BIOORGANIC CHEMISTRY 12, 312–328 (1984)

Pentalenene Biosynthesis and the Enzymatic Cyclization of Farnesyl Pyrophosphate: Proof that the Cyclization Is Catalyzed by a Single Enzyme

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Received April 18, 1984

A cell-free preparation from *Streptomyces* UC5319 has been developed which catalyzes the conversion of *trans, trans*-farnesyl pyrophosphate (**1**) to pentalenene (**2**), the parent hydrocarbon of the pentalenolactone family of sesquiterpene antibiotics. Incubation of [9-$^{3}H_2$, 12,13-^{14}C]farnesyl pyrophosphate with the pentalenene synthetase gave [1,8-$^{3}H_2$, 14,15-^{14}C]pentalenene, as established by a combination of chemical and microbial degradation methods. The retention of both equivalents of tritium in the enzymatically derived pentalenene establishes that the cyclization of *trans,trans*-farnesyl pyrophosphate to **2** is catalyzed by a single enzyme.

The study of terpenoid biosynthesis has entered a particularly exciting phase in recent years as attention has increasingly focused on the enzymes catalyzing the key cyclization reactions which characterize these nearly ubiquitous metabolic pathways (*1, 2*). Thus, cell-free studies have not only confirmed the long-held belief that geranyl and farnesyl pyrophosphate are the precursors of cyclized monoterpenes (*3*) and sesquiterpenes (*4*), respectively, but have provided heretofore inaccessible mechanistic and stereochemical details about these multistep carbocyclization reactions. The enzymatic cyclization of farnesyl pyrophosphate (**1**) (Scheme 1) to pentalenene (**2**), the parent hydrocarbon of the pentalenolactone family of antibiotics, has provided a particularly rich opportunity for the study of terpenoid cyclization reactions. We report below our studies of pentalenene synthetase, including results which establish that a single enzyme catalyzes the transformation of the acyclic farnesyl precursor to the tricyclic sesquiterpene hydrocarbon.

Several years ago we began a study of the biosynthesis of pentalenolactone (**3**), a terpenoid antibiotic which had been isolated from cultures of a variety of *Streptomyces* species (*5–7*). Using ^{13}C-labeled precursors we were able to establish the mevalonoid origin of pentalenolactone, and to suggest the basic outlines of the biosynthetic pathway leading to the formation of this novel metabolite. At the same time, independent investigations by Seto and his collaborators had resulted in the isolation and structure elucidation of a series of related metabolites repre-

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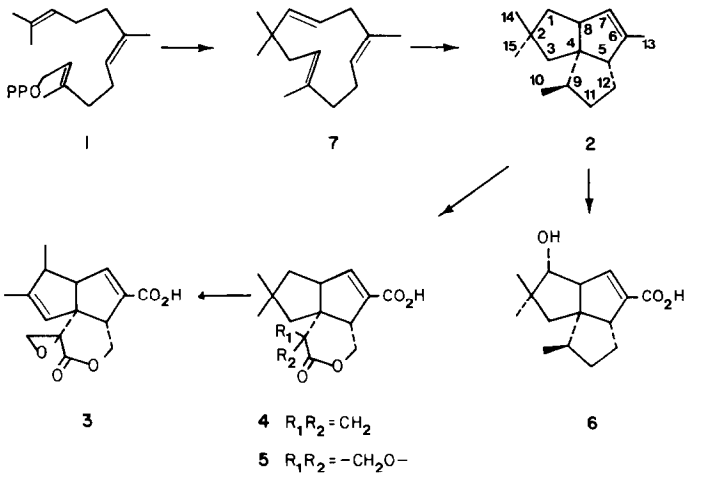
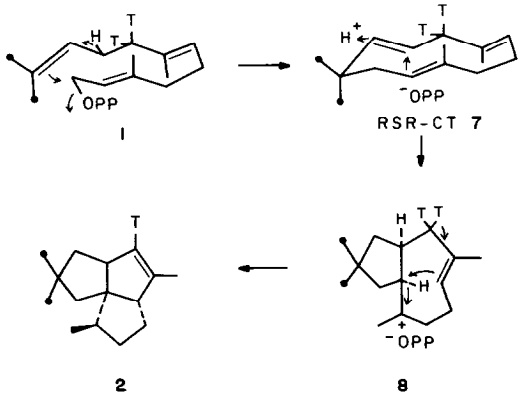
0045-2068/84 $3.00



SCHEME 1

senting potential intermediates or shunt metabolites of the latter stages of the biosynthetic pathway (*9, 10*). Of greatest significance was the reported isolation of the parent hydrocarbon itself, pentalenene (**2**) (*11*). The latter compound had, in fact, previously been prepared in racemic form by Shirahama and Matsumoto as part of an extensive program of biogenetically modelled cyclizations of humulene-derived substrates (*12*). Using a variation of this synthesis, we subsequently prepared [1,13-^{3}H]pentalenene, which was fed to intact cells of *Streptomyces* UC5319 (*13*). The labeled pentalenene was incorporated into the more oxidized pentalenanes, including pentalenolactone (**3**), pentalenolactones E (**4**) and F (**5**), and pentalenic acid (**6**), and the site of tritium labeling in the latter metabolite was unambiguously confirmed by chemical degradation.

With the intermediacy of pentalenene in the pentalenolactone biosynthetic pathway firmly established, we turned our attention to the study of the cyclization of farnesyl pyrophosphate to pentalenene by cell-free extracts of *Streptomyces* UC5319 (*13*). Eventually, conditions were found under which [8-^{3}H,12,13-^{14}C]farnesyl pyrophosphate (atom ratio ^{3}H/^{14}C, 2:2) was converted to labeled pentalenene (atom ratio ^{3}H/^{14}C, 0.9:2). Chemical degradation served to locate the tritium label at the expected position, C-7, of pentalenene. These results are consistent with the mechanism illustrated in Scheme 2 in which *trans, trans*-farnesyl pyrophosphate undergoes ionization to the corresponding allylic cation, which cyclizes to humulene (**7**) by electrophilic attack at C-11 and loss of a proton from C-9. Our earlier ^{13}C-labeling studies had previously established that cyclization takes place on the *si*-face of the distal 10,11-double bond of farnesyl pyrophosphate (*8*). Reprotonation of the humulene at C-10 and transannular cyclization generates the cation **8**, which can undergo hydride migration and further cyclization with loss of one of the H-8 protons of the farnesyl precursor to yield pentalenene. Based on the observed relative and absolute configuration of the



SCHEME 2

pentalenene, it is inferred that the postulated humulene intermediate would be folded in the RSR-CT conformation, as illustrated.[3]

