Biosynthesis of Pentalenene and Pentalenolactone

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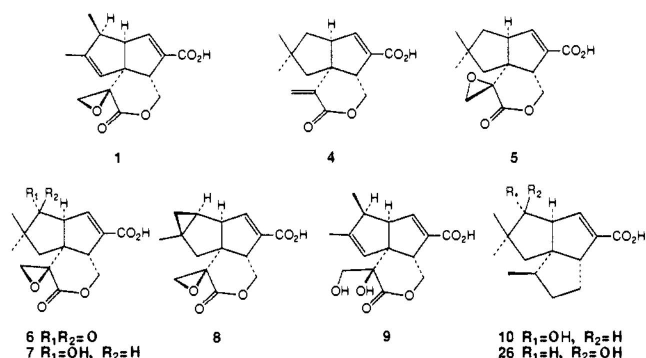
Abstract: Pentalenene synthase catalyzes the cyclization of farnesyl pyrophosphate (**2**) (FPP) to the sesquiterpene hydrocarbon pentalenene (**3**). Incubation of both (9*R*)- and (9*S*)-[9-^{3}H,4,8-$^{14}C_2$]FPP (**2c** and **2d**) with pentalenene synthase isolated from *Streptomyces* UC5319 and analysis of the derived labeled pentalenene by a combination of chemical and microbial methods established that H-9 *re* of **2** becomes H-8 of pentalenene, while H-9 *si* undergoes net intramolecular transfer to H-1 of **3**. The stereochemistry of the proton transfer was determined by incubation of [10-^{2}H,11-^{13}C]FPP (**2e**) with pentalenene synthase and analysis of the product by a combination of ^{2}H and ^{13}C NMR spectroscopy, which established that the deuterium atom occupied exclusively the predicted H-1 *si* (H-1β) position in pentalenene. Deuterium NMR analysis of pentalenene derived from both (1*S*)- and (1*R*)-[1-^{2}H]FPP (**2h** and **2i**) established that the cyclization takes place with inversion of configuration at C-1 of FPP. These results are fully consistent with a stereochemical model of the cyclization reaction in which FPP is cyclized to humulene (**11**), folded in the *RSR*-CT conformation. The humulene is in turn protonated on the 10 *re* face of the C-9,10 double bond, leading, after further cyclization and rearrangement, to the formation of pentalenene (**3**). The stereochemical course of the oxidative metabolism of pentalenene was also examined. The hydroxylation at C-1 that leads to the formation of pentalenic acid (**10**) was shown to take place with retention of configuration. The fact that tritium label from either (1*R*)-[1,8-$^3H_2$]- or (1*R*)-[1-^{3}H]pentalenene (**3b** or **3d**) was lost upon formation of pentalenic acid but was retained in the eventually formed pentalenolactone (**1**) ruled out **10** as an intermediate in the formation of **1**. Administration of both (3*S*)- and (3*R*)-[3-^{3}H,14,15-$^{14}C_2$]pentalenene (**3j** and **3k**) to cultures of *Streptomyces* UC5319 and analysis of the ^{3}H/^{14}C ratio in the derived pentalenolactone established that the A-ring rearrangement takes place with stereospecific loss of H-3 *re* of **3**, anti to the migrating methyl group.

Pentalenolactone (1), also known as arenemycin E and PA-132, is an antibiotic produced by a variety of *Streptomyces* species. It was first isolated in 1957 and shown to be active against a number of eukaryotic microorganisms.[1] The antibiotic was independently reisolated in 1969 as an inhibitor of nucleic acid synthesis in bacterial cells[2] and in 1970 during a screening for antitumor agents.[3] Recent studies have shown that pentalenolactone is a potent and specific inhibitor of glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in the glycolytic pathway.[4] Pentalenolactone also possesses antiviral activity.[5]

The sesquiterpenoid biosynthetic origin of pentalenolactone (**1**) was first demonstrated by growth of *Streptomyces* UC5319 in media containing [U-$^{13}C_6$]glucose, which acts as an in vivo precursor to [1,2-$^{13}C_2$]acetate, which in turn is converted to mevalonate and thence to dimethylallyl, isopentenyl, and farnesyl pyrophosphates and eventually to **1**.[6] Although none of these latter intermediates are incorporated into pentalenolactone by whole-cell cultures, the labeling pattern in the product is consistent with the proposed isoprenoid pathway. Cell-free extracts prepared from this organism catalyze the cyclization of farnesyl pyrophosphate (**2**), the univeral biosynthetic precursor of the sesquiterpenes, to pentalenene (**3**).[7] The cyclase, pentalenene synthase, has recently been purified to homogeneity.[8] Pentalenene itself was isolated as a natural product in 1980 by Seto and Yonehara.[9]

Streptomyces strains that produce pentalenolactone (**1**) also produce a variety of related metabolites, including pentalenolactones E (**4**),[10] epi-F (**5**),[11] G (**6**),[12] H (**7**),[13] P (**8**),[14] and O (**9**),[14] as well as pentalenic acid (**10**).[13] Many of these compounds have



been proposed as potential biosynthetic intermediates between pentalenene and pentalenolactone. Consistent with this notion, labeled pentalenene has been incorporated into **4**, **5**, and **10** as well as into pentalenolactone itself.[7]

We have undertaken an extensive investigation of the enzyme-catalyzed conversion of farnesyl pyrophosphate (**2**) to pentalenene (**3**) as well as the subsequent biological oxidations by which pentalenene is converted to members of the pentalenolactone family of compounds. With a crude cell-free system from *Streptomyces* UC5319, conversion of [8,8-$^3H_2$, 12,13-$^{14}C_2$]farnesyl pyrophosphate (**2a**) to labeled pentalenene **3a** and of [9,9-$^3H_2$, 12,13-$^{14}C_2$]farnesyl pyrophosphate (**2b**) to **3b** was demonstrated[7,15] (Scheme I). The labeling patterns, determined by a combination of chemical and microbiological degradation

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(7) Cane, D. E.; Tillman, A. M. *J. Am. Chem. Soc.* **1983**, *105*, 122.
(8) (a) Cane, D. E.; Pargellis, C. *Arch. Biochem. Biophys.* **1987**, *254*, 421. (b) Cane, D. E.; Oliver, J. S.; Harrison, P. H. M.; Hubbard, B. R. Unpublished work.
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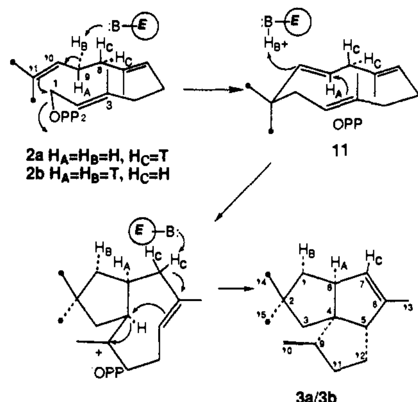
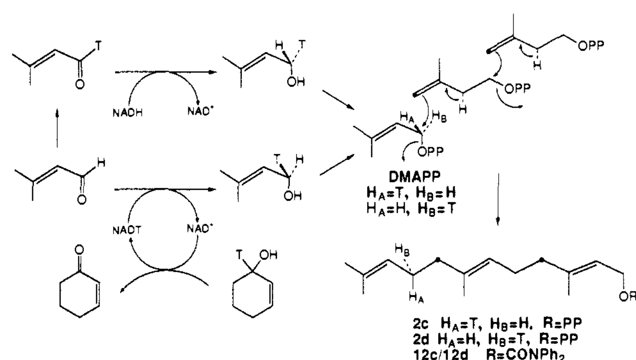
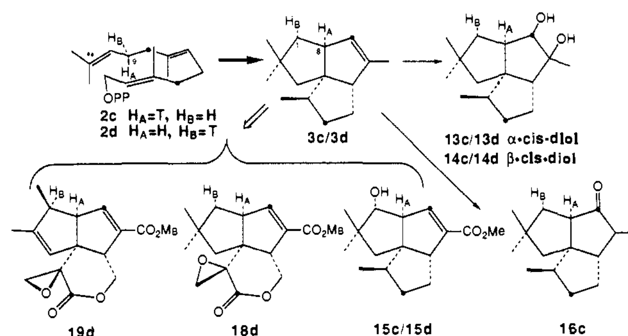
Scheme I



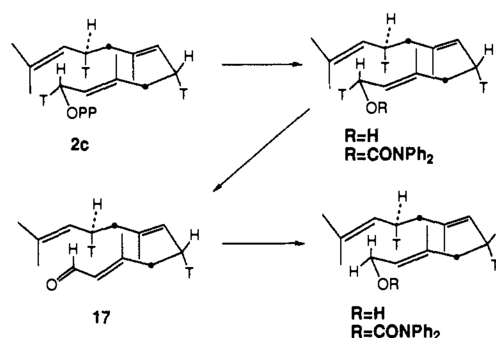
Scheme II



Scheme III



Scheme IV



methods, were interpreted in terms of initial cyclization of **2** to give humulene (**11**), folded in the *RSR*-CT conformation.[16] Reprotonation of **11**, followed by cyclization, hydride shift, further cyclization, and proton loss, would generate **3**. The observed retention of both H-9 *re* and H-9 *si* of farnesyl pyrophosphate in the resulting pentalenene showed that conversion of **2** to **3** must occur on a single enzyme, without the intermediacy of free humulene.

To probe further the mechanistic details of the cyclization, we have also investigated the cryptic stereochemical fate of the enantiotopic protons at both C-1 and C-9 of FPP during cyclization to pentalenene and reported these results in preliminary communications.[17,18] We now report a more detailed description of these earlier experiments as well as the results of investigations that lend further support to the proposed stereochemical model for FPP cyclization and which demonstrate that the oxidation of pentalenene to pentalenic acid proceeds with net retention of configuration at C-1 of **3**. We have also established that the conversion of pentalenene to pentalenolactone involves elimination of H-3 *re*, corresponding to an antiperiplanar methyl migration and deprotonation.

Results

Cyclization of Farnesyl Pyrophosphate to Pentalenene. Stereochemistry of the Deprotonation at C-9. Cyclization of FPP to pentalenene involves removal of one of the two protons at C-9. This same proton has been shown to be transferred to the adjacent carbon atom, presumably by protonation of the proposed humulene intermediate.[15] To investigate the stereochemical fate of the enantiotopic H-9 protons, both (9*R*)- and (9*S*)-[9-^{3}H,4,8-$^{14}C_2$]-farnesyl pyrophosphate were prepared. For the preparation of the 9*R* isomer, 3-methyl-2-butenal was reduced with horse liver alcohol dehydrogenase (HLADH) by using catalytic NAD$^+$ and [1-^{3}H]cyclohexenol as the tritium source[19,20] (Scheme II). The resulting (1*R*)-[1-^{3}H]dimethylallyl alcohol was converted to (1*R*)-[1-^{3}H]dimethylallyl pyrophosphate (DMAPP) by the method of Cramer and Böhm.[21] Incubation of (1*R*)-[1-^{3}H]DMAPP and [4-^{14}C]isopentenyl pyrophosphate (IPP) with avian liver prenyltransferase gave (9*R*)-[9-^{3}H,4,8-$^{14}C_2$]farnesyl pyrophosphate (FPP) (**2c**).[22] Partial purification by anion-exchange chromatography on DEAE-Sephadex gave the desired FPP along with unreacted DMAPP and IPP. A portion of this material was treated with acid phosphatase and the resulting farnesol, after addition of inactive carrier, was converted to the diphenylurethane (**12c**),[23] which was recrystallized to constant activity and isotope ratio (^{3}H/^{14}C atom ratio 1:2).

For the (9*S*)-[9-^{3}H,4,8-$^{14}C_2$]FPP isomer (**2d**), reduction of 3-methyl-2-butenal with sodium [^{3}H]borohydride followed by oxidation with pyridinium chlorochromate (PCC) gave [1-^{3}H]-3-methyl-2-butenal. Stereospecific reduction of [1-^{3}H]-3-methyl-2-butenal with HLADH and excess NADH gave (1*S*)-[1-^{3}H]dimethylallyl alcohol, which was converted to the corresponding pyrophosphate ester.[21] Incubation of (1*S*)-[1-^{3}H]-DMAPP and [4-^{14}C]IPP with avian liver prenyltransferase gave (9*S*)-[9-^{3}H,4,8-$^{14}C_2$]FPP (**2d**). The FPP was carefully separated

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(16) The designation *RSR* refers to the chiralities of the three double bonds, $\Delta^{2,3}$, $\Delta^{6,7}$, and $\Delta^{9,10}$, respectively. C and T indicate crossed and parallel arrangements of the $\Delta^{9,10}$–$\Delta^{2,3}$ and $\Delta^{2,3}$–$\Delta^{6,7}$ double-bond pairs, respectively. See: Sutherland, J. K. *Tetrahedron* **1974**, *30*, 1651.
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(19) The stereospecificity of the coupled enzymatic reduction was verified by acid phosphatase catalyzed hydrolysis of the derived (1*R*)-[1-^{3}H]DMAPP, mixture of the resulting (1*R*)-[1-^{3}H]dimethylallyl alcohol with [1-^{14}C]dimethylallyl alcohol, and oxidation of a portion of the alcohol with HLADH and excess NAD$^+$. (See Experimental Section.) Comparison of the ^{3}H/^{14}C ratio of the [1-^{14}C]-3-methyl-2-butenal semicarbazone (0.04) with that of the dinitrobenzoate ester of (1*R*)-[1-^{3}H,1-^{14}C]dimethylallyl alcohol (5.23) confirmed that the alcohol consisted of >99% of the expected (1*R*)-[1-^{3}H] enantiomer. The analysis also confirmed that the pyrophosphorylation reaction had taken placed without disturbing the configuration at C-1 of the substrate.
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from DMAPP, IPP, and GPP by reverse-phase ion-pairing HPLC,[24] followed by anion-exchange chromatography on DEAE-Sephadex. The derived farnesyl diphenylurethane (**12d**) was recrystallized to constant activity and isotope ratio ($^3H/^{14}C$ atom ratio 1:2).

Incubation of (9*R*)-[9-^{3}H,4,8-$^{14}C_2$]FPP (**2c**) with crude pentalenene synthase gave labeled **3c** ($^3H/^{14}C$ atom ratio 1:2), which was diluted with inactive carrier pentalenene (Scheme III). Treatment of a portion of **3c** with OsO_4 gave the diastereomeric *cis*-diols **13c** ($^3H/^{14}C$ atom ratio 1:2) and **14c** ($^3H/^{14}C$ atom ratio 1:2), each of which was recrystallized to constant activity.[7,15] The precise location of the tritium in the labeled pentalenene was established by a combination of microbiological and chemical methods.[15] Thus, when the labeled pentalenene **3c** was fed to intact cultures of *Streptomyces* UC5319, the $^3H/^{14}C$ atom ratio (1:2) of the resulting labeled pentalenic acid methyl ester (**15c**) was unchanged, consistent with the absence of tritium at C-1 of pentalenene. Hydroboration of **3c** followed by oxidation of the resulting alcohols gave the ketone **16c** with an unchanged $^3H/^{14}C$ ratio. Treatment of **16c** with sodium deuterioxide in D_2O resulted in complete exchange of the protons α to the ketone (H-6 and H-8), as shown by the disappearance of the corresponding resonances in the ^{1}H NMR spectrum. On the other hand, the resulting deuterated ketone had lost only 69% of the tritium label ($^3H/^{14}C$ atom ratio 0.31:2), instead of the expected value of 100%. Further investigation showed that the FPP substrate unexpectedly carried ^{3}H at both C-1 and (presumably) C-5 as well as at the intended site, C-9. Thus oxidation by HLADH and NAD^+ of farnesol that had been recovered from the incubation of labeled FPP **2c** with pentalenene synthase gave farnesal (**17**), which was reduced with $NaBH_4$ and converted to the corresponding diphenylurethane ($^3H/^{14}C$ atom ratio 0.88:2) (Scheme IV). Comparison of the $^3H/^{14}C$ ratio of this compound with that of the diphenylurethane derivative of the original recovered farnesol showed that 12% of the tritium in the FPP substrate was located at H-1 *re*. The scrambling of label in the substrate was most likely due to isomerization of contaminating labeled DMAPP to IPP by endogenous isomerase present in the crude *Streptomyces* extract, followed by incorporation of this IPP into FPP by prenyltransferase. In fact, both isomerase and prenyltransferase activities were subsequently confirmed to be present in the crude pentalenene synthase preparation. The resulting (1*R*,5*R*,9*R*)-[1,5,9-^{3}H]FPP must therefore contain a total of 24% ^{3}H at H-1 *re* and H-5 *re*, based on an equal distribution over both positions, leaving 76% ^{3}H at H-9 *re*. Thus, the base-catalyzed exchange of the ketone **16c** had in fact resulted in loss of 90% of the theoretical amount of tritium label actually located at H-9 *re*.

