid of tritium activity, indicating that the $LiAl^2H_4$ used contained no ^3H isotope.[79] This preliminary assay was important because any tritium in this isopentenol would alter the result of eventual chiral acetate assay: ^1H NMR ($CDCl_3$) δ 1.75 (s, CH_3, 3 H), 2.1 (b s, CH, 1 H), 2.27 (b s, CH_2, 2 H), 4.82 (b s, CH_2=C, 2 H), 3.77 (t, CH_2OH, 2 H) (observed only in the undeuterated alcohol); IR ν_{max} ($CHCl_3$) 3580, 3420 (OH), 1645 (C-H_2=C), 1445, 1382, 1050, 905 cm^{-1}.

(c) Oxidation of [1,1-$^2H_2$]Isopentenol to [1-^2H]-3-Methylbut-3-en-1-al (Isopentenal). Pyridinium chlorochromate[80] (182 mg, 0.84 mmol) was suspended in 1 mL of methylene chloride, and the heterogeneous mixture was stirred vigorously at room temperature for a few minutes to disperse the orange-red oxidizing reagent. A solution containing [1,1-$^2H_2$]isopentenol (38 mg, 0.43 mmol) in 1.5 mL of methylene chloride was added, and the mixture was stirred at room temperature under nitrogen. TLC showed that more than half of the starting material had been oxidized within 1 h. A second portion of pyridinium chlorochromate (100 mg, 0.46 mmol) was added, and the mixture was stirred at room temperature under nitrogen for 10 h. Anhydrous methylene chloride (5 mL) was added to the reaction mixture, and the resulting black solution was allowed to pass through a column of silica gel (70–270 mesh) packed in a disposable pipette. The colorless eluate was collected and concentrated to ca. 0.5 mL at 0 °C and subsequently flushed with nitrogen to remove all solvent. The crude product could be further purified by a second pipette column chromatography with pure methylene chloride. Each 2-mL portion of eluate was collected in 3-mL vials and each vial assayed by TLC (methylene chloride, R_f (isopentenol) 0.16, R_f (isopentenal) 0.68, R_f (3-methylbut-2-en-1-al) 0.4). Vials containing the required isopentenal were combined, and solvent was again evaporated at 0 °C under reduced pressure. ^1H NMR spectra showed that the ratio of isopentenal to the conjugated aldehyde was 4:1. Reaction carried out at 0 °C gave better ratios but proceeded at a much slower rate. Isopentenal is very unstable to heat or base, and isomerization to the conjugated aldehyde is observed during storage. Usually this oxidation reaction was carried out immediately before the utilization of [1-^2H]isopentenal in the next step.

(d) Enzymic Reduction of [1-^2H]Isopentenal to (1*R*)-[1-^2H,^3H]Isopentenol.[37] NAD^+ (125 mg, 0.2 mmol) was dissolved in 12.5 mL of 0.05 M Tris–HCl buffer (pH 8.5) at 0 °C. To this aqueous solution 0.3 mL of 0.1 M $NaBH_4$ solution (prepared by dissolving 38 mg of $NaBH_4$ in 10 mL of distilled water at 0 °C) was added with vigorous stirring.[81] After 10 min, 0.1 mL of 0.1 M aqueous acetone was added, and 30 min later, 0.5 mL of 0.1 M $NaBH_4$ was again added. After an additional 10 min 0.1 mL of acetone solution was again added and the reaction was stirred for 30 min at 0 °C. The reduction was terminated by addition of 0.5 mL of 1 M aqueous $BaCl_2$ followed by 100 mL of ethanol. The resulting mixture was cooled to −10 °C for 3 h to allow the NADH barium salt to precipitate. The solvent was removed by filtration and the precipitate was thoroughly washed with cold ethanol (3 × 20 mL) and cold ether (3 × 20 mL). This yellow NADH barium salt was dried at high vacuum at 0 °C for at least 4 h and 140 mg of crude NADH barium salt was collected. The yield of this reduction was 67%, based on the absorption of NADH at 340 nm. With [^3H]$NaBH_4$ (25 mCi) 400 mg of NAD^+ was reduced to give 500 mg of crude NAD^3H barium salt.