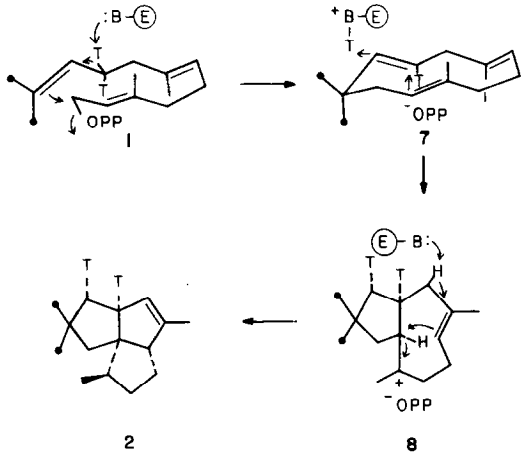
It is noteworthy that the absolute sense of folding of both the farnesyl pyrophosphate precursor and the postulated humulene intermediate are identical. We have previously noted a similar correlation between the conformations of farnesyl pyrophosphate and humulene in the biosynthesis of a number of related humulene-derived sesquiterpenes (*14*), including fomannosin, illudins, marasmic acid, hirsutic acid, coriolin, and, most recently, quadrone (*15*). The correlation is striking, since humulene, an achiral molecule, could in principle be generated by farnesyl pyrophosphate folded in either of two enantiomeric conformations. We have previously suggested that the basis for the observed correlation is that the humulene must be generated and further cyclized at the same active site (*8, 13, 14*). With the availability of an active preparation of pentalenene synthetase, it became possible to test this general prediction experimentally.

RESULTS

Circumstantial evidence against the existence of free humulene was obtained by incubating pentalenene synthetase with [1-^{3}H]farnesyl pyrophosphate for 15 min, and adding inactive carrier humulene to the quenched reaction mixture before extraction with pentane. The reisolated humulene, which was recrystallized as the silver nitrate complex [mp 175°C (dec); lit. (*16*) mp 173–174°C (dec)], was inactive, thereby establishing that free humulene does not accumulate during the enzymatic cyclization.

The latter experiment, of course, merely sets an upper limit on the amount of free humulene, and does not settle the issue as to whether one or two enzymes are

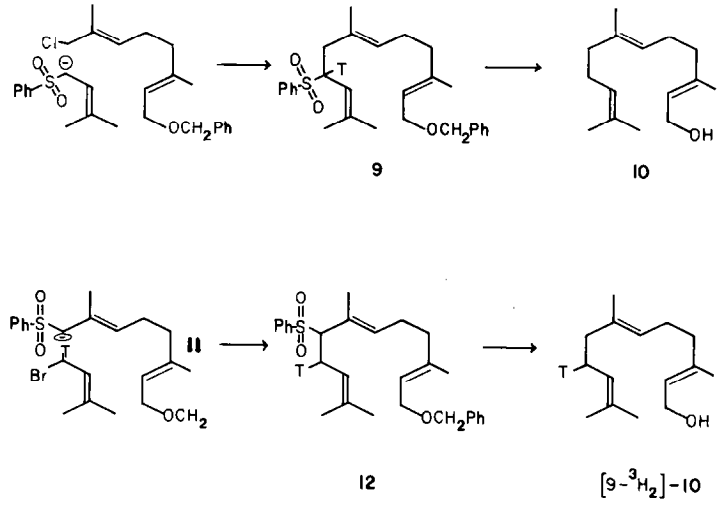
[3] The designation *RSR* refers to the chiralities of the three double bonds, $\Delta^{2,3}$, $\Delta^{6,7}$, and $\Delta^{9,10}$, respectively. C and T indicate crossed and parallel arrangements of the $\Delta^{9,10}$–$\Delta^{2,3}$ and $\Delta^{2,3}$–$\Delta^{6,7}$ double-bond pairs, respectively. See J. K. Sutherland (1974) *Tetrahedron* **30**, 1651.



SCHEME 3

required for the enzymatic conversion of farnesyl pyrophosphate to pentalenene. In order to make this distinction we examined the fate of the proton removed from C-9 of farnesyl pyrophosphate in the course of the initial cyclization step (Scheme 3). Should the resultant humulene be cyclized by a separate pentalenene synthetase, the proton in question would be irreversibly lost. On the other hand, should further cyclization take place at the same active site, there is a chance that some fraction of the time the proton abstracted from C-9 of farnesyl pyrophosphate would be involved in the reprotonation at C-10 of the intermediate humulene. The extent of net proton return would depend on the competition between external exchange of the transiently generated conjugate acid of the enzyme base and reprotonation of the humulene 9,10-double bond. It is also conceivable that the 10- and 9-humulyl cations might simply be interconverted by a hydride shift, resulting in complete retention of the original farnesyl H-9 proton, without generation of a neutral, enzyme-bound humulene species. In either case, full or partial retention of the latter proton would be consistent only with the action of a single cyclase.

In order to test for the postulated proton return, we required a sample of [9-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate. Previously, we had prepared [8-^3H]farnesyl pyrophosphate by coupling of the lithio anion of dimethylallyl phenylsulfone with [8-^3H]-8-chlorogeranyl benzyl ether, followed by reduction of the resulting farnesyl 9-phenylsulfone derivative with lithium in ethylamine (*13*). Although it was found that [9-$^3H_2$]-9-benzenesulfonyl farnesyl benzyl ether (**9**) could be readily obtained by quenching of the corresponding 9-lithio anion with tritiated water, the tritium label was nearly completely lost by exchange during the subsequent reduction step (Scheme 4). The desired [9-$^3H_2$]farnesol (**10**) was eventually prepared by coupling of the lithio anion of 8-benzenesulfonyl geranyl benzyl ether (**11**) with [1-^3H]dimethylallyl bromide, and lithium/ethylamine reduction of the resulting [9-$^3H_2$]-8-benzenesulfonyl farnesyl benzyl ether (**12**) (*17–19*). The labeled dimethylallyl bromide was, in turn, obtained by treatment of the corresponding [1-^3H]dimethylallyl alcohol with phosphorus tribromide. The tritiated farnesol was

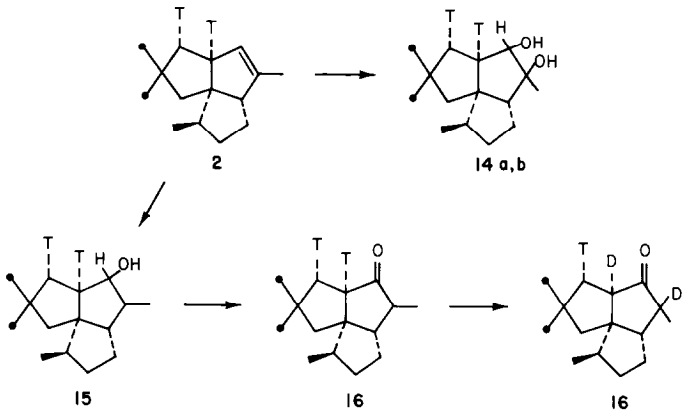


SCHEME 4

mixed with [12,13-^{14}C]farnesol, prepared as previously described (*20*), and a portion of this mixture was converted to farnesyl diphenylurethane (**13**), which was recrystallized to constant activity and isotope ratio (^{3}H/^{14}C 4.96, atom ratio 2 : 2). The remainder of the [9-$^{3}H_2$,12,13-^{14}C]farnesol was converted to the pyrophosphate ester via farnesyl bromide by the method of Poulter (*21*).