To avoid a repetition of such complications, cyclization of rigorously purified (9*S*)-[9-^{3}H,4,8-$^{14}C_2$]FPP (**2d**) was carried out with 130-fold purified pentalenene synthase that had been shown to be free of contaminating phosphatase, prenyltransferase, and isomerase activities.[25] Half of the resulting labeled pentalenene (**3d**) ($^3H/^{14}C$ atom ratio 1:2) was diluted with inactive pentalenene, and the derived diols **13d** ($^3H/^{14}C$ atom ratio 1:2) and **14d** ($^3H/^{14}C$ atom ratio 1:2) were each recrystallized as before (Scheme III). The remaining half of the pentalenene **3d** was administered to actively producing cultures of *Streptomyces* UC5319. Extraction of the acidic metabolites and methylation with diazomethane followed by HPLC separation on silica gel gave pentalenic acid methyl ester (**15d**) ($^3H/^{14}C$ atom ratio 0.03:2), epipentalenolactone F methyl ester (**18d**) ($^3H/^{14}C$ atom ratio 1.1:2), and pentalenolactone methyl ester (**19d**) ($^3H/^{14}C$ atom ratio 1:2). While the latter two metabolites had unchanged $^3H/^{14}C$ ratios, the pentalenic acid methyl ester was devoid of tritium, thereby locating the tritium at C-1 of pentalenene, as expected.

Cyclization of Farnesyl Pyrophosphate to Pentalenene. Stereochemistry of the Intramolecular Proton Transfer. The above results demonstrate that H-9 *si* of FPP becomes one of the two diastereotopic protons at C-1 of pentalenene. Protonation of humulene folded in the *RSR*-CT conformation would be expected to occur on the 10 *re* face of the C-9,10 double bond. One would therefore expect H-1 *si* of the resultant pentalenene to be derived from H-10 of FPP, while H-1 *re* of **3** would originate from H-9 *si* of the precursor. This prediction can be tested by cyclizing [10-^{2}H,11-^{13}C]FPP and analyzing the product by ^{2}H and ^{13}C NMR spectroscopy (Scheme V). With this objective in mind, we therefore carried out a complete assignment of the proton and carbon NMR spectra of pentalenene, using a combination of ^{1}H-^{1}H COSY,[26] INEPT, and ^{1}H-^{13}C heteronuclear shift correlation[27] experiments (Table I).

Assignment of the ^{1}H and ^{13}C NMR Spectra of Pentalenene. In the ^{13}C NMR spectrum of pentalenene, C-6 was readily identified as the only quaternary olefinic carbon; the remaining olefinic carbon signal must therefore have been due to C-7. Similarly, H-7 could be assigned to the only olefinic proton resonance. The quaternary signals in the ^{13}C NMR spectrum corresponding to C-2 and C-4 were differentiated on the basis of the expected downfield shift of C-4 relative to C-2.[28] Of the four signals in the ^{1}H NMR spectrum arising from methyl protons, the allylic methyl H-13 appeared furthest downfield. H-14 and

Scheme V

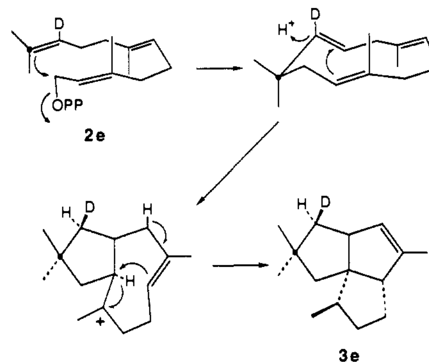

Table I. Assignment of the ^{13}C and ^{1}H NMR Spectra of Pentalenene (3)

^{13}C δ, ppm[a]	carbon	type[b]	^{1}H δ[c] (mult, *J*)[d]
140.57	6	C	
129.55	7	CH	5.15 (dqm, 1.9, 1.3)
64.73	4	C	
62.04	5[e]	CH	2.54 (b d, 8.9)
59.36	8[e]	CH	2.66 (m)
48.92	3[e]	CH_2 *re*	1.73 (dd, 12.9, <1)
		CH_2 *si*	1.35 (dd, 13.0, <1)
46.81	1[e]	CH_2 *re*	1.60 (ddd, 12.5, 9.1, 1.0)
		CH_2 *si*	1.17 (ddd, 12.6, 5.1, 0.7)
44.59	9	CH	1.84 (m)
40.51	2	C	
33.51	11[e]	CH_2	1.61 (m)
			1.27 (m)
29.94	14[f]	CH_3	0.98 (s)
29.11	15[f]	CH_3	0.99 (s)
27.59	12	CH_2	1.77 (m)
			1.33 (m)
17.01	10	CH_3	0.89 (d, 7.1)
15.50	13	CH_3	1.61 (m, small)

[a] 100.6-MHz ^{13}C NMR spectrum in $CDCl_3$ with solvent reference at 77.00 ppm. [b] Based on INEPT and {^{1}H}-^{13}C NOE. [c] 400-MHz ^{1}H NMR spectrum in $CDCl_3$ with internal TMS at 0.00 ppm. [d] Multiplicities (mult) and coupling constants (*J*, in Hz). [e] Assignments for these ^{13}C NMR signals differ from those of ref 9. [f] These assignments may be reversed.

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(25) Pentalenene synthase was purified to a specific enzyme activity of 545 nmol h^{-1} (mg of protein)$^{-1}$ (cf. ref 8b).

(26) (a) Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Magn. Reson.* **1976**, *64*, 2229. (b) Nagayama, K.; Kumar, A.; Wuthrich, K.; Ernst, R. R. *J. Magn. Reson.* **1980**, *40*, 321.

(27) Bax, A.; Morris, G. *J. Magn. Reson.* **1981**, *42*, 501.

(28) This assignment was subsequently confirmed by the labeling of C-2 of **3** by [11-^{13}C]FPP, as described.

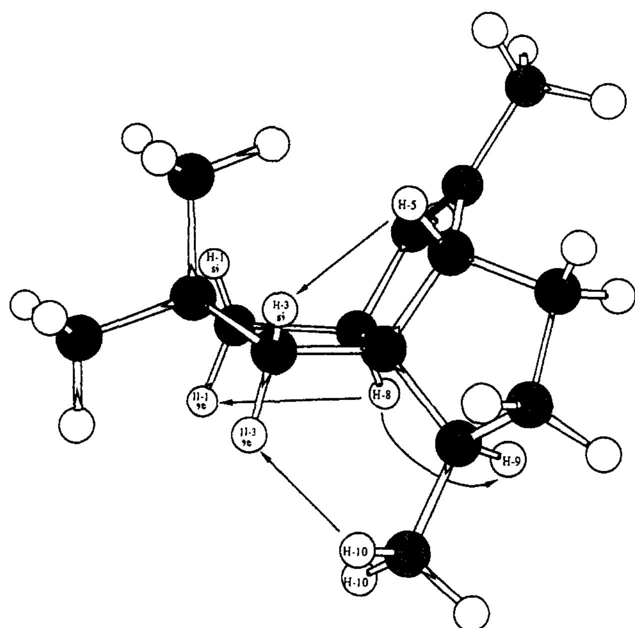


Figure 1. Conformation of pentalenene calculated with MacroModel showing observed NOEs. Nonbonded distances (Å): H-1 *si*/H-5, 4.50; H-1 *si*/H-8, 2.79; H-1 *re*/H-8, 2.30; H-3 *si*/H-5, 2.30; H-3 *si*/H-10, 3.18; H-3 *re*/H-5, 3.59; H-3 *re*/H-8, 3.47; H-3 *re*/H-9, 3.60; H-3 *re*/H-10, 2.23; H-8/H-9, 2.30; H-8/H-10, 2.95. Calculated dihedral angles: H-1 *si*/H-8, 90°; H-1 *re*/H-8, 28°.

H-15 each gave rise to singlets, and H-10 appeared as a doublet. C-10, C-13, C-14, and C-15 were thus readily assigned from the ^{1}H-^{13}C heteronuclear shift correlation. The diastereotopic methyl groups CH_3-14 and CH_3-15 could not be individually assigned in this series of experiments. From the COSY spectrum, CH_3-10 was seen to be coupled to a multiplet, corresponding to H-9; the assignment of C-9 follows from the ^{1}H–^{13}C correlation spectrum. The two allylic proton signals, H-5 and H-8, each showed coupling in the COSY spectrum to two other protons, CH_2-12 and CH_2-1 respectively. These methylenes were differentiated on the basis of their coupling patterns. Thus, CH_2-1 gave rise to two clean doublets of doublets, while CH_2-12 appeared as a pair of multiplets. Assignment of C-5 and C-8 followed readily from the heteronuclear correlation spectrum. The HETCOSY experiment also confirmed that the pairs of protons assigned to CH_2-1 and CH_2-12 were indeed each attached to the indicated carbon atoms, thereby allowing assignment of C-1 and C-12. A pair of coupled multiplets in the ^{1}H NMR spectrum at δ 1.27 and 1.61 that correlated with the ^{13}C NMR signal at 33.51 ppm were assigned to CH_2-11 on the basis of coupling in the COSY spectrum to CH_2-9 and CH_2-12. The remaining methylene, CH_2-3, gave rise to an AB pattern of doublets correlated to the ^{13}C signal at 48.92 ppm.

The individual proton NMR signals due to the diastereotopic H-1 protons were assigned on the basis of difference NOE experiments. Thus, irradiation of H-8 gave rise to an NOE (4%) at H-1 *re*. The observed coupling constants ($J_{H\text{-}1re\text{-}H\text{-}8}$ = 9.1 Hz, $J_{H\text{-}1si\text{-}H\text{-}8}$ = 5.1 Hz) are also consistent with this assignment. The signals from the H-3 *re* and H-3 *si* protons were also assigned on the basis of NOE effects: H-3 *re* showed an NOE (4%) upon irradiation of CH_3-10, while H-3 *si* exhibited an NOE (2%) upon irradiation of H-5. The observed NOEs and coupling constants are in accord with predictions based on nonbonded distances and dihedral angles calculated with the MacroModel molecular mechanics program using an MM-2 force field[29] (Figure 1). For example, the MacroModel structure indicated that H-8 and H-9 should be 2.30 Å apart, close enough for the observation of an NOE. Irradiation of H-8 did in fact produce an NOE at H-9.

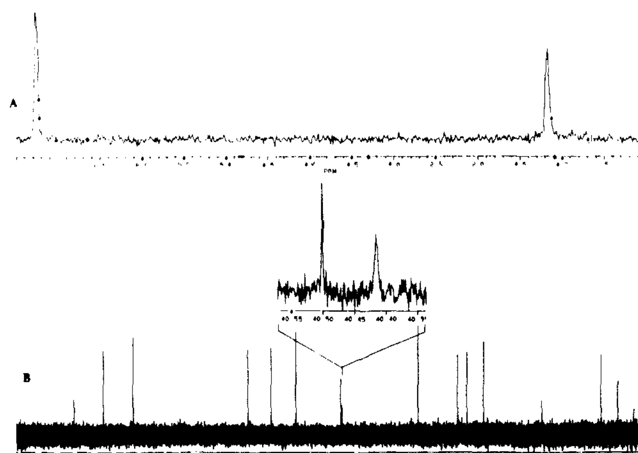
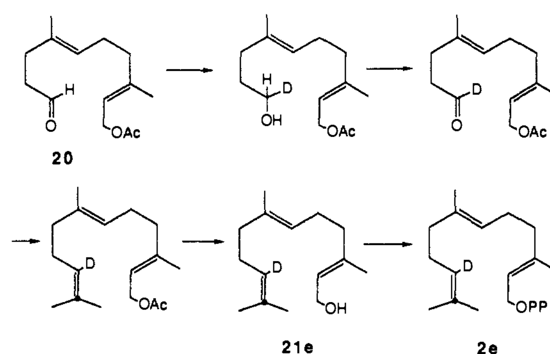


Figure 2. (A) ^{2}H NMR spectrum (61.4 MHz) of pentalenene (**3e**) derived from [10-^{2}H,11-^{13}C]FPP (**2e**). (B) ^{13}C NMR spectrum of **3e**.

Scheme VI



These results confirmed that our assignments for H-8 and H-5 were indeed correct, the reverse of those reported earlier by Seto.[9]

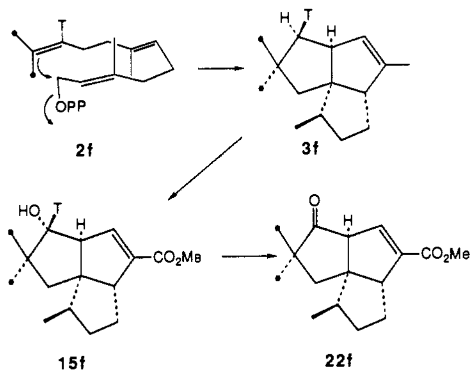
Conversion of [10-^{2}H]FPP to Pentalenene and Determination of the Position and Stereochemistry of Labeling. In order to determine the fate of the proton at C-10 of FPP, [11-^{13}C]FPP was synthesized as a mixture of species carrying deuterium and tritium at C-10. Treatment of the trisnoraldehyde of farnesyl acetate (**20**), prepared as previously described,[23] with $NaBD_4$ or $NaBH_3T$ gave the corresponding labeled alcohols, which were mixed and oxidized with PDC to produce the desired [10-^{2}H-(^{3}H)]trisnoraldehyde acetate (Scheme VI). Treatment of [1-^{13}C]ethyl iodide with triphenylphosphine gave [1-^{13}C]ethyltriphenylphosphonium iodide, which was deprotonated and quenched with methyl iodide.[23] Wittig reaction of the resulting isopropyltriphenylphosphonium salt with the labeled trisnoraldehyde acetate **20**, followed by deacetylation with K_2CO_3, gave [10-^{2}H-(^{3}H),11-^{13}C]farnesol (**21e**), which was purified by chromatography over silver nitrate impregnated silica gel to remove demethylfarnesols. Conversion to the pyrophosphate by the methyl of Poulter[30] followed by anion-exchange chromatography gave [10-^{2}H(^{3}H),11-^{13}C]FPP (**2e**), which was converted to pentalenene (**3e**) (216 nmol) by using pentalenene synthase that had been purified through the methylagarose step[8b] (Scheme V).