Crude NAD^3H barium salt (400 mg, 0.42 mmol) was dissolved in 20 mL of 0.1 M potassium phosphate buffer at pH 7.0. Liver alcohol dehydrogenase suspension (0.5 mL, 12 units), dialyzed to remove ethanol stabilizer, and [1-^2H]isopentenal (50 mg, 0.59 mmol), contaminated with less than 10% of conjugated aldehyde, were then added, and the mixture was incubated at 37 °C in a water bath with occasional stirring. By following the disappearance of the 340-nm UV absorption of NADH, it was found that the reduction had almost ceased after less than 2 h. The incubation was nevertheless continued for another 7 h under the same conditions. The reduction was terminated by cooling the incubation mixture to −78 °C. The frozen solution could be stored at −25 °C. Before isolation, inactive carrier isopentenol (25.3 mg, 0.29 mmol) was added. The mixture was divided into two 10-mL portions which were each subjected to continuous extraction with 40 mL of ether for 24 h. The combined ethereal extract was dried over sodium sulfate and evaporated under reduced pressure at 0 °C to about 1 mL. The ether-containing crude (1*R*)-[1-^2H,^3H]isopentenol was used directly in the next step without purification. A similar reduction reaction carried out on inactive material gave a 3:2 ratio of the two alcohols isopentenol and dimethylallyl alcohol.[37]

(e) (5*R*)-[5-^2H,^3H]Mevalonolactone. The procedure was that of Cornforth.[37] The crude alcohols from the previous reaction were transferred to a solution containing *N*-bromosuccinimide (142.7 mg, 0.8 mmol) in 2 mL of water. The solution was stirred in the dark at room temperature for 2 h. After saturating with solid sodium chloride, the aqueous solution was extracted with ether (3 × 10 mL). The combined ethereal extract was dried over sodium sulfate and evaporated to dryness under reduced pressure to give the crude bromohydrin which was not further purified but dissolved in 2 mL of methanol and reacted with 60 mg (0.92 mmol) of potassium cyanide at room temperature overnight. After evaporation of the solvent, the residue was extracted with anhydrous ether (2 × 10 mL) and insoluble materials were removed by filtration. The ethereal extract was dried over sodium sulfate and subsequently evaporated under reduced pressure to give crude (1*R*)-[1-^2H,^3H]-3-methyl-4-cyanobutan-1,3-diol. Subsequent investigation revealed that this reaction can lead to formation of variable amounts of 3,4-epoxyisopentenol. A tenfold excess of cyanide appeared to avoid this undesirable side product.

The crude cyanohydrin was hydrolyzed by refluxing in 2 mL of 2 N NaOH for 22 h after which the mixture was acidified with 4 N sulfuric acid to pH 3, saturated with sodium chloride, and continuously extracted with chloroform for 20 h. The chloroform extract was dried over sodium sulfate and evaporated to dryness under reduced pressure. PLC separation of this crude residue (benzene–ethyl acetate, 1:1; R_f 0.15) gave (5*R*)-[5-^2H,^3H]mevalonolactone (4.3 mg, 0.033 mmol). This partially purified mevalonolactone was mixed with 7.8 mg of inactive carrier and recrystallized from acetone–ether at −78 °C to give crystalline (5*R*)-[5-^2H,^3H]mevalonolactone (8.1 mg, 0.06 mmol, total activity = 1 × 10^8 dpm). This purified mevalonolactone was redissolved in 1 mL of benzene and stored frozen at −25 °C.

(f) [1-^3H]Isopentenol. 3-Methylbut-3-enoic acid (59 mg, 0.69 mmol) containing ca. 10% of the conjugated isomer was dissolved in 10 mL of anhydrous tetrahydrofuran and reacted with 4.9 mg (0.12 mmol) of lithium aluminum hydride with vigorous stirring under nitrogen at room temperature for 30 min. Tritiated lithium aluminum hydride (5.9 mg, 0.155 mmol, 25 mCi) was then added, and the reaction mixture was again stirred at room temperature under nitrogen for 5 h after which an additional portion of lithium aluminum hydride (11.8 mg, 0.31 mmol) was added and the reaction mixture left stirring at room temperature for 10 h. A second portion of 3-methylbut-3-enoic acid (30 mg, 0.3 mmol) in 5 mL of tetrahydrofuran was added to the reaction mixture by syringe followed 2 h later by 12.3 mg (0.32 mmol) of lithium aluminum hydride. After 5 h the reduction was terminated by adding 3 drops of water and 3 drops of 1 N sodium hydroxide, and the product [1-^3H]isopentenol was isolated as usual.