Further work with the cyclase had been hampered by the extremely low specific activity of the extracts (ca. 0.003 nmol pentalene mg protein^{-1} h^{-1}) and the pronounced instability (loss of 50% of cyclase activity after only 4 h at 4°C) (*13*). An additional complication was the competing hydrolysis of the farnesyl pyrophosphate substrate by contaminating phosphatase–pyrophosphatase activities. We therefore set out to improve the activity, stability, and purity of the pentalenene synthetase preparation. Substitution of a soluble fermentation medium in place of the previously employed production medium proved to be beneficial by allowing a substantial reduction in the volume of buffer required to suspend the harvested *Streptomyces* mycelium during cell disruption. The buffer itself was modified by reduction of the original phosphate concentration to 50 mM and an increase in the concentration of glycerol and dithioerythritol (DTE). Treatment of the crude cell extract with insoluble polyvinylpolypyrrolidone (PVPP) also resulted in improved yields of cyclase. Finally, it was found that when the 7800*g* supernatant was adjusted to 50% saturation with ammonium sulfate, the redissolved protein pellet, containing 50% of the total protein, retained greater than 90% of the pentalenene synthetase activity but less than 10% of the original phosphatase activity. The latter preparation had a specific activity of 1 nmol pentalenene mg protein^{-1} h^{-1} and retained 85% of its cyclase activity after 5 days at 4°C.

For the preparative scale incubation, cell-free extract (15 ml) from 2.4 liters of a 60-h culture of *Streptomyces* UC5319 was treated with 100 mg PVPP, and the supernatant after centrifugation (135 mg protein) was sparged with nitrogen, sup-



SCHEME 5

plemented with 0.1 mmol magnesium chloride, and incubated at 30°C for 1.5 h with 0.86 μmol [9-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate (5×10^5 dpm ^{14}C/μmol). The resulting recovered, purified pentalenene (2×10^4 dpm ^{14}C) was diluted to 48 mg with synthetic (±)-pentalenene. Half of the labeled pentalenene was converted to the mixture of *cis*-6,7-diols (**14a** and **14b**) by treatment with osmium tetroxide (Scheme 5, Table 1). Recrystallization of each diol isomer to constant activity gave ^{3}H/^{14}C ratios of 4.31 (**14b**–β isomer)[4] and 4.27 (**14a**—α isomer), respectively (atom ratio, 1.7 : 2). Reaction of the remaining 24 mg of labeled pentalenene with borane–THF (tetrahydrofuran) and oxidation of the resulting borane complex with basic hydrogen peroxide gave labeled 7-hydroxypentalenane (**15**). Pyridinium chlorochromate oxidation of **15** gave pentalen-7-one (**16**) (^{3}H/^{14}C = 4.11; atom ratio, 1.7 : 2), which was subjected to exchange with sodium deuteroxide in dioxane/D_2O. After reflux for 12 h, one-half of the reaction mixture was removed, and the ketone **16** was reisolated and purified (^{3}H/^{14}C = 2.12). The base-catalyzed exchange was allowed to continue for 24 h, and the resulting ketone, after purification, maintained a ^{3}H/^{14}C ratio of 2.27 (atom ratio, 0.9 : 2).

The above experiments indicated that pentalenene derived from [9-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate retained nearly all the original tritium, and that half this label was located at C-8. According to Scheme 3, the remainder of the tritium label would be expected to be at C-1. In order to establish the presence of label at this ordinarily unreactive site, we took advantage of the ability of cultures of *Streptomyces* UC5319 to convert pentalenene to more oxidized pentalenane metabolites (*13*) (Scheme 6). Accordingly, a second series of incubations was carried out on a total of 0.99 μmol [9-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate using 47 mg pentalenene synthetase preparation obtained from the 50% ammonium sulfate pellet. The latter incubations were carried out for between 2 and 5 h at 24°C, since

[4] The structure and relative configuration of **14b** were established by X-ray diffraction analysis (P. G. Williard, D. E. Cane, C. Abell, and A. M. Tillman, to be reported). These results also confirmed the previously assigned relative configuration of the C-9 secondary methyl [Cf. Ref. (*12*) and G. D. Annis and L. A. Paquette (1982) *J. Amer. Chem. Soc.* **104**, 4504].

TABLE 1

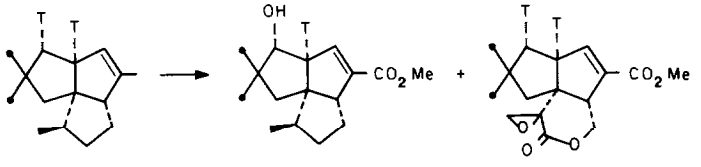
CONVERSION OF [9-$^3H_2$,12,13-^{14}C]FARNESYL PYROPHOSPHATE TO PENTALENENE (**2**) BY PENTALENENE SYNTHETASE, AND DISTRIBUTION OF THE LABEL IN **2**

Compound	^{14}C specific activity (dpm/mmol)	$^3H/^{14}C$	Atom ratio
1[a]	5.0×10^8	4.96 ± 0.1[b]	2:2
2[c]	8.0×10^4[d]		
14a	6.10×10^4	4.27 ± 0.17	1.7:2
14b	6.04×10^4	4.31 ± 0.20	1.7:2
15	7.42×10^4	4.38 ± 0.18	1.8:2
16[e]	7.03×10^4	4.11 ± 0.16	1.7:2
16[f]	6.83×10^4	2.12 ± 0.09	0.9:2
16[g]	6.48×10^4	2.27 ± 0.09	0.9:2

[a] Amount incubated, 4.2×10^5 dpm ^{14}C.
[b] Based on recrystallization of farnesyl diphenylurethane. (cf. Footnote 5).
[c] Total recovered activity, 2.0×10^4 dpm ^{14}C.
[d] Diluted to 50 mg.
[e] Before base-catalyzed exchange.
[f] After exchange with NaOD in D_2O/Dioxane for 12 h.
[g] After exchange for 24 h.

the use of reduced temperature had been found to prolong enzyme lifetime with a corresponding increase in substrate turnover. The resulting labeled pentalenene was diluted with 7 mg (±)-pentalenene, a portion of which was further diluted with carrier and converted to the crystalline *cis*-6,7-diols **14a** ($^3H/^{14}C$ = 4.07) and **14b** ($^3H/^{14}C$ = 3.97). The remaining labeled pentalenene (6 mg) was dissolved in 6.0 ml absolute ethanol and administered in 0.5-ml portions to 100-ml fermentation cultures of *Streptomyces* UC5319 which had been grown for 24 h at 28°C and 300 rpm prior to the addition of labeled precursor. Half the cultures were incubated for an additional 36 h, and the remainder for 60 h before harvesting and isolation of the more oxidized pentalenane derivatives. The latter acids were methylated with diazomethane and purified by flash chromatography on silica gel and HPLC. The $^3H/^{14}C$ ratios of the pentalenolactone F methyl esters (**5-Me**) obtained from both the 60- and 84-h cultures were unchanged compared to the administered enzymatically synthesized pentalenene, whereas the corresponding samples of pentalenic acid methyl ester (**6-Me**) retained only half the original tritium activity ($^3H/^{14}C$ = 2.14 and 2.08, respectively; atom ratio, 0.85:2) (Table 2).

