# Cyclization of farnesyl diphosphate to pentalenene. Orthogonal stereochemistry in an enzyme-catalyzed $S_{E'}$ reaction[1]

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*This paper is dedicated to Professor Ian D. Spenser*

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Pentalenene synthase catalyzes the cyclization of farnesyl diphosphate (**1**) to the sesquiterpene hydrocarbon pentalenene (**4**). Separate incubations of (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]farnesyl diphosphate (**1***a*) and (4*R*,8*R*)-[4,8-$^3H_2$, 4,8$^{14}C_2$]farnesyl diphosphate (**1***b*) with pentalenene synthase isolated from *Streptomyces* UC5319 and analysis of the derived labeled pentalenenes, **4***a* and **4***b*, respectively, by chemical degradation established that H-8*si* of FPP was lost upon cyclization to pentalenene. Consideration of the plausible conformations of the enzymatic cyclization intermediates indicates that the electrophilic allylic addition–elimination ($S_{E'}$) reaction in which the C-4,5 bond of pentalenene is formed involves an orthogonal relationship between the C—C bond being formed and the C—H bond that is ultimately broken.

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La pentalènène synthase catalyse la cyclisation du diphosphate de farnésyle (**1**) en hydrocarbure sesquiterpénique pentalènène (**4**). Des incubations séparées des diphosphates de (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]farnésyle (**1***a*) et de (4*R*,8*R*)-[4,8$^3H_2$, 4,8-$^{14}C_2$]farnésyle (**1***b*) avec la pentalènène synthase isolée de *Streptomyces* UC5319 et une analyse des pentalènènes marqués, **4***a* et **4***b* respectivement, par une dégradation chimique a permis d'établir que le H-8*si* du diphosphate de farnésyle est perdu lors de la cyclisation en pentalènène. Une considération des conformations plausibles des intermédiaires enzymatiques de la cyclisation indique que la réaction électrophile d'addition–élimination allylique ($S_{E'}$), au cours de laquelle se forme la liaison C-4,5 du pentalènène, implique une relation orthogonale entre la liaison C—C qui se forme et la liaison C—H qui sera finalement brisée.

[Traduit par la rédaction]

The formation of carbon–carbon bonds by the electrophilic attack of carbenium ions on double bonds is one of the most important reactions in the biosynthesis of hundreds of isoprenoid metabolites, including linear polyprenols (dolichols, ubiquinones), cyclic terpenes (mono-, sesqui-, and diterpenes), and steroids and other triterpenes (cholesterol, α-amyrin) (1). Although this process has considerable precedent in solution phase chemistry and much is known about the physical organic chemistry of carbenium ions themselves, the enzymatic reactions remain poorly understood, both with respect to the means by which such reactive species are manipulated and stabilized by protein catalysts and the manner in which enzymes exercise regiochemical and stereochemical control over such reactions (2).

One particularly important subclass of these electrophilic reactions involves the quenching of positive charge resulting from the initial addition to the double bond by the removal of one of the original allylic protons. This allylic addition–elimination formally corresponds to an $S_{E'}$ process. Cornforth and Popjak have shown that the key chain elongation step of polyprenol biosynthesis, typified by the farnesyl diphosphate (FPP, 1) synthase reaction, involves a pair of net *syn* allylic addition–elimination reactions (3). (Scheme 1). By contrast, the closely analogous prototropic rearrangement of isopentenyl diphosphate (IPP, 2) to dimethylallyl diphosphate (DMAPP, 3) has been shown to occur with net *anti* stereochemistry (4). In more recent studies of the cyclization of farnesyl diphosphate (1) to the sesquiterpene hydrocarbon pentalenene (4), we have shown that the initial $S_{E'}$ reaction takes place with net *anti* stereochemistry (5) (Scheme 2). Overton has described model experiments in which an intramolecular $S_{E'}$ reaction was shown to proceed with clean *syn* stereochemistry (6), in contrast to arguments based on MO theory that had predicted a preferred *anti* pathway (7). On the other hand, the possibility could not be excluded that the observed stereochemistry might have resulted from a conformational bias in the substrate rather than from a stereoelectronic imperative. Since that time, there have been several documented examples of (predominant) *anti* stereochemistry in the mechanistically related alkylative or prototropic desilylation of allylsilanes (8). The divergent stereochemical course of various biological allylic addition–elimination reactions suggests that the precise position of substrates relative to enzymic bases may be more important to the outcome than an inherent physicochemical preference for one or the other stereochemistry. To explore this issue further, we have examined a second $S_{E'}$ reaction in the cyclization of FPP to pentalenene.

Pentalenene synthase is a soluble, monomeric enzyme of $M_r$ 42.5 kDa obtained from *Streptomyces* UC5319 (9). A divalent metal cation, either $Mg^{2+}$ or $Mn^{2+}$, is the only cofactor required. Extensive studies carried out in this laboratory support at mechanism in which FPP, folded as illustrated in Scheme 2, is initially cyclized to humulene (**5**), which undergoes further cyclization initiated by reprotonation at C-10 (5, 10). Consistent with this mechanistic and stereochemical model is the demonstration that cyclization of FPP takes place with net inversion of configuration at C-1 (5). Electrophilic attack on the *si* face of the C-10, 11 double bond is followed by removal the H-9*si* proton. The proton that is removed does not undergo exchange with the medium, but is redonated to the *re* face of the C-9,10 double bond of the intermediate humulene, leading to formation of the protoilludyl cation **6** (5, 10). Following a 1,2-hydride shift, the resultant cation **7** attacks the remaining double bond, leading, after loss of one of the original H-8 protons of FPP, to formation of pentalenene (**4**). We now report experiments which establish that this latter $S_{E'}$ reaction proceeds with neither *syn* nor *anti*

[1] Dedicated with great affection and warm admiration to Professor Ian D. Spenser.

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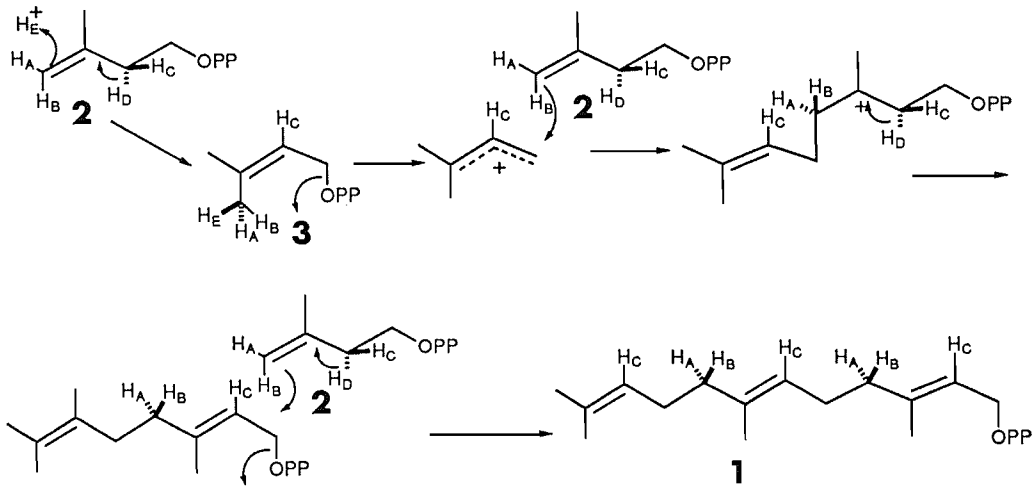
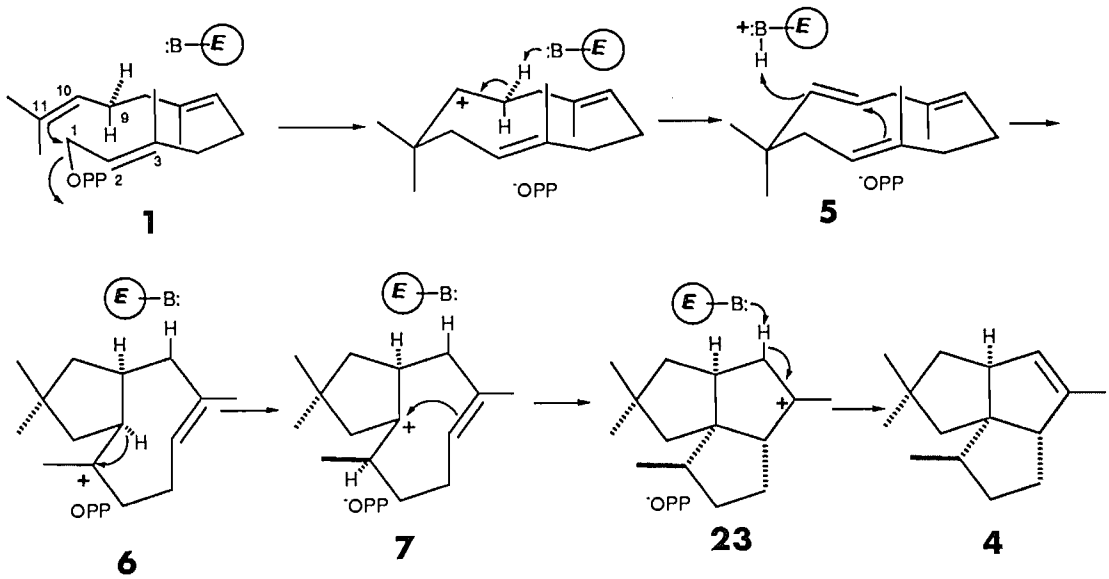
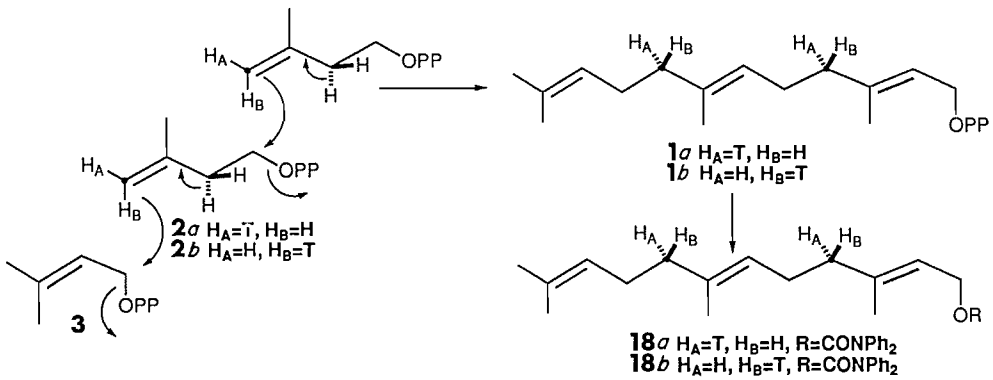