(g) [1-^3H]Isopentenal. The crude [1-^3H]isopentenol from the previous reaction was diluted with inactive isopentenol (50 mg, 0.58 mmol) and dissolved in 1.5 mL of methylene chloride and reacted with 200 mg (0.93 mmol) of pyridinium chlorochromate in 1 mL of methylene chloride with vigorous stirring under nitrogen at room temperature. After 1 h another portion of pyridinium chlorochromate (200 mg, 0.93 mmol) in 1 mL of CH_2Cl_2 was added to the reaction mixture. The last step was repeated twice more so that a total of 800 mg of pyridinium chlorochromate (3.7 mmol) was utilized. The reaction mixture contained no starting material detectable by TLC. The black reaction mixture was allowed to pass through a small column packed with silica gel (70–270 mesh) by eluting with methylene chloride. The black material was completely retained in the column, and the eluate was concentrated at 0 °C to dryness. (This evaporation inevitably caused serious loss.) TLC (methylene chloride, R_f 0.68) showed a mixture of two aldehyde compounds, isopentenal and its conjugated isomer, 3-methylbut-2-enal, but no starting material. The ratio of these two radioactive aldehyde isomers was not determined at this stage. TLC also showed some of the corresponding acids as side products due to over oxidation. This oxidation was carried out for a much longer time than usual to ensure that no [1-^3H]isopentenol survived since any trace of (1*RS*)-[1-^3H]isopentenol would alter the result of the eventual chiral acetate assay.

(h) (1*S*)-[1-^2H,^3H]Isopentenol.[37,82] The partially purified [1-^3H]isopentenal was transferred to a test tube containing 3.0 mL of 0.1 M phosphate buffer (pH 7.0), 1 mL of ethanol-free horse liver alcohol

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dehydrogenase (27 units), NAD^+ (12.3 mg, 0.019 mmol, 92% purity), and 1 mL of ethanol-d_6 (801 mg, 15.4 mmol). The contents were incubated at 27 °C in a water bath with occasional stirring. After 2.5 h another 1 mL of ethanol-d_6 (801 mg, 15.4 mmol), 0.5 mL of liver alcohol dehydrogenase (13.5 units) and NAD^+ (4.3 mg, 0.0068 mmol) were added to the reaction mixture. The reaction was terminated after 44-h incubation at 27 °C by adding inactive isopentenol (20 mg, 0.23 mmol) and immediately freezing the contents to −78 °C. This mixture could be stored at −25 °C before continuous extraction with 50 mL of ether for 36 h. Because the mixture contained 2 mL of ethanol-d_6, the efficiency of this continuous extraction was somewhat low. The ethereal extract was dried over sodium sulfate, and the solvent was evaporated at 0 °C under reduced pressure. A significant loss of product inevitably occurred in this evaporation because the extract also contained a very high concentration of ethanol-d_6 and was thus hard to evaporate without simultaneous loss of (1*S*)-[1-^{2}H,^{3}H]-3-methylbut-3-en-1-ol. It was subsequently found that complete evaporation of the solvent was not essential for the subsequent steps, thereby allowing a significant improvement in yield.

(i) (5*S*)-[5-^{2}H,^{3}H]Mevalonolactone. The transformation of the (1*S*)-[1-^{2}H,^{3}H]isopentenol to (5*S*)-[5-^{2}H,^{3}H]mevalonolactone was carried out by essentially the same procedures as described for (5*R*)-[5-^{2}H,^{3}H]mevalonolactone.[37] The crude (5*S*)-[5-^{2}H,^{3}H]-mevalonolactone was partially purified by PLC as before to give 13.7 mg of product. This mevalonolactone was diluted with inactive carrier (5.3 mg, 0.041 mmol) and again purified by a second PLC to give (5*S*)-[5-^{2}H,^{3}H]mevalonolactone (11 mg, 0.085 mmol, total activity = 2×10^7 dpm) which was stored as a frozen solution in 1 mL of benzene at −25 °C.

Incorporation of (5*R*)-[5-^{2}H,^{3}H]- and (5*S*)-[5-^{2}H,^{3}H]Mevalonates into Cyclonerodiol by *G. fujikuroi*. Cultures of *G. fujikuroi* were grown in the usual manner in 500-mL Delong flasks containing 100 mL each of nutrient broth. Labeled mevalonates were fed after 5 days according to the previously described procedure,[10] and the resulting labeled cyclonerodiol was isolated and purified by the usual procedures without addition of inactive carrier. Thus feeding of 5.7×10^7 dpm of (3*RS*,5*R*)-[5-^{2}H,^{3}H]mevalonate as the sodium salt to a total of 2.5 L of *G. fujikuroi* culture yielded 38.5 mg of purified cyclonerodiol (2.90×10^6 dpm/mmol), corresponding to a 1.6% incorporation. Similarly 1.43×10^7 dpm of sodium (3*RS*,5*S*)-[5-^{2}H,^{3}H]mevalonate added to 0.9 L of fungal culture gave 10.6 mg of cyclonerodiol (1.92×10^6 dpm/mmol, 1.2% incorporation).