[5] The apparent loss of tritium in the conversion of farnesyl pyrophosphate to pentalenene may in part be due to contamination of the [9-$^3H_2$,12,13-^{14}C]-*trans,trans*-farnesyl pyrophosphate substrate with the corresponding [9-$^3H_2$]-*trans,cis*-farnesyl pyrophospate isomer. Alkaline pyrophosphate hydrolysis of the pyrophosphate ester and gas chromatographic analysis of the resulting farnesol indicated the presence of up to 10% geometric isomers as contaminants, although the isotopic composition of the individual components was not determined. The *trans,cis*-farnesyl isomer is not completely removed by recrystallization of the diphenylurethane derivative, suggesting that the measured $^3H/^{14}C$ ratio could be 5–10% too high.



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SCHEME 6

DISCUSSION

The above results conclusively demonstrate that, in the conversion of [9-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate (**1**) to pentalenene (**2**) by pentalenene synthetase, the pentalenene is equally distributed between C-8 and C-1. Furthermore, if one makes the reasonable assumption that introduction of the hydroxyl oxygen atom at C-1 of pentalenic acid has taken place with the usually observed retention of configuration (*22*), the loss of half the tritium label in the conversion of pentalenene to pentalenic acid suggests that the tritium in pentalenene has the H-1_a configuration. The latter point is under further investigation. Nonetheless, the latter configuration is that predicted based on reprotonation on the 10*re* face of the presumed humulene intermediate folded in the RSR-CT conformation.

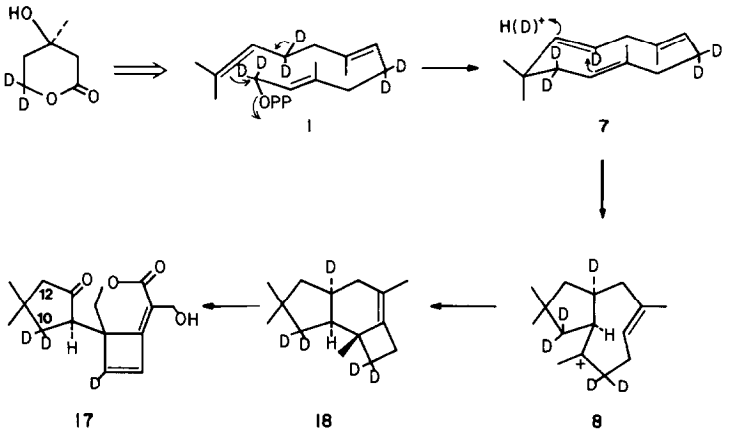
The essentially complete retention of both equivalents of tritium demonstrates that the cyclization of farnesyl pyrophosphate to pentalenene takes place at a single active site, without allowing a distinction between the alternative deprotonation–reprotonation and hydride shift mechanisms. For the moment we favor the deprotonation–reprotonation sequence based on analogy to the biosynthesis of the related sesquiterpene metabolite, fomannosin (**17**), which is believed to be derived from farnesyl pyrophosphate by protonation of humulene folded in the RSR-CT conformation to give the seco-protoilludyl cation **8** (Scheme 7) (*14*). We have previously reported that feeding of [5-$^2H_2$]mevalonic acid to *Fomes annosus* gave fomannosin which bore detectable label only at C-5 and C-10, as determined by ^{2}H NMR spectroscopy (*23*). Although the presence of a full equivalent of

TABLE 2

FEEDING OF [1,8-$^3H_2$,14,15-^{14}C]PENTALENENE[a] TO *Streptomyces* UC5319

	^{3}H/^{14}C (Incorporation)	
Compound	60 h	84 h
5-Me	4.07 ± 0.05 (1.0%)	4.12 ± 0.03 (2.0%)
6-Me	2.14 ± 0.01 (9.5%)	2.08 ± 0.02 (2.0%)

[a] ^{3}H/^{14}C of derived **14a**, 4.07 ± 0.02 (atom ratio 1.7 : 2); **14b**, 3.97 ± 0.05 (atom ratio 1.6 : 2).



SCHEME 7

deuterium at C-12 was firmly excluded by this experiment, the extremely low level of deuterium enrichment precluded the detection of as much as 20% deuterium at the latter site. Interestingly, the corresponding incorporations of [5-^{3}H,2-^{14}C]mevalonate and 3*R*,5*S*-[5-^{3}H],3*RS*-[2-^{14}C]mevalonate gave samples of fomannosin which retained 3.2 and 1.2 equivalents of tritium, respectively, based on ^{3}H/^{14}C ratios. Unfortunately, the latter data are only suggestive since no degradations were carried out to establish the distribution of the labels. Although we cannot as yet exclude the possibility that the cyclization of farnesyl pyrophosphate to pentalenene, on the one hand, and to the protoilludene (**18**) precursor of fomannosin on the other (*24*), take place by fundamentally different mechanisms, for the moment we prefer to believe that the two cyclizations both involve protonation–reprotonation sequences which differ only in the extent to which the transiently generated conjugate acid of the enzyme base can exchange with the external medium. Further work will be required to make a definitive distinction between the two competing mechanisms. In the meantime, it is worth noting that the presumed enzyme base is suitably positioned for the final deprotonation from C-7, which ultimately quenches the tricyclic carbocation and generates pentalenene itself.

MATERIALS AND METHODS

The preparation of [1-^{3}H]farnesyl pyrophosphate and of [12,13-^{14}C]farnesyl pyrophosphate have been described previously (*20*). (±)-Pentalenene was synthesized by the method of Shirahama and Matsumoto (*12*). Casein hydrolysate, Type I, and glycerol (>99%) were obtained from Sigma. Lithium aluminum [^{3}H]hydride (100 mCi/mmol) was purchased from New England Nuclear.

Flash chromatography on silica gel was carried out according to Still (*25*). Radioactivity measurements were performed as previously described (*8*). Cultures of *Streptomyces* UC5319, originally obtained from L. J. Hanka of the Up-

john Company, were stored over liquid nitrogen and used to prepare vegetative inocula as previously described (*8*).

Enzyme preparation. A fermentation medium consisting of 2.0 g black strap molasses, 20 g dextrin, 2.0 g sodium chloride, 1.25 g calcium carbonate, 4.17 g casein hydrolysate and 3.33 g Bacto-dextrose/L distilled water, adjusted to pH 7.2 with 10% sodium hydroxide, was distributed in 22 500-ml DeLong flasks (100 ml/flask), autoclaved at 120°C for 20 min, and inoculated with 1.0-ml portions of a vegetative inoculum of *Streptomyces* UC5319, grown as previously described (*8*). The cultures were incubated at 28°C and 300 rpm for 60 h, after which the mycelium was harvested by centrifugation (8 min, 6000*g*, 4°C). The supernatant was decanted, and the cells were washed successively with cold glass-distilled water, 1 M potassium chloride, and 0.8 M sodium chloride (*26*). After each washing the supernatant was discarded. The washed cells were combined and made into a slurry with approx 25 ml 50 mM potassium phosphate buffer, pH 7.2, containing 5 mM DTE, 1.0 mM EDTA, and 10% (v/v) glycerol.