SCHEME 1



SCHEME 2

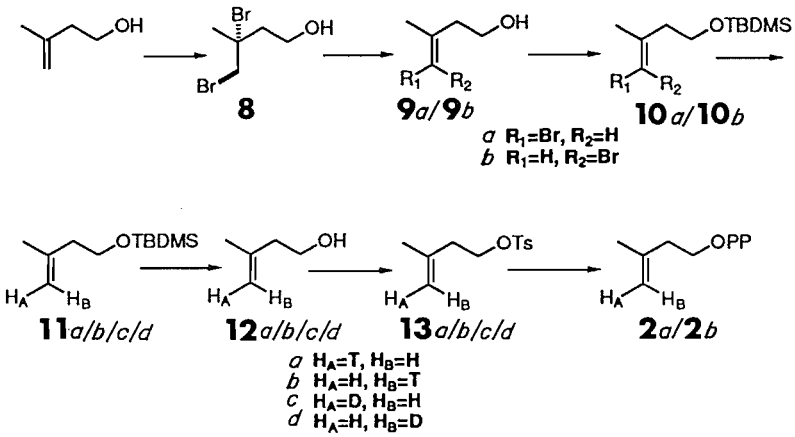



SCHEME 3

stereochemistry but involves a, presumably stepwise, orthogonal electrophilic addition – allylic proton elimination (11).

## Results

The stereochemical course of the final deprotonation step was determined by incubation of samples of FPP, stereospecifically tritiated at C-8, with pentalenene synthase obtained from *Streptomyces* UC5319. The requisite labeled samples of FPP could be conveniently prepared by incubation of DMAPP and stereospecifically tritiated IPP with avian liver prenyl transferase (Scheme 3). Thus, condensation of DMAPP (**3**) and *E*-[4-$^3$H]IPP (**2***a*) was expected to afford (4*S*,8*S*)-[4,8-$^3H_2$]FPP

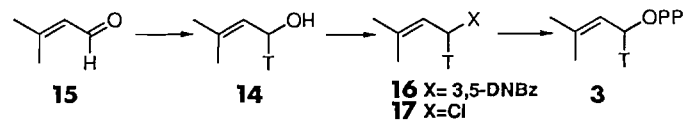



SCHEME 4

(**1***a*), whereas DMAPP (**3**) and *Z*-[4-$^3$H]IPP (**2***b*) would yield (4*R*,8*R*)-[4,8-$^3H_2$]FPP (**1***b*) (3). The required samples of IPP labeled with tritium at either the 4*E* or 4*Z* positions were themselves prepared by a modification of the method of Ogura and coworkers (12), initially using deuterium in order to optimize both the incorporation of label and the degree of stereospecificity (Scheme 4).

Bromination (3, 12) of isopentenyl alcohol yielded the corresponding dibromide **8** in 92% yield. Dehydrobromination of **8** with 5 M methanolic KOH gave a 4:1 mixture of *E*- and Z-4-bromo-3-methyl-3-buten-1-ol, **9***a* and **9***a*, respectively, which were separated by flash chromatography on silica gel followed by medium pressure liquid chromatography (MPLC) on silica gel and converted into their respective *tert*-butyldimethylsilyl ethers **10***a* and **10***b* by treatment with *tert*-butyldimethylsilylchloride ((TBDMSCl) and imidazole in DMF. Metallation of **10***a* and **10***b* with 2.2 equivalents of *tert*-butyllithium at −78°C followed by quenching of the resulting bright yellow solution with trifluoroacetic acid 1-*d* (99.8 at.% *d*) at −78°C afforded stereospecifically deuterated isopentenyl *tert*-butyldimethylsilyl ethers **11***c* and **11***d*, respectively. Desilylation of **11***c* and **11***d* with tetra(*n*-butyl)ammonium fluoride (TBAF) in THF followed by treatment of the corresponding 4*E*- and 4*Z*-deuterated isopentenols, **12***c* and **12***d*, and *p*-toluenesulfonyl chloride in pyridine, yielded the tosylates **13***c* and **13***d* in 20% average overall yield from **10***a* and **10***b*. The individual steps of silylation and tosylation were achieved with yields of 80–93% and 80–100%, respectively. **13***c* was 96% deuterated, based on integration of the $^1$H NMR signals for the *Z* and *E* olefinic protons at δ 4.67 and 4.78. Integration of the two olefinic signals in the $^2$H NMR spectrum of **13***c* showed the *E*- to *Z*-deuterium ratio to be 92:8. By similar analysis, tosylate **13***d* was found to contain 85% deuterium, exclusively in the expected *Z* geometry.

Having worked out conditions for efficient and stereospecific labeling, both *E*- and *Z*-[4-$^3$H]-3methyl-3-buten-1-yl tosylates, **13***a* and **13***b*, were synthesized following the procedure outlined in Scheme 4, using tritiated trifluoroacetic acid (45 μCi/μmol).[3] Radiolabeled tosylates **13***a* (21.9 μCi/μmol) and **13***b* (41.2 μCi/μmol) were obtained in 34% and 23% overall yield, respectively, from the *tert*-butyldimethylsilyl ethers **11***a* and **11***b*. **13***a* and **13***b* were each converted to the corresponding samples of IPP, **2***a* and **2***b*, upon treatment with tris(tetra-(*n*-butyl)ammonium) hydrogen pyrophosphate in acetonitrile (13).




SCHEME 5

[1-$^3$H]DMAPP (**3**) was prepared from [1-$^3$H]dimethylallyl alcohol (**14**), readily obtained by reduction of 3-methyl-2-butenal (**15**) with sodium borotritide in methanol (Scheme 5). Unlabeled carrier dimethylallyl alcohol was added, and a portion of the isotopically diluted alcohol was derivatized as the 3,5-dinitrobenzoate **16**, which was recrystallized to constant specific activity (0.29 nCi/μmol). Treatment of **14** with triphenylphosphine in hexachloroacetone (HCA) (14) generated the allylic chloride **17**, which was converted to [1-$^3$H]-**3** with tris(tetra(*n*-butyl)ammonium)hydrogen pyrophosphate in acetonitrile (13).

For the prenyl transferase-catalyzed preparation of stereospecifically tritiated FPP, **2***a* and **2***b* were each mixed with commercially available [4-$^{14}$C]IPP. An equimolar mixture of *E*-[4-$^3$H, 4-$^{14}$C]IPP (**2***a*) (337 μM) and DMAPP (**3**) was incubated with avian liver prenyl transferase to give (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***a*) (3, 12).[4] Under similar conditions an equimolar mixture of *Z*-[4-$^3$H, 4-$^{14}$C]IPP (**2***b*) (309 μM) and DMAPP (**3**) yielded (4*R*,8*R*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***b*). **1***a* and **1***b* were each purified by successive Sephadex G-25 gel filtration, C-18 reverse phase HPLC (16), and DEAE–Sephadex anion exchange chromatography. Since the $^3$H specific activities of **2***a* and **2***b* were each greater than that of **3** by at least a factor of $10^6$, the contribution of **3** to the measured $^3$H/$^{14}$C ratio in **1***a* and **1***b* was negligible. The precise $^3$H/$^{14}$C ratios for **1***a* and **1***b* were determined by hydrolysis of small samples of each

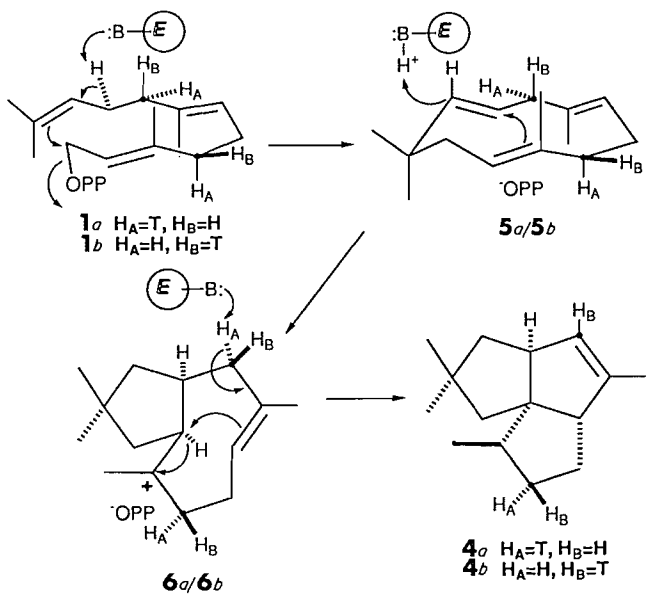
[3]Tritiated trifluoroacetic acid is conveniently prepared by reaction of trifluoroacetic anhydride with tritiated water.