Kuhn–Roth Oxidations of [1-^{2}H,^{3}H]Cyclonerodiols. Each of the above prepared samples of cyclonerodiol was subjected to Kuhn–Roth oxidation in the manner described above. Thus 28 mg (0.12 mmol) of cyclonerodiol derived from incorporation of (5*R*)-[5-^{2}H,^{3}H]mevalonate was oxidized with 12 mL of the standard oxidizing solution. Steam distillation of the resulting acetic acid and collection of a total volume of 70 mL of distillate were followed by titration with 47.2 mL of 7.8 mM KOH, corresponding to an 82% yield of acetate (more than 90% of this acetate was contained in the first 30 mL of distillate). This chiral acetate sample was mixed with a suitable amount of [2-^{14}C]acetate and a small portion of the resultant [2-^{2}H,^{3}H,^{14}C]acetate was converted to the *p*-bromophenacyl ester which was recrystallized to constant activity and ^{3}H/^{14}C ratio (1.73×10^5 dpm/mmol, ^{3}H; ^{3}H/^{14}C = 3.38). Similarly oxidation of 7.3 mg of cyclonerodiol, derived from feeding (5*S*)-[5-^{2}H,^{3}H]mevalonate, was accomplished by using 3.0 mL of the standard oxidant solution to give a 70% titrated yield of potassium acetate (8.4 mg dry weight). Mixture of this sample with [2-^{14}C]acetate and derivatization of an aliquot gave the *p*-bromophenacyl ester (1.16×10^5 dpm/mmol of ^{3}H; ^{3}H/^{14}C = 3.88).

Chiral Acetate Assays. Each of the above-prepared samples of [2-^{2}H,^{3}H,^{14}C]acetate was subjected to the standard chiral acetate assay as described above. Control samples of authentic (2*R*)-[2-^{2}H,^{3}H]- and (2*S*)-[2-^{2}H,^{3}H]acetate, generously provided by Professor Duilio Arigoni, as well as [2-^{3}H,^{14}C]acetate were also analyzed and the results of the various assays are summarized in Table IV.

Sodium [1-^{18}O$_2$]Acetate.[39] [^{18}O]Water (90.43% ^{18}O atom excess, 50 μL, 2.5 mmol) was stirred with a mixture of 4 mL of dry triethyl orthoacetate and 0.2 mg of *p*-toluenesulfonic acid monohydrate until one phase had formed. Sodium methoxide (2.63 N, formed by dissolving freshly cut sodium in 10 mL of rigorously dried methanol) was added to the labeled ethyl acetate followed by another 50 μL of [^{18}O]water in 200 μL of anhydrous THF. Sodium acetate precipitated almost instantly and formed a white crystalline mass. The reaction mixture was left standing at room temperature for 4 h. The unreacted alcohols and triethyl orthoformate were removed, first on the rotary evaporator and then on the high vacuum pump. The sodium acetate was dried overnight at 70 °C under vacuum to yield 215 mg (80%) of sodium [2-^{18}O$_2$]acetate.

***trans,trans*-Farnesyl Chloride.** *trans,trans*-Farnesol (300 mg, 1.35 mmol) was stirred with *s*-collidine (0.27 mL, 2.03 mmol) at 0 °C under nitrogen. Dry lithium chloride (210 mg, 4.86 mmol) was dissolved in 3 mL of dry dimethylformamide at 0 °C and then added to the farnesol mixture.[38] To the white slurry was added slowly methanesulfonyl chloride (0.18 mL, 2.03 mmol), and the pale yellow precipitate was stirred at 0 °C for 2 h. The reaction was quenched by the addition of water, and the mixture was stirred at room temperature for 2 h. The aqueous layer was extracted with pentane–ether (1:1; 3×). The combined organic extracts were first washed with saturated copper nitrate solution until no further intensification of the blue color occurred, washed successively with 5% aqueous sodium hydroxide, water and saturated sodium chloride, then dried, and concentrated. The crude farnesyl chloride (326 mg) was used without further purification: TLC hexane–ethyl acetate, 10:1; R_f 0.68; ^{1}H NMR ($CDCl_3$) δ 1.8 (s, CH_3, 12 H), 2.03 (b, CH_2, 8 H), 4.05 (d, J = 8 Hz, CH_2Cl, 2 H), 4.9–5.6 (overlapping t, vinyl, 3 H); IR ν_{max} ($CHCl_3$) 1675 (double bond) cm^{-1}.