The suspended cells were ruptured by rapid stirring with 0.1 to 0.15-mm glass beads in a 50-ml jacketed cell. A 15-s on, 15-s off cycle and use of an ice/alcohol cooling jacket reduced the risk of a deleterious temperature increase. The total cycle time was 6 min. The homogeneous slurry from the beater was decanted into small centrifuge tubes and centrifuged at 14,500*g* to deposit cell-wall material and the glass beads. Polyvinylpolypyrrolidone (3 mg/ml) was then added to the supernatant, and the mixture was centrifuged (15 min, 14,500*g*). The supernatant was decanted from the PVPP and recentrifuged (60 min, 47,800*g*) to give 28 ml of enzyme solution (1.4 mg protein/ml).[6]

A portion (21 ml) of the clear supernatant obtained from the final centrifugation was treated with finely ground ammonium sulfate (344 mg/ml). The ammonium sulfate was added slowly over 40 min with stirring at 0°C. After the addition, the solution was stirred for an additional 60 min and then centrifuged (60 min, 47,800*g*). The protein pellet was resuspended in 5.0 ml phosphate buffer containing DTE, EDTA, and glycerol, as above (2.4 mg protein/ml). Magnesium chloride (75 μl of a 1.0 M solution) was added, and the solution was purged with nitrogen. Preparative-scale enzyme reactions were initiated by addition of [9-$^3H_2$,12,13-^{14}C]farnesyl pyrophosphate (20–100 μM), and the incubation was carried out at 24°C for 2–5 h. Acetone (2 ml) was added to terminate the incubation, and the organic products were extracted (3×) into pentane. Carrier (±)pentalenene (1–3 mg) was added at this stage. The pentane extract was purified by flash chromatography (silica, 230–400 mesh; column, 1 × 15 cm; pentane as eluant; fraction size, 4 ml). Radioactive pentalenene, free from farnesol, was isolated in the 3rd or 4th fraction.

Enzyme assay. Assays were typically carried out on 1-ml volumes of enzyme solution which were incubated for 30 min before quenching with 0.5 ml acetone. Synthetic (±)pentalenene (0.2 g) in pentane (1 ml) was added, and the two layers were mixed thoroughly. A portion (0.5 ml) of the pentane extract was withdrawn

[6] Protein concentration was determined by the method of Lowry [O. A. Bessey, O. H. Lowry, and R. H. Love (1949) *J. Biol. Chem.* **180,** 755] or by the dye binding assay, Bio-Rad Laboratories [M. Bradford (1976) *Anal. Biochem.* **72,** 248; T. Spector (1977) *ibid.* **83,** 773].

and passed through a 2-cm column of TLC-grade silica gel in a Pasteur pipet. The eluate was collected, and the column was washed with an additional 1 ml pentane. A 100-μl aliquot of the combined pentane eluate, containing only pentalenene, was withdrawn for scintillation counting. The total extractable radioactive product from the incubation was determined by counting 50 μl of the crude pentane extract.

Feeding of [1,8-^{3}H]pentalenene to Streptomyces UC5319. Labeled pentalenene (3 mg) was dissolved in absolute ethanol (3 ml). The solution was divided equally among six 100-ml cultures of *Streptomyces* UC5319 which had been grown for 24 h on a fermentation medium consisting of 2.0 g black strap molasses, 20 g cornstarch, 10 g corn gluten meal, 5.0 g calcium carbonate, 2.0 g sodium chloride, and 2.25 g Bacto-dextrose/L distilled water, pH adjusted to 7.2. After incubation for an additional 36 or 60 h, the mycelium and insoluble ingredients in the medium were separated from the remainder of the broth by centrifugation (8 min, 6000*g*). The precipitate was washed with water and recentrifuged. The washings were combined with the broth, saturated with sodium chloride, and acidified to pH 2.5 with dilute sulfuric acid. The aqueous solution was extracted with dichloromethane (2 × 300 ml). Emulsions were efficiently broken by passage through Celite under suction. The combined organic extracts were washed with brine and dried. Concentration *in vacuo* gave an oil which was dissolved in tetrahydrofuran (2 ml) and treated with an excess of a distilled ethereal solution of diazomethane. Residual diazomethane was destroyed after 10 min by addition of acetic acid. Removal of the solvent *in vacuo* left a brown oil, which displayed two major spots by TLC (5 : 1 benzene–ethyl acetate). The methyl esters of pentalenolactone (**3-Me**) and pentalenolactone E (**4-Me**) cochromatographed (R_f 0.5), as did the methyl esters of pentalenic acid (**6-Me**) and pentalenolactone F (**5-Me**) (R_f 0.25).

Preliminary purification by flash chromatography (1 × 15-cm column; 8 : 1 benzene–ethyl acetate; fraction size, 4 ml) separated the two pairs of metabolites, which were collected in fractions 4–5 and 9–14, respectively. Each mixture was then further purified by HPLC on a 20 cm × 7.8 mm μ-Porisil column (4 : 1, hexane–ethyl acetate). Approximately 1 mg of crude mixture was applied per injection. Fraction 4–5: Flow rate, 2 ml/min; pentalenolactone methyl ester (**3-Me**) (retention time, 14.6 min) was isolated pure on one pass through the column. Pentalenolactone E methyl ester (**4-Me**) (r.t., 15.4 min) was only 85% pure after one pass, and was reinjected. Fraction 9–14: Flow rate, 7 ml/min; pentalenic acid methyl ester (**6-Me**) (r.t. 5.0 min) and pentalenolactone F methyl ester (**5-Me**) (r.t. 11.3 min).

*Conversion of pentalenene to cis-6,7-diols (**14a**) and (**14b**).* Pentalenene (21 mg, 0.103 mmol) in 0.3 ml pyridine was treated with 26.9 mg (0.106 mmol) osmium tetroxide for 12 h under nitrogen. Sodium bisulfite (183 mg) (*27*) dissolved in 3 ml water and 0.5 ml pyridine was added, and the solution was stirred for 0.5 h. Water was added, and the aqueous layer was extracted with ether. The ether layer was washed with brine and dried over $MgSO_4$. After concentration, the residue was dissolved in ether and washed with 0.05 M copper sulfate solution, and the ether layer was dried over $MgSO_4$. The crude diols were purified by flash chromatography (7 : 3, hexanes–ethyl acetate) to provide 20.4 mg (0.086 mmol, 84%) of solid.