As illustrated in Figure 2A, the ^{2}H NMR spectrum of **3e** showed a single resonance at δ 1.18, the chemical shift previously assigned to H-1 *si*. Due to extensive overlap of the resonances in this region of the ^{1}H NMR spectrum, however, this experiment alone did not allow unambiguous proof that the 1 *si* position was labeled. Confirmation of the site of labeling was obtained by analysis of the ^{13}C NMR spectrum (Figure 2B), which exhibited an isotopically shifted signal 0.08 ppm upfield from the natural abundance signal corresponding to C-2 of the carrier pentalenene. The observed β-deuterium isotope shift established that deuterium

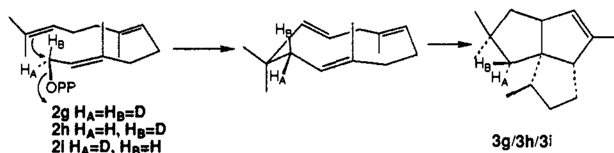
(29) Still, W. C.; Richards, N. G. J.; Guida, W. C.; Lipton, M.; Liskamp, R.; Chang, G.; Hendrickson, T. *MacroModel V2.0*; Department of Chemistry, Columbia University: New York, 1988.

(30) Davisson, V. J.; Zabriskie, T. M.; Poulter, C. D. *Bioorg. Chem.* **1986**, *14*, 46. Poulter, C. D.; Dixit, V. M.; Laskovics, F. M.; Noall, W. *J. Org. Chem.* **1981**, *46*, 1967.

Scheme VII



Scheme VIII



must be located at either C-1, C-3, C-14, or C-15 of **3e**. Since the proton resonances for H-1 *re*, H-3, H-14, and H-15 appear at chemical shifts readily distinguished from that due to H-1 *si*, the combined ^{2}H NMR and ^{13}C NMR experiments do in fact demonstrate unambiguously that H-10 of FPP becomes H-1 *si* of pentalenene.

Stereochemical Course of the Oxidation of Pentalenene to Pentalenic Acid. In order to establish the stereochemical course of the C-1 hydroxylation of pentalenene during the oxidative formation of pentalenic acid, FPP labeled with a mixture of tritium and deuterium at C-10 was used to prepare a mixture of (1*R*)-[1-^{2}H]- and (1*R*)-[1-^{3}H]pentalenene (Scheme VII). The position and stereochemistry of isotopic labeling was reconfirmed by ^{2}H NMR. Starting from [10-^{2}H(^{3}H),12,13-^{14}C$_2$]FPP (**2f**), the labeled pentalenene (**3f**) (^{3}H/^{14}C atom ratio 1:2) that was obtained was converted to pentalenic acid by incubation with growing cultures of *Streptomyces* UC5319. The derived pentalenic acid, isolated as the methyl ester (**15f**) (^{3}H/^{14}C atom ratio 0.84:2), retained 84 ± 4% of the original tritium label, based on comparison of the ^{3}H/^{14}C ratio with that of the farnesyl diphenylurethane **12f** (^{3}H/^{14}C atom ratio 1:2) prepared from the FPP precursor. The assigned site of tritium labeling in **15f** was confirmed by oxidation of the methyl ester with PCC to give the ketone **22f** (^{3}H/^{14}C atom ratio 0.1:2), which had lost 87 ± 5% of the original tritium. These results established that the microbial oxidation of pentalenene takes place, as expected,[31] with net retention of configuration at C-1.

Cyclization of Farnesyl Pyrophosphate to Pentalenene. Inversion of Configuration at C-1 of FPP. The stereochemistry of the initial displacement of the pyrophosphate moiety and concomitant C–C bond-forming reaction in the enzymatic conversion of FPP to pentalenene was established by ^{2}H NMR spectroscopic analysis of pentalenene derived from FPP that had been labeled stereospecifically at C-1 with deuterium.

To this end, [1,1-^{2}H$_2$,12,13-^{14}C$_2$]farnesyl pyrophosphate (**2g**) was prepared by reduction of farnesal (**17**) with sodium borodeuteride, oxidation to [1-^{2}H]farnesal with MnO$_2$, and reduction with NaBD$_4$. Addition of [12,13-^{14}C$_2$]farnesol to facilitate monitoring subsequent reactions and pyrophosphorylation[30] gave **2g**. Incubation with pentalenene synthase for 1 h at 25 °C yielded 90 nmol of pentalenene (**3g**), as determined from the measured ^{14}C incorporation. After dilution of the product with carrier (±)-pentalenene, the ^{2}H NMR spectrum of **3g** confirmed the incorporation of deuterium at the expected sites, H-3 *re* and H-3 *si* (Scheme VIII and Figure 3A).

(31) Hayaishi, O. *Oxygenases*; Academic Press: New York, 1962. Gautier, A. E. Dissertation, ETH Zurich, 1980, No. 6583.

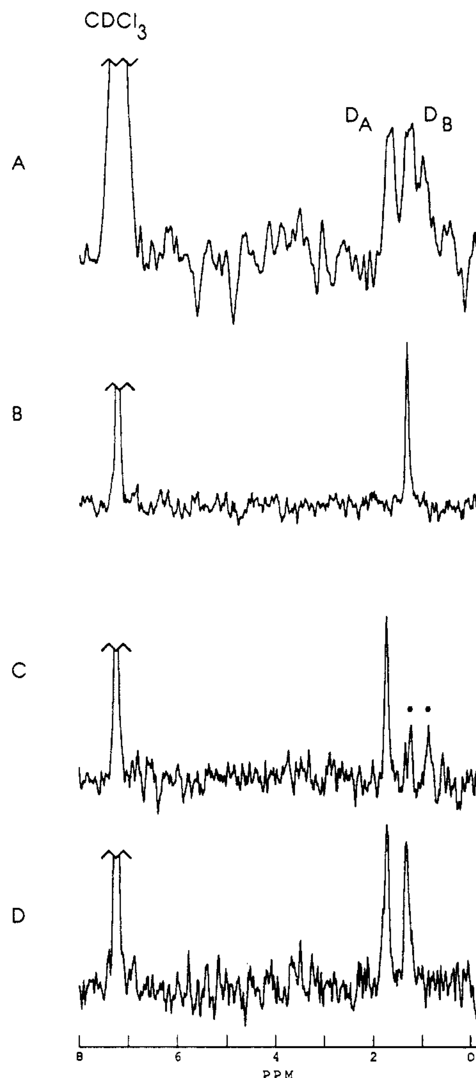


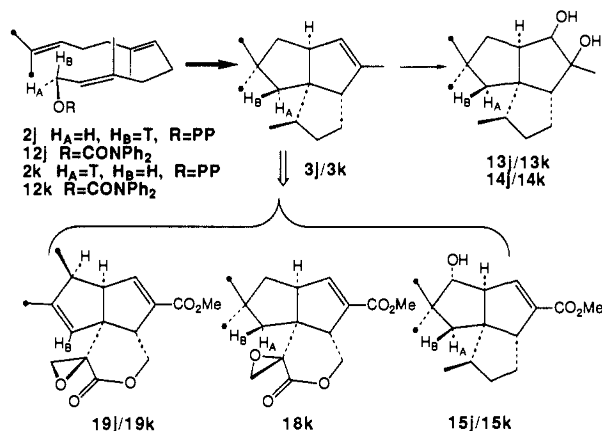
Figure 3. ^{2}H NMR spectra of pentalenene derived from (A) [1,1-^{2}H$_2$]FPP (**2g**), (B) (1*S*)-[1-^{2}H]FPP (**2h**), (C) (1*R*)-[1-^{2}H]FPP (**2i**), and (D) an equimolar mixture of **2h** and **2i**. Shifts are relative to natural abundance CDCl$_3$ at 7.24. Peaks marked with an asterisk are from hexane and are removed after repeated concentration from CHCl$_3$ (see spectrum D).

The requisite sample of (1*S*)-[1-^{2}H,12,13-^{14}C$_2$]FPP (**2h**) was next prepared by reduction of [1-^{2}H]farnesal with HLADH and NADH, after which [12,13-^{14}C$_2$]farnesol was added as an internal standard and the mixture was converted to the pyrophosphate ester.[21,23] After addition of (1*RS*)-[1-^{3}H]FPP to aid in monitoring the chromatographic purification, the resulting mixture of oligophosphate esters was separated by ion-exchange chromatography to afford **2h**. To obtain the 1*R* isomer **2i**, farnesal was treated with HLADH in a coupled reduction using catalytic NAD$^+$ and excess [1-^{2}H]cyclohexenol (**23**). Pyrophosphorylation of the resulting (1*R*)-[1-^{2}H]farnesol gave (1*R*)-[1-^{2}H,12,13-^{14}C$_2$]FPP (**2i**). Both **2h** and **2i** were converted to pentalenene (**3h**, 170 nmol, and **3i**, 585 nmol) by incubation with crude pentalenene synthase for 3 days at 4 °C.

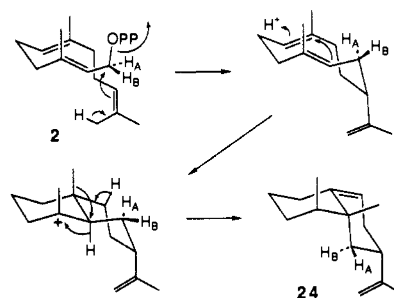
The ^{2}H NMR spectra of the resulting samples of pentalenene showed that the 1*S* isomer **2h** incorporated deuterium only into the 3 *si* position of pentalenene **3h**, while the 1*R* isomer **2i** yielded pentalenene (**3i**) that displayed a single resonance corresponding to deuterium in the 3 *re* configuration (Figure 3). These results establish that the conversion of FPP to pentalenene takes place with net inversion of configuration at C-1 of FPP.

Conversion of Pentalenene to Pentalenolactone. Stereochemistry of Proton Loss from C-3 of Pentalenene. Having established the stereochemistry of the enzymatic cyclization of FPP to pentalenene, it was possible to prepare samples of pentalenene stereospecifically labeled with tritium at C-3 and of known config-

Scheme IX



Scheme X



uration. Incubation of these samples with *Streptomyces* UC5319 revealed the stereochemistry of the proton elimination accompanying the metabolic conversion of pentalenene to pentalenolactone

The required (1*S*)-[1-^{3}H]FPP was prepared by reduction of [1-^{3}H]farnesal with HLADH and NADH followed by pyrophosphorylation of the resulting (1*S*)-[1-^{3}H]farnesol.[21,23] Purification by anion-exchange chromatography, after addition of [12,13-$^{14}C_2$]farnesyl pyrophosphate, gave **2j**. The derived diphenylurethane derivative **12j** was recrystallized to constant ^{3}H/^{14}C atom ratio (1:2). Pentalenene synthase, purified through the Sephadex G-100 gel filtration step,[8b] was used to convert **2j** to (3*S*)-[3-^{3}H,14,15-$^{14}C_2$]pentalenene (**3j**) (Scheme IX). A portion of **3j** was converted to *cis*-diols **13j** (^{3}H/^{14}C atom ratio 1:2) and **14j** (^{3}H/^{14}C atom ratio 1:2). The remainder of the labeled pentalenene **3j** was administered to cultures of *Streptomyces* UC5319, and the resulting oxidized metabolites were isolated and purified as the derived methyl esters. The recovered pentalenolactone methyl ester (**19j**) had a ^{3}H/^{14}C atom ratio of 1:2. As a control, pentalenic acid methyl ester (**15j**) (^{3}H/^{14}C atom ratio 1:2) was also isolated.

For the complementary series of experiments, (1*R*)-[1-^{3}H]-farnesol was prepared by reduction of farnesal with HLADH, NAD^+, and [1-^{3}H]cyclohexenol and then pyrophosphorylated as before.[21,23] Purification by anion-exchange chromatography, after addition of [12,13-$^{14}C_2$]FPP, gave **2k**. The diphenylurethane derivative **12k** was recrystallized to constant activity and isotope ratio (^{3}H/^{14}C atom ratio 1:2). Incubation of **2k** with pentalenene synthase gave (3*R*)-[3-^{3}H,14,15-$^{14}C_2$]pentalenene (**3k**), a portion of which was converted in the usual manner to the corresponding diols **13k** (^{3}H/^{14}C atom ratio 1:2) and **14k** (^{3}H/^{14}C atom ratio 1:2) (Scheme IX). Administration of pentalenene **3k** to cultures of *Streptomyces* UC5319 and purification of the resulting oxidized metabolites gave pentalenolactone methyl ester (**19k**) (^{3}H/^{14}C atom ratio 0:2), which retained only 1.6% of the original tritium label. By contrast, the pentalenic acid methyl ester (**15k**) (^{3}H/^{14}C atom ratio 0.9:2) and epipentalenolactone F methyl ester (**18k**) (^{3}H/^{14}C atom ratio 0.9:2) isolated from the same incubation each retained the bulk of the original tritium label.

Discussion

Stereochemistry of the Enzymatic Cyclization of Farnesyl Pyrophosphate to Pentalenene. As a result of extensive prior investigations, we have proposed a simple mechanistic and stereochemical model, illustrated in Scheme I, to account for the enzymatic conversion of *trans,trans*-farnesyl pyrophosphate (**2**) to the sesquiterpene hydrocarbon pentalenene (**3**).[7,15,32] The results described above are fully consistent with this picture and provide further details of the multistep cyclization process.

According to the mechanism of Scheme I, cyclization of farnesyl pyrophosphate is initiated by ionization of the pyrophosphate moiety and electrophilic attack of the resulting allylic cation on the *si* face of the C-10,11 double bond.[6] We have now shown that displacement of the pyrophosphate and formation of the new C–C bond take place with net *inversion* of configuration at C-1 of the precursor. Thus ^{2}H NMR analysis has shown unambiguously that (1*S*)-[1-^{2}H]FPP (**2h**) is converted exclusively to (3*S*)-[3-^{2}H]-pentalenene (**3h**), while (1*R*)-[1-^{2}H]FPP (**2i**) is transformed to the corresponding (3*R*)-[3-^{2}H]pentalenene (**3i**) (Scheme VIII). The observed inversion of configuration is analogous to the action of prenyltransferase, which catalyzes the stepwise condensation of the allylic pyrophosphate substrates dimethylallyl and geranyl pyrophosphate with isopentenyl pyrophosphate to give geranyl and farnesyl pyrophosphate, respectively[22] (Scheme II). Recent studies of aristolochene synthase, which catalyzes the cyclization of FPP to aristolochene (**24**) by way of a 10-membered ring intermediate, have established that this transformation also takes place with inversion of configuration at C-1 of FPP[33] (Scheme X). By contrast, cyclization of FPP and GPP to 6-membered rings in sesquiterpenes and monoterpenes, respectively, requires initial isomerization to the corresponding tertiary allylic isomers, nerolidyl and linalyl pyrophosphate,[32,34-36] and results in net *retention* of configuration at C-1 of the allylic pyrophosphate precursor.[20,36]

As illustrated in Scheme I, electrophilic attack on C-11 of FPP is followed by loss of a proton from C-9 to generate the 11-membered ring hydrocarbon humulene (**11**). Reprotonation of humulene at C-10 initiates further cyclization leading ultimately to generation of pentalenene.[37] From the relative and absolute configuration of the resultant pentalenene, we have inferred that the intermediate humulene must be folded in the *RSR*-CT conformation.[7,15,32] The initial electrophilic addition–proton elimination step, a formal $S_{E'}$ process, is in fact analogous to the corresponding condensation–elimination reaction catalyzed by prenyltransferase,[38] a transformation that has previously been shown to take place with net syn stereochemistry[22] (Scheme II). By incubation of (9*R*)- and (9*S*)-[9-^{3}H,12,13-$^{14}C_2$]FPP with pentalenene synthase and analysis of the resultant labeled pentalenenes by a combination of chemical and microbial degradation methods, we have previously shown that one of the two hydrogen atoms originally located at C-9 of FPP indeed becomes H-8 of pentalenene, whereas the second hydrogen is transferred without significant exchange with the medium to C-1 of **3**.[15] The latter observation was interpreted as indicating that in the postulated deprotonation–reprotonation sequence the proton that is removed from C-9 of the substrate is insulated from the bulk medium such that protonation at C-10 of the intermediate humulene is rapid compared to exchange.[37] On the basis of the proposed *RSR*-CT

(32) Cane, D. E. *Acc. Chem. Res.* **1985**, *18*, 220.