[4]IPP can exhibit substrate inhibition by competing with DMAPP or geranyl diphosphate for binding to the allylic substrate region of the active site (cf. ref. 15). To minimize this problem, IPP and DMAPP are incubated at equimolar concentrations, rather than in the 2:1 ratio required by the stoichiometry of the FPP synthase reaction.

TABLE 1. Enzymatic cyclization of (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***a*) and (4*R*,8*R*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***b*) to pentalenenes **4***a* and **4***b* and determination of the distribution of label

| Compound | $^{14}C$ specific activity (pCi/μmol) | $^3H/^{14}C$ | Atom ratio | Compound | $^{14}C$ specific activity (pCi/μmol) | $^3H/^{14}C$ | Atom ratio |
|---|---|---|---|---|---|---|---|
| **1***a* | | 6.02±0.01[a] | 2:2 | **1***b* | | 6.95±0.11[b] | 2:2 |
| **19***a* | 899±34 | 3.17±0.02 | 1.05:2 | **19***b* | 580±6 | 6.64±0.04 | 1.91:2 |
| **21***b* | 831±7 | 2.99±0.05 | 0.99:2 | **20***b* | 569±25 | 6.58±0.04 | 1.89:2 |
| | | | | **21***b* | 555±9 | 3.46±0.06 | 1.00:2 |

[a]Based on recrystallization of farnesyl diphenylurethane **18***a*.
[b]Based on recrystallization of farnesyl diphenylurethane **18***b*.




SCHEME 6

labeled FPP with acid phosphatase, followed by the addition of unlabeled carrier *trans,trans*-farnesol and treatment with *N,N*-diphenyl carbamoyl chloride in pyridine (17) to give the diphenylurethanes **18***a* and **18***b*, respectively. **18***a* and **18***b* were each recrystallized to constant $^3H/^{14}C$ ratio, specific activity, and melting point (Scheme 3 and Table 1).

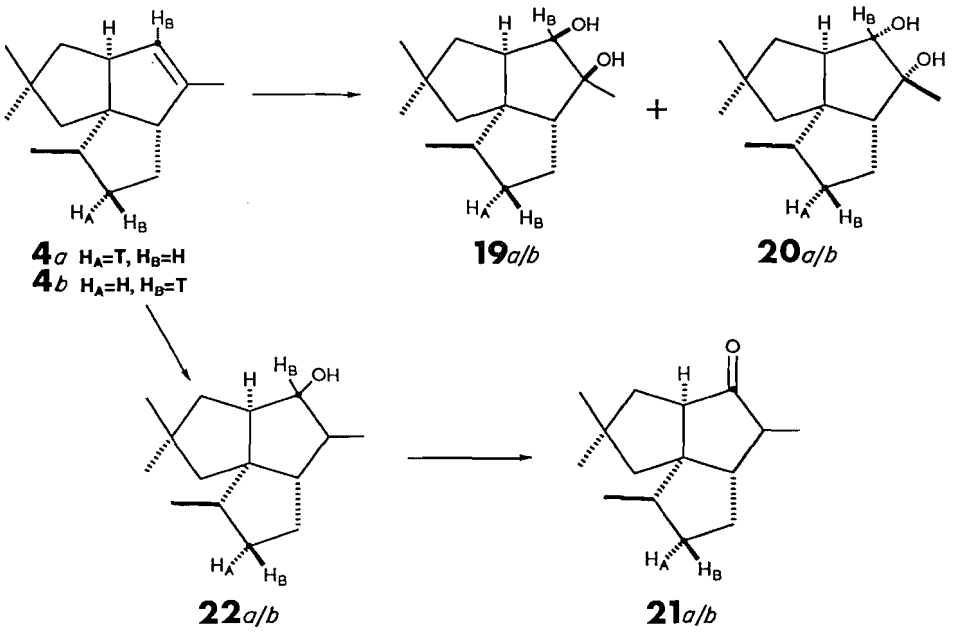
When (4*R*,8*R*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP, **1***b* ($^3H/^{14}C$ 6.95 ± 0.11), was incubated with crude pentalenene synthase obtained from cell-free extracts of *Streptomyces* UC5319, the resulting **4***b* retained 95% of the original tritium label, based on the $^3H/^{14}C$ ratio of the derived pair of diastereomeric *cis* 6,7-diols (10) **19***b* (β isomer) ($^3H/^{14}C$ 6.64 ± 0.04) and **20***b* (α isomer) ($^3H/^{14}C$ 6.58 ± 0.04), obtained after dilution of the enzymatically generated product with synthetic (±)-pentalenene (18) followed by treatment with osmium tetraoxide in pyridine (Scheme 7 and Table 1). The site of tritiation was established by hydroboration–oxidation followed by PCC oxidation (10) to give 7-keto pentalenane **21***b* ($^3H/^{14}C$ 3.46 ± 0.06). The isotope ratio found in the ketone was roughly half that of both **19***b* and **20***b* as well as 7-hydroxypentalenane (**22***b*), indicating that half the tritium label resided at C-7 of pentalenene (**4***b*) (Table 1).

In a complementary set of experiments, (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***a*) ($^3H/^{14}C$ 6.02 ± 0.01) was cyclized to pentalenene (**4***a*) by incubation with crude pentalenene synthase (Scheme 6). After dilution with unlabeled (±)-pentalenene, the derived product was converted to the corresponding mixture of diastereomeric diols **19***a* and **20***a* (Scheme 7). The β isomer **19***a* was recrystallized to constant specific activity, $^3H/^{14}C$ ratio (3.17 ± 0.02), and melting point. These results indicated that 53% of the original tritium label had been retained in the enzymatically generated pentalenene. Hydroboration–oxidation followed by oxidation with PCC as before yielded 7-keto pentalenane (**21***a*) of essentially unchanged $^3H/^{14}C$ ratio (2.99 ± 0.05). These results established that H-8*si* of FPP was lost upon cyclization to pentalenene.

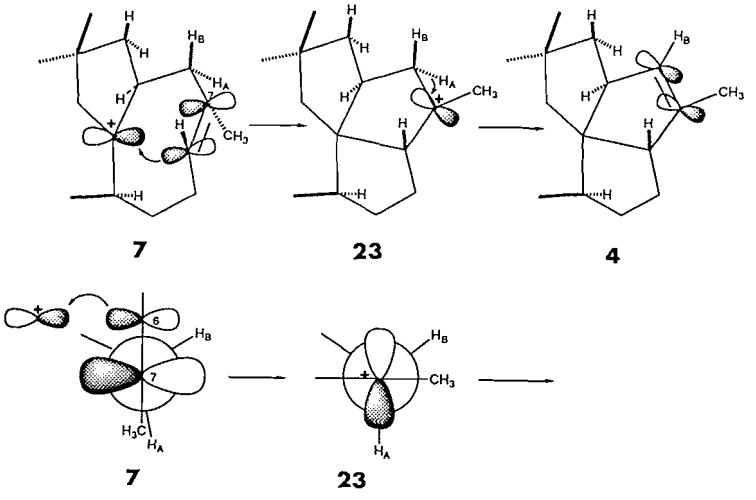
## Discussion

Having identified which of the two diastereotopic H-8 protons of FPP is lost in the formation of pentalenene, it is possible to analyze the overall stereochemical course of the final electrophilic allylic addition–elimination reaction, which leads to formation of 4. From the absolute configuration of pentalenene (9, 10), it is evident that the cyclization of cation **7** involves attack on the *si* face of the *trans*-6,7 double bond (FPP numbering, Scheme 8). The *p* orbitals of **7** must be aligned in a parallel orientation for the electrophilic reaction to occur and allow formation of the carbon–carbon σ bond. In principle, formation of the new double bond in pentalenene could involve removal of either of the original allylic protons, $H_A$ or $H_B$. If $H_B$ (H*re*) were removed, the *p* orbital of the pentalenyl cation 23 would only have to rotate 30° in order to achieve the necessary parallel alignment with the C-$H_B$ σ bond (Scheme 8). Since in fact $H_A$ (H*si*) is removed, this *p* orbital must instead undergo a rotation of 90° prior to the final deprotonation. The final $S_{E'}$ reaction in the enzymic conversion of FPP to pentalenene cannot therefore be described as proceeding with either net *syn* or net *anti* stereochemistry. Intially, both $H_A$ and $H_B$ are *anti* to the newly formed carbon–carbon σ bond. When $H_A$ (corresponding to H-8*si* of FPP) is eventually removed after internal bond rotations, it is actually *orthogonal* to the newly formed 5,6 σ bond of pentalenene.

Although a rotation of only 30° would have sufficed for proper overlap of the vacant *p*-orbital of **22** with the adjacent β proton, why then is a rotation of 90° required for deprotonation to occur? The most likely explanation is that the stereochemistry of deprotonation is determined by the relative positioning of the substrate and the relevant enzymic base. Intriguingly, this final deprotonation occurs immediately adjacent to the sites of deprotonation and reprotonation involved in the formation and further cyclization of the presumed humulene intermediate (5, 10). While the three C—H bonds in question, H-8*si* of FPP ($H_A$ of **7**), H-9*si* of FPP, and H-1*re* of pentalenene are not parallel to each other, they do extend in the same general direction in the various intermediates (5, 10). It is tempting to speculate, therefore, that there is in fact a *single base* at the active site of pen-




SCHEME 7




SCHEME 8

talenene synthase that is responsible for deprotonation, reprotonation, and deprotonation at C-9, C-10, and C-8 of the original farnesyl skeleton. We recently succeeded in cloning the relevant structural gene for pentalenene synthase and in obtaining high-level expression of recombinant cyclase in *Escherichia coli* (19). It should therefore be possible to test these predictions directly and to obtain additional insights into the mechanism and stereochemistry of the enzymatic conversion of farnesyl diphosphate to pentalenene.