Fractional recrystallization from hexanes–methylene chloride provided 3.9 mg of the higher melting diol (**14b**). Recrystallization from hexanes of the residue after concentration of the mother liquor yielded 12.3 mg of the lower melting diol **14a.** α-Diol (**14a**): ^{1}H NMR ($CDCl_3$) δ 3.77 (bt, J = 7.9 Hz, H-7, 1H), 1.5–2.2 (m, methylene and methine H, 10H), 1.20 (s, H-13, 3H), 1.11 (s, H-14 or H-15, 3H), 1.02 (s, H-14 or H-15, 3H), 0.87 (d, J = 6.8 Hz, H-10, 3H); ^{13}C NMR (MeOD) δ 84.7 (s, C-6), 79.1 (d, C-7), 64.0 (s, C-4), 60.9 (d, C-5 or C-8), 58.4 (d, C-5 or C-8, 50.4 (s, C-1), 47.1 (d, C-9), 43.3 (t, C-11), 41.9 (s, C-2), 35.7 (t, C-3), 27.1 (t, C-12), 30.6 (q, C-13), 28.9, 24.5 (2 q, C-14 and C-15), 17.9 (C-10); IR ($CHCl_3$): 3425 cm^{-1}, 2955, 2870, 1358, 1369, 1454; R_f = 0.37, 7 : 3 hexanes–ethyl acetate; mp 89.5–91.5°C (previously reported as 74.5–75°C (*13*)); *m/e* M^+-H_2O. *Anal.* Calcd for $C_{15}H_{24}O$: 220.1827. Found: 220.1822. β-Diol (**14b**): ^{1}H NMR ($CDCl_3$) δ 3.81 (bt, J = 8.4 Hz, H-7, 1H), 1.3–2.5 (m, methylene H) and 1.24 (s, H-13), overlapping integration, 13H, 1.06 (s, H-14 or H-15, 3H), 0.97 (s, H-14 or H-15, 3H), 0.91 (d, J = 6.9 Hz, H-10, 3H); ^{13}C NMR (MeOD) δ 87.8 (d, C-7), 80.8 (s, C-6), 63.7 (s, C-4), 61.3 (d, C-5 or C-8), 57.3 (d, C-5 or C-8), 48.1 (t, C-1), 45.9 (t, C-11), 44.2 (d, C-9), 42.1 (s, C-2), 36.7 (t, C-3), 22.6 (t, C-12), 32.7 (q, C-13), 31.9, 26.7 (2 q, C-14 and C-15); R_f = 0.37, 7 : 3 hexanes–ethyl acetate; mp 135–137°C.; *m/e* H^+-H_2O. *Anal.* Calcd for $C_{15}H_{24}O$: 220.1827. Found: 220.1841.

[1,8-^{3}H,14,15-^{14}C]cis-6,7-Dihydroxypentalenane (***14a***)/(***14b***). [1,8-^{3}H,14,15-^{14}C]Pentalenene (26 mg, 0.125 mmol; ^{3}H, 2.8 × 10^5 dpm/mmol; ^{14}C, 8 × 10^4 dpm/mmol) was treated with 37 g osmium tetroxide in 0.65 ml pyridine as described. The resulting crude diols (**14a**) and (**14b**) were initially purified by flash chromatography (7 : 3, hexanes–ethyl acetate), separated by fractional recrystallization, and recrystallized to constant activity.

Hydroboration/oxidation of pentalenene. To a stirred solution of 73 mg (0.36 mmol) pentalenene (**2**) in 1 ml THF at 0°C was added 0.4 ml (0.4 mmol) 1.0 M BH_3–THF (*28*). The reaction mixture was stirred for 0.5 h, and then quenched with a drop of water followed by 0.4 ml 3 M sodium hydroxide and 0.4 ml 30% H_2O_2. The reaction mixture was heated to an oil-bath temperature of 55°C for 1 h. Water was added, and the aqueous solution was extracted with ether. The ethereal extracts were combined, washed with saturated sodium bisulfite and brine, and dried over $MgSO_4$. The crude alcohols were purified by flash chromatography (5 : 1, hexanes–ethyl acetate) to give 56.9 mg (0.26 mmol, 71%) of a 20 : 1 mixture (as determined by ^{1}H NMR) of the 6,7-diastereomers of 7-hydroxypentalenane (**15**). ^{1}H NMR (major epimer $CDCl_3$) δ 3.52 (dd, J = 7.7 Hz, H-7, 1H), 2.2–1.2 (m, methylene and methine H, 12H), 1.09 (s, H-14 or H-15, 3H), 1.02 (d, J = 6.1 Hz, H-13) and 1.00 (s, H-14 or H-15) overlapping integration, 6H, 0.88 (d, J = 7.1 Hz, H-10, 3H); ^{13}C NMR ($CDCl_3$) δ 87.4 (d, C-7), 62.9 (s, C-4), 59.2 (d, C-8), 57.0 (d, C-6 or C-5), 51.3 (d, C-5 or C-6), 47.1 (t, C-1), 45.2 (t, C-11), 42.9 (d, C-9), 41.2 (s, C-2), 34.5 (t, C-3), 31.5 (q, C-14 or C-15), 31.3 (q, C-14 or C-15), 28.0 (t, C-12), 16.9 (q, C-10 or C-13), 15.4 (q, C-10 or C-13); IR (film): 3325 cm^{-1}, 2950, 2865, 1460, 1365; R_f = 0.21, 9 : 1 hexanes–ethyl acetate. *m/e* M^+. *Anal.* Calcd for $C_{15}H_{26}O$: 222.1984. Found: 222.1985.

Hydroboration/oxidation of [1,8-^{3}H,14,15-^{14}C]pentalenene to [1,8-^{3}H,14,15-^{14}C]7-hydroxypentalenane (***15***). [1,8-^{3}H,14,15-^{14}C]Pentalenene (20 mg, 0.098

mmol; 7×10^3 dpm ^{14}C) in 1 ml THF was treated with 0.5 ml (0.4 mmol) 0.8 M BH_3–THF as described. The crude alcohol was purified by flash chromatography (5:1, hexanes–ethyl acetate) to afford 8 mg [1,8-^{3}H,14,15-^{14}C]-7-hydroxypentalenane (**15**) (3.27×10^5 dpm/mmol ^3H, 7.42×10^4 dpm/mmol ^{14}C; $^3H/^{14}C = 4.38$).

Pentalen-7-one (***16***). Pyridinium chlorochromate (20 mg) was reacted with 10.3 mg (0.046 mmol) 7-hydroxypentalenane (**15**) in 1 ml methylene chloride for 1.5 h at room temperature before dilution with ether. The resulting sludge-like material was passed through a short column of Florisil to remove the chromium salts, and the column was washed with ether. The ethereal solution was concentrated to give 9.5 mg (0.043 mmol, 93%) pentalen-7-one (**16**). ^1H NMR ($CDCl_3$) δ 2.51 (dd, J = 7.2, 1.9 Hz, H-8, 1H), 2.1–1.1 (m, methylene and methine H, 11H), 1.09 (d, J = 6.6 Hz, H-13, 3H), 1.00 (s, H-14 or H-15) and 0.98 (d, J = 6.6 Hz, H-10) overlapping integration, 6H, 0.85 (s, H-14 or H-15, 3H); ^{13}C NMR ($CDCl_3$) δ 222.2 (C-7), 59.7 (C-4), 58.1 (C-8), 55.1 (C-5 or C-6), 53.1 (C-5 or C-6), 46.97 (C-1), 43.6 (C-11), 43.1 (C-9), 40.9 (C-2), 35.1 (C-3), 29.9 (C-12), 29.8 (C-14 or C-15), 28.9 (C-14 or C-15), 14.5 (C-13 or C-10), 13.6 (C-13 or C-10); IR (film): 2955 cm^{-1}, 2870, 1738, 1455, 1465; R_f = 0.42, 5:1 hexanes–ethyl acetate; *m/e* M^+. *Anal.* Calcd for $C_{15}H_{24}O$: 220.1827. Found: 220.1850.