(33) Cane, D. E.; Prabhakaran, P. C.; Salaski, E. J.; Harrison, P. H. M.; Noguchi, H.; Rawlings, B. J. *J. Am. Chem. Soc.* **1989**, *111*, 8914.

(34) Cane, D. E. *Tetrahedron* **1980**, *36*, 1109.

(35) Croteau, R. *Chem. Rev.* **1987**, *87*, 929.

(36) Cane, D. E.; Ha, H. J. *J. Am. Chem. Soc.* **1988**, *110*, 6865.

(37) The generation of the 9-humulyl cation could in principle also take place by a hydride shift mechanism, without intervention of the neutral humulene intermediate, thereby explaining the absence of exchange between the original H-9 *si* proton of FPP and the medium. For a discussion, see ref 15. This alternative mechanism would not alter any of the stereochemical conclusions drawn in this and related papers.

(38) Poulter, C. D.; Rilling, H. C. *Acc. Chem. Res.* **1978**, *11*, 307. Poulter, C. D.; Argyle, J. C.; Mash, E. A. *J. Biol. Chem.* **1978**, *253*, 7227. Poulter, C. D.; Wiggins, P. L.; Le, A. T. *J. Am. Chem. Soc.* **1981**, *103*, 3926. Mash, E. A.; Gurria, G. M.; Poulter, C. D. *J. Am. Chem. Soc.* **1981**, *103*, 3927.

conformation of the latter intermediate, the transferred proton would be expected to attack the 10 *re* face of the humulene double bond and thereby be located at H-1 *si* (H-1α) of the resultant pentalenene. The essentially complete retention of tritium from [9-^{3}H,12,13-^{14}C$_2$]FPP also established for the first time that the conversion of FPP to pentalenene is catalyzed by a single enzyme, a conclusion subsequently confirmed by the purification of pentalenene synthase to homogeneity.[8b]

The full stereochemical details of the condensation–deprotonation–reprotonation sequence have now been established. Thus separate incubations of (9*R*)- and (9*S*)-[9-^{3}H]FPP (**2c** and **2d**) with pentalenene synthase and analysis of the sites of labeling in the derived samples of pentalenene established that H-9 *re* of **2** becomes H-8 of pentalenene while H-9 *si* undergoes net intramolecular transfer to H-1 of **3** (Scheme III). The formal S_E' reaction therefore is seen to take place with net anti stereochemistry. Although the demonstrated stereochemical course of the addition–elimination sequence is opposite to that observed with prenyltransferase,[22] the result is fully consistent with the postulated conformation of the cyclizing substrate, FPP, which prevents access by any enzymic base to the H-9 *re* proton. To establish the stereochemistry of the reprotonation reaction, we carried out an incubation of [10-^{2}H,11-^{13}C]FPP (**2e**) with pentalenene synthase (Scheme V). Analysis of the product by a combination of ^{2}H and ^{13}C NMR established that the deuterium atom occupied exclusively the predicted H-1 *re* (H-1β) position in pentalenene, consistent with transfer of the original H-9 *si* proton of FPP to the 10 *re* face of the humulene double bond, as illustrated. Further investigations of the mechanism of the cyclization reaction are in progress, including a determination of the stereochemistry of the final deprotonation event that generates the C-6,7 double bond of pentalenene.

Mechanism and Stereochemistry of the Oxidative Metabolism of Pentalenene. Pentalenolactone-producing cultures have been shown to produce a variety of oxidized metabolites, several of which have been proposed as plausible intermediates in the conversion of pentalenene to pentalenolactone. Although labeled pentalenene has indeed been incorporated into several of these metabolites, including pentalenic acid (**10**) and pentalenolactones E (**4**) and epi-E (**5**) as well as pentalenolactone (**1**), it has so far not been possible to confirm the proposed biosynthetic relationships among the various oxidized metabolites. The majority of these substances retain the *gem*-methyl substitution pattern found in the parent hydrocarbon pentalenene. It has been proposed that formation of the rearranged skeleton of pentalenolactone results from generation of a positive charge at C-1 of some intermediate, followed by sequential migration of the adjacent β-methyl group (C-14) and loss of a proton from C-3. Moreover, it has been attractive to speculate that the requisite cation could be generated by ionization of the *trans*-α-hydroxyl group found in metabolites such as pentalenic acid (**10**) or pentalenolactone H (**7**). Indeed, in vitro studies of the synthesis of pentalenolactone, carried out by Shirahama and Matsumoto,[39] have demonstrated the feasibility of such a rearrangement.

With a knowledge of the detailed origins of the diastereotopic hydrogen atoms attached to C-1 of pentalenene, it became possible to investigate the stereochemical course of the oxidation of pentalenene to pentalenic acid. To this end, a mixture of [10-^{2}H,12,13-^{14}C$_2$]- and [10-^{3}H,12,13-^{14}C$_2$]FPP (**2f**) was incubated with pentalenene synthase to give (1*S*)-[1-^{2}H,14,15-^{14}C$_2$]- and (1*S*)-[1-^{3}H,14,15-^{14}C$_2$]pentalenene (**3f**), the configuration of the deuterium being confirmed by ^{2}H NMR analysis. When the labeled pentalenene was administered to cultures of *Streptomyces* UC5319, the resultant pentalenic acid methyl ester (**15f**) retained 84% of the original tritium label, based on the observed change in ^{3}H/^{14}C ratio (Scheme VII). Oxidation of **15f** gave 1-oxopentalenic acid methyl ester (**22f**) that had lost 87% of the tritium, confirming the presence of tritium at C-1 of pentalenic acid and establishing that the microbial hydroxylation has taken place with net retention of configuration. This result complemented the finding that 1 equiv of tritium was lost upon formation of pentalenic acid from either (1*R*)-[1,8-^{3}H$_2$]- or (1*R*)-[1-^{3}H]pentalenene (**3b** or **3d**), obtained by enzymatic cyclization of (9*RS*)-[9-^{3}H]- or (9*S*)-[9-^{3}H]FPP (**2b** or **2d**), respectively (Scheme I and III). The net retention of configuration demonstrated for this hydroxylation reaction is consistent with the known course of numerous biological oxidations at unactivated methylene groups.[31]

Although the pentalenic acid methyl ester (**15d**) obtained after microbial oxidation of (1*R*)-[1-^{3}H,7,11-^{14}C$_2$]pentalenene (**3d**) (^{3}H/^{14}C atom ratio 1:2), itself produced by cyclization of (9*S*)-[9-^{3}H,4,8-^{14}C$_2$]FPP (**2d**) (^{3}H/^{14}C atom ratio 1:2), had lost all tritium (^{3}H/^{14}C atom ratio 0.03:2), the corresponding sample of pentalenolactone methyl ester (**19d**) isolated from the same incubation had an unchanged ^{3}H/^{14}C value (atom ratio 1:2). This observation corroborated the results of incorporation experiments in which [1,8-^{3}H$_2$,14,15-^{14}C$_2$]pentalenene (**3b**) (^{3}H/^{14}C atom ratio 2:2), derived from (9*RS*)-[9-^{3}H,12,13-^{14}C$_2$]FPP (**2b**) (^{3}H/^{14}C atom ratio 2:2), was converted by intact cells of *Streptomyces* UC5319 to pentalenic acid (Me ester, **15b**) (^{3}H/^{14}C atom ratio 1:2), which had lost half the original tritium, and pentalenolactone (Me ester, **19b**) (^{3}H/^{14}C atom ratio 1.7:2), which retained 85.5% of the original tritium label. In both sets of incorporation experiments, the ^{3}H/^{14}C ratio in the resulting epipentalenolactone F methyl esters (**18d** and **18b**) was unchanged compared to that of the pentalenene precursor. Since the tritium originally at H-1α in pentalenene is *lost* in pentalenic acid but *retained* in pentalenolactone, it is evident that pentalenic acid can be unambiguously excluded as an intermediate in the biosynthesis of pentalenolactone. Since pentalenolactone H (**7**) also possesses an α-hydroxyl group, it is also unlikely that **7** serves as a precursor of pentalenolactone, although there are as yet no direct data available to support this prediction.

Having established that cyclization of FPP to pentalenene takes place with inversion of configuration at C-1 of the precursor, it became possible to investigate the stereochemistry of the deprotonation at C-3 of the pentalenane skeleton, which is involved in the formation of pentalenolactone. To this end, (3*S*)-[3-^{3}H,14,15-^{14}C$_2$]pentalenene (**3j**) was prepared by the cyclization of (1*S*)-[1-^{3}H,12,13-^{14}C$_2$]FPP (**2j**) and fed to cultures of *Streptomyces* UC5319 (Scheme IX). The resulting labeled sample of pentalenolactone methyl ester (**19j**) retained all the tritium from the precursor. On the other hand, pentalenolactone **19k** derived from (3*R*)-[3-^{3}H,14,15-^{14}C$_2$]pentalenene (**3k**), itself prepared by cyclization of (1*R*)-[1-^{3}H,12,13-^{14}C$_2$]FPP (**2k**), had lost >98% of the original tritium label. Formation of the A-ring of pentalenolactone therefore occurs with stereospecific loss of H-3 *re* of **3**. This proton is anti to the 14-methyl group, which has been shown to migrate suprafacially to C-1.[6] Taken together with the fact that the formation of pentalenolactone also involves removal of H-1 *si* of pentalenene, it can be seen that the protons that are lost from C-1 and C-3 of pentalenene are on opposite faces of the A-ring.

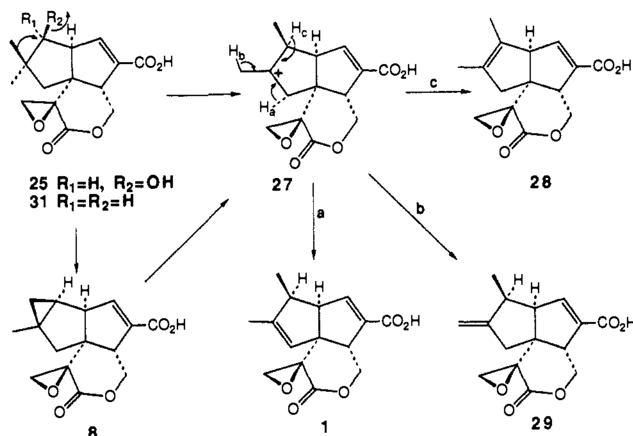
Since pentalenic acid (and, by extension, pentalenolactone H) has been excluded as an intermediate in the conversion of pentalenene to pentalenolactone, it is conceivable that **1** is derived from the as yet unobserved metabolites 1-epipentalenolactone H (**25**)[40] or 1-epipentalenic acid (**26**). Protonation or other activation of the hydroxyl group of **25** followed by ionization and net syn migration of the *vic*-methyl group would generate the tertiary carbocation **27**, which would undergo elimination of H-3α leading to the characteristic A-ring substitution pattern of pentalenolactone (Scheme XI). Alternatively, cation **27** might be generated by protonation of the cyclopropane ring of pentalenolactone P (**8**), itself derived from **25** by ionization of the hydroxyl group and insertion of the resultant cation into the *syn*-methyl group.[41] We

(39) Ohtsuka, T.; Shirahama, H.; Matsumoto, T. *Chem. Lett.* **1984**, 1923.

(40) Epipentalenolactone H has been reported as the product of treatment of pentalenolactone G with $NaBH_4$.[12,13]

(41) Cf. the intermediacy of cycloartenol in the conversion of squalene oxide to phytosterols: Goad, L. J. *Symp. Biochem. Soc.* **1970**, *29*, 45. Altman, L. J.; Han, C. Y.; Bertolino, A.; Hanly, G.; Laungani, D.; Muller, W.; Schwartz, S.; Shanker, D.; deWolf, W. H.; Yang, F. *J. Am. Chem. Soc.* **1978**, *100*, 3235.

Scheme XI



have recently isolated two additional metabolites, pentalenolactones A (**28**) and B (**29**), from cultures of *Streptomyces* UC5319.[42] The latter two substances are likely to be formed by loss of a proton from C-1 and C-15, respectively, of **25**. It is also conceivable that these rearrangements are initiated by oxidative removal of H-1 *si* from pentalenolactone F (**31**), which we have also recently isolated,[42] without intervention of the corresponding hydroxylated intermediate **25**.

Summary and Prospect

The above-described labeling experiments have provided a detailed picture of the mechanism and stereochemistry of the enzymatic conversion of farnesyl pyrophosphate to the sesquiterpene hydrocarbon pentalenene. This multistep cyclization, catalyzed by a single enzyme, is believed to be typical of a wide family of such transformations catalyzed by sesquiterpene synthases. In spite of the significant advances that have been made over the last several years in the isolation of terpenoid synthases and in the study of their characteristic reactions, essentially nothing is known about the active sites of these enzymes, nor have any of the amino acids participating in catalysis been identified. The means by which the cyclase enforces a particular folding of the substrate are as yet unknown, as is the manner in which the various reactive intermediates are stabilized. The answers to these and related questions are the subject of current investigation.

Experimental Section

General Procedures. All reactions requiring nonaqueous conditions were performed in oven-dried glassware under a positive pressure of nitrogen. All solvents were distilled. All reagents were recrystallized or were reagent grade or better. All buffers were prepared with deionized water from a Barnstead Nanopure system. The term in vacuo refers to the removal of solvent on a rotary evaporator followed by evacuation to constant sample weight (<0.05 mmHg). All reactions were followed by thin-layer chromatography (TLC) using either UV fluorescence or staining with iodine, arsenomolybdic acid, or *p*-anisaldehyde for visualization. Commercial TLC plates were Merck 60F-254. Flash chromatography was performed according to the method of Still et al.[43] on Merck Type 60 silica gel, 230–240 mesh. Melting points (mp) were determined on a Thomas-Hoover apparatus using open capillary tubes and are uncorrected. Liquid scintillation spectrometry was performed on a Beckman LS5801 scintillation counter, using Optifluor scintillation cocktail.

Biochemical Methods. Growth of *Streptomyces* UC5319 as well as preparation and assay of cell-free extracts of pentalenene synthase was as previously described.[15] Incorporation of labeled samples of pentalenene into oxidized metabolites by *Streptomyces* UC5319 was carried out as described.[7,15] Homogeneous avian prenyltransferase used in the preparation of **2c** was a generous gift from Professor C. D. Poulter of the University of Utah. Prenyltransferase used for the preparation of **2d** was isolated from chicken liver (Pel-Freeze Biologicals) and purified through the hydroxylapatite step to a specific activity of 30 nmol min^{-1} (mg of protein)$^{-1}$ (0.03 unit/mg).[44] Protein concentrations were determined by the Bradford dye-binding assay (Bio-Rad Laboratories).[45]

NMR Methods. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker WH-250 or AM-400 instruments in the specified deuterated solvent with tetramethylsilane (TMS) as internal standard for ^1H spectra and $CDCl_3$ as internal standard for ^{13}C spectra. Phosphorus-31 spectra were recorded at 162 MHz with 85% H_3PO_4 as external standard at a chemical shift of 0 ppm. Deuterium NMR spectra were recorded at 61 MHz in an unlocked mode with ^1H broad-band decoupling; the pulse width was 45° or 90° and acquisition time was 0.75 s. No relaxation delay was used. Natural abundance $CDCl_3$ (δ 7.24) was used as internal standard.