## Experimental

*Materials and methods*

Melting points were measured on a Thomas–Hoover capillary melting point apparatus and are uncorrected. Proton Fourier transform NMR spectra were routinely obtained at 250 MHz on a Bruker WM250 spectrometer, or at 400 MHz on a Bruker AM-400 spectrometer. Chemical shifts were referenced with respect to TMS at $\delta = 0$ or with respect to HOD at $\delta = 4.60$. $^{13}C$ NMR spectra were obtained at 100 MHz with broadband $^{1}H$ decoupling and chemical shifts were referenced with respect to the center of the triplet of $CDCl_3$ at $\delta = 77.0$. $^{2}H$ NMR spectra were obtained at 61 MHz, and chemical shifts were referenced with respect to $CDCl_3$ at $\delta = 7.24$. Infrared spectra were recorded on a Perkin–Elmer 1600 series FTIR spectrometer. Liquid films were scanned between sodium chloride plates. Radioactive samples were counted for both $^{3}H$ and $^{14}C$ activity by dissolving the sample in 5 mL Packard Opti-Fluor liquid scintillation fluid and measuring the activity on a Beckman LS 5801 liquid scintillation counter. For

determining specific activities and isotope ratios, the activity was measured to within a standard deviation of ±1%. Lyophilization of aqueous samples was achieved at (7–10) × $10^{-3}$ Torr (1 Torr = 133.3 Pa) on a Labconco lyophilizer. Centrifugation at 4°C was performed in a Sorvall RC-5 refrigerated centrifuge. Measurements of pH were obtained using a Corning pH/ion meter 150, which was calibrated with pH 4.01 and pH 7.41 buffers. Nanopure water was obtained from a Barnstead NANOpure II apparatus. Radiochemicals were purchased from ICN Radiochemical division (Costa Mesa, Calif.) and other chemicals were routinely purchased from Aldrich (Milwaukee, Wis.). Routine flash chromatography was performed on EM-Science Silica Gel 60 (particle size 40–63 μm, 200–400 mesh), and thin-layer chromatography (TLC) was routinely performed on Kieselgel-60 precoated silica gel plates. For reactions that required anhydrous conditions, glassware was routinely oven dried at 110°C, then cooled either under an atmosphere of dry nitrogen, or in a desiccator containing calcium sulfate (Drierite). Ether and THF were dried and distilled from sodium – benzophenone ketyl. Pentane, hexane, and methylene chloride were distilled from calcium hydride. Pyridine was distilled from calcium hydride and stored over KOH.

E- *and Z-4-Bromo-3-methyl-3-buten-1-ol (**9**a and **9**b) (3, 12)*

To 3,4-dibromo-3-methyl-buten-1-ol (**8**) (5.0 g, 19.4 mmol), prepared as previously described (3, 12), was added 5 M KOH in MeOH (25% excess) at 0°C under a slight positive pressure of nitrogen. The white milky reaction mixture was allowed to stir for 2 h at 25°C, then ether (5–10 mL) was added and the mixture was dried over anhydrous $MgSO_4$ and filtered. The filter cake was washed with several small portions of ether and the combined filtrates were neutralized with a few drops of glacial acetic acid. Solvent was removed via rotovap leaving 2.68 g of a yellow oil (83.9% crude yield). Partial separation of the *E* and *Z* isomers of **9** was achieved by flash chromatography on silica gel (2:1 v:v hexane:ethyl acetate). From the flash column, pure *E* isomer **9***a* could be separated from a mixture of *E* and *Z* isomers (1.51 g, 47.1% yield. A mixture of the *E* and *Z* isomers (223 mg, 1.35 mmol) was loaded onto a size B Lobar medium pressure column prepacked with Si 60 silica gel (particle size 40–63 μm) and eluted with 9:1 v:v hexane:ethyl acetate (5 mL/min, 20 psi (1 psi = 6.9 kPa)). The effluent was monitored by TLC (1:1 v:v hexane:ethyl acetate, visualized by *p*-anisaldehyde). The *Z* isomer **9***b*, $R_f$ = 0.33, was obtained as a colorless oil (55 mg, 335 μmol) in 25% yield. $^1$H NMR (250 MHz, $CDCl_3$) δ: 1.85 (s, 3H, $CH_3$), 2.53 (t, $J$ = 6.7 Hz, 2H, $CH_2CH_2O$), 3.78 (t, $J$ = 6.7 Hz, 2H, $CH_2O$), 6.01 (s, 1H, C*H*=C). The *E* isomer **9***a*, $R_f$ = 0.29, was obtained as a colorless oil (142 mg, 860 μmol) in 64% yield. $^1$H NMR (250 MHz, $CDCl_3$) δ: 1.83 (s, 3H, $CH_3$), 2.38 (t, $J$ = 6.2 Hz, 2H, $CH_2CH_2O$), 3.72 (t, $J$ = 6.3 Hz, 2H, $CH_2O$), 6.01 (s, 1H, C*H*=C).

E-*4-Bromo-3-methyl-3-buten-1-yl*-tert-*butyldimethylsilyl ether (**10**a)*

To a solution of *E*-4-bromo-3-methyl-3-buten-1-ol (**9***a*) (249 mg, 1.51 mmol) in 10 mL dry DMF were added imidazole (1.07 g, 15.3 mmol) and *tert*-butyldimethylsilyl chloride (Aldrich, 904 mg, 5.99 mmol). The reaction mixture was shaken vigorously for 5 min, effecting a complete consumption of starting material. To the reaction mixture was added 20 mL ether, followed by washing with 3 × 20 mL water. The aqueous layer was saturated with brine and back-extracted with ether. The ether layer was dried over anhydrous $MgSO_4$, and solvent was removed via rotovap. The colorless residue was purified by flash chromatography on silica gel (hexane, followed by 99:1 v:v hexane:ethyl acetate) to afford **10***a* as a colorless oil (391 mg,1.40 mmol) in 93% yield. IR (neat, $cm^{-1}$): 3074, 2954, 2929, 2896, 2857, 2738, 1632, 1472, 1431, 1408, 1381, 1361, 1288, 1256, 1213, 1161, 1105, 1006, 956, 939, 836, 775, 713, 662. $^1$H NMR (250 MHz, $CDCl_3$) δ: 0.02 (s, 6H, $(CH_3)_2Si$), 0.86 (s, 9H, $(CH_3)_3CSi$), 1.78 (s, 3H, $CH_3$), 2.29 (t, $J$ = 6.6 Hz, 2H, $CH_2CH_2OSi$), 3.66 (t, $J$ = 6.6 Hz, 2H, $CH_2OSi$), 5.92 (s, 1H, C*H*=C). $^{13}$C NMR (100 MHz, $CDCl_3$) δ: −5.4, 18.2, 19.4, 25.9, 41.4, 61.2, 102.7, 138.9. MS ($CI^+/CH_4$) (M + 1) ($^{79}$Br): 279. HRMS, calcd. for $C_{11}H_{23}OSiBr$: 279.0773; found: 279.0791.

Z-*4-Bromo-3-methyl-3-buten-1-yl*-tert-*butyldimethylsilyl ether (**10**b)*

To a solution of *Z*-4-bromo-3-methyl-3-buten-1-ol (**9***b*) (61 mg, 372 μmol) in 1 mL dry DMF were added imidazole (122 mg, 1.74 mmol) and *tert*-butyldimethylsilyl chloride (222 mg, 1.48 mmol, Aldrich). The reaction was carried out as described for the *E* isomer **10***a*. **10***b* was obtained as a colorless oil (81 mg, 290 μmol) in 78% yield. $^1$H NMR (250 MHz, $CDCl_3$) δ: 0.04 (s, 6H, $(CH_3)_2Si$), 0.87 (s, 9H, $(CH_3)_3CSi$), 1.61 (s, 3H, $CH_3$), 2.42 (t, $J$ = 6.9 Hz, 2H, $CH_2CH_2OSi$), 3.70 (t, $J$ = 6.9 Hz, 2H, $CH_2OSi$), 5.90 (s, 1H, C*H*=C).

E-*[4-$^2$H]-3-Methyl-3-buten-1-yl*-tert-*butyldimethylsilyl ether (**11**c) (12)*

An oven-dried, nitrogen-cooled flask was charged with *E*-4-bromo-3-methyl-3-buten-1-yl-*tert*butyldimethylsilyl ether **10***a* (93 mg, 332 μmol), which had been dried at atmospheric pressure in a desiccator containing $P_2O_5$ for 12 h prior to use. Dry *N,N,N′,N′* tetramethylethylenediamine (TMEDA, 0.20 mL) and 1.5 mL dry ether were added via syringe. The reaction mixture was cooled to −78°C before the addition of *tert*-butyllithium (0.43 mL, 1.7 M in pentane, 731 μmol) via syringe. Upon addition of *tert*-butyllithium, the colorless solution turned bright yellow. After 5 min the reaction mixture was quenched with trifluoroacetic acid 1-*d* (Aldrich, 99.8 at.% *d*, 100 μL, 1.3 mmol). After an additional 5 min, the reaction mixture was warmed to 0°C, and 2 mL sodium phosphate buffer, pH 7.0, was added. The reaction mixture was warmed to room temperature and was extracted with 3 × 3 mL ether. The ether extract was washed with brine and solvent was removed via rotovap. The colorless oily residue was purified by flash chromatography on silica gel (5 g, hexane, then 99:1 v:v hexane:ethyl acetate), and solvent was removed in vacuo to afford 39 mg of a colorless oil (58% crude yield). $^1$H NMR (250 MHz, $CDCl_3$) δ: 0.03 (s, 6H, $(CH_3)_2Si$), 0.87 (s, 9H, $(CH_3)_3CSi$), 1.71 (s, 3H, $CH_3$), 2.21 (t, $J$ = 7.0 Hz, 2H, $CH_2CH_2OSi$), 3.69 (t, $J$ = 7.0 Hz, 2H, $CH_2OSi$), 4.66 (s, 1H, C*H*=C), 4.73 (s, 0.04 H, 4*Z*-C*H*=C). In addition, the following impurity peaks, each integrating to ca. 0.1 H, were present: 0.12 (s), 1.06 (s), 1.52 (s, $H_2O$), 1.65 (s), 1.69 (s), 2.10 (t), 2.45 (t), 3.61 (dt).