[1,8-^{3}H,14,15-^{14}C]Pentalen-7-one (***16***). [1,8-^{3}H,14,15-^{14}C]-7-Hydroxypentalenane (**15**) (8 mg) was oxidized with 20 mg pyridinium chlorochromate in 1.5 ml methylene chloride. The crude product was purified by flash chromatography (10:1, hexanes–ethyl acetate). One-half of the ketone was used in the subsequent exchange reaction, while the other half was repurified by a short silica gel column, and an aliquot of the repurified ketone was counted for radioactivity (3.04×10^5 dpm/mmol ^3H, 7.30×10^4 dpm/mmol ^{14}C; $^3H/^{14}C = 4.16$).

[1-^{3}H,6,8-$^2H_2$,14,15-^{14}C]Pentalen-7-one (***16***). The ketone (**16**) (about 4 mg) was dissolved in 0.75 ml dioxane, and 0.6 ml NaOD (prepared from 45 mg sodium and 2.5 ml D_2O) was added. The clear solution became pale yellow after addition of the base. The reaction mixture was stirred at an oil-bath temperature of 110°C for 12 h under nitrogen. One-half of the reaction mixture was withdrawn and diluted with D_2O, and the aqueous layer was extracted with ether. The combined ethereal extracts were washed with brine and dried over magnesium sulfate. The exchanged ketone (**16**) was purified by flash chromatography (10:1, hexanes–ethyl acetate), and an aliquot was counted for activity. To the remainder of the reaction was added 0.5 ml NaOD in D_2O, and the mixture was allowed to stir at reflux for 12 h longer, after which the exchanged ketone was recovered and purified as described above.

[^{3}H]Lithium aluminum hydride reduction of dimethylacrylic acid. Dimethylacrylic acid (190.8 mg, 1.91 mmol) in ether at 0°C was treated with 15 mg (0.39 mmol) lithium aluminum hydride followed by 5.5 mg [^{3}H]lithium aluminum hydride (25 mCi). After 10 min, a further 125 mg of unlabeled reducing agent was added. After 20 min, the reaction was quenched by the addition of 0.1 ml water and 0.1 ml of a 1 M sodium hydroxide solution. Celite was added, and the reaction mixture was filtered, the filter pad being washed thoroughly with ether. The ethereal solution of [1-^{3}H]dimethylallyl alcohol was dried over $MgSO_4$, and was used without further purification in the subsequent reaction.

[1-^{3}H]-1-Bromo-3-methyl-2-butene. The ethereal solution of [1-^{3}H]dimethylallyl alcohol was treated at 0°C with 0.1 ml phosphorus tribromide in 1 ml ether. The reaction was complete after 15 min, and the reaction mixture was poured into ice water. The aqueous layer was saturated with solid sodium chloride and then extracted with ether. After drying the ethereal extracts over $MgSO_4$, the solvent was carefully removed by rotary evaporation and the resulting volatile [^{3}H]-1-bromo-3-methyl-2-butene was used immediately in the subsequent coupling reaction.

8-Benzenesulfonyl geranyl benzyl ether (***10***). To a stirred solution of 1.04 g (4 mmol) 8-hydroxygeranyl benzyl ether, prepared as previously described (*17*), in 10 ml ether at 0°C, was added 0.4 ml phosphorus tribromide in 1 ml ether. The reaction mixture was stirred under nitrogen for 1 h and then poured into ice water, and the aqueous layer was extracted with ether. The combined ether extracts were washed with brine and dried over $MgSO_4$. The 8-bromogeranyl benzyl ether (1.32 g) was dissolved in 5 ml DMF and reacted with 1.06 g (6.46 mmol) of the sodium salt of benzenesulfinic acid for 6 h at room temperature. The reaction mixture was poured into saturated sodium chloride, and the aqueous layer was extracted with ether. The combined ether extracts were washed with water and brine, and dried over magnesium sulfate. The crude sulfone was purified by flash chromatography (4 : 1, hexanes–ethyl acetate) to give a yellow oil, 8-benzenesulfonyl geranyl benzyl ether (**11**) (745 mg, 1.94 mmol; 50% from the alcohol). ^{1}H NMR ($CDCl_3$) δ 7.24–7.9 (m, aromatic H, 10H); 5.29 (bt, J = 12 Hz, H-6, 1H); 5.03 (bt, J = 12 Hz, H-2, 1H); 4.49 (s, -CH_2Ph, 2H); 3.98 (d, J = 7.6 Hz, -CH_2OBz, 2H); 3.70 (s, -CH_2SO_2Ph, 2H); 1.8–2.11 (m, methylene H, 4H); 1.75 (s, H-3′, 3H); 1.58 (s, H-7′, 3H); ^{13}C NMR ($CDCl_3$) δ 139.3, 135.6, 133.5, 128.9 (2 carbons), 128.5 (2 carbons), 128.4 (2 carbons), 127.8 (2 carbons), 127.6, 121.4, 72.2, 66.6, 66.3, 38.5, 26.7, 16.8, 16.5; IR ($CHCl_3$) 2920 cm^{-1}, 1444, 1304, 1148, 1085; R_f = 0.21, 7 : 3 hexanes–ethyl acetate; *m/e* M^+-CH_2Ph. *Anal.* Calcd for $C_{16}H_{21}O_3S$: 293.1211. Found: 293.1210.

8-Benzenesulfonyl farnesyl benzyl ether (***12***). *n*-Butyllithium (1.7 M, 0.30 ml, 0.51 mmol) was added to 186.7 mg (0.486 mmol) 8-benzenesulfonyl geranyl benzyl ether (**11**) in 2 ml THF and 0.1 ml HMPA at −78°C, and the reaction mixture was stirred at −78°C for 0.5 h. A solution of 1-bromo-3-methyl-2-butene (66.4 mg, 0.44 mmol) in 0.5 ml THF was added, and the reaction mixture was allowed to stir at −78°C for 0.5 h and then at −20 to −30°C for 1 h. The reaction mixture was cooled to −78°C and quenched with 1 : 1 ether–methanol. After warming to room temperature, the reaction mixture was poured into water and extracted with ether. The combined ether extracts were washed twice with water and once with brine before drying over $MgSO_4$. Chromatographic purification (7 : 3, hexanes–ethyl acetate) yielded 148.6 mg (0.33 mmol, 74%) 8-benzenesulfonyl farnesyl benzyl ether (**12**).