***trans,trans*-Farnesyl [1-^{18}O,*acyl*-^{18}O]Acetate.** A mixture of crude farnesyl chloride (326 mg, 1.35 mmol), sodium [1-^{18}O$_2$]acetate (133 mg, 1.58 mmol), and sodium iodide (20 mg, 0.13 mmol) in 5 mL of dry DMF was stirred overnight under nitrogen at room temperature.[83] The reaction mixture was poured into water, and the aqueous layer was extracted with ether (3×). The combined organic extracts were washed with water, and the crude ester was purified by PLC (hexane–ethyl acetate, 10:1; R_f 0.48) to give 278 mg of *trans,trans*-farnesyl [1-^{18}O,-*acyl*-^{18}O]acetate (78% overall yield from farnesol).

***trans,trans*-Farnesyl [1-^{18}O]Pyrophosphate.** *trans,trans*-Farnesyl [1-^{18}O,*acyl*-^{18}O]acetate (272 mg, 1.03 mmol) was hydrolyzed by the usual procedure to give 220 mg (97%) of [1-^{18}O]-*trans,trans*-farnesol. The latter compound was phosphorylated in the usual manner to yield 76 mg of *trans,trans*-farnesyl [1-^{18}O]pyrophosphate. A portion of the recovered [1-^{18}O]farnesol was reconverted to farnesyl acetate in the usual manner and the ^{18}O content determined by mass spectrometry: 70.0 ± 2.3 atom excess % ^{18}O, average of three determinations.

Incubation of [12,13-^{14}C]-*trans,trans*-Farnesyl [1-^{18}O]Pyrophosphate. *trans,trans*-Farnesyl [1-^{18}O]pyrophosphate (76 mg) was mixed with [12,13-^{14}C]-*trans,trans*-farnesyl pyrophosphate (0.54 mg, 5.7×10^5 dpm). A 6-mg aliquot of this farnesyl pyrophosphate mixture was hydrolyzed with alkaline phosphatase for 3 h at 37 °C and then overnight at room temperature. The crude [1-^{18}O,12,13-^{14}C]farnesol isolated from this incubation was converted to the corresponding crystalline farnesyl diphenylurethane which was recrystallized from methanol to constant activity (2.85×10^6 dpm/mmol).

The remainder of the [^{14}C]farnesyl [^{18}O]pyrophosphate (70 mg) was incubated with the S_{AS} cell-free extract from a total of 13 L of *G. fujikuroi* (585 mg of protein) for 3 h at 26 °C. The resulting crude ether extract was purified by PLC (methylene chloride–ether, 3:1; 2×) to give 1.43 mg of cyclonerodiol (**3**) (R_f 0.25, activity = 1.69×10^4 dpm) and 16 mg of farnesol (R_f 0.6). A portion of the reisolated farnesol was converted to its acetate, and the mass spectrum was recorded (parent peak M, m/e 264, 70.2 ± 1.3 atom excess % ^{18}O, average of three determinations).

A portion of the isolated [^{18}O]cyclonerodiol (0.5 mg) was converted to its bis(dinitrobenzoate) ester in the usual manner. After PLC purification the yield of ester was calculated to be 693 μg, based on the 230- and 215-nm absorbances of six independent UV measurements recorded in cyclohexane and standardized against authentic solutions of bis(dinitrobenzoate) ester. Aliquots of these bis(dinitrobenzoate) ester solutions were withdrawn and counted to determine their activity (2.86×10^6 dpm/mmol).

3-(Trimethylsilyl)oxylactone (10). (Trimethylsilyl)imidazole (6 mg, 0.042 mmol) dissolved in 100 μL of pyridine[84] was added to the [3-^{18}O]norlactone (**7**) obtained from 0.9 mg of [3-^{18}O]cyclonerodiol by osmium tetroxide–sodium periodate treatment followed by oxidation with Jones reagent as described above. The solution was stirred for 4 h at room temperature under nitrogen. After removal of the solvent the crude **10** was purified by PLC (methylene chloride–ether, 3:1; R_f 0.6). The mass spectrum of the isolated **10** was recorded (Tables V and VI).

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