For the 2D ^1H-^{13}C heteronuclear shift correlation of pentalenene (**3**), 128 experiments were performed on a AM-400 instrument accumulating 128 scans per experiment in a 2K data block over a 5435-Hz sweep width centered at δ 39.5 (F2). The proton sweep width was 800 Hz centered at δ 1.8 (F1). The relaxation delay was 1 s, and the value of $J_{^{13}C-^1H}$ selected was 131 Hz. The data were zero-filled to 2K in F2 and to 1K in F1 and subjected to Fourier transformation using Lorentzian data manipulation.

For the 2D ^1H–^1H COSY of pentalenene (**3**), 128 experiments were performed on a AM-400 instrument accumulating 16 scans per experiment in a 2K data block over a 800-Hz sweep width centered at δ 3.01. The relaxation delay was 0.5 s. The data were zero-filled to 1K in F1 and subjected to Fourier transformation. The spectrum was symmetrized to improve appearance; this did not affect the overall results obtained.

The NOE difference spectra were recorded with the Bruker NOEDIFF.AUR program on an AM-400 instrument, using a 90° pulse, relaxation of 0.5 s, acquisition time of 4.5 s per scan, and 2–3-s irradiation time at a decoupler power of 25 μW (DP = 39L). A total of 4 dummy scans and 16 scans at each frequency irradiated and at a control frequency were collected during each experiment, and 40–128 experiments were performed. Each FID was subjected to Fourier transformation, and the spectrum at the control frequency was subtracted to give the difference spectrum.

[1-^{3}H]Cyclohex-2-en-1-ol. Cyclohexenone (50 mg, 520 μmol) and cerium(III) chloride heptahydrate (194 mg, 500 μmol) were dissolved in methanol (1.3 mL), and the solution was cooled to 0 °C. $NaBH_3T$ (2.97 mg, 25 mCi, 80 μmol) was added. The mixture was allowed to stand at 0 °C for 30 min, warmed to room temperature, and stirred for 3 h. The solution was extracted with hexanes (3 × 1 mL). The organic layers were dried ($MgSO_4$) and loaded onto a flash chromatography column. Elution with 15% ethyl acetate/hexanes gave labeled fractions, which were pooled and concentrated to give 3.21 mCi (13%) of [1-^{3}H]-cyclohex-2-en-1-ol that was dissolved in 0.05 M phosphate buffer, pH 7.4 (4 mL). TLC and NMR showed the product to be free of cyclohexenone and cyclohexanol: ^1H NMR ($CDCl_3$, 250 MHz) δ 5.83 (abdt, J_{ab} = 10.0, J_d = 0.8, J_t = 3.8 Hz, 1 H, CH=C*H*CHOH), 5.75 (abdt, J_{ab} = 10.0, J_d = 2.0, J_t = 3.0 Hz, 1 H, C*H*=CHCHOH), 4.20 (br m, 1 H, C*H*OH), 2.1–1.5 (m, 7 H, 3CH_2 and O*H*).

(1*R*)-[1-^{3}H]-3-Methyl-2-buten-1-ol. 3-Methyl-2-buten-1-al (10 μL, 106 μmol), NAD^+ (0.5 mg, 0.75 μmol), and HLADH (2 mg, 3 units) were dissolved in 0.1 M phosphate buffer, pH 7.4 (3 mL). A solution of [1-^{3}H]cyclohex-2-en-1-ol in buffer (0.5 mL, 1.4 × 10^{10} dpm, 8 μmol) was added, and the mixture was kept in the dark at room temperature for 24 h. A second portion of HLADH (1 mg, 1.5 units) was added and the mixture was incubated a further 24 h. Methanol (4 mL) was added and the mixture was extracted with diethyl ether (3 × 5 mL). The extracts were dried (Na_2SO_4) and concentrated by distillation through a Vigreux column to give 1.8 × 10^9 dpm (13%) of crude alcohols.

(1*R*)-[1-^{3}H]-3-Methyl-2-buten-1-yl 3,5-Dinitrobenzoate. A mixture of (1*R*)-[1-^{3}H]-3-methyl-2-buten-1-ol (2.65 × 10^7 dpm), 3-methyl-2-buten-1-ol (20 μL, 200 μmol), and 3,5-dinitrobenzoyl chloride (200 mg, 870 μmol) in pyridine (1 mL) was stirred for 1 h at room temperature and then heated at 60 °C for 15 min. Diethyl ether (50 mL) was added, and the mixture was washed with 2 M HCl (3 × 20 mL), saturated $NaHCO_3$ (3 × 20 mL), and brine (3 × 20 mL). The organic layers were dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was recrystallized to constant specific activity from hexanes (5 mL): 1.8 × 10^4 dpm/μmol; mp 71–72 °C. The radiochemical purity of the alcohol was therefore 14%.

(1*R*)-[1-^{3}H]-3-Methyl-2-buten-1-yl Pyrophosphate. A mixture of (1*R*)-[1-^{3}H]-3-methyl-2-buten-1-ol (2.7 × 10^9 dpm), 3-methyl-2-buten-1-ol (10 μL, 100 μmol), bis(triethylammonium) phosphate (75 mg, 250 μmol), and trichloroacetonitrile (80 μL, 800 μmol) in dry acetonitrile (0.4 mL) was stirred at room temperature for 3 h. A further aliquot of CCl_3CN (100 μL, 1 mmol) was added and the solution was stirred a

(42) Cane, D. E.; Sohng, J.-K. Unpublished results.
(43) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, *43*, 2923.
(44) Reed, B. C.; Rilling, H. C. *Biochemistry* **1975**, *14*, 50.
(45) Bradford, M. *Anal. Biochem.* **1976**, *72*, 248. Spector, T. *Anal. Biochem.* **1977**, *83*, 773.

further 7 h. The mixture was diluted (H_2O, 3 mL) and extracted (Et_2O, 3 × 3 mL). The aqueous phase was chromatographed on a column of DEAE-Sephadex A-25 (20 mL) eluted with a triethylammonium bicarbonate gradient (0.05–1 M, pH 8, 360 mL). Fractions containing the pyrophosphate were combined and lyophilized. The product (3.2 × 10^7 dpm, 1%) was stored in dilute ammonia at 4 °C.

Preparation of (1*R*)-[1-^{3}H,1-^{14}C]-3-Methyl-2-buten-1-ol from (1*R*)-[1-^{3}H]-3-Methyl-2-buten-1-yl Pyrophosphate. A mixture of the pyrophosphate (50 μL, 1.14 × 10^6 dpm) and acid phosphatase (17 mg, 7 units) in 0.2 M acetate buffer, pH 5.5, containing 10 mM $MgCl_2$ (2 mL) was incubated at 30 °C for 1.5 h. Ethanol (2 mL) was added and the solution was extracted with diethyl ether (2 × 4 mL). The extracts were washed (brine, 3 × 2 mL), dried (Na_2SO_4), and mixed with 0.5 μmol (9 × 10^4 dpm) of [1-^{14}C]-3-methyl-2-buten-1-ol [prepared by treatment of ethereal isobutenylmagnesium chloride with [^{14}C]CO_2,[46,47] isomerization of the resulting [1-^{14}C]-3-methyl-3-butenoic acid with 25% aqueous KOH at reflux for 18 h, esterification with diazomethane, and reduction with $LiAl(OEt)H_3$[48]] to give a solution of (1*R*)-[1-^{3}H,1-^{14}C]-3-methyl-2-buten-1-ol.

(1*R*)-[1-^{3}H,1-^{14}C]-3-Methyl-2-buten-1-yl 3,5-Dinitrobenzoate. To half of the above solution were added 3-methyl-2-buten-1-ol (20 μL, 200 μmol), pyridine (1 mL), and 3,5-dinitrobenzoyl chloride (200 mg, 870 μmol). Reaction and workup as above gave the crude ester, which was recrystallized to constant specific activity; ^{3}H/^{14}C 5.23.

[1-^{14}C]-3-Methyl-2-buten-1-al and Semicarbazone Derivative. The remaining solution of (1*R*)-[1-^{3}H,1-^{14}C]-3-methyl-2-buten-1-ol was concentrated at 1 atm and treated with HLADH (1.6 units) and NAD^+ (5 mg) in 0.1 M glycine buffer, pH 8.8 (2 mL), for 10 min at room temperature. Carrier 3-methyl-2-buten-1-al (20 mg) was added, followed by a solution of semicarbazide hydrochloride (0.5 g, 4.5 mmol) in water (2 mL), and the precipitate was collected and washed (2 × H_2O, 1 mL). The crude product was recrystallized from ethanol to constant specific activity; ^{3}H/^{14}C 0.04; mp 220 °C dec (lit.[49] mp 221–222 °C).

(9*R*)-[9-^{3}H,4,8-$^{14}C_2$]Farnesyl Pyrophosphate (2c). A mixture of (1*R*)-[1-^{3}H]DMAPP (50 μL, 1.6 × 10^6 dpm), isopentenyl pyrophosphate (197 μg, 0.7 μmol), [4-^{14}C]isopentenyl pyrophosphate (50 μL, 1.6 × 10^6 dpm), and homogeneous avian prenyltransferase (2 μL, 16 μg) in 10 mM phosphate buffer, pH 7.4, containing 1 mM $MgCl_2$, 1 μM NaN_3, and 1 mM DTT (1 mL) was incubated at 30 °C for 24 h. The solution was applied to an anion-exchange column of DEAE-Sephadex and eluted with a gradient of triethylammonium bicarbonate (0.05–1.0 M), as described above for DMAPP. Partial separation of IPP and DMAPP from FPP was obtained. The FPP-containing fractions were pooled and lyophilized to give 154 nmol (44% based on IPP) of (9*R*)-[9-^{3}H,4,8-$^{14}C_2$]farnesyl pyrophosphate (**2c**).

(9*R*)-[9-^{3}H,4,8-$^{14}C_2$]Farnesyl Diphenylurethane (12c). Phosphatase-catalyzed hydrolysis of (9*R*)-[9-^{3}H,4,8-$^{14}C_2$]farnesyl pyrophosphate (0.1 μmol) in the manner described for DMAPP gave (9*R*)-[9-^{3}H,4,8-$^{14}C_2$]-farnesol, which was isolated after addition of carrier farnesol (30 mg, 135 μmol). The derived diphenylurethane was prepared by reaction with diphenylcarbamoyl chloride (70 mg, 300 μmol) and pyridine (2 mL) at 105 °C for 14 h. The mixture was cooled, diluted with HCl (5 mL), and extracted (Et_2O, 3 × 5 mL). The combined organic extracts were washed (saturated $CuSO_4$, 5 mL, then brine, 5 mL), dried ($MgSO_4$), filtered, and concentrated in vacuo. Flash chromatography (hexanes, then EtOAc/hexanes containing 1%, 2%, 3%, 4%, 5%, and 6% EtOAc) of the resulting oil gave 47 mg (113 μmol, 84%) of **12c**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.14–7.35 (m, 10 H, Ar*H*), 5.32 (td, *J* = 7.0, 1.0 Hz, 1 H, C=C*H*-2), 5.08 (tm, *J* = 6.7 Hz, 2 H, 2C=C*H*), 4.68 (d, *J* = 6.9 Hz, 2 H, C*H*$_2$O), 2.02 (m, 8 H, C*H*$_2$), 1.67 (s, 6 H, C*H*$_3$), 1.59 (s, 3 H, C*H*$_3$), 1.58 (s, 3 H, C*H*$_3$). The diphenylurethane was recrystallized from MeOH to constant melting point and ^{3}H/^{14}C ratio: mp 61–62 °C (lit.[50] mp 61–63 °C); ^{3}H/^{14}C (average of last three crystallizations) 1.43 ± 0.02.

[8-^{3}H,7,11-$^{14}C_2$]Pentalenene (3c). A cell-free extract of *Streptomyces* UC5319 was prepared as described previously[8,15] and purified through the 65% ammonium sulfate step to give 25 mg of protein. The pellet was resuspended in 50 mM phosphate buffer, pH 7.2, containing 5 mM DTE, 1 mM EDTA, 10% (v/v) glycerol, and 15 mM $MgCl_2$. The (9*R*)-[9-^{3}H,4,8-$^{14}C_2$]farnesyl pyrophosphate (**2c**) (9.2 × 10^5 dpm of ^{14}C) was added, and the mixture was incubated at 25 °C for 2 h. Acetone (20 mL) was added, and the mixture was extracted with pentane (3 × 50 mL) containing carrier pentalenene (3 mg) and carrier farnesol (30 μL). The extracts were dried and concentrated on the rotary evaporator. Flash chromatography (silica; pentane, then diethyl ether) gave pentalenene (**3c**) (3.5 × 10^4 dpm of ^{14}C, 3.8%; ^{3}H/^{14}C 1.48) and farnesol (6.3 × 10^4 dpm of ^{14}C, 7%).

Conversion of [8-^{3}H,7,11-$^{14}C_2$]Pentalenene to [8-^{3}H,7,11-$^{14}C_2$]-(7*S*,8*R*)- and -(7*R*,8*S*)-7,8-Dihydroxypentalenanes (13c and 14c). Labeled pentalenene (**3c**) (1.12 × 10^4 dpm of ^{14}C) was diluted with carrier (21 mg, 103 μmol) and treated with OsO_4 (31.8 mg, 118 μmol) in pyridine (0.3 mL) under nitrogen for 14 h at room temperature. The mixture was diluted with a solution of $NaHSO_3$ (200 mg) in H_2O (2 mL), stirred for 60 min, and extracted with Et_2O (3 × 5 mL). The combined extracts were washed with saturated $CuSO_4$ and then brine (5 mL each), dried ($MgSO_4$), and concentrated in vacuo to give 23.5 mg (99 μmol, 96%) of crude diols. The mixture of diols was separated by fractional crystallization, and each was recrystallized to constant melting point, ^{14}C specific activity, and ^{3}H/^{14}C ratio. The minor β-isomer **14c** was crystallized from 50% CH_2Cl_2/hexanes: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.81 (d, *J* = 8.9 Hz, 1 H, C*H*OH), 1.1–2.45 (m, 13 H, C*H*, C*H*$_2$, O*H*), 1.24 (s, 3 H, C*H*$_3$), 1.06 (s, 3 H, C*H*$_3$), 0.97 (s, 3 H, C*H*$_3$), 0.91 (d, *J* = 7.0 Hz, 3 H, C*H*$_3$); mp 147–147.5 °C; 124.3 ± 5.3 dpm/μmol ^{14}C; ^{3}H/^{14}C 1.49. The major α-isomer **13c** was crystallized from hexanes: ^{1}H NMR δ 3.77 (d, *J* = 8.4 Hz, 1 H, C*H*OH), 1.1–2.45 (m, 13 H, C*H*, C*H*$_2$, O*H*), 1.20 (s, 3 H, C*H*$_3$), 1.11 (s, 3 H, C*H*$_3$), 1.03 (s, 3 H, C*H*$_3$), 0.86 (d, *J* = 6.9 Hz, 3 H, C*H*$_3$); mp 87–89 °C; 126.3 ± 0.4 dpm/μmol ^{14}C; ^{3}H/^{14}C 1.47.