Alternatively, it was found that the anion of the C_{10} sulfone **11** could be generated by addition of lithium diisopropylamide (LDA) (*29*). The LDA was prepared by adding 0.5 ml (0.7 mmol) 1.4 M *n*-butyllithium to 0.1 ml diisopropylamine in 1 ml THF at 0°C. After 0.5 h at 0.5°C, the base was added to the sulfone **11** in THF at −78°C. Under these conditions, the dimethylallylbromide could be added immediately after LDA. Alternatively, the bromide could be mixed with the sulfone,

and the LDA added directly to this mixture. ^{1}H NMR ($CDCl_3$) δ 7.2–7.9 (m, aromatic H, 10H), 5.28 (bt, J = 12.8 Hz, olefinic H, 1H), 5.03 (bt, J = 13.3 Hz, olefinic H, 1H), 4.85 (bt, J = 12.8 Hz, olefinic H, 1H), 4.49 (s, CH_2Ph, 2H), 4.47 (dd, J = 11.7 Hz, $CHSO_2Ph$, 1H), 3.98 (d, J = 6.7 Hz, $-CH_2OBz$, 2H), 2.5–2.95 (m, $-CH_2CHSO_2Ph$, 2H), 1.7–2.15 (m, methylene H, 4H), 1.65 (bs, H-3′ and H-12, 6H), 1.59 (s, H-11′, 3H), 1.57 (s, H-7′, 3H); ^{13}C NMR ($CDCl_3$) δ 139.4 (s), 138.3 (s), 135.4 (d), 134.7 (s), 133.3 (d), 128.9 (2 carbons, d), 128.7 (2 carbons, d), 128.4 (2 carbons, s), 127.8 (s), 127.0 (d), 127.6 (s), 121.3 (d), 118.9 (d), 74.2 (d), 72.3 (t), 66.6 (t), 38.5 (t), 26.5 (t), 25.7 (q), 24.7 (t), 18.0 (q), 16.4 (q), 13.8 (q): IR (film): 3058 cm^{-1}, 2915, 2850, 1444, 1305, 1134, 1085; R_f = 0.32, 7:3 hexanes–ethyl acetate; *m/e* M^+-CH_2Ph. *Anal.* Calcd for $C_{21}H_{29}O_3S$: 361.1841. Found: 361.1841.

[9-$^3H_2$]-8-Benzenesulfonyl farnesyl benzyl ether (**12**). To two solutions at −78°C of 108 mg (0.28 mmol) and 50 mg (0.13 mmol) 8-benzenesulfonyl geranyl benzyl ether (**11**) in 1 and 0.5 ml THF, respectively, each containing 0.2 ml hexamethylphosphoramide (HMPA), were added 0.65 and 0.4 ml of 0.75 M LDA in THF. A solution of [1-^{3}H]-1-bromo-3-methyl-2-butene in THF was added to each flask, and the reactions were allowed to warm to room temperature over 1 h. The reactions were then quenched at −78°C with 1:1 ether–methanol. Water was added, and the reaction mixtures were combined and extracted with ether. The ethereal extracts were washed with water and brine, dried over $MgSO_4$, and concentrated. The crude product was purified by flash chromatography (9:1 hexanes–ethyl acetate) to provide 30.7 mg (0.068 mmol; ^{3}H 4.5×10^9 dpm/mmol) [9-$^3H_2$]-8-benzenesulfonyl farnesyl benzyl ether (**12**).

[9-$^3H_2$]trans,trans-Farnesol (**9**). Lithium (6 mg) in freshly distilled ethylamine (15 ml) was reacted with 30.7 mg (0.068 mmol) [9-$^3H_2$]-8-benzenesulfonyl farnesyl benzyl ether (**12**) in 0.5 ml THF. After 20 min, the reaction was quenched by addition of 0.1 ml 3-hexyne, and the temperature of the mixture was brought to 25°C. Water was added, and the volatile solvents were removed by rotary evaporation. The aqueous solution was extracted with ether, and the combined organic extracts were washed with brine and dried over $MgSO_4$. The residue after evaporation was purified by flash chromatography (5:1, hexanes–ethyl acetate) to give 11 mg (0.05 mmol, 75%; 4.3×10^9 dpm/mmol) [9-$^3H_2$]-*trans,trans*-farnesol (**10**).

[9-$^3H_2$,12,13-^{14}C]Farnesyl pyrophosphate (*21*). To 11 mg (0.05 mmol) [9-$^3H_2$]farnesol (**10**) (4.3×10^9 dpm/mmol) was added 2 mg [12,13-^{14}C]farnesol (4.8×10^8 dpm/mmol). An aliquot was diluted with 53 mg unlabeled farnesol and converted to the crystalline [9-$^3H_2$,12,13-^{14}C]farnesyl diphenylurethane (**13**) ($^3H/^{14}C$ = 4.96) as previously described (*20*). The remaining alcohol was reacted for 10 min at 0°C in 10 ml anhydrous ether with 0.05 ml phosphorus tribromide. The mixture was poured into ice water, and the aqueous layer was extracted with ether. The combined ether extracts were washed with saturated sodium bicarbonate and brine, and then dried over magnesium sulfate. The ether was removed by rotary evaporation without warming, and the farnesyl bromide was used immediately for the subsequent reaction. The allylic bromide was dissolved in 1 ml dry acetonitrile, and 80 mg (0.087 mmol) tris-[tetra-*n*-butylammonium] hydrogen pyrophosphate was added. The slightly turbid, yellow solution was allowed to stir under nitrogen at room temperature for 18 h. The solvent was removed by rotoevapora-

tion (without warming), and the residue was dissolved in 0.5 ml methanol–water (3 : 1). This solution was applied to a 1 × 13-cm column of Dowex AG 1-X8 (200–400 mesh, formate form), which had been preequilibrated by elution with 30 ml 50 mM ammonium formate in methanol–water–ammonium hydroxide (95 : 5 : 0.5). The column was washed with 150 ml 50 mM ammonium formate in methanol. The pyrophosphate ester was eluted from the column using 600 mM ammonium formate in methanol–water–ammonium hydroxide. Four-milliliter fractions were collected, and an aliquot from each was counted for radioactivity. On the basis of radioactivity, fractions 1–10 (from washing with the 600 mM ammonium formate) were combined, and the methanol was removed by lyophilization. The resulting white crystalline material was washed with cold methanol. The residue was centrifuged, and the methanol was removed from the pellet and saved. The resulting pellet was treated again with cold methanol. This procedure was continued until a very fine white solid remained. TLC analysis of this material showed the absence of the characteristic yellow spot (as visualized with arsenomolybdate spray) corresponding to ammonium formate, which has an R_f just below that of the pyrophosphate ester (which appears as a blue spot). The methanol washes were combined and concentrated by lyophilization, and the same procedure was repeated to give additional pyrophosphate ester (5×10^5 dpm/μmol ^{14}C).

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health, GM22172. The Bruker WM-250 NMR spectrometer used in this work was purchased with funds provided by the National Science Foundation and the Montedison Group of Milan. High-resolution mass spectra were obtained on a VG Micromass 7070H at the University of Pennsylvania. We thank Professor Haruhisa Shirahama of Hokkaido University for providing us with a detailed experimental description of the synthesis of **2** reported in Ref. (*12*).

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