E-*[4-$^2$H]-3-Methyl-3-buten-1-yl-tosylate (**13**c)*

To the TBDMS ether **11***c* (39 mg, 0.19 mmol, assuming 100% purity) was added tetra(*n*-butyl)ammonium fluoride (TBAF, 1.0 M in THF, 400 μL, 0.40 mmol), and the reaction mixture was allowed to stir for 15 min. Then 1 mL water was added and the mixture was extracted with 5 × 2 mL $CH_2Cl_2$. The organic layer was washed with brine and dried over anhydrous $Na_2SO_4$. The organic layer was then concentrated to a volume of <1 mL by simple distillation of the solvent using a Vigreux column. Pyridine (1 mL) and *p*-toluenesulfonyl chloride (*p*-TsCl, recrystallized, 241 mg, 1.16 mmol) were added and the solution was stirred under nitrogen for 13 h. Solvent was partially removed via rotovap and the residual mixture was cooled to 0°C, mixed with 2 mL distilled water, and extracted with 2 × 3 mL ether. The organic layer was washed successively with 10% $NaHCO_3$ and saturated $CuSO_4$:$5H_2O$, and the aqueous washings were back- extracted with ether. The combined organic layers were washed successively with water and brine and then dried over anhydrous $Na_2SO_4$. Solvent was removed via rotovap and the residue was purified by flash chromatography on silica gel (5 g, 98:2 v:v hexane: ethyl acetate). **13***c* was obtained as a colorless oil (7 mg, 29 μmol) in 24% yield from **11***c*. (Unlabeled isopentenyl tosylate was obtained in 35% overall yield from pure, unlabeled isopentenyl-*tert*-butyldimethylsilyl ether, using the above procedure.) $^1$H NMR (250 MHz, $CDCl_3$) δ: 1.54 (s, $H_2O$), 1.64 (s, 3H, $CH_3$), 2.33 (t, $J$ = 6.8 Hz, 2H, $CH_2CH_2OTs$), 2.43 (s, 3H, Ar$CH_3$), 4.10 (t, $J$ = 6.9 Hz, 2H, $CH_2OTs$), 4.64 (s, 1H, C*H*=C), 4.75 (s, 0.04 H, 4*E*-C*H*=C), 7.30 (d, $J$ = 8.0 Hz, 2H, Ar*H*), 7.76 (d, $J$ = 8.3 Hz, 2H, Ar*H*). $^2$H NMR (61 MHz, $CHCl_3$) δ: 4.71 (small br s), 4.83 (s). The ratio of areas of resonances at 4.83 and 4.71 in the $^2$H NMR spectrum was 92:8.

Z-*[4-$^2$H]-3-Methyl-3-buten-1-yl*-tert-*butyldimethylsilyl ether (**11**d) (12)*

To an oven-dried, nitrogen-cooled flask charged with *Z*-4-bromo-

3-methyl-3-buten-1-yl-*tert*-butyldimethylsilyl ether **10***b* (23 mg, 83 μmol), which had been dried at room temperature and at atmospheric pressure in a desiccator containing $P_2O_5$ for 12 h prior to use, was added dry TMEDA (80 μL) and 0.5 mL dry ether. The reaction mixture was cooled to −78°C before the addition of *tert*-butyllithium (0.11 mL, 1.7 M in pentane, 187 μmol) via dry syringe. Upon addition of *tert*-butyllithium, the reaction mixture became a bright yellow color. After 5 min, the reaction was quenched with trifluoroacetic acid 1-*d* (99.8 at.% *d*, Aldrich, 48 μL, 636 μmol). The product was isolated and purified in a manner completely analogous to that described for **11***c* to give a colorless oil (16 mg, 81 μmol if 100% pure) in 98% crude yield. The product was not analyzed by $^1$H NMR at this point, but was used as is for making the tosylate **13***d*.

*Z-[4-$^2$H]-3-Methyl-3-buten-1-yl-tosylate (**13**d)*

To crude **11***d* (16 mg, 81 μmol) was added TBAF (1.0 M in THF, 160 μL, 160 μmol). The reaction was allowed to proceed for 10 min, after which 1 mL water was added. The aqueous solution was saturated with sodium chloride and extracted with 5 × 2 mL ether. Concentration of the ether extract and conversion to the tosylate **13***d* was carried out as described for the preparation of **13***c*. Purification by flash chromatography on silica gel (5 g, 98:2 v:v hexane:ethyl acetate) afforded **13***d* as a colorless oil (3 mg, 12 μmol) in 15% overall yield from **10***b*. $^1$H NMR (250 MHz, $CDCl_3$) δ: 1.54 (s, 2H, $H_2O$), 1.63 (s, 3H, $CH_3$), 2.33 (t, $J$ = 6.9 Hz, 2H, $CH_2CH_2OTs$), 2.43 (s, 3H, Ar$CH_3$), 4.11 (t, $J$ = 6.9 Hz, 2H, $CH_2OTs$), 4.64 (s, 0.14 H, 4*Z*-C*H*=C), 4.75 (s, 1H, C*H*=C), 7.31 (d, $J$ = 8.0 Hz, 2H, Ar*H*), 7.76 (d, $J$ = 8.3 Hz, 2H, Ar*H*). $^2$H NMR (61 MHz, $CHCl_3$) δ: 4.74 (s), 7.28 (s, $CDCl_3$).

*Z-[4-$^3$H]-3-Methyl-3-buten-1-yl tosylate (**13**b)*

To a solution of *Z*-4-bromo-3-methyl-3-buten-1-yl-*tert*-butyldimethylsilyl ether (**10***b*) (80 mg, 288 μmol, dried over $P_2O_5$) in 1.5 mL dry ether was added 0.20 mL dry TMEDA. The reaction mixture was cooled to −78°C under a slight positive pressure of nitrogen, and *tert*-butyllithium (0.38 mL, 1.7 M in pentane, 0.65 mmol) was added. A bright yellow color was immediately observed. Within 5 min, the reaction mixture was rapidly quenched with 60 μL (89 mg, 780 μmol) of tritiated trifluoroacetic acid (45 μCi/μmol) at −78°C. After an additional 5 min, the reaction mixture was warmed to 0°C and 2 mL of sodium phosphate buffer, pH 7.0, were added. The product, **11***b*, was isolated as described for **11***c*. The desilylation of **11***b* with TBAF (1.0 M in THF, 600 μL, 600 μmol) and the tosylation of crude alcohol **12***b* using 0.5 mL dry pyridine and *p*-TsCl (recrystallized, 297 mg, 1.56 mmol) were carried out as described for the preparation of **13***d*. Counting of an aliquot indicated a total activity for **13***b* of 6.01 × $10^9$ dpm (2.73 mCi). The tosylate was recovered in 23% chemical yield (16 mg, 66 μmol, 41.2 μCi/μmol) from **10***b*. $^1$H NMR (250 MHz, $CDCl_3$) δ: 1.64 (s, 3H, $CH_3$), 2.33 (t, $J$ = 6.8 Hz, 2H, $CH_2CH_2OTs$), 2.43 (s, 3H, Ar$CH_3$), 4.10 (t, $J$ = 6.9 Hz, 2H, $CH_2OTs$), 4.66 (s, 1H, *Z*-C*H*), 4.77 (s, 1H, *E*-C*H*), 7.31 (d, $J$ = 8.0 Hz, 2H, Ar*H*), 7.77 (d, $J$ = 8.5 Hz, 2H, Ar*H*).

E-*[4-$^3$H]-3-Methyl-3-buten-1-yl tosylate (**13**a)*

*E*-4-Bromo-3-methyl-3-buten-1-yl-*tert*-butyldimethylsilyl ether **10***a* (82 mg, 292 μmol, dried over $P_2O_5$) was metallated at −78°C with *tert*-butyllithium (0.42 mL, 1.7 M in pentane, 0.71 mmol) in TMEDA–ether and quenched with tritiated trifluoroacetic acid (45 mCi/mmol, 85 μL, 127 mg, 1.11 mmol) as described for the preparation of **11***c*. Desilylation of **11***c* with TBAF (1.0 M in THF, 600 μL, 600 μmol) gave the alcohol **12***a*, which was converted in the usual manner to the corresponding tosylate **13***a* (26 mg, 107 μmol, 21.9 μCi/μmol) in 34% chemical yield from **10***a*. $^1$H NMR (250 MHz, $CDCl_3$) δ: 1.64 (s, 3H, $CH_3$), 2.33 (t, $J$ = 6.8 Hz, 2H, $CH_2CH_2OTs$), 2.43 (s, 3H, Ar$CH_3$), 4.10 (t, $J$ = 6.9 Hz, 2H, $CH_2OTs$), 4.66 (s, 1H, *Z*-C*H*), 4.77 (s, 1H, *E*-C*H*), 7.31 (d, $J$ = 8.0 Hz, 2H, Ar*H*), 7.77 (d, $J$ = 8.5 Hz, 2H, Ar*H*).