[8-^{3}H,7,11-$^{14}C_2$]-7-Hydroxypentalenane. Labeled pentalenene (**3c**) (1.12 × 10^4 dpm of ^{14}C) was diluted with carrier (21 mg, 103 μmol) and dry THF (0.5 mL) and treated with 1 M borane in THF (500 μL, 500 μmol) under nitrogen for 1 h at 0 °C. A further portion of 1 M borane in THF (500 μL, 500 μmol) was added and the reaction continued for 1 h. Water (150 μL), 10% NaOH (1 mL), and 30% H_2O_2 (1 mL) were added and the mixture was heated at 55 °C for 1.5 h. The solution was extracted with diethyl ether (3 × 4 mL), and the extracts were washed with saturated $NaHSO_3$ (3 × 2 mL) and brine (3 × 2 mL), dried (Na_2SO_4), filtered, and concentrated in vacuo to give 20.2 mg (88%) of [8-^{3}H,7,11-$^{14}C_2$]-7-hydroxypentalenane: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 3.52 (dd, *J* = 9.4, 7.7 Hz, 1 H, C*H*OH), 1.15–2.0 (m, 13 H, C*H*, C*H*$_2$), 1.09 (s, 3 H, C*H*$_3$), 1.02 (d, *J* = 6.5 Hz, 3 H, C*H*$_3$), 1.00 (s, 3 H, C*H*$_3$), 0.88 (d, *J* = 6.9 Hz, 3 H, C*H*$_3$); 114.8 dpm/μmol ^{14}C; ^{3}H/^{14}C 1.43.

[8-^{3}H,7,11-$^{14}C_2$]Pentalen-7-one (16c). Labeled 7-hydroxypentalenane (13.8 mg, 62 μmol) in CH_2Cl_2 (2 mL) was treated with pyridinium chlorochromate (30 mg, 139 μmol). After 2 h, diethyl ether (2 mL) was added, and the mixture was filtered through Florisil. The filtrates were concentrated in vacuo to give 12.0 mg (88%) of [8-^{3}H,7,11-$^{14}C_2$]pentalen-7-one (**16c**): ^{1}H NMR ($CDCl_3$, 250 MHz) δ 2.52 (ddd, *J* = 9.7, 5.0, 1.4 Hz, 1 H, C*H*CO), 1.0–2.2 (m, 11 H, C*H*, C*H*$_2$), 1.08 (d, *J* = 8.6 Hz, 3 H, C*H*$_3$), 1.00 (s, 3 H, C*H*$_3$), 0.98 (d, *J* = 6.6 Hz, 3 H, C*H*$_3$), 0.85 (s, 3 H, C*H*$_3$); 113.6 dpm/μmol ^{14}C; 3H/^{14}C 1.46.

[6,8-$^2H_2$,7,11-$^{14}C_2$]Pentalen-7-one. Pentalen-7-one (**16c**) (10.2 mg, 46.3 μmol), dioxane (1.5 mL), and 0.7 M NaOD in D_2O (1.2 mL) were heated at 110 °C for 12 h. The cooled solution was saturated with NaCl and extracted with diethyl ether (2 × 4 mL). The extracts were washed until neutral with brine, dried (Na_2SO_4), filtered, and evaporated to give 8.9 mg (86%) of [6,8-$^2H_2$,7,11-$^{14}C_2$]pentalen-7-one: ^{1}H NMR ($CDCl_3$, 400 MHz) showed a mixture of diastereomers, the major isomer being identical with the starting material [the signal at δ 2.52 (H-8) had disappeared, and δ 1.08 (C*H*$_3$-13) collapsed from a doublet to a singlet due to complete exchange of H-6 and H-8]; 98.5 dpm/μmol ^{14}C; ^{3}H/^{14}C 0.49. A second exchange as above with NaOH/H_2O gave a sample with 102.4 dpm/μmol ^{14}C and ^{3}H/^{14}C 0.45.

Enzymatic Oxidation of Farnesol and Chemical Reduction. Farnesol (1 × 10^4 dpm of ^{14}C, 20 μmol) recovered from the incubation of (9*R*)-[9-^{3}H,4,8-$^{14}C_2$]FPP (**2c**) with pentalenene synthase was oxidized with HLADH and NAD^+ as described for 3-methyl-2-buten-1-ol. After addition of carrier farnesal (20 mg), flash chromatography (silica; 30% EtOAc/hexanes) gave farnesal (5000 dpm, 50%). Reduction with $NaBH_4$ (20 mg, 530 μmol) in EtOH (2 mL) yielded 21.5 mg (100%) of farnesol after the solution was quenched with saturated NH_4Cl and extracted into ether.

Farnesyl Diphenylurethane Derivatives. The diphenylurethane derivative **12c** of farnesol recovered from the incubation of (9*R*)-[9-^{3}H,4,8-$^{14}C_2$]FPP with pentalenene synthase was prepared as described above (sample A, 85%). In like manner, the farnesol that had been subjected to HLADH oxidation and $NaBH_4$ reduction was also converted to the diphenylurethane (sample B, 37%). Each sample was recrystallized 8 times, and the average of the constant ^{3}H/^{14}C ratios for crystallizations 5–8 was determined: sample A, 1.45; sample B, 1.28.

Incorporation of [8-^{3}H,7,11-$^{14}C_2$]Pentalene (3c) into Oxidized Metabolites by *Streptomyces* UC5319. Labeled pentalenene (**3c**) was ad-

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ministered to 20 500-mL flasks of *Streptomyces* UC5319 as previously described.[15] The recovered pentalenic acid methyl ester (**15c**) (21.5 mg, 85.3 μmol) was purified in the usual manner (8000 dpm of ^{14}C, 9.4 mg): 3.9 dpm/μmol ^{14}C; $^{3}H/^{14}C$ 1.44.

[1-^{3}H]-3-Methyl-2-Buten-1-ol. To sodium borotritide (25 mCi, 48 μmol) at 0 °C was added a solution of 3-methyl-2-butenal (10 mg, 120 μmol) in ethanol (0.4 mL). After 12 h, water (0.5 mL) was added, and the mixture was extracted with diethyl ether (3 × 5 mL). The extracts were dried (Na_2SO_4), filtered, and concentrated at 1 atm to give 7.31 mCi of crude [1-^{3}H]-3-methyl-2-buten-1-ol.

[1-^{3}H]-3-Methyl-2-Butenal. To a stirred suspension of pyridinium chlorochromate (100 mg, 464 μmol) in CH_2Cl_2 (2 mL) was added a solution of [1-^{3}H]-3-methyl-2-buten-1-ol (7.31 mCi) in CH_2Cl_2 (1 mL). The mixture was stirred for 1 h and diethyl ether (25 mL) was added. The suspension was filtered through Florisil and the solvent removed under nitrogen at 1 atm. Flash chromatography (silica; 10% Et_2O/hexanes) gave 1.22 mCi (17%) of [1-^{3}H]-3-methyl-2-butenal.

(1*S*)-[1-^{3}H]-3-Methyl-2-buten-1-ol. To a solution of [1-^{3}H]-3-methyl-2-butenal (1.22 mCi) and NADH (107 mg, 137 μmol) in 0.05 M phosphate buffer, pH 8.0 (400 mL), was added HLADH (20 mg, 30 units). After incubation at 30 °C for 4 h, additional NADH (100 mg) and HLADH (20 mg) were added, and the incubation was continued for 12 h. The solution was saturated with NaCl and extracted with diethyl ether (8 × 50 mL). The extracts were dried (Na_2SO_4), filtered, and concentrated at 1 atm under nitrogen. Flash chromatography (silica; 10% Et_2O/hexanes) gave 57 μCi (5%) of recovered [1-^{3}H]-3-methyl-2-butenal and 203 μCi (17%) of (1*S*)-[1-^{3}H]-3-methyl-2-buten-1-ol.

(1*S*)-[1-^{3}H]-3-Methyl-2-buten-1-yl Pyrophosphate. Pyrophosphorylation as for (1*R*)-[1-^{3}H]DMAPP gave 3.1 μCi (1.5%) of (1*S*)-[1-^{3}H]-3-methyl-2-buten-1-yl pyrophosphate from 203 μCi of (1*S*)-[1-^{3}H]-3-methyl-2-buten-1-ol.

(9*S*)-[9-^{3}H,4,8-$^{14}C_2$]Farnesyl Pyrophosphate (2d). (1*S*)-[1-^{3}H]-3-Methyl-2-buten-1-yl pyrophosphate (79 nCi, 1.26 nmol) and [4-^{14}C]IPP (53 nCi, 1.05 nmol) were incubated with partially purified avian prenyltransferase (0.13 unit, specific activity 0.03 unit/mg) for 2 h at 37 °C in 6 mL of 20 mM bicyclo[2.2.1]hept-2-ene-2,3-dicarboxylic acid (BHDA) buffer,[51] pH 7.0, containing 1.0 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.01% bovine serum albumin. Assay for FPP demonstrated the production of 0.324 nmol of **2d**. The material from four such incubations in buffer (10.1 mL), containing 3.28 × 10^5 dpm of ^{3}H and 1.97 × 10^5 dpm of ^{14}C, was diluted with methanol (1.73 mL), and the protein was removed by ultrafiltration through an Amicon YM-30 membrane. After the membrane was washed with 15% methanol in 20 mM BHDA, pH 7.4, 85% (2 × 1.55 mL), the clear ultrafiltrate (12.85 mL) contained 4.57 × 10^5 dpm of ^{3}H (70%) and 2.61 × 10^5 dpm of ^{14}C (70%). Most of the ^{14}C was shown to be acid labile. The ultrafiltrate was diluted with stock solution 1 (0.31 mL), prepared from tetra-*n*-butylammonium hydrogen sulfate (6.8 g) and dipotassium hydrogen phosphate (5.5 g) in water (100 mL), adjusted to pH 8.0 with 5 M KOH, and filtered through a Millipore HA filter. The mixture was injected directly onto a C_{18} reverse-phase ion-pairing HPLC column, which was developed with a linear gradient as previously described.[24] Fractions were assayed for ^{14}C and ^{3}H by liquid scintillation spectrometry. Unreacted [^{14}C]IPP and [^{3}H]DMAPP were not retained on the column. The (9*S*)-[9-^{3}H,4,8-^{14}C2]farnesyl pyrophosphate (**2d**) eluted at approximately 1 mM Bu_4N^+. Combined fractions were lyophilized to a small volume to give 40 900 ± 900 dpm of ^{3}H and 70 900 ± 1200 dpm of ^{14}C (27% of applied ^{14}C counts). DEAE-Sephadex chromatography as for **2c** gave farnesyl pyrophosphate **2d**: 10 700 dpm of ^{14}C; specific activity 100 μCi/μmol ^{14}C; 0.32 nmol; $^{3}H/^{14}C$ 0.577 ± 0.007.

(9*S*)-[9-^{3}H,4,8-$^{14}C_2$]Farnesol. Radioactive farnesyl pyrophosphate (**2d**, 7000 dpm of ^{14}C) was treated with acid phosphatase as described above. Addition of carrier farnesol (71.8 mg) and workup afforded 64.5 mg (90% chemical recovery) of farnesol: 4430 ± 70 dpm of ^{14}C, 62% radiochemical yield; specific activity 3.3 pCi/μmol ^{3}H, 6.9 pCi/μmol ^{14}C; $^{3}H/^{14}C$ 0.481 ± 0.009.

(9*S*)-[9-^{3}H,4,8-$^{14}C_2$]Farnesyl Diphenylurethane (12d). The above sample of labeled farnesol (61.9 mg, 278 μmol, 4260 dpm of ^{14}C) was converted to the diphenylurethane **12d**: 95.2 mg (228 μmol, 82%); 3350 ± 50 dpm of ^{14}C, 79% radiochemical yield; $^{3}H/^{14}C$ 0.512 ± 0.010. The material was recrystallized 4 times from MeOH to constant melting point, ^{3}H and ^{14}C specific activities, and $^{3}H/^{14}C$ ratio: specific activity 3.4 pCi/μmol ^{3}H, 6.7 pCi/μmol ^{14}C; $^{3}H/^{14}C$ (average of last three crystallizations) 0.497 ± 0.009.

Conversion of (9*S*)-[9-^{3}H,4,8-$^{14}C_2$]Farnesyl Pyrophosphate (2d) to (1*R*)-[1-^{3}H,7,11-$^{14}C_2$]Pentalenene (3d) by Pentalenene Synthase. (9*S*)-[9-^{3}H,4,8-$^{14}C_2$]Farnesyl Pyrophosphate (**2d**, 10 600 dpm of ^{14}C, 0.40 nmol) was dissolved in 200 mM Tris buffer, pH 8.4, containing 20 mM $MgCl_2$ and 5 mM β-mercaptoethanol (300 μL), to a concentration of 1.3 μM FPP. Partially purified pentalenene synthase (specific activity 545 nmol mg^{-1} h^{-1}, total activity 13.6 nmol/h) was added, and the mixture was incubated at 30 °C for 1 h. Carrier pentalenene (2.7 mg) in hexanes (1 mL) was added, and the phases were separated after mixing. After further extraction with hexanes (4 × 1 mL), the combined hexanes layers were passed through a flash chromatography column (hexanes). Fractions were concentrated on the rotary evaporator at room temperature to afford **3d**: 5110 ± 60 dpm of ^{14}C, 48% radiochemical yield; $^{3}H/^{14}C$ 0.502 ± 0.006.

Conversion of (1*R*)-[1-^{3}H,7,11-$^{14}C_2$]Pentalene (3d) to (1*R*)-[1-^{3}H,7,11-$^{14}C_2$]-(7*S*,8*R*)- and -(7*R*,8*S*)-7,8-Dihydroxypentalenanes (13d and 14d). Labeled pentalenene (**3d**, 2550 dpm of ^{14}C) was diluted with carrier (41.9 mg, 205 μmol) and treated with OsO_4 (78.2 mg, 308 μmol) in pyridine (0.6 mL) under nitrogen for 14 h at room temperature. The mixture was diluted with pyridine (0.65 mL) and a solution of $NaHSO_3$ (425 mg) in H_2O (5 mL), stirred for 30 min, and extracted with Et_2O (3 × 5 mL). The combined extracts were washed with H_2O (10 mL), dried ($MgSO_4$), and concentrated in vacuo to give 47.2 mg (198 μmol, 97%) of crude diols. Flash chromatography (30/70 EtOAc/hexanes) afforded 37.8 mg (159 μmol, 77%) of the mixture of diastereomeric diols: 1940 dpm of ^{14}C (76% radiochemical yield); $^{3}H/^{14}C$ 0.477 ± 0.011. The diols were separated by fractional crystallization, and each was recrystallized to constant melting point, ^{3}H and ^{14}C specific activity, and $^{3}H/^{14}C$ ratio: 46 ± 1 pCi/μmol ^{3}H, 97 ± 1 pCi/μmol ^{14}C; $^{3}H/^{14}C$ ratio (**13d**) 0.478 ± 0.010, (**14d**) 0.486 ± 0.009.

Incorporation of (1*R*)-[1-^{3}H,7,11-$^{14}C_2$]Pentalenene (3d) into Oxidized Metabolites by *Streptomyces* UC5319. Labeled pentalenene (**3d**, 5300 dpm of ^{14}C, 3.2 mg) was administered as described above to six culture flasks of *Streptomyces* UC5319 to give the following products. Pentalenolactone methyl ester (**19d**): 11.2 mg; 400 dpm of ^{14}C; 4.64 ± 0.07 pCi/μmol ^{14}C; specific incorporation 3.0%; $^{3}H/^{14}C$ 0.483 ± 0.008. Epipentalenolactone F methyl ester (**18d**): 0.6 mg; 41 dpm of ^{14}C; 9.1 ± 0.2 pCi/μmol ^{14}C; specific incorporation 6%; $^{3}H/^{14}C$ 0.525 ± 0.023. Pentalenic acid methyl ester (**15d**): 1.3 mg; 187 dpm of ^{14}C; 16.3 ± 0.2 pCi/μmol ^{14}C; specific incorporation 11%; $^{3}H/^{14}C$ 0.014 ± 0.006.