E-*[4-$^3$H]-3-Methyl-3-buten-1-yl diphosphate (**2**a) (13)*

To a solution of *E*-[4-$^3$H]-3-methyl-3-buten-1-yl tosylate **13***a* (2 mg, 8 μmol, 21.9 μCi/μmol) in 200 μL dry acetonitrile was added tris(tetra(*n*-butyl)ammonium) hydrogen pyrophosphate (101 mg, 112 μmol). The reaction was carried out for 2 h at room temperature under nitrogen, after which the reaction mixture was partitioned between 1 mL hexane and 1 mL water. The two layers were vortexed and a 10-μL aliquot of each layer was counted for $^3$H activity. By comparison of the $^3$H activity of the aqueous layer ((4.11 ± 0.04) × $10^4$ dpm) with that of the hexane layer ((1.63 ± 0.07) × $10^3$ dpm), it was determined that pyrophosphorylation had gone to 96% completion. Solvent was removed by passing a stream of nitrogen over the surface of the liquid, followed by drying at high vacuum, and the white solid residue was taken up to 1 mL 0.05 M triethylammonium bicarbonate, pH 8.0. This solution was applied to a DEAE–Sephadex anion exchange column previously equilibrated with 0.05 M triethylammonium bicarbonate at 4°C. The product was eluted using a linear gradient of 0.05–1.0 M triethylammonium bicarbonate. A total of 67 fractions (250 drops perfraction) was collected and a 10-μL aliquot of every other fraction was counted for radioactivity. Fractions 29–33 were combined and lyophilized at (5–10) × $10^{-3}$ Torr. Two 5-mL portions of 1.0 M ammonium hydroxide were added and the resulting solution was lyophilized each time to near dryness. The fluffy white solid was then taken up in 2 mL isopropanol:acetonitrile:0.1 M ammonium bicarbonate (4.5:2.5:3.0, v:v:v) and the solution was applied to a flash column of EM-Avicel Microcrystalline Cellulose (1.5 cm × 10 cm) previously equilibrated with 4 column volumes of the above elution buffer. The column was run at room temperature, a total of 60 fractions (3 mL each) was collected, and a 20-μL aliquot of every other fraction was counted for radioactivity. Fractions 5–19 were combined, a few drops of 1.0 M ammonium hydroxide were added, and the solution was concentrated to about half its original volume via rotovap over a period of 1 h. A few drops of 1.0 M ammonium hydroxide were added after approximately 30 min to keep the solution slightly basic. Acetonitrile and isopropanol were completely removed from the concentrated solution via SpeedVac over a period of 2 h, during which time a few drops of 1.0 M ammonium hydroxide was added intermittently. The resulting aqueous solution was lyophilized to dryness, then taken up in 5 mL 0.2 M ammonium hydroxide. **2***a*, total $^3$H activity 3.51 × $10^8$ dpm (160 μCi, 7.28 μmol), 92% yield from **13***a*. $^1$H NMR (250 MHz, $D_2O/NH_4OD$) δ: 1.60 (s, 3H, $CH_3$), 2.22 (t, $J$ = 6.6 Hz, 2H, $CH_2CH_2OPP$), 3.69 (dt, $J_1 = J_2$ = 6.5 Hz, 2H, $CH_2OPP$), 4.60 ($H_2O$ very intense, obscuring the olefinic protons).

*Z-[4-$^3$H]-3-Methyl-3-buten-1-yl diphosphate (**2**b) (13)*

**13***b* (6 mg, 25 μmol, 41.2 μCi/μmol) in 400 μL dry acetonitrile was reacted with tris(tetra(*n*-butyl)ammonium) hydrogen pyrophosphate (202 mg, 223 μmol) for 6 h at room temperature. The product was isolated and purified as described for **2***a* to give **2***b*, which was taken up in 10 mL 0.1 M ammonium hydroxide. Total $^3$H activity 6.52 × $10^8$ dpm (294 μCi, 7.13 μmol), 29% overall yield from **13***b*. $^1$H NMR (250 MHz, $D_2O/NH_4OD$) δ: 1.60 (s, 3H, $CH_3$), 2.22 (t, $J$ = 6.6 Hz, 2H, $CH_2CH_2OPP$), 3.69 (dt, $J_1 = J_2$ = 6.5 Hz, 2H, $CH_2OPP$), 4.60 ($H_2O$ very intense, and obscuring the olefinic protons).

*[1-$^3$H]-3-Methyl-3-buten-1-ol (**14**)*

To a solution of 3-methyl-2-butenal (**15**) (15 mg, 180 μmol, Aldrich) in 250 μL methanol at 0°C, was added $NaBH_3T$ in methanol (316.7 μCi/μmol, 50 μCi/μL, 5 μL, 0.79 μmol), and the reaction was allowed to proceed at 0°C for 1 h. Unlabeled $NaBH_4$ was added (15 mg, 385 μmol), and the reaction was allowed to proceed for an additional 5–10 min, followed by the dropwise addition of 5% HCl (1 mL). The aqueous solution was extracted with 6 × 1 mL pentane. The combined pentane extracts were filtered through a Pasteur pipet containing anhydrous $MgSO_4$ into a flask charged with unlabeled carrier 3-methyl-2-buten-1-ol (516 mg, 5.99 mmol). Solvent was removed via rotovap for 5–10 min. The alcohol was stored at −10°C. $^1$H NMR analysis of a sample of **14** prepared under the same conditions with unlabeled $NaBH_4$ indicated the absence of any 1,4 reduction.

The corresponding crystalline 3,5-dinitrobenzoate ester derivative **16**, which was obtained by reaction of a portion of the tritiated alcohol

(152 mg, 1.76 mmol) and 3,5-dinitrobenzoyl chloride (423 mg, 1.97 mmol) in dry pyridine (1.5 mL) for 3.5 h, was recrystallized to constant specific activity ($2.28 \times 10^3$ dpm $^3$H/mg, 0.29 nCi/μmol) and mp (67.5–69.0°C) from benzene–hexane. $^1$H NMR (250 MHz, $CDCl_3$) δ: 1.55 (br, $H_2O$), 1.81 (s, with fine splitting, 6H, $CH_3$), 4.95 (d, $J$ = 7.4 Hz, 2H, $CH_2O$), 5.49 (t with splitting, $J$ = 7.4 Hz, 1H, C*H*=C), 9.17 (s with fine splitting, 2H, Ar*H*), 9.21 (s with fine splitting, 1H, Ar*H*).

*[1-$^3$H]-3-Methyl-2-buten-1-yl diphosphate (**3**) (13, 14)*

To a solution of [1-$^3$H]-3-methyl-2-buten-1-ol (**14**) (200 mg, 2.32 mmol, 0.29 nCi/μmol) in hexachloroacetone (1.7 mL, 975 mg, 3.68 mmol) at 0°C was added triphenylphosphine (645 mg, 2.46 mmol) in small portions over a period of 10 min. The reaction was allowed to proceed at room temperature for 20 min. Analysis of the reaction mixture by TLC (1:1 v:v hexane:ethyl acetate) showed that the alcohol had been completely consumed. The resulting thick dark brown slurry was cooled to −78°C, and the reaction flask was attached to a flash distillation apparatus. While the receiver flask was kept at room temperature, the distillation system was evacuated to 0.01–0.02 Torr for 1–2 min, then closed to the vacuum pump but kept under vacuum. The receiver flask was cooled to −78°C and the reaction flask was warmed to room temperature. The flash distillation proceeded for 1 h, at which time the system was bled to the atmosphere. When the reaction was carried out on unlabeled alcohol, the recovered 1-chloro-3-methyl-2-butene was analysed by $^1$H NMR (250 MHz, $CDCl_3$) δ: 1.55 (br, <1H, $H_2O$), 1.73 (d, $J$ = 11.2 Hz, 6H, $CH_3 \times 2$), 4.07 (d, $J$ = 8.0 Hz, 2H, $CH_2Cl$), 5.44 (t, with fine splitting, $J$ = 8.0 Hz, 1H, C*H*=C). Also present were two impurity peaks: 2.17 (s, 0.2 H), and 6.74 (s, 0.03 H). The labeled chloride **17**, without any further purification or analysis, was taken up in 1 mL dry acetonitrile and tris(tetra(*n*-butyl)ammonium) hydrogen pyrophosphate (1.60 g, 1.77 mmol) was added. The reaction proceeded at room temperature for 12 h. Analysis of an aliquot of the reaction mixture partitioned between 1 mL water and 1 mL hexane showed that the reaction had only gone to two-thirds completion. The volatile components of the reaction mixture were evaporated with a stream of nitrogen and the resulting thick brown oil was dissolved in 1 mL 0.05 M triethylammonium bicarbonate, pH 8.0, and then extracted with 3 × 1 mL hexane to remove any unreacted allylic chloride. A stream of nitrogen was passed over the aqueous layer to remove any traces of hexane. The aqueous solution was applied to a DEAE–Sephadex anion exchange column equilibrated with 0.05 M triethylammonium bicarbonate at 4°C. The product was eluted by a linear gradient of 0.05–1.0 M triethylammonium bicarbonate pH 8.0. A total of 52 fractions (250 drops each) was collected and a 100-μL aliquot of every other fraction was counted for radioactivity. Fractions 25–30 were combined and lyophilized to near dryness. Two portions of ammonium hydroxide (5 mL, 1.0 M) were added and each time the solution was lyophilized to near dryness. The white solid residue was taken up in 2 mL isopropanol:acetonitrile:0.1 M ammonium bicarbonate (4.5:2.5:3.0, v:v:v) and applied to a flash column of cellulose (1.5 cm × 8 cm) previously equilibrated with the same elution buffer. A total of 36 fractions (3 mL each) was collected and fractions 4–12 were combined. Solvent was removed successively by rotovap, SpeedVac, and lyophilization just to dryness. Intermittently, a few drops of 1 M ammonium hydroxide were added to keep the pH slightly basic. The recovered **3** was taken up in 2 mL 0.25 M ammonium hydroxide. Total $^3$H activity $1.21 \times 10^5$ dpm (54.5 μCi, 190 μmol), 8% overall yield from the alcohol. $^1$H NMR (250 MHz, $D_2O/ND_4OD$) δ: 1.37 (s, 3H, $CH_3$), 1.41 (s, 3H, $CH_3$), 4.09 (dd, $J_1 = J_2$ = 6.7 Hz, 2H, $CH_2OPP$), 5.09 (t with fine splitting $J$ = 7.2 Hz, 1H, C*H*=C).

*(4R,8R)-[4,8-$^3H_2$,4,8-$^{14}C_2$]FPP (**1**b) (11)*

A mixture of *Z*-[4-$^3$H]-IPP (**2***b*, 41.2 μCi/μmol, 80.4 nCi/μL, 513 μL, 1.00 μmol), [4-$^{14}$C]IPP (47.9 μCi/μmol, 20 nCi/μL, 400 μL, 0.17 μmol), and [1-$^3$H]DMAPP (**3**, 0.29 nCi/μmol, 12.9 nmol/μL, 1.17 μmol) in 2.40 mL 20 mM Tris-HCl, pH 7.72, containing 10 mM $MgCl_2.6H_2O$ and 7 mM DTT, was incubated in a total volume of 3.8 mL with avian liver prenyl transferase (400 μL) that had been purified by literature methods (20) through the DE-52 step to a specific activity of 43.5 nmol min$^{-1}$ (mg protein)$^{-1}$ (13.0 pmol min$^{-1}$ μL$^{-1}$). The concentration of each substrate in the incubation mixture was 307 μM. A 20-μL aliquot of the mixture was counted for activity: $^3$H $5.84 \times 10^5$ dpm, $^{14}$C $9.55 \times 10^4$ dpm. After 2 h at 30°C, two separate 20-μL aliquots of the incubation mixture were withdrawn. One aliquot was mixed with 500 μL 1:1 v:v EtOH:6 N HCl and extracted with 1 mL hexane. The hexane layer was filtered through a Pasteur pipet column of silica gel, and the silica gel column was washed with 1 mL ether. The ether–hexane eluate (acid labile, hexane-extractable) was counted for radioactivity: $^3$H $2.09 \times 10^5$ dpm, $^{14}$C $2.97 \times 10^4$ dpm. The other aliquot was mixed with 500 μL 1:1 v:v EtOH:$H_2O$ and extracted with 1 mL hexane. The hexane layer was filtered through a Pasteur pipet column of silica gel, and the column of silica gel was washed with 1 mL ether. The ether–hexane eluate (blank) was counted for radioactivity: $^3$H $1.10 \times 10^4$ dpm, $^{14}$C $1.63 \times 10^3$ dpm. Based on $^{14}$C activity, 30% of the IPP has been turned over by the enzyme. After 2.5 h of incubation, the reaction mixture was applied to a column (20 cm × 2.5 cm) of Sephadex G-25 (100–300 μm bead size) swollen in Eluant One (see below). A total of 48 fractions (2–3 mL each) was collected, and a 10-μL aliquot of every other fraction was counted for activity. Fractions 14–36 were combined and stored at 4°C until further purification. The preparation of the various buffers for C-18 Reverse Phase HPLC (12) was as follows:

Solution A: $(n\text{-Bu})_4NHSO_4$ (6.8 g) and $K_2HPO_4$ (5.5 g) in nanopure water (100 mL) adjusted to pH 8.0. The solution was vacuum filtered through a Millipore HA filter.

Solution B: 10 mL solution A diluted with 490 mL nanopure water and vacuum filtered through a Millipore HA filter.

Solution C: 10 mL solution A diluted with 490 mL HPLC-grade methanol. The precipitated phosphate was removed by vacuum filtration through a Millipore FH filter. The filtrate was then diluted with 1470 mL Millipore FH-filtered methanol.

Eluant One: a mixture of solution C with solution B in a ratio of 15:85 v:v filtered by suction through a Millipore GV filter.

Eluant Two: a mixture of solution C with solution B in a ratio of 70:30 v:v filtered by suction through a Millipore FH filter.

Fractions 14–36 in Eluant One were loaded onto a Waters C-18 μ-Bondapak reverse phase HPLC column in a Z-module. The column was developed at 1.5 mL/min for 5 min using 100% Eluant One followedby a linear gradient up to 100% Eluant Two over a period of 15.4 min. The column was run with a flow rate of 1.5 mL/min using 100% Eluant Two for an additional 30 min. A total of 84 fractions of 50 drops each was collected. A 10-μL aliquot of every other fraction was counted for radioactivity. Fractions 37–43 were combined, mean retention time 28 min, corresponding to the retention time of [1-$^3$H]FPP used as a standard. Fractions 37–43 were loaded onto a column of DEAE–Sephadex previously swollen and equilibrated with 0.05 M triethylammonium bicarbonate pH 8.0 at 4°C. A linear gradient was established with 1.0 M triethylammonium bicarbonate pH 8.0. A total of 70 fractions of 250 drops each was collected. A 10-μL aliquot of every other fraction was counted for activity. Fractions 39–44 were combined, and the sample lyophilized almost to dryness at a pressure of $(7–10) \times 10^{-3}$ Torr. The residue was taken up in ca. 9 mL of 0.1 M ammonium hydroxide and lyophilized almost to dryness. Finally, the residue was taken up in ca. 5 mL 0.1 M ammonium hydroxide and lyophilized just to dryness. The sample was taken up in 1 mL 0.05 M ammonium hydroxide. **1***b*, total $^3$H activity $9.60 \times 10^6$ dpm (9% radiochemical yield, based on [4-$^3$H]IPP (**2***b*)).

*(4S,8S)-[4,8-$^3H_2$,4,8-$^{14}C_2$]FPP (**1**a)*

A mixture of *E*-[4-$^3$H]-IPP (**2***a*, 21.9 μCi/μmol, 39.9 nCi/μL, 548 μL, 1.0 μmol), [4-$^{14}$C]IPP (47.9 μCi/μmol, 20 nCi/μL, 218 μL, 0.09 μmol), and [1-$^3$H]DMAPP (**3**, 0.29 nCi/μmol, 12.9 nmol/μL, 1.08 μmol) in 1.95 mL 20 mM Tris-HCl, pH 7.72, containing 10 mM $MgCl_2.6H_2O$ and 7 mM DTT, was incubated with avian liver prenyl transferase (400 μL) in a total volume of 3.20 mL at 30°C for 2.5 h. The concentration of each substrate was 339 μM. The desired product, (4*S*,8*S*)-[4,8-$^3H_2$,4,8-$^{14}C_2$]FPP (**1***a*), was separated by successive

Sephadex G-25, C-18 reverse phase HPLC, and DEAE–Sephadex anion exchange chromatography as described above for **1***b* (10% radiochemical yield, based on [4-$^3$H]IPP (**2***a*)).

*(4*R,*8*R*)-[4,8-$^3H_2$,4,8-$^{14}C_2$]Farnesyl diphenylurethane (**18**b) (17)*

An aliquot of a dilute solution of (4*R*,8*R*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***b*, $6.39 \times 10^2$ dpm $^3$H/μL, 150 μL, total $^3$H = $9.55 \times 10^4$ dpm) was taken up in NaOAc/HOAc buffer, pH 5.5, and incubated with acid phosphatase (Sigma, 8.5 mg) at 30°C for 3.5 h. Subsequently 1 mL EtOH was added, followed by unlabeled *trans,trans*-farnesol (Aldrich, 50 mg, 225 μmol) in 2 mL hexane. The mixture was extracted, and the hexane layer was filtered through a Pasteur pipet containing anhydrous $MgSO_4$. The mixture was further extracted with $5 \times 2$ mL hexane. Each hexane extract was filtered through anhydrous $MgSO_4$. Solvent was removed via rotovap. The residue was then taken up in 2 mL hexane and a 20-μL aliquot had $^3$H $7.57 \times 10^2$ dpm, $^{14}$C $1.11 \times 10^2$ dpm, corresponding to a total $^3$H activity of $7.57 \times 10^4$ dpm (80% radiochemical yield from **1***b*). The radioactive farnesol was dissolved in 1 mL dry pyridine and reacted with *N,N*-diphenyl carbamoyl chloride (recrystallized from hexane, 205 mg, 882 μmol). The mixture was heated on an oil bath at 104°C for 11 h. The reaction mixture was cooled to room temperature and 1 mL water was added, followed by extraction with $5 \times 2$ mL ether. The ether extracts were washed with three portions of saturated $CuSO_4.5H_2O$ to remove the pyridine, and the $CuSO_4$ layer was back-extracted with ether. The combined ether extracts were washed with brine and dried over anhydrous $MgSO_4$. Solvent was removed via rotovap. The residue was purified by flash chromatography on silica gel (5 g, 98:2 v:v hexane:ethyl acetate) to give **18***b* as a white solid (74 mg, 178 μmol) in 80% yield. $^1$H NMR (250 MHz, $CDCl_3$) δ: 1.59 (s, 6H, $CH_3 \times 2$), 1.67 (s, 6H, $CH_3 \times 2$), 1.93–2.10 (m, 8H, $CH_2$, at C4, C5, C8, and C9), 4.69 (d, $J$ = 6.8 Hz, 2H, $CH_2O$), 5.08 (bt, $J$ = 6.7 Hz, 2H, C*H*=C, at C6 and C10), 5.33 (bt, $J$ = 6.9 Hz, 1H, C*H*=C at C2), 7.15–7.35 (m, 10H, Ar*H*). **18***b* was recrystallized 4 times from methanol to constant specific activity, isotope ratio, and mp. $^3$H/$^{14}$C 6.95 ± 0.10, mp 60.5–61.0°C (lit. (17) mp 61–62°C), 174 ± 0.4 pCi $^3$H/μmol and 25.2 ± 0.5 pCi $^{14}$C/μmol. From the isotope ratio and the specific activity of the diphenylurethane, the $^3$H specific activity of *Z*-[4-$^3$H, 4-$^{14}$C]IPP (**2***b*) was calculated to be 36.7 μCi/μmol, and the $^3$H specific activity of (4*R*,8*R*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***b*) was calculated to be 73.3 μCi/μmol.