(1*RS*)-[1-^{2}H(^{3}H)]-10-Acetoxy-4,8-dimethyldeca-4,8-dien-1-ol. Unlabeled trisnoraldehyde acetate (**20**) (143 mg, 601 μmol), prepared as described previously,[23] was dissolved in MeOH and cooled to 0 °C before addition of $NaBD_4$ (13.2 mg, 315 μmol). After the solution was stirred for 5 min at 0 °C, 1 M HCl (5 mL) was added and the reaction mixture was extracted with hexanes (3 × 5 mL). After addition of alcohol prepared in like manner from trisnoraldehyde acetate (2.4 mg, 10 μmol), $NaBH_3T$ (30 μg, 0.78 μmol, 319 mCi/mmol), and $NaBH_4$ (2 mg, 52 μmol), the combined organic layers were dried over $MgSO_4$ and concentrated by rotary evaporation to give 136 mg of alcohol (564 μmol, 94%): radiochemical yield 25.2 μCi, 10%; specific activity 44 nCi/μmol; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.33 (tq, J = 7.1, 1.2 Hz, 1 H, C=C*H*CH_2OAc), 5.13 (tq, J = 6.9, 1.1 Hz, 1 H, C=C*H*), 4.59 (d, J = 7.0 Hz, 2 H, CH_2OAc), 3.59 (tt, J = 6.9 Hz, $^{2}J_{H-D}$ = 1.2 Hz, 1 H, C*H*DOH), 2.12 (tt, J = 7.6, 7.0 Hz, 2 H, CH_2), 2.06 (s, 3 H, CH_3CO_2), 2.06 (m, 4 H, 2CH_2), 1.70 (s, 3 H, CH_3), 1.67 (dt, J = 6.9, 6.9 Hz, 2 H, CH_2CHDOH), 1.61 (s, 3 H, CH_3), 1.54 (br s, 1 H, O*H*).

[10-^{2}H(^{3}H)]Farnesol Trisnoraldehyde Acetate (20). A mixture of the above-prepared [1-^{2}H(^{3}H)]alcohol (136 mg, 5.64 μmol, 5.63 × 10^7 dpm) and pyridinium dichromate (1.29 g, 3.43 mmol) in CH_2Cl_2 was stirred at room temperature overnight. Diethyl ether (5 mL) was added and the mixture was filtered through Celite. The filtrate was concentrated in vacuo. Flash chromatography (silica; 10% EtOAc/hexanes, then 30% EtOAc/hexanes) gave aldehyde **20** (76.4 mg, 320 mmol, 56%; 3.32 × 10^7 dpm, 59% radiochemical yield) and then recovered alcohol (26%). **20**: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 9.67 (t, J = 1.9 Hz, 0.086 H, C*H*O, 91% CDO), 5.26 (tq, J = 7.1, 1.2 Hz, 1 H, C=C*H*CH_2OAc), 5.05 (tq, J = 6.0, 1.3 Hz, 1 H, C=C*H*), 4.51 (d, J = 7.1 Hz, 2 H, CH_2OAc), 1.9–2.5 (m, 8 H, 4CH_2), 1.97 (s, 3 H, CH_3CO_2), 1.62 (br s, 3 H, CH_3), 1.54 (s, 3 H, CH_3).

[10-^{2}H(^{3}H)]Farnesol. Ethyltriphenylphosphonium bromide (79.2 mg, 213 μmol) in THF (1 mL) was treated with *n*-butyllithium (1.55 M in hexane, 137 μL, 213 μmol) for 1 h at 0 °C. A solution of methyl iodide (13.3 μL, 30.2 mg, 213 μmol) in THF (0.5 mL) was added, and the mixture was stirred for 30 min at 0 °C. The mixture was treated with *n*-butyllithium (1.55 M in hexane, 137 μL, 213 μmol) at 0 °C, followed by a solution of aldehyde **20** (51 mg, 213 μmol) in THF (0.66 mL). The solution was allowed to warm to room temperature overnight and then quenched with 40/30/30 MeOH/saturated NH_4Cl/water (5 mL) before extraction with pentane (3 × 5 mL). The extracts were washed with the methanolic NH_4Cl solution (5 mL), dried over $MgSO_4$, filtered, and concentrated in vacuo. The residue was treated with anhydrous K_2CO_3

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(40 mg) in methanol (1 mL) for 5 h. Methanol was removed by rotary evaporation, and the residue was triturated with 30% EtOAc/hexanes. Flash chromatography (silica; 30% EtOAc/hexanes) of the organic solution gave crude farnesol (28.8 mg). Chromatography on 10% silver nitrate impregnated silica gel (Aldrich) in 50% EtOAc/hexanes afforded 12.2 mg of pure farnesol (54.7 μmol, 26%; 5.14 $\times$ 10^6 dpm, 23% radiochemical yield): ^{1}H NMR (CDCl$_3$, 250 MHz) δ 5.41 (t, J = 7.0 Hz, 1 H, C=C*H*CH$_2$OH), 5.11 [tm, J = 6.7 Hz, 1.16 H, 1 H of C=C*H*, 0.16 H of C=C*H*(D)], 4.15 (dd, J = 6.9, 0.6 Hz, 2 H, C*H*$_2$OH), 1.2–2.2 (m, 8 H, 4C*H*$_2$), 1.66 (s, 6 H, 2C*H*$_3$), 1.60 (s, 6 H, 2C*H*$_3$), 1.33 (s, 1 H, O*H*).

[10-^{2}H(^{3}H),12,13-^{14}C$_2$]Farnesyl Pyrophosphate (2f). [10-^{2}H(^{3}H)]-Farnesol (12.2 mg, 54.7 μmol, 2.32 μCi) was mixed with [12,13-^{14}C$_2$]-farnesol (50 mCi/mmol, 2.32 μCi, 46 nmol), triphenylphosphine (25 mg, 95.4 μmol), and CCl$_4$ (200 μL). The mixture was refluxed for 4 h and the solvent removed under nitrogen. The residue was triturated with pentane (3 $\times$ 1 mL) and the precipitate removed by filtration. The filtrate was concentrated in vacuo to give crude chloride. Acetonitrile (300 μL) and tris(tetrabutylammonium) hydrogen pyrophosphate (50 mg, 55.5 μmol) were added, and the mixture was stirred for 4 h. The solvent was removed under nitrogen, and water (2 mL) was added. Extraction with diethyl ether (2 $\times$ 3 mL) and chromatography on DEAE-Sephadex as for **2c** gave [10-^{2}H(^{3}H),12,13-^{14}C$_2$]farnesyl pyrophosphate (**2f**): 24 μmol; 1.01 mCi of ^{3}H (44%); 1.16 mCi of ^{14}C (50%); ^{3}H/^{14}C 0.873 $\pm$ 0.002.

[10-^{2}H(^{3}H),12,13-^{14}C$_2$]Farnesyl Diphenylurethane (12f). [10-^{2}H-(^{3}H),12,13-^{14}C$_2$]Farnesyl pyrophosphate (**2f**) (0.48 μmol) was hydrolyzed with acid phosphatase to farnesol (97% radiochemical yield; ^{3}H/^{14}C 0.858 $\pm$ 0.010) and converted to the diphenylurethane **12f** after addition of carrier farnesol (82.3 mg, 370 μmol): 99.1 mg, 237 μmol, 64%; ^{3}H/^{14}C ratio 0.854 $\pm$ 0.008.

(1*S*)-[1-^{2}H(^{3}H),14,15-^{14}C$_2$]Pentalenene (3f). [10-^{2}H(^{3}H),12,13-^{14}C$_2$]Farnesyl pyrophosphate (**2f**) (2.0 μmol) was cyclized to pentalenene (**3f**) (34% radiochemical yield; ^{3}H/^{14}C 0.843 $\pm$ 0.005) with pentalenene synthase purified through the methylagarose step[8b] (total activity 1 μmol/h). After addition of carrier pentalenene (1 mg), the spectrum of **3f** was recorded: ^{2}H NMR (CHCl$_3$, 61 MHz) δ 1.15.

Incorporation of (1*S*)-[1-^{2}H(^{3}H),14,15-^{14}C$_2$]Pentalenene (3f) into Oxidized Metabolites by *Streptomyces* UC5319. Pentalenene (**3f**) (3.6 $\times$ 10^4 dpm) was converted in the usual manner to give labeled pentalenic acid methyl ester (**15f**): 72 dpm of ^{14}C; ^{3}H/^{14}C 0.710 $\pm$ 0.035.

Oxidation of (1*S*)-[1-^{2}H(^{3}H),14,15-^{14}C$_2$]Pentalenic Acid Methyl Ester (15f). Pentalenic acid methyl ester (**15f**) (40 dpm) was dissolved in CH$_2$Cl$_2$ (200 μL), and PCC was added (10 mg). After being stirred for 1 h, the reaction mixture was diluted with diethyl ether (2 mL) and filtered before removal of solvent in vacuo. The residue was triturated with 10% EtOAc/hexanes, and the supernatant was purified by flash chromatography on silica gel to give [14,15-^{14}C$_2$]-1-oxopentalenic acid methyl ester (**16f**): 19.6 dpm of ^{14}C, 49%; ^{3}H/^{14}C 0.093 $\pm$ 0.039.

[10-^{2}H(^{3}H),11-^{13}C]Farnesol. Reaction of 40 mg (95.4 μmol) of [1-^{13}C]ethyltriphenylphosphonium bromide (prepared from [1-^{13}C]ethyl iodide) with methyl iodide (6.6 μL, 15.1 mg, 106 μmol) and aldehyde **20** (25.4 mg, 106 μmol) in the manner described above afforded 3.0 mg of [10-^{2}H(^{3}H),11-^{13}C]farnesol (13.4 μmol, 14%; 2.13 $\times$ 10^6 dpm, 19% radiochemical yield): ^{1}H NMR (CDCl$_3$, 400 MHz) was the same as that of [10-^{2}H(^{3}H)]farnesol except for δ 1.68 [d, (J obscured), 3 H, C*H*$_3$^{13}C=C], 1.59 [d, (J obscured), 3 H, C*H*$_3$^{13}C=C]; ^{2}H NMR (CHCl$_3$, 61 MHz) δ 5.10 (s, ^{13}C=C*D*); ^{13}C NMR (CDCl$_3$, 100 MHz) showed enhanced signals at δ 131.35 [(CH$_3$)$_2$^{13}*C*=CH, 0.14 C] and 131.25 [(CH$_3$)$_2$^{13}*C*=CD, 0.86 C].

[10-^{2}H(^{3}H),11-^{13}C]Farnesyl Pyrophosphate (2e). [10-^{2}H(^{3}H),11-^{13}C]Farnesol (3.0 mg, 0.96 μCi) was converted to [10-^{2}H(^{3}H),11-^{13}C]-farnesyl pyrophosphate (**2e**) as for **2f**: 0.58 μCi (61%); 13.7 μmol.

(1*S*)-[1-^{2}H(^{3}H),2-^{13}C]Pentalenene (3e). [10-^{2}H(^{3}H),11-^{13}C]Farnesyl pyrophosphate (**2e**) (1.4 μmol) was cyclized by pentalenene synthase to pentalenene (**3e**) (216 nmol, 15% radiochemical yield), and carrier pentalenene (3.6 mg) was added: ^{2}H NMR (CHCl$_3$, 61 MHz) δ 1.18; ^{13}C NMR (CDCl$_3$, 100 MHz) identical with that of unlabeled pentalenene except for an additional peak at δ 40.41.

(1*RS*)-[1-^{2}H]Farnesol. A solution of farnesal (95.1 mg, 432 μmol) in CD$_3$OD (2 mL) was treated with NaBD$_4$ (14.7 mg, 352 μmol). After 5 min, water (5 mL) was added and the mixture was extracted (hexanes, 3 $\times$ 5 mL). The combined organic layers were dried (MgSO$_4$), filtered, and concentrated in vacuo to give 74.8 mg (335 μmol, 78%) of (1*RS*)-[1-^{2}H]farnesol: ^{1}H NMR (CDCl$_3$, 250 MHz) δ 5.34 (br d, J = 6.8 Hz, 1 H, C=C*H*CHDOH), 5.04 (m, 2 H, 2C=C*H*), 4.05 (br d, J = 6.3 Hz, 1 H, C*H*D), 1.8–2.2 (m, 8 H, C*H*$_2$), 1.61 (s, 3 H, C*H*$_3$), 1.60 (s, 3 H, C*H*$_3$), 1.53 (s, 6 H, 2C*H*$_3$).

[1,1-^{2}H$_2$]Farnesol. Oxidation of (1*RS*)-[1-^{2}H]farnesol (52.5 mg, 235 μmol) with activated MnO$_2$ in hexanes gave 46.2 mg (209 μmol, 88%) of [1-^{2}H]farnesal: ^{1}H NMR (CDCl$_3$, 400 MHz) identical with that of farnesal except for signals at δ 9.88 (0.15 H) and 5.78 (q, J = 1.2 Hz). Reduction with NaBD$_4$ gave 35.2 mg (157 μmol, 75%) of [1,1-^{2}H$_2$]farnesol (85 atom % ^{2}H).

[1,2-^{2}H$_2$,12,13-^{14}C$_2$]Farnesyl Pyrophosphate (2g). [1,1-^{2}H$_2$]Farnesol (20.8 mg, 93 μmol) was mixed with [12,13-^{14}C$_2$]farnesol (1 mg, 1.50 $\times$ 10^6 dpm) and converted to the pyrophosphate as described above to give **2g**: 1.37 $\times$ 10^5 dpm of ^{14}C; 8.5 μmol; 9.1%.

[3,3-^{2}H$_2$,14,15-^{14}C$_2$]Pentalenene (3g). FPP (**2g**) (2 μmol, 3.24 $\times$ 10^4 dpm) was cyclized to pentalenene (**3g**) by incubation with pentalenene synthase for 1 h at 27 °C in the usual manner. Carrier pentalenene (7.5 mg, 37 μmol) was added: 1100 dpm of ^{14}C; 90 nmol; ^{2}H NMR (CHCl$_3$, 61 MHz) δ 1.71, 1.31.

[1-^{2}H]Cyclohex-2-en-1-ol (23). To a solution of cyclohex-2-en-1-one (228 mg, 2.37 mmol) in diethyl ether (10 mL) at −10 °C was added lithium aluminum deuteride (49.8 mg, 1.19 mmol) in one portion. The reaction was stirred for 45 min and then quenched with water. The resulting mixture was extracted with diethyl ether (2 $\times$ 10 mL) and the combined organic layers were dried (MgSO$_4$), filtered, and concentrated on the rotary evaporator. Flash chromatography (10/90 diethyl ether-/pentane) gave 153 mg (65%) of **23**: ^{1}H NMR (CDCl$_3$, 400 MHz) δ 5.80 (*a*bt, J_{ab} = 10.1, J_t = 3.5 Hz, 1 H, C*H*=CHCDOH), 5.73 (*a*bt, J_{ab} = 10.1, J_t = 1.7 Hz, 1 H, CH=C*H*CDOH), 2.91 (br s, 1 H, O*H*), 1.5–2.1 (m, 6 H, 3C*H*$_2$); ^{13}C NMR (CDCl$_3$, 100 MHz) δ 129.90, 129.85 (*C*H=*C*H), 64.65 (t, $J_{^{13}C-^{2}H}$ = 22 Hz, *C*DOH), 31.55, 24.81, 18.84 (3*C*H$_2$).

(1*R*)-[1-^{2}H]Farnesol. Farnesal (22.6 mg, 103 μmol) mixed with 1 drop of Tween 30 was suspended in incubation buffer (60 mM KH$_2$PO$_4$, pH 7.2, 20 mL). NAD$^+$ (3.8 mg, 5.7 μmol), [1-^{2}H]cyclohex-2-en-1-ol (**23**) (10 mg, 101 μmol), and HLADH (17.7 mg, 37.2 units) were added, and the mixture was incubated in the dark at room temperature for 2 days. The mixture was extracted with 5/95 ether/hexanes (3 $\times$ 15 mL) and the organic layers were dried (MgSO$_4$), filtered, and rotary evaporated. Flash chromatography (10/90 ethyl acetate/hexanes) afforded 9.3 mg (42%) of (1*R*)-[1-^{2}H]farnesol: ^{1}H NMR (CDCl$_3$, 250 MHz) identical with that of farnesol except for δ 4.05 (br d, J = 6.3 Hz, 1 H, C*H*D). Integration showed that the sample was >95 atom % deuterated.

(1*RS*)-[1-^{3}H]-(1*R*)-[1-^{2}H,12,13-^{14}C$_2$]Farnesyl Pyrophosphate (2i). A mixture of (1*R*)-[1-^{2}H]farnesol (9.3 mg, 41.8 μmol) and [12,13-^{14}C$_2$]-farnesol (1 mg, 1.8 $\times$ 10^6 dpm) was converted to the pyrophosphate by the method of Cramer and Böhm.[21] After addition of (1*RS*)-[1-^{3}H]-farnesyl pyrophosphate (3.0 $\times$ 10^5 dpm, 2.4 nmol), the aqueous phase was applied to a column of DEAE-Sephadex A-25, which was developed as described for **2c** to give farnesyl pyrophosphate **2i**: 2.8 $\times$ 10^5 dpm of ^{14}C, 6.5 μmol, 15%; 2.3 $\times$ 10^5 dpm of ^{3}H.

(3*RS*)-[3-^{3}H]-(3*R*)-[3-^{2}H,14,15-^{14}C$_2$]Pentalenene (3i). **2i** (2.3 μmol, 9.9 $\times$ 10^4 dpm of ^{14}C) was incubated with pentalenene synthase for 3 days at 4 °C,[52] and the resulting pentalenene was isolated after addition of carrier ($\pm$)-pentalenene (3.0 mg, 15 μmol): 7316 dpm of ^{14}C; 170 nmol; ^{2}H NMR (CHCl$_3$, 61 MHz) δ 1.71.

(1*S*)-[1-^{2}H]Farnesol. The [1-^{2}H]farnesal (200 mg, 899 μmol) was dissolved in dioxane (1 mL) and the mixture suspended in incubation buffer (100 mM glycine, pH 8.8, 60 mL). NADH (100 mg, 140 μmol), HLADH (10 mg, 21 units), and ethanol (2 mL) were added, and the mixture was incubated in the dark at room temperature for 4 days. After extraction with 5/95 ether/hexanes (3 $\times$ 50 mL), the organic layers were dried (MgSO$_4$), filtered, and concentrated in vacuo. Flash chromatography (10/90 ethyl acetate/hexanes) afforded 30.0 mg (135 μmol, 15% based on farnesal) of (1*S*)-[1-^{2}H]farnesol: ^{1}H NMR (CDCl$_3$, 250 MHz) identical with that of (1*R*)-[1-^{2}H]farnesol. Integration showed that the sample was 90 atom % deuterated.

(1*RS*)-[1-^{3}H]-(1*S*)-[1-^{2}H,12,13-^{14}C$_2$]Farnesyl Pyrophosphate (2h). A mixture of (1*S*)-[1-^{2}H]farnesol (30 mg, 135 μmol) and [12,13-^{14}C$_2$]-farnesol (1 mg, 1.8 $\times$ 10^6 dpm) was converted to the pyrophosphate **2h** as for **2i**. After addition of (1*RS*)-[1-^{3}H]farnesyl pyrophosphate (3.0 $\times$ 10^5 dpm, 2.4 nmol), the aqueous phase was applied to a column of DEAE-Sephadex A-25, which was developed as described for **2c** to give farnesyl pyrophosphate (**2h**): 1.84 $\times$ 10^5 dpm of ^{14}C, 13.8 μmol, 10.2%; 1.6 $\times$ 10^5 dpm of ^{3}H.

(3*RS*)-[3-^{3}H]-(3*S*)-[3-^{2}H,14,15-^{14}C$_2$]pentalenene (3h). FPP (**2h**) (5.0 μmol, 6.5 $\times$ 10^4 dpm ^{14}C) was incubated with pentalenene synthase for 3 days at 4 °C[52] in the usual manner to give pentalenene (**3h**): 7844 cpm of ^{14}C; 585 nmol; ^{2}H NMR (CHCl$_3$, 61 MHz) δ 1.33.

(1*R*)-[1-^{3}H]Farnesol. Farnesal (10.0 mg, 45.4 μmol) in 1 drop of Tween 30 was suspended in incubation buffer (50 mM KH$_2$PO$_4$, pH 7.4,

(52) Although the V_{max} for pentalenene synthase at 4 °C is 0.25 times that at 30 °C, the enzyme half-life could be extended from <30 min to >3 days at the lower temperature, resulting in an increase in the net turnover of FPP to **3**.

25 mL). NAD$^+$ (4.0 mg, 6.0 μmol), [1-^{3}H]cyclohex-2-en-1-ol (0.5 mCi), and HLADH (3.0 mg, 6.0 units) were added, and the mixture was incubated in the dark at room temperature for 6 h. The mixture was extracted with hexanes (2 × 30 mL) and the organic layers were dried ($MgSO_4$), filtered, and concentrated by rotary evaporation. After addition of carrier farnesol (1.8 mg, 8.1 μmol), flash chromatography (gradient from hexanes to 10/90 ethyl acetate/hexanes) afforded 116 μCi (46%) of (1*R*)-[1-^{3}H]farnesol.

(1*R*)-[1-^{3}H,12,13-$^{14}C_2$]Farnesyl Pyrophosphate (2k). (1*R*)-[1-^{3}H]-Farnesol (116 μCi) was converted to the pyrophosphate as for **2g**. After addition of [12,13-$^{14}C_2$]farnesyl pyrophosphate (9.6 × 10^5 dpm, 8.7 nmol), purification on a column of DEAE-Sephadex A-25 gave farnesyl pyrophosphate (**2k**): 3.5 × 10^7 dpm of ^{3}H (14%), 8.96 × 10^5 dpm of ^{14}C.

(1*R*)-[1-^{3}H,12,13-$^{14}C_2$]Farnesyl Diphenylurethane (12k). Hydrolysis of **2k** (2.4 × 10^4 dpm of ^{14}C) with acid phosphatase gave [1-^{3}H,12,13-$^{14}C_2$]farnesol (1.6 × 10^4 dpm of ^{14}C, 68% radiochemical yield, ^{3}H/^{14}C 4.72 ± 0.13), which was converted to the corresponding diphenylurethane after dilution with unlabeled farnesol. **12k**: 8680 dpm of ^{14}C, 59% radiochemical yield; ^{3}H/^{14}C 5.05 ± 0.14. The product was further purified by preparative thin-layer chromatography (three plates, 1000 μm, three elutions of 4% EtOAc/hexanes) before recrystallization from MeOH to constant melting point, ^{3}H and ^{14}C specific activities, and ^{3}H/^{14}C ratio: specific activity 62 pCi/μmol ^{3}H, 15.1 pCi/μmol ^{14}C; ^{3}H/^{14}C (average of last three crystallizations) 4.10 ± 0.02.

Cyclization of (1*R*)-[1-^{3}H,12,13-$^{14}C_2$]Farnesyl Pyrophosphate (2k) to (3*R*)-[3-^{3}H,14,15-$^{14}C_2$]Pentalenene (3k). (1*R*)-[1-^{3}H,12,13-$^{14}C_2$]Farnesyl pyrophosphate (**2k**, 1.57 × 10^5 dpm of ^{14}C) was dissolved in 200 mM Tris buffer, pH 8.4, containing 20 mM $MgCl_2$ and 5 mM β-mercaptoethanol (5 mL). Pentalenene synthase purified through the G-100 size exclusion step (50 μL, total activity 26 nmol/h)[8b] was added, and the mixture was incubated at 30 °C for 2 h. Carrier pentalenene (2.0 mg) in hexanes (5 mL) was added and the mixture extracted. After further extraction with hexanes (2 × 5 mL), the hexanes layers were passed through a flash chromatography column (hexanes). Fractions were concentrated on the rotary evaporator at room temperature to afford **3k**: 6.2 × 10^4 dpm of ^{14}C, 39% radiochemical yield; ^{3}H/^{14}C 4.09 ± 0.04.

(3*R*)-[3-^{3}H,14,15-$^{14}C_2$]-(7*S*,8*R*)- and -(7*R*,8*S*)-7,8-Dihydroxypentalenanes (13k and 14k). Labeled pentalenene (**3k**) (1.54 × 10^4 dpm of ^{14}C) was diluted with carrier (±)-pentalenene (40 mg, 196 μmol) and converted to the mixture of *cis*-diols, which was purified by flash chromatography (30/70 EtOAc/hexanes) to afford 35.7 mg (150 μmol, 55%) of product: 1.2 × 10^4 dpm of ^{14}C, 78% radiochemical yield; ^{3}H/^{14}C 4.25 ± 0.05. The individual diols were separated by fractional crystallization and recrystallized to constant melting point, ^{3}H and ^{14}C specific activity, and ^{3}H/^{14}C ratio: **13k**, 4.02 ± 0.10, and **14k**, 4.10 ± 0.14, respectively.

Incorporation of (3*R*)-[3-^{3}H,14,15-$^{14}C_2$]Pentalenene (3k) into Oxidized Metabolites by *Streptomyces* UC5319. Feeding of labeled pentalenene (**3k**) (1.0 mg, 3.11 × 10^4 dpm of ^{14}C) to 12 flasks of *Streptomyces* UC5319 in the usual manner gave the following products. Pentalenolactone methyl ester (**19k**): 5.6 mg; 1660 dpm of ^{14}C; 3.7 pCi/μmol ^{14}C; specific incorporation 1.4%; ^{3}H/^{14}C 0.065 ± 0.002. Epipentalenolactone F methyl ester (**18k**): 0.4 mg; 153 dpm of ^{14}C; 43 pCi/μmol ^{14}C; specific incorporation 1.5%; ^{3}H/^{14}C 3.73 ± 0.05. Pentalenic acid methyl ester (**15k**): <100 μg; 223 dpm of ^{14}C; ^{3}H/^{14}C 3.62 ± 0.03.

(1*S*)-[1-^{3}H]Farnesol. A solution of farnesal (80 mg, 363 μmol) in methanol (1 mL) was reduced with $NaBH_3T$ (3 mg, 24 mCi). After 3 h, excess $NaBH_4$ was added and the mixture was worked up as before to give (1*RS*)-[1-^{3}H]-*trans,trans*-farnesol (33.0 mg, 149 μmol, 41%): 8.63 mCi, 36% radiochemical yield; specific activity 58 mCi/mmol. Oxidation of 15 mg (67.6 μmol, 3.92 mCi) of the alcohol with MnO_2 gave [1-^{3}H]farnesal (5.1 μmol, 295 μCi, 7.5%), of which 1.12 mg (5.09 μmol, 295 μCi) was enzymatically reduced with HLADH to give (1*S*)-[1-^{3}H]farnesol (32 μCi, 11%), as described for the corresponding sample of (1*S*)-[1-^{2}H]farnesol.

(1*S*)-[1-^{3}H,12,13-$^{14}C_2$]Farnesyl Pyrophosphate (2j). (1*S*)-[1-^{3}H]-Farnesol (32 μCi) was converted to the pyrophosphate, mixed with [12,13-$^{14}C_2$]FPP (6.44 × 10^5 dpm, 1.26 μmol), and purified as described for **2k** to yield 768 nmol of **2j**: 1.49 × 10^6 dpm of ^{3}H (2.1%), 3.87 × 10^5 dpm of ^{14}C; ^{3}H/^{14}C 3.56 ± 0.015.

(1*S*)-[1-^{3}H,12,13-$^{14}C_2$]Farnesyl Diphenylurethane (12j). Hydrolysis of **2j** with acid phosphatase gave farnesol, which was converted to the corresponding diphenylurethane **12j**: 1.21 × 10^4 dpm of ^{14}C, 45% radiochemical yield. Recrystallization from MeOH to constant melting point, ^{3}H and ^{14}C specific activities, and ^{3}H/^{14}C ratio gave a ^{3}H/^{14}C ratio of 2.17 ± 0.02.

Cyclization of (1*S*)-[1-^{3}H,12,13-$^{14}C_2$]Farnesyl Pyrophosphate (2j) to (3*S*)-[3-^{3}H,14,15-$^{14}C_2$]Pentalenene (3j) by Pentalenene Synthase. (1*R*)-[1-^{3}H,12,13-$^{14}C_2$]Farnesyl pyrophosphate (**2j**) (3.9 × 10^4 dpm of ^{14}C) was cyclized in the usual manner to pentalenene, which was purified after addition of carrier pentalenene (2.75 mg): 1.26 × 10^4 dpm of ^{14}C, 33% radiochemical yield; ^{3}H/^{14}C 2.50 ± 0.02.

(3*S*)-[3-^{3}H,14,15-$^{14}C_2$]-(7*S*,8*R*)- and -(7*R*,8*S*)-7,8-Dihydroxypentalenanes (13j and 14j). Labeled pentalenene (**3j**) (41 mg, 201 μmol, 1.05 × 10^4 dpm of ^{14}C) was converted to 25.5 mg of the *cis*-diol mixture: 107 μmol, 53%; 6460 dpm of ^{14}C, 62% radiochemical yield; ^{3}H/^{14}C 2.34 ± 0.03. The individual diols were separated by fractional crystallization, and each was recrystallized to constant melting point, ^{3}H and ^{14}C specific activity, and ^{3}H/^{14}C ratio: **13j**, 2.19 ± 0.04, and **14j**, 2.24 ± 0.04.

Incorporation of (3*S*)-[3-^{3}H,14,15-$^{14}C_2$]Pentalenene (3j) into Oxidized Metabolites by *Streptomyces* UC5319. Labeled pentalenene (**2j**) (1.2 × 10^4 dpm of ^{14}C, 2.75 mg) of ethanol (600 μL) was added (100 μL/flask) to six flasks of *Streptomyces* UC5319 as described above, and the resulting oxidized metabolites were isolated and purified as the corresponding methyl esters. Pentalenolactone methyl ester (19j): 3.0 mg, 468 dpm of ^{14}C; 10.2 pCi/μmol ^{14}C; specific incorporation 2.4%; ^{3}H/^{14}C 2.25 ± 0.03. Epipentalenolactone F methyl ester (**18j**): 0.9 mg; 106 dpm of ^{14}C; 15.5 pCi/μmol ^{14}C; specific incorporation 3.7%; ^{3}H/^{14}C 1.62 ± 0.03. Pentalenic acid and methyl ester (**15j**): 4 mg; 246 dpm of ^{14}C; 14 pCi/μmol ^{14}C; specific incorporation 3.3%; ^{3}H/^{14}C 2.38 ± 0.02.

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Synthesis and Chemistry of a Quinone Methide Model for Anthracycline Antitumor Antibiotics

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Abstract: In an effort to understand the chemistry of anthracycline antitumor antibiotic, a simple stable *o*-quinone methide has been constructed and fully characterized. The reaction of the quinone methide with nucleophiles, including 2′,3′-isopropylideneadenosine has been examined.

In 1977 Moore put forth a theory of "bioreductive alkylation" to rationalize the biological activity of quinonoid antitumor compounds.[1] This theory, drawing on the earlier work of Lin and Sartorelli,[2] calls for several hundred structurally similar anthracyclines to derive their antitumor activity via the same general type of process: reduction of a quinone to a hydroquinone followed by the loss of a leaving group in the benzylic position, usually a sugar, to afford a quinone methide. The quinone methide

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