*(4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]Farnesyl diphenylurethane (**18**a) (17)*

An aliquot of a solution of (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***a*) was converted to the corresponding (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]farnesyl diphenylurethane (**18***a*) as described above for **18***b*. The diphenylurethane was recrystallized 4 times from methanol to constant specific activity, isotope ratio, and mp. $^3$H/$^{14}$C 6.02 ± 0.01, 104 ± 1 pCi $^3$H/μmol, 17.6 ± 0.5 pCi $^{14}$C/μmol, mp 60.5–61.5°C (lit. (17) mp 61–62°C). Based on the isotope ratio and the specific activity of the diphenylurethane, the $^3$H specific activity of *E*-[4-$^3$H, 4-$^{14}$C]IPP (**2***a*) was calculated to be 20.4 μCi/μmol, and the $^3$H specific activity of (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***a*)was calculated to be 40.7 μCi/μmol.

*Isolation of pentalenene synthase from* Streptomyces *UC5319 (5, 9, 10)*

A vegetative medium consisting of Pharmamedia (2.5 g) and Bactodextrose (2.5 g) in 100 mL nanopure water, pH adjusted to 7.2, in a 500-mL flask was inoculated with spores of *Streptomyces* UC5319 and the culture incubated at 26°C at 300 rpm for 1 day and at 28°C at 300 rpm for an additional 1.5 days. A 40-mL portion of this 2.5-day-old vegetative culture was used to inoculate a production culture containing NaCl (8 g), $CaCO_3$ (5 g), Casein (acid type) (16.68 g), Bactodextrose (13.32 g), molasses (10 g), and Dextrin (corn type 3) (80 g) in 4 L nanopure water, pH adjusted to 7.2, in a 8-L cylindrical flask. The culture was shaken at 30 cycles/min at 32°C with continuous aeration (10 psi) for 2.5 days, then aeration was discontinued and the culture shaken at 30 cycles/min at 32°C for an additional 24 h. The contents of the flask were centrifuged (GS 3 rotor, 7500 rpm, 10 100 × *g*) at 4°C for 20 min and the mycelial pellet was suspended in 1.0 M KCl and recentrifuged at 10 100 × *g* at 4°C. The supernatant was decanted from the mycelial pellet. The above procedure was repeated successively with 0.8 M NaCl, nanopure water, and breaking buffer. The breaking buffer consisted of 50 mM $K_2HPO_4$, 10% glycerol (by volume), 1 mM EDTA – $Na_2$ in nanopure water. This solution was degassed in vacuo with vigorous stirring for 45 min, then chilled to 4°C, the pH adjusted to 7.2, and β-mercaptoethanol was added to a final concentration of 5 mM. The mycelial pellet was resuspended in 200 mL of breaking buffer, and phenylmethylsulfonyl fluoride (PMSF) (3.5 mg PMSF/1 mL acetone, 5 μL, final concentration 0.5 μM) was added. The resulting cell suspension was poured into a 350mL polycarbonate chamber containing a 100-ML volume of glass beads (0.5 mm diameter). Breaking buffer was added to barely cover the surface of the beads and air bubbles were removed with a glass stirring rod before the suspension was poured. The polycarbonate chamber was cooled with an ice-water jacket, and the cells were broken in a Biospec bead beater under a 15 s on/15 s off cycle for 6 min. The beads and the cellular debris were removed by centrifugation (GS 3 rotor, 7500 rpm, 10 100 × *g*) at 4°C for 20 min. The supernatant was decanted into a glass jar and stored at 4°C. Pentalenene synthase activity was assayed with [1-$^3$H]FPP (8.0 μM, $5.0 \times 10^5$ dpm/10 μL, 56.3 μCi/μmol, 10 μL, 4.0 nmol), in 480 μL 20 mM Tris buffer, pH 8.2, containing 10 μL of the cell-free extract. The mixture was incubated at 30°C for 10 min, then extracted with 1.5 mL hexane. The hexane extract was filtered through a Pasteur pipet of silica gel and the silica gel was washed with an additional 1.5 mL hexane. The hexane eluate was counted for radioactivity: $^3$H $6.94 \times 10^3$ dpm corresponding to pentalenene synthase activity in crude cell-free extracts of 5.5 nmol/mL cell-free extract per 10 min.

*Preparative-scale cyclization of (4*R,*8*R*)-[4,8-$^3H_2$, 4,8$^{14}C_2$]FPP (**1**b) to pentalenene (**4**b)*

A mixture of (4*R*,8*R*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***b* 73.3 μCi $^3$H/μmol, $9.59 \times 10^4$ dpm $^3$H/10 μL, 700 μL, 41.2 nmol, final concentration 5.9 μM), Tris pH 8.2 (4.90 mL), and cell-free extract (1.40 mL), was incubated at 30°C for 2 h. The incubation was quenched after 2 h by addition of 3 mL ethanol along with 99 mg synthetic (±)-pentalenene in 10 mL of pentane. The mixture was extracted and the pentane layer was filtered through silica gel. The aqueous phase was further extracted with $2 \times 10$ mL pentane and each pentane extract was passed through the silica gel column. Counting of an aliquot indicated a total $^3$H activity of $2.59 \times 10^6$ dpm and $^{14}$C activity of $4.10 \times 10^5$ dpm (39% radiochemical yield). The solution was concentrated to ca. 2 mL by rotovap and stored at −10°C until further use.

*Enzymatic cyclization of (4*S,*8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1**a) to pentalenene (**4**a)*

A mixture of (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***a*, 40.7 μCi $^3$H/μmol, $3.29 \times 10^4$ dpm $^3$H/10 μL, 1.20 mL, 43.7 nmol, final concentration 7.3 μM), Tris pH 8.2 (3.6 mL), and cell-free extract of *Streptomyces* UC5319 (1.20 mL) was incubated at 30°C for 2 h. After addition of carrier (±)-pentalenene (49 mg), the cyclization product was isolated as described above: total $^{14}$C activity $2.49 \times 10^5$ dpm (39% radiochemical yield). The pentane extracts were concentrated to ca. 2 mL by rotovap and stored at −10°C until further use.

*6,7-Dihydroxypentalenane (**19**b) and (**20**b) (10)*

A 1.50-mL portion of a solution of pentalene (**4***b*) (74 mg, 363 μmol) derived from (4*R*,8*R*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***b*) was converted to the corresponding mixture of diastereomeric *cis*-6,7-diols **19***b* and **20***b* as previously described (10). The resulting diastereomers were separated by fractional recrystallization from $CH_2Cl_2$–hexane and each diastereomer was recrystallized to constant activity (**19***b*, $^3$H $(3.85 \pm 0.02) \times 10^3$ pCi/μmol, $^{14}$C $(5.80 \pm 0.06) \times 10^2$ pCi/μmol; **20***b*, $^3$H $(3.74 \pm 0.18) \times 10^3$ pCi/μmol, $^{14}$C $(5.69 \pm 0.25) \times 10^2$ pCi/μmol), and isotope ratio (Table 1) (**19***b* mp 147–148°C (lit. (9) mp 145–146°C); **20***b* mp 75–76.5°C (lit. (9, 10) mp 74.5–75°C)).

*Conversion of pentalenene derived from (4*R,8R*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]-FPP (**1**b) to 7-hydroxypentalenane (**22**b) (9, 10)*

A 500-μL aliquot of a pentane solution of pentalenene (25 mg, 120 μmol) derived from **1***b* was converted to 7-hydroxypentalenane (**22***b*) in 64.0% yield as previously described (9, 10).

*7-Ketopentalenane (**21**b) (9, 10)*

Oxidation of **22***b* with PCC as previously described (10) gave the corresponding ketone **21***b*. $^3H/^{14}C$ 3.46 ± 0.11, $^3H$ (1.92 ± 0.02) × $10^3$ pCi/μmol, $^{14}C$ (5.55 ± 0.09) × $10^2$ pCi/μmol (Table 1).

*6,7-Dihydroxypentalenanes (**19**a and **20**a)*

**19***a* and **20***a* were prepared from pentalenene (**4***a*) derived from (4*S*,8*S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]FPP (**1***a*) by procedures identical to those described above. The individual diastereomers were separated by fractional recrystallization and the β diastereomer (**19***a*) was recrystallized to constant activity ($^3H$ (2.84 ± 0.09) × $10^3$ pCi/μmol, $^{14}C$ (8.99 ± 0.34) × $10^2$ pCi/μmol) and isotope ratio (Table 1); mp 147–148°C (9).

*Conversion of pentalenene derived from (4*S,8S*)-[4,8-$^3H_2$, 4,8-$^{14}C_2$]-FPP (**1**a) to 7-hydroxypentalenane (**22**a)*

The conversion of pentalenene (**4***a*) (10 mg, 49 μmol), derived from **1***a*, to **22***a* was carried out as described above.

*Preparation of 7-ketopentalenane (**21**a)*

**21***a* was prepared from the corresponding alcohol **22***a* as described above. $^3H$ (2.49 ± 0.02) × $10^3$ pCi/μmol, $^{14}C$ (8.31 ± 0.07) × $10^2$ pCi/μmol (Table 1